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Micropropagation, Genetic Engineering, and Molecular Biology of *Populus*

Ned B. Klopfenstein, Young Woo Chun,
Mee-Sook Kim, and M. Raj Ahuja, editors

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Abstract:

Thirty-four *Populus* biotechnology chapters, written by 85 authors, are comprised in 5 sections: 1) *in vitro* culture (micropropagation, somatic embryogenesis, protoplasts, somaclonal variation, and germplasm preservation); 2) transformation and foreign gene expression; 3) molecular biology (molecular/genetic characterization); 4) biotic and abiotic resistance (disease, insect, and pollution); and 5) biotechnological applications (wood properties, flowering, phytoremediation, breeding, commercialization, economics, and bioethics).

Keywords: plant transformation; gene expression; tree biotechnology; molecular genetics; bioethics; plant tissue culture

Editors' note: The views expressed in each chapter are those of the author(s) and not necessarily those of the USDA Forest Service. Trade names are used for the information and convenience of the reader and do not imply endorsement or preferential treatment by the USDA Forest Service.

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Micropropagation, Genetic Engineering, and Molecular Biology of *Populus*

Ned B. Klopfenstein, Young Woo Chun, Mee-Sook Kim, and M. Raj Ahuja, Editors

Madelyn C. Dillon, Richard C. Carman, and Lane G. Eskew, Technical Editors

Contents

Preface
N.B. KLOPFENSTEIN, Y.W. CHUN, M.-S. KIM, and M.R. AHUJA iv

Introduction - Sentinels of the Prairie Speak (by *Populus* Species)
H.S. McNABB, Jr. 1

Section I. *In Vitro* Culture

Chapter 1 - Poplar Shoot Cultures: Their Generation and Use in Biotechnology
B.H. McCOWN 5

Chapter 2 - Somatic Embryogenesis From Poplar Leaf Tissue
Y.G. PARK and S.H. SON 10

Chapter 3 - Plant Regeneration Through Organogenesis in Poplar
H. KANG and Y.W. CHUN 13

Chapter 4 - Protoplast Isolation and Culture
J. RUSSELL KIKKERT 24

Chapter 5 - Somaclonal Variation in *Populus*: An Evaluation
D.J. FRY, G.C. DOUGLAS, and N. Th. SAIEED 33

Chapter 6 - Germplasm Preservation of *Populus* Through *In Vitro* Culture Systems
S.H. SON, Y.G. PARK, Y.W. CHUN, and R.B. HALL 44

Section II. Transformation and Foreign Gene Expression

Chapter 7 - *Agrobacterium*-mediated Transformation of *Populus* Species
M.-S. KIM, N.B. KLOPFENSTEIN, and Y.W. CHUN 51

Chapter 8 - Direct Gene Transfer in Poplar
P.J. CHAREST, Y. DEVANTIER, C. JONES, J.C. SELLMER, B.H. McCOWN, and D.D. ELLIS 60

Chapter 9 - Gene Expression Studies
L. JOUANIN and G. PILATE 65

Chapter 10 - Differential Expression of *aro*A Gene in Transgenic Poplar: Influence of Promoter and Ozone Stress
D.F. KARNOSKY, G.K. PODILA, D. SHIN, and D.E. RIEMENSCHNEIDER 70

Chapter 11 - Growth and Development Alteration in Transgenic *Populus*: Status and Potential Applications
B. SUNDBERG, H. TUOMINEN, O. NILSSON, T. MORITZ, C.H.A. LITTLE, G. SANDBERG, and O. OLSSON 74

Chapter 12 - Transgene Expression in the Field-Grown Poplar
G. PILATE, D.D. ELLIS, and S. HAWKINS 84

Chapter 13 - Transgenes and Genetic Instability
M.R. AHUJA 90

Section III. Molecular Biology

Chapter 14 - Applications of Molecular Marker Technologies in *Populus* Breeding
M.T. CERVERA, M. VILLAR, P. FAIVRE-RAMPANT, M.-C. GOUÉ, M. VAN MONTAGU, and W. BOERJAN 101

Chapter 15 - Differentiation of Poplar Clones Using Random Amplified Polymorphic DNA Fingerprinting
D.C. LIN, M. HUBBES, and L. ZSUFFA 116

Chapter 16 - Seasonal Vegetative Storage Proteins of Poplar
G.D. COLEMAN 124

Chapter 17 - Poplar Seed Storage Proteins
T.L. BEARDMORE, S. WETZEL, and S.M. REGAN 131

Chapter 18 - Molecular Genetic Analysis of *Populus* Chloroplast DNA
E.W. NOH and J.S. LEE 143

Chapter 19 - Molecular Marker Techniques to Study Variability of *Populus* Pathogens
G.M. TABOR and H.S. McNABB, Jr. 150

Section IV. Biotic and Abiotic Resistance

Chapter 20 - *In Vitro* Screening and Selection for Disease Resistance
M.E. OSTRY 155

Chapter 21 - Transformation of Hybrid Aspen for Resistance to Crown Gall Disease
H. EBINUMA, E. MATSUNAGA, K. YAMADA, and M. YAMAKADO 161

Chapter 22 - Designing Small Antimicrobial Peptides and Their Encoding Genes
W.A. POWELL and C.A. MAYNARD 165

Chapter 23 - Potential of Proteinase Inhibitors for Enhanced Resistance to *Populus* Arthropod and Pathogen Pests
S.A. HEUCHELIN, L. JOUANIN, N.B. KLOPFENSTEIN, and H.S. McNABB, Jr. 173

Chapter 24 - Expression of Transgenic *Bacillus thuringiensis* δ-endotoxin in Poplar
D.D. ELLIS and K.F. RAFFA 178

Chapter 25 - Genetic Engineering for Air-Pollutant Resistance in Hybrid Aspen
S. ENDO, E. MATSUNAGA, K. YAMADA, and H. EBINUMA 187

Section V. Biotechnological Applications

Chapter 26 - Genetic Modification of Lignin Biosynthesis in Quaking Aspen and Poplar
W. BOERJAN, M. BAUCHER, B. CHABBERT, M. PETIT-CONIL, J.-C. LEPLÉ, G. PILATE, D. CORNU, B. MONTIES, D. INZÉ, J. VAN DOORSSELAERE, L. JOUANIN, and M. VAN MONTAGU; with C.-J. TSAI, G.K. PODILA, and V.L. CHIANG 193

Chapter 27 - Potential Use of *Populus* for Phytoremediation of Environmental Pollution in Riparian Zones
M.E. DIX, N.B. KLOPFENSTEIN, J.W. ZHANG, S.W. WORKMAN, and M.-S. KIM 206

Chapter 28 - Poplar Genetically Engineered for Reproductive Sterility and Accelerated Flowering
R. MEILAN and S.H. STRAUSS 212

Chapter 29 - Integration of Molecular and Classical Genetics: A Synergistic Approach to Tree Improvement
R.J. DINUS and G.A. TUSKAN 220

Chapter 30 - Application of Tissue Culture Systems for Commercial Plant Production
K.A. LOUIS and L.E. EILS 236

Chapter 31 - Economics of Producing *Populus* Biomass for Energy and Fiber Systems
C.H. STRAUSS and S.C. GRADO 241

Chapter 32 - Environmental Risk Assessment and Deployment Strategies for Genetically Engineered Insect-Resistant *Populus*
K.F. RAFFA, K.W. KLEINER, D.D. ELLIS, and B.H. McCOWN 249

Chapter 33 - Biotechnology Risk Assessment: A View From Plant Pathology
X.B. YANG 264

Chapter 34 - Biotechnology, Biodiversity, and Bioethics
M.R. AHUJA 273

Section VI. Appendices

Authors and Affiliations 281

Reviewer Acknowledgment 288

Scientific and Common Names of Biological Organisms Associated With *Populus* Biotechnology
M.C. DILLON, R.C. CARMAN, and M.-S. KIM 290

Glossary of *Populus* Tissue Culture and Molecular Biology
M.-S. KIM, M.C. DILLON, and R.C. CARMAN 295

Index of *Populus* 311

General Index 313

Preface

Species of the genus *Populus* (poplars and aspens) are interspersed primarily in temperate and cold-region forests of the northern hemisphere. They are relatively short-lived, fast-growing trees that can grow on marginal soils and are widely adaptable. *Populus* systems are rapidly being developed for application in fiber, fuel, and environmental plantings. In addition, contributions of *in vitro* and molecular biological studies of *Populus* are apparent in virtually every area of forest biology and ecology. The future of *Populus* research appears bright and promises significant biotechnological advances toward our understanding of processes and interactions in forest ecosystems.

The concepts for this book were generated from a survey that identified the need to synthesize diverse, yet related, information from the rapidly developing studies on *Populus* molecular biology and *in vitro* culture. The topics included were designed to complement the recently published book, *Biology of Populus and its implications for management and conservation* (Stettler, R.F.; Bradshaw, H.D., Jr.; Heilman, P.E.; Hinckley, T.M., eds. 1996. Ottawa, Ontario, Canada: NRC Research Press. 539 p.). Although we attempted to direct chapters toward a "more general" audience, not all topics were amenable. Lead authors were invited and encouraged to collaborate with co-authors working in similar areas, and we were especially supportive of collaboration among diverse, international laboratories. We thank all authors for their stellar efforts and valuable contributions. We also appreciate the constructive advice from peer reviewers, who are listed in the appendix, on all chapters.

This book was designed to foster information exchange and to encourage research cooperation. U.S. Department of Agriculture (USDA) funding was obtained to distribute this publication at a minimal cost and to broaden information access. Unfortunately, hampered by USDA Forest Service budget cuts and downsizing, associated publication delays ensued. These delays were minimized by the efforts and perseverance of the Rocky Mountain Forest and Range Experiment Station publication staff. We appreciate their patience during the extended publication process.

Administrative support is essential for the success of all projects, especially those of substantial proportion. USDA Forest Service administrative support for this significant endeavor was provided by Dr. Barbara C. Weber (Associate Deputy Chief for Research, USDA Forest Service), Dr. Calvin F. Bey (Director, Forest Management Research, USDA Forest Service, retired), Dr. J. Robert Bridges (Staff Research Entomologist, Forest Insect and Disease Research), Dr. Denver P. Burns (Director, Rocky Mountain Forest and Range Experiment Station [RMFRES]), Dr. Brian M. Kent (Assistant Director for Research, RMFRES), Dr. Michele M. Schoeneberger (Project Leader, RM-4551, RMFRES), and Mr. Lane G. Eskew (Supervisory Technical Publications Editor, RMFRES). We appreciate the administrative support from these people that helped this project proceed during difficult times.

The diligent perseverance of several people provided the driving force behind this publication and was instrumental in improving overall quality. The proficient efforts of Richard C. Carman were especially outstanding. Richard maintained general organization in the multi-step process, conducted correspondence, performed preliminary technical editing, prepared the index, and sustained the voluminous records. Special recognition is extended to Madelyn C. Dillon, who conducted the primary technical editing and ensured continued progress with her devoted vigilance and persistence. Karen Mora helped to produce this publication's high-quality visual information and professional layout. Joyce Patterson designed and created the book's aesthetically pleasing cover art. Rudy M. King provided the biometrics review of all chapters. Loretta J. Ulibarri with the assistance of Derald L. Dunagan, and Sharon M. Powers (U.S. Geological Survey, Water Resources Division) completed the indexing process, a task of monumental proportions. We are grateful to all these people for their valuable contributions.

We especially thank our friends and colleagues for their overall assistance, and hope that this publication will serve as a basis for future research with *Populus* and other tree genera.

Ned B. Klopfenstein
Young Woo Chun
Mee-Sook Kim
M. Raj Ahuja

Introduction

Sentinels of the Prairie Speak (by *Populus* Species)

Harold S. McNabb, Jr.

This chapter is dedicated to the late Dr. Marie-France Michel, Station d'Amelioration des Arbres Forestiers, INRA, Centre de Recherches d'Orleans, Ardon - 45160 Olivet, France. Dr. Michel bridged the old and the new, first researching the crown gall disease of poplar in nurseries, then helping to identify strains of the causal agent, *Agrobacterium tumefaciens*, which were ideal for foreign gene transfer.

As we approach the 21st century, our American Indian friends should be proud of us poplars. As the research writings in the following pages show, we are the model system for new scientific advances in woody plant biology. We continue to be the "guinea pig" of forest-tree breeding (Pauley 1949). This distinction means more than the mere fact that humans can easily manipulate our germplasm to meet their needs. The following is our attempt to convey the mystique poplars have possessed in recent human history.

Although we resemble our ancestors in the Salicaceae from approximately 58 million years ago, we are comprised of around 29 different species (Eckenwalder 1996). Poplars were selected as the symbol of liberty during the French Revolution of 1789, later becoming the banner of the resulting new republic (Tucker 1989). During the 18th and 19th centuries, poplars contributed to the overall beauty, stability, and economy of rural life. Our wood was important to the everyday lives of people; shoes in the lowland areas of western Europe, fuel and building material for rural areas, and later, matches that fueled specialized industrial development. Poplars were also part of an established economic system that designated boundaries between landowners and tenants. Monet's famous poplar series from the 1890s illustrate the significance of poplars to people of that period. Our importance is conveyed to subsequent societies as people continue to enjoy the spirituality of these works of art (Tucker 1989).

Monet's tall, slender images of poplars suggests an early selection of *Populus* x *euramericana*. A number of us were first recognized in 1775 as "hybrids spontanés" between the recently imported *P. deltoides* and the European native *P. nigra* (Muhle Larsen 1960). Then, we were called black Italian poplars (*P. serotina* Hartig). The hybrid name *P.* x *canadensis* was introduced in 1795 by Mönch (Pauley 1949). Members of these early spontaneous hybrids are used by our human friends throughout the world and constitute a vast network of our poplar clan today.

Many of our natural hybrids were identified and used during the 19th century. However, in the beginning of the 20th century, artificial breeding was introduced at Kew Gardens when Professor Henry fertilized flowers of *P. angulata* with pollen from *P. trichocarpa*. The resulting progeny were subsequently named *P. generosa* (Muhle Larsen 1960). Later, many breeding programs were initiated with our species. For example, the Oxford Paper Co. in the northeastern United States in 1924 with A. B. Stout and E. J. Schreiner, C. Syrach Larsen in Denmark in 1926, P. Guinier in France in 1926, and C. Heimburger and his colleagues in Canada in 1930. Probably the most intensive breeding efforts occurred in Italy where the Institute of Poplar Culture at Casale Monferrato was founded in 1948 by Professor G. Piccarolo. A similar institute was established in 1949 by the l'Union Allumettiere, S.A. (UNAL), a match producing group, at Geraardsbergen (Grammont), Belgium. C. Muhle Larsen came from Denmark to head this new Belgium institute.

Vic Steenackers, recently retired head of the Belgian laboratory, remarked once while showing colleagues his famous female poplar parents, "My supervisors always wanted to know when I would begin biotechnology research?" My answer was, "What do you think I have been doing during my career? Isn't breeding a form of biotechnology?" Although the modern biotechnology tools enable the geneticist to quickly and comprehensively understand germplasm questions, early poplar selection, breeding, and basic genetic studies produced valuable practical and scientific benefits and were the basis of today's work. Current use of the word biotechnology implies use of molecular tools, which was absent from early poplar selection, breeding, and basic genetic studies.

We poplars believe that molecular tools will help alleviate many problems, such as disease susceptibility and insect injury, with establishment, maintenance, and use of our productive systems. Our interpreter, H. S. McNabb, Jr., and his colleagues believe that host resistance provides the fundamental basis of management strategies for these

damaging agents. Breeding programs with our species have recognized this principle and have used pest resistance as a major selection criteria. Consequently, people look to genetic engineering and gene mapping as tools to develop resistance in our poplar descendants. Molecular tools should also reduce the time needed by breeders to produce individuals with the desired traits.

In early genetic engineering discussions about foreign, pest-resistance genes, our interpreter expressed concern about using genes that could promote higher applications of silvicultural pesticides (e.g., herbicide tolerance genes) (Fillatti et al. 1987) or genes that coded for constitutive internal production of pesticides (e.g., the *Bt* endotoxin genes) (Raffa 1989). Based on their interactions with insects and pathogens, resistant (R) genes can be classified as either subtle or radical (Klopfenstein et al. 1992; McNabb et al. 1990). For example, subtle R genes (e.g., proteinase inhibitor genes) are generally nonspecific and may reduce growth and/or reproduction of pests, making them more susceptible to parasites, predators, and adverse environments. Alternatively, radical R genes (e.g., *Bt* endotoxin genes) are typically pest-specific and pesticidal in their mode of action. Misuse of these radical/specific genes can place high selection pressure on pest populations; whereas, subtle/nonspecific genes are less likely to be rapidly overcome by shifts in pest populations.

Attempts to reduce selection pressure has fostered interest in wound-inducible constructs such as the Proteinase Inhibitor II (*PIN2*) gene. Such constructs were used in genetic engineering research with three of our hybrid relatives (Chun et al. 1988; Heuchelin et al. 1997; Kang et al. 1997; Klopfenstein et al. 1989, 1991, 1997) and were tested in an early field planting of transgenic poplar in July 1989 (Klopfenstein et al. 1991; McNabb et al. 1991). However, questions remain: Do subtle reductions in pest populations significantly diminish the selection pressure on pests? Would subtle reduction be sufficient to manage these pest populations below economic loss levels? The age-old questions remain about the proper planting design for pest resistant poplars in woody crop systems.

Although we poplars believe that pest problems are potentially the most serious, molecular techniques can also help in other areas. An intriguing topic concerns competition among ourselves. The phytochrome system has been shown to be associated with a "communication mechanism" among adjacent individuals (Smith 1995). Poplars "talk" to each other. In competition with each other, we compensate by favoring height growth instead of diameter growth. But, if this effect was mitigated, you humans might achieve greater biomass production from us. Genetic modification of the phytochrome recognition of infra-red reflectance could change this competitive growth response. The potential importance of this research area has prompted the recent establishment of an issue team at Iowa State University led by R. B. Hall. People continue to unravel our secrets.

This book addresses the multi-faceted biological studies that are inspired and challenged by molecular tools. Use of these tools will rapidly convey greater understanding about us poplars, our relatives, and our role in the diverse natural ecosystems in which we reside. Concurrently, our natural ecosystems can be preserved when humans develop special needs and artificial systems, such as fiber farms, for us. However, appropriate precautions are needed to prevent pollution of our native germplasm via nonindigenous seed and pollen. As more molecular techniques are applied to poplars, the resulting information will become a vital part of our genetic and breeding programs around the world. This new biology does not exist in a vacuum. If people wish to optimize poplar potential for sustainable growth in these artificial systems, future research must be integrated into system development. This publication attempts to promote this integration.

This book is divided into 5 general sections each containing chapters on related topics. The initial section discusses what has been achieved with *in vitro* cultures of our poplar relatives including micropropagation, somatic embryogenesis, protoplast culture, somaclonal variation, and germplasm preservation. The second section concerns methods of genetic engineering and evaluation of transgene expression. Poplar researchers have included topics on genetic marker technologies and molecular characterization of *Populus* in the third section. Methods to improve our resistance to insects, pathogens, and air pollution are addressed in the fourth section. We find the fifth section most interesting. These chapters include the biotechnological applications of modification of our wood properties, alteration of our flowering processes, integration of molecular techniques into breeding programs, the commercialization of propagule production, bioethics, and economic considerations. We speak for all members of our *Populus* genus when we say that this new treatment of poplar biology is a welcome event. Humans have honored us as woody plant leaders in the basic biological knowledge explosion.

This introductory chapter closes by asking the following questions: With these new techniques, will humans be able to determine our gender before our reproductive structures develop? At the turn of the 20th century, when our ancestors along the Platte River were cut, the loggers always harvested the female trees because they believed that the wood was stronger. Is this true? And, if so, why? As humans unravel more and more of our secrets, will they have the same reverence for the natural systems as the American Indian people possessed? Will we remain the "Sentinels of the Prairie?"

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Section I *In Vitro* Culture

Chapter 1

Poplar Shoot Cultures: Their Generation and Use in Biotechnology[1]

Brent H. McCown

Introduction

Although most biotechnologists would welcome an opportunity to transform a plant, such as poplar, without resorting to *in vitro* techniques (microculture), currently microculture is central to any genetic engineering protocol commonly used for plants. Functional microculture methods are more essential to tree biotechnology than to annual crop biotechnology. Unlike annual crops where seed-based technologies can be used for various aspects of biotechnology, many tree selections are clonally propagated. Thus, access to uniform and responsive tissues can most readily be achieved only through *in vitro* culture. Although the need for microculture is highest in tree biotechnology, optimizing such procedures for woody perennials is among the most challenging of any *in vitro* work.

Fortunately, a microculture tool that is relatively easy to perfect and is useful for various aspects of tree biotechnology is shoot culture (figure 1A). In this chapter, a shoot culture is defined as an *in vitro* culture that: 1) is derived from shoot explants that contain at least 1 preformed meristem (shoot-tip or node); new shoots are derived solely from preformed meristems (apical or nodal) and adventitious meristem generation is avoided; and 2) remains actively growing through most or all of the culture cycle; the culture can be maintained indefinitely by subculturing shoot explants. Since poplar has become the model species for tree biotechnology, detailed aspects of poplar microculture have been extensively reviewed (Ahuja 1987; Chun 1993; Douglas 1986; Ernst 1993). This chapter examines the various uses of shoot cultures to complement tree biotechnological efforts and summarizes major factors and challenges involved in establishing shoot cultures of trees, especially poplar.

Use of Shoot Cultures in Tree Biotechnology

The responsiveness, stability, and reproducibility of shoot cultures are particularly suitable for tree biotechnology. However, the complex physiological growth cycles of woody perennials is a major limiting factor. The phase change and mid-seasonal growth cycles are of interest. The vegetative life cycle is exemplified by strong juvenile and adult phases, the former is typified by rapid vegetative growth and the latter by flowering (Hackett 1987). As a woody plant progresses through the juvenile and into the adult phase, its tissue responsiveness to microculture manipulation usually decreases markedly (Bonga 1987; Francelot et al. 1987; Hackett 1987). Strong physiological changes accompany seasonal growth cycles (e.g., spring flush, bud set, and dormancy) and confound this progressive life cycle change. For microculture, the most responsive tissues are usually obtained from the spring flush growth. Shoot cultures of trees are a powerful research tool because, once established, they suspend these progressive changes in a more or less permanent condition equivalent to the juvenile phase and the spring flush of growth. Thus, a well-established shoot culture will provide a tissue source that typically is more responsive to various biotechnological manipulations than most other sources, excluding embryonic materials that are usually unsuitable for clonal crops.

Besides providing responsive tissues, shoot cultures stop progression through life and seasonal cycles. Plant shoot growth is essentially suspended while plants are maintained as shoot cultures. For example, shoot cultures of a birch clone (*Betula populifolia* cv. 'Whitespire') and a poplar clone (*Populus tremula* cv. 'Erecta') have been maintained continuously for more than 15 years without noticeable

[1] Klopfenstein, N.B.; Chun, Y. W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

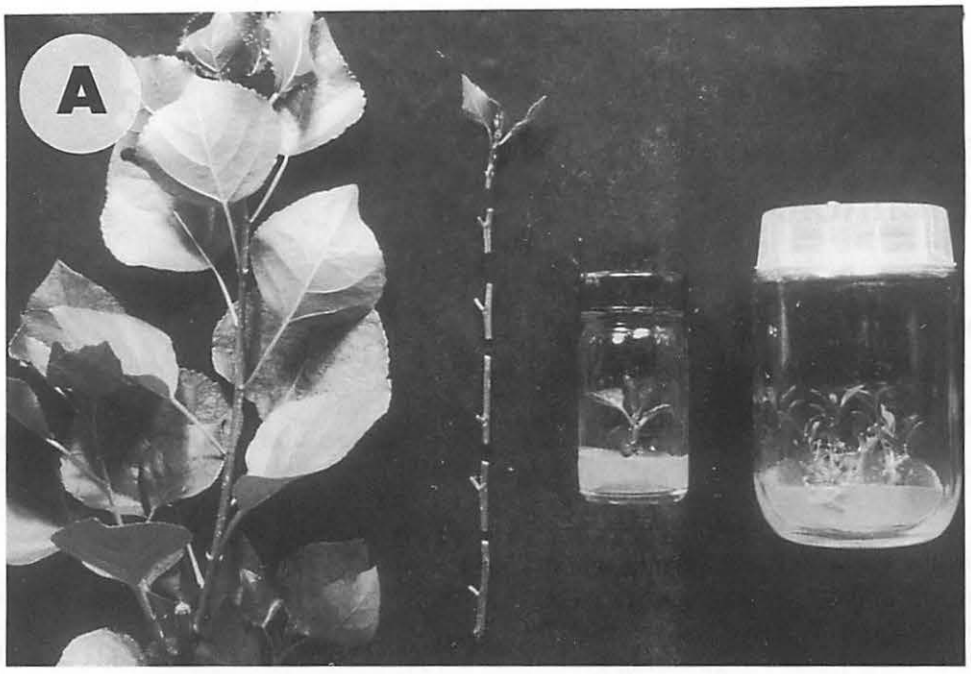


Figure 1A. Steps in establishing a shoot culture of a *Populus alba* hybrid clone. Nodal and tip explants are taken from actively growing shoots, sterilized, and placed in culture. New shoot growth is rapidly subcultured until uniform and continuous growth is obtained.



Figure 1B. Microcuttings (center) are harvested from mature shoot cultures and rooted/acclimated *ex vitro* to produce young plants for testing and evaluation.

changes in their shoot growth characteristics (Deborah D. McCown, Knight Hollow Nursery, Middleton, WI, USA, personal communication). Plants derived from these cultures appear to begin their *ex vitro* growth at the same life-cycle stage (i.e., as a seedling-like plant). Thus, shoot cultures can supply tissues of relatively uniform and reproducible physiology throughout the year.

A second aspect of stability and reproducibility is genetics. Because shoot culture depends on growth from preformed meristems (apical or nodal) of subcultured explants, any single cell mutations and other aberrations rarely develop into aberrant shoots. Mutated shoots should appear at a rate approximately equal to the rate that such aberrant shoots appear in source plants grown *ex vitro*. Some plant genotypes, especially those that are chimeric in character, may be inherently genetically unstable, whether grown as shoot cultures or in the field. However, a vast majority of plants are genetically stable when maintained properly (i.e., without adventitious shoot generation) as shoot cultures. For example, no aberrant shoots have been seen in the long-term shoot cultures of the poplar and birch clones mentioned previously.

Because of their responsiveness, stability, and reproducibility, shoot cultures are an excellent tissue source for various *in vitro* manipulations. For poplar, shoot cultures provide useful source tissues to establish cell/callus/nodule cultures (McCown et al. 1988), protoplast cultures (Chun 1985; McCown 1985; Russell and McCown 1986; Smith and McCown 1983), and genetic engineering. Tissues from shoot cultures were proven adaptable to vector (Confalonieri et al. 1994; DeBlock 1990; Fillatti et al 1987; Riemenschneider and Haissig 1991) and particle-bombardment transformation of *Populus* (McCown et al. 1991).

Conducting routine assays can be hampered by complex secondary compounds (e.g., phenolics or tannins) often present in the tissues of many woody perennials. Protein isolations, DNA extractions, or common assays of gene activity (e.g., the reporter gene *GUS*) (Vainstein et al. 1993) are frequently difficult with woody perennials. Culture-derived shoots usually contain few interfering compounds and often provide the best source tissue for such assays (Francis 1996). With at least some poplar genotypes, DNA for molecular analyses is more readily obtained from shoot cultures than greenhouse- or field-grown leaves (Francis 1996). However, unlike many other woody plants, there were no compounds interfering with the *GUS* reporter gene assays detected in poplar leaf tissues, even in tissues collected from field plants (Francis 1996).

Shoot cultures also provide the basis for the most widely used method of micropropagation in commerce today. Shoot cultures provide uniform microcuttings that can be rooted and acclimated (figure 1B) to produce liners that are handled like seedlings in container and field plantings (Douglas 1986). Micropropagation is particularly useful to provide a multitude of uniform plants in a relatively short time for use in biotechnological tests. Transgene expression in poplar was tested under laboratory conditions (Confalonieri et al. 1994; Francis 1996), in growth chambers/greenhouses (McCown et al. 1991; Riemenschneider and Haissig 1991; Robison et al. 1994), and in the field (Kleiner et al. 1995) using shoot cultures directly or plants derived from shoot cultures.

Finally, shoot cultures provide a convenient way to store selections under evaluation. Cultures can be continuously subcultured and/or maintained under cold storage conditions (Chun 1993). We have routinely maintained more than 100 transformants as shoot cultures, which preserves these unique genotypes and provides a convenient "on demand" source of new plants for further testing or distribution.

Generation of Shoot Cultures

As previously described (McCown and McCown 1987), new isolations from most woody perennials pass through an *in vitro* stage called stabilization before reproducible and uniform shoot growth is achieved. Physiological processes of the stabilization phase are not well understood; however, rejuvenation apparently is involved (reviewed in McCown 1986; for poplar example, see Whitehead and Giles 1977). Stabilized shoot cultures are achieved most rapidly when the explant sources are juvenile materials such as collar shoots, adventitious shoots, or rejuvenated plants (Hartmann et al. 1990). However, gradual rejuvenation often can be achieved by repeated subculturing of new shoots (Mullins 1987).

Once stabilized, the growth and multiplication rate of the shoot cultures can be optimized in the production phase (McCown and McCown 1987). Shoots can be multiplied by either cytokinin stimulation of axillary bud growth, often in conjunction with the loss of apical dominance (shoot tip removal), or by manually dividing a shoot explant into its component nodes during subculture. Both methods are useful with poplars (e.g., Chun 1993; Sellmer et al. 1989).

With *Populus*, the optimal growth and multiplication regime usually depends on the specific genotype. Although many specific differences and variations are observed, 3 general groups of poplars can be discerned from the literature.

1. Many clones, particularly those in the Leuce (currently termed Populus) section can be successfully cultured as shoot cultures grown on standard MS medium (Murashige and Skoog 1962) supplemented with the cytokinin benzyladenine (Sellmer et al. 1989). Auxins are usually not required.

2. Some clones, especially those of *P. tremula* and *P. tremuloides* species/hybrids, often perform better as shoot cultures on media with a lower salt formulation, such as Woody Plant Media (WPM) (Lloyd and McCown 1980; McCown and Sellmer 1987) or its derivatives (Ahuja 1987; Sellmer et al. 1989), supplemented with benzyladenine. Again, auxins are usually not required.

3. Other clones, especially those belonging to the Aigeiros and Tacamahaca sections, do not perform particularly vigorously as shoot cultures on any medium so far defined. Some selections may not be amenable to long-term maintenance as shoot cultures (Sellmer et al. 1989). For others, acceptable shoot cultures may be generated using more complex cultural regimes. MS medium supplemented with cytokinins and auxins may be helpful (Whitehead and Giles 1977). For some selections, benzyladenine may be phytotoxic, but naturally-occurring cytokinins, such as zeatin, may be stimulatory (Ernst 1993). Multistage protocols separating bud stimulation and elongation, each with a separate medium requirement, was useful in other work (Chun 1993; Ernst 1993; Whitehead and Giles 1977). DeBlock (1990) used a buffered medium supplemented with calcium gluconate to overcome shoot-tip necrosis (Sha et al. 1985) problems associated with some poplar clones.

Conclusion

Although not a comprehensive remedy for the difficulties associated with working with trees for biotechnological research, shoot cultures are a major and often essential tool. Shoot cultures provide tissues to begin manipulations and offer an effective avenue for moving plant materials from culture to testing or production. For a program beginning work on a selection of poplar, establishing the genotype in shoot culture will sensitize researchers to its idiosyncrasies in the microculture environment.

Acknowledgments

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Chapter 2

Somatic Embryogenesis From Poplar Leaf Tissue[1]

Young Goo Park and Sung Ho Son

Introduction

An efficient *in vitro* regeneration system with cell and tissue cultures is a prerequisite for biotechnological applications to plant improvement programs. Although whole plants have been regenerated by several techniques from various species and explants, somatic embryogenesis is one of the most powerful morphogenetic schemes. First reported using carrot (Steward et al. 1958), somatic embryogenesis was later defined by Haccius (1978) as a nonsexual developmental process leading to the differentiation of zygotic embryo-like structures from somatic cells. Two advantages of somatic embryogenesis are that: 1) genetically identical propagules are provided by avoiding genetic recombination that occurs during meiosis in sexual reproduction; and 2) it provides a fast, reliable, reproducible method for mass production. In contrast to organogenesis, somatic embryogenesis exhibits a bipolar structure with a closed vascular system and differentiated shoot and root meristems.

Poplars, considered an economically important species in many countries, are usually propagated by cuttings and/or root suckers. Thus, cloning techniques can propagate superior genotypes that show heterosis in the F_1 progeny from artificial hybridization. For interspecific crosses, some species from the Leuce (currently termed Populus) section have superior traits such as a straight trunk and rapid growth (Jiang et al. 1980). Nevertheless, most hybrids from this section have been difficult to propagate asexually using standard techniques. Tissue culture systems, especially somatic embryogenesis are advantageous for application to mass cloning. Techniques developed for poplar may also be applied to related tree species such as willow (*Salix* spp.).

Literature Review

Studies on poplar tissue culture began in the early 1930s (Gautheret 1934). After 30 years, Mathes (1964), Winton (1968), and Wolter (1968) have established plant regeneration methods based on callus culture. In their systems, shoots and roots were induced through organogenesis from different callus sites at different developmental stages. Since then, similar morphogenetic responses were reported by other authors (Chen and Huang 1980; Sellmer et al. 1989; Whitehead and Giles 1977). Because of the potential applications associated with somatic embryogenesis, many studies have focused on crop, horticultural species, and woody perennials (Gingas and Lineberger 1989; Tremblay 1989).

Pioneering studies on somatic embryogenesis in poplar were reported by Michler and Bauer (1987, 1991), Park and Son (1988), Cheema (1989). Somatic embryogenesis at relatively high frequency was obtained directly and/or indirectly by leaf-tissue and cell culture of hybrid aspen (*Populus alba* x *P. grandidentata* cl. 'NC5339'). With this hybrid, short-term auxin treatment stimulated embryo maturation and eventually allowed plants to harden under greenhouse conditions (Michler and Bauer 1991). Cheema (1989) obtained callus and cell suspension cultures using semi-organized leaf culture from mature Himalayan poplars (*P. ciliata*). Similar to most other species, somatic embryogenesis was induced in *P. ciliata* by exposure to a high level of 2,4-dichlorophenoxyacetic acid (2,4-D), followed by reduced auxin concentration. Callus retained its embryonic potential over a year; however, embryogenic suspension cultures lost this ability after 6 subcultures. In another study, organogenesis and somatic embryogenesis occurred simultaneously when punctured leaves of hybrid poplar (*Populus nigra* x *P. maximowiczii*) were cultured (Park and Son 1988).

[1] Klopfenstein, N.B.; Chun, Y. W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

With *Populus*, embryogenic capacity has been associated with leaf-tissue explants. For most other species including woody plants, immature or mature zygotic embryos are commonly used as explants to initiate somatic embryogenesis (Tremblay 1989). For many woody plant species, immature embryos are available only once a year for a very short time. Using leaf tissue, especially that grown *in vitro*, for somatic embryogenesis provides *Populus* species with a substantial advantage.

Poplar Somatic Embryogenesis

In poplar, the developmental process, morphology, and physiology of somatic embryogenesis is similar to zygotic embryogenesis. Because reports on somatic embryogenesis of poplar are limited, summarizing a general method is difficult. However, based on our research and other reports, some critical steps for somatic embryogenesis in *Populus* spp. are described. The steps in somatic embryogenesis from leaf explants of poplar are: 1) explant preparation and adjustment to culture conditions; 2) induction of direct and/or indirect embryogenesis; and 3) maturation and germination.

Explant Preparation and Culture Conditions

As donor plants, greenhouse- or field-grown plants can provide explants to initiate *in vitro* plantlets or callus. Typically, stem-node sections with or without buds are disinfested using 0.5 to 1 percent of sodium hypochlorite for 5 to 10 min. Murashige and Skoog (1962) (MS) culture medium with a low concentration of cytokinin (6-benzyladenine; BA), alone and in combination with high levels of auxin (2,4-D), is used to induce *in vitro* bud break and callus induction, respectively. To multiply the shoots for source material, apices are excised from shoots before subculturing on proliferation medium containing 0.88 μM BA. A 16-h photoperiod provided by cool-white, fluorescent light is used for shoot elongation and direct embryogenesis. Complete darkness is used to establish and maintain callus and embryogenic cell suspension cultures. Suspension cultures are routinely agitated at 100 to 120 rpm using a gyratory shaker, and subcultured at 2-week intervals.

Direct Embryogenesis

Isolated leaves (< 2 cm in diameter) from *in vitro* cultured plantlets are cultured on MS medium supplemented with BA and 2,4-D. Leaves are further prepared by puncturing with a pin or slicing into leaf disks with a cork borer (1 cm in diameter). Amendments such as coconut milk, malt extract, and glutamine may be used to stimulate somatic embryogenesis. In this method, direct and indirect embryogenesis and organogenesis can occur simultaneously. To maintain somatic embryos or embryogenic callus for further development, embryoids and/or embryogenic callus must be selected at an early stage.

Indirect Embryogenesis

Isolated embryogenic callus or embryogenic callus-derived cell suspension cultures can be used for indirect somatic embryogenesis. A simple, reliable method for indirect somatic embryogenesis is obtained by altering the 2,4-D level. A semi-solid agar medium can support further development of embryos harvested at an early developmental stage. Overall, the culture media for maintenance of embryogenic cell lines are similar to induction media except that plant growth regulator levels are lower.

Maturation and Germination

Harvested embryos require several washes in MS liquid medium without plant growth regulators. Somatic embryos originating directly or indirectly from poplar leaf cultures typically do not exhibit dormancy. Maturation of somatic embryos in poplar can be triggered by the changing plant growth regulators. In 2 cases, the maturation medium included BA, alone or combined with α-naphthaleneacetic acid. Increased levels of osmoticum may effectively stimulate maturation.

Limitations

Although somatic embryogenesis was successfully demonstrated in *Populus* species, the induced somatic embryo is strikingly plastic in its development. Many of the induced embryos exhibited abnormal growth characteristics including: 1) bearing multiple secondary embryoids among the embryonic axes; 2) rapid growth of nonembryonic callus during embryogenesis; 3) abnormal cotyledons growth; and 4) growth failure of either cotyledon or root radical. This plasticity may result from the altered gene expression during development. Another problem associated with poplar embryogenesis is its low success rate and poor reproducibility in comparison with carrot. Because all stages from induction to maturation are very labor intensive, large-scale propagation through somatic embryogenesis is impractical without improving overall efficiency. A critical limitation is lack of knowledge on the somatic embryogenic processes of poplar. Continued intensive studies are needed.

Conclusion and Prospects

Somatic embryogenesis can be induced from leaf explants of poplar. Many factors such as basal media composition, undefined substances, amino acids, plant growth regulator dosage, and treatment methods influence somatic embryogenesis. Among these factors, 2,4-D plays a paramount role as an auxin source. However, a phytohormone imbalance usually causes the formation of abnormal or incomplete structures. Because the role of auxins in embryoid formation is complex, purification and identification of various auxin receptors and understanding their molecular action will contribute to our knowledge of cellular differentiation mechanisms related to somatic embryogenesis (LoSchiavo 1995). Using leaf explants of poplar, the somatic embryogenic response was examined using inverted stereo microscopy (Michler and Bauer 1991; Park and Son 1988). When embryos were produced from cultured leaves, they appeared to arise directly or via callogenesis from wounded portions or from sliced leaf ends.

In recent years, embryogenesis through cell suspension culture has provided opportunities for gene transformation by microparticle bombardment of intact cells or direct gene transfer using protoplasts. Liquid plating of protoplast allows stringent selection of transformed cells. Cell suspension culture-based somatic embryogenesis is also applicable to germplasm preservation and mutant selection. If successfully developed, these technologies can be employed in bioreactor systems for mass production. Attempts at large-scale embryogenesis are being applied to various bioreactor systems such as air-lift, impeller, and hybrid types.

Induction of somatic embryogenesis and organogenesis have usually occurred simultaneously, but embryoid formation efficiency was relatively low. Abnormalities in morphology and growth were evident. It is our hope that this summary of somatic embryogenesis methods for poplar will be useful to future studies. Continued studies are needed to improve culture systems and for further understanding of somatic embryogenesis for application to poplar and other woody plants.

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Chapter 3

Plant Regeneration Through Organogenesis in Poplar[1]

Hoduck Kang and Young Woo Chun

Introduction

Among woody genera, *Populus* has been extensively studied as a model system for biotechnological research. This genus of the Salicaceae family, widely distributed across the Northern Temperate Zone (FAO 1980), is composed of 5 sections: Leuce (currently termed Populus), Aigerios, Tacamahaca, Turanga, and Leucoides (Dickman and Stuart 1983). Leuce is a large group that includes aspen and white poplars, which have great economic importance. Aigeiros, considered the "true poplars," include the cottonwoods and black poplars. Tacamahaca, containing the balsam poplars, is the largest poplar species group. The remaining 2 sections (Turanga and Leucoides) have minor economic importance.

Populus species and hybrids are ideal for plywood and lumber production, and woody biomass production because of rapid growth, ease of establishment through stem or root cuttings, and relative ease of coppice regeneration (Ahuja 1987; Behrens and Melchior 1978; Hall et al. 1989; Herrmann and Seuthe 1982). Trees of this genus represent wide genetic diversity, exhibit widely ranging site requirements, and respond well to cultural input (Schreiner 1974). Many new, high-quality, rapidly growing poplar clones were developed in various breeding programs from diverse geographic regions (Hall et al. 1989). Vegetatively propagated *Populus* species and hybrid clones have been planted extensively in many countries (Zsuffa 1985). For the difficult-to-root poplar (section Leuce), tissue culture techniques for micro-propagation have become a primary alternative to more conventional propagation procedures. *In vitro* propagation techniques were applied to poplars to overcome limitations such as production cost and availability of specific genotypes for planting material (Ahuja 1987). Furthermore, as a model system for biotechnology programs, poplars have been intensively studied for *in vitro* micropropagation, genetic transformation, and gene expression.

The objectives of this review are to summarize: 1) organogenic regeneration of poplar by adventitious bud induction from various explants; and 2) physiological malformation of *in vitro* plantlets, which is a fundamental problem in poplar micro-propagation.

In Vitro Organogenesis

Since 1980, poplars have been intensively studied for *in vitro* establishment (Chun et al. 1988). Such studies reveal that *Populus* tissue exhibits a high degree of developmental plasticity, similar to tobacco in the herbaceous species. Many *in vitro* cloning techniques were developed that provide an integral basis for biotechnological applications. Published reports on *in vitro* regeneration of poplars are summarized in table 1. Types of shoot regeneration with organs or tissues are ordinarily classified as: 1) adventitious shoot formation or 2) axillary shoot induction. Adventitious shoot formation is amorphous shoots originating from the various explants of leaf, internode, catkin, immature embryo, pollen, vascular or bark cambium, root, etc. Cultures for axillary shoot induction are composed of morphous shoots originating from an apical meristem or axillary bud. Apical meristems containing shoot primordia can produce multiple shoots. For more information on *in vitro* morphogenesis of *Populus* hybrids and species, refer to reviews by Ahuja (1987, 1993), Chun (1993), and Ernst (1993).

Adventitious Shoot Organogenesis Through *In Vitro* Cultured Explants

Adventitious poplar shoots can be induced from *in vitro* cultured leaf, internode, petiole, and root explants that

[1] Klopfenstein, N.B.; Chun, Y.W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

Table 1. Published reports on *in vitro* establishment with *Populus* species.

Culture type	Species	Reference
Leaf culture		
immature lamina discs	*P. ciliata*	Mehra and Cheema 1980
leaf, internode, root	*P. alba* x *P. grandidentata*	Chun 1987
leaf discs	*P. maximowiczii* x *P. trichocarpa*	Michler and Bauer 1988
leaf, stem, root	*P. maximowiczii* x *P. trichocarpa*	Kang 1991
Root culture		
bud, stem, leaf, root	*P. tremula*	Ahuja 1983
bud, stem, leaf, root	*P. tremuloides*	Ahuja 1983
root	*P. alba* x *P. grandidentata*	Son and Hall 1990
leaf, stem, root	*P.* x *euramericana*	Kang 1991
Anther culture		
anther	*P. glandulosa*	Kim et al. 1983
anther	*P. deltoides*	Uddin et al. 1988
Embryo culture		
immature embryo	*P. deltoides*	Kouider et al. 1984
immature embryo	*P. deltoides*	Savka et al. 1987
ovary, embryo	*P. alba*	Raquin et al. 1993
ovary, embryo	*P. deltoides*	Raquin et al. 1993
ovary, embryo	*P. trichocarpa*	Raquin et al. 1993
ovary, embryo	*P. lasiocarpa*	Raquin et al. 1993
ovary, embryo	*P. trichocarpa* x *P. deltoides*	Raquin et al. 1993
immature ovary	*P. deltoides*	Kang and Hall 1996
Callus culture		
callus	*P. tremuloides*	Winton 1968
callus	*P. tremuloides*	Wolter 1968
callus	*P. tremuloides*	Winton 1970
callus	*P. tremuloides*	Wolter and Gordon 1975
callus	*P. tremuloides*	Noh and Minocha 1986
callus	*P. maximowiczii* x *P. trichocarpa*	Ostry and Skilling 1988
callus	*P. nigra* var. *charkowiensis* x *P. nigra* var. *caudina*	Ostry and Skilling 1988
callus	*P. nigra* var. *betulifolia* x *P. trichocarpa*	Ostry and Skilling 1988
callus	*P. nigra* var. *charkowiensis* x *P. deltoides*	Ostry and Skilling 1988
callus	*P. nigra* var. *betulifolia* x *P. nigra*	Ostry and Skilling 1988
callus	*P. nigra* x *P. maximowiczii*	Park and Son 1988
cell suspension	*P. ciliata*	Cheema 1989
callus	*P. deltoides*	Coleman and Ernst 1990
callus	*P. alba* x *P. grandidentata*	Michler and Bauer 1991

normally never reproduce vegetatively *in vivo* (Ahuja 1993; Chun 1993; Ernst 1993). Most aspen, cottonwood, and their hybrids produce adventitious shoots in Murashige and Skoog (MS) (Murashige and Skoog 1962) media or Woody Plant Media (WPM) (Lloyd and McCown 1980) supplemented with 0.2 to 0.5 mg/l, 6-benzyladenine (BA) or 2.0 mg/l zeatin as a cytokinin source. Many *Populus* species were successfully regenerated through adventitious shoot induction from cultured explants including node, internode, leaf, and root segment (Agrawal and Gupta 1991; Chun 1990; Coleman and Ernst 1990; Douglas 1984; Kim et al. 1994a, 1994b; Nadel et al. 1992; Rutledge and Douglas 1988).

Adventitious regeneration *in vitro* may generate a much higher rate of shoot production than proliferation of axillary shoots. Commercial-scale regeneration systems were

established from leaf and root cultures of European aspen (*P. tremula*), quaking aspen (*P. tremuloides*), and their hybrids (Ahuja 1984). Plant regeneration from stem internodes was accomplished in *P. trichocarpa* x *P. tacamahaca* (Douglas 1984). Park and Son (1988) demonstrated that an average of 178 shoots was directly produced from a punctured leaf of *P. nigra* x *P. maximowiczii* after 6-weeks culture.

Morphogenetic responses of explants from *in vitro* cultured poplar plantlets depend on the explant source and the combination of exogenously applied plant growth regulators. Chun (1990) demonstrated that abaxial side culture of whole-leaf explants was best to induce adventitious shoot buds from a hybrid aspen (*P. alba* x *P. grandidentata*) plantlet. Kim et al. (1994a) showed that the form and concentration of nitrogen sources (ammonium and nitrate) and combination of BA with naphthaleneacetic acid (NAA) greatly affect adventitious shoot induction from leaf-explant cultures of *P. davidiana*.

Kang and Hall (1996b) successfully established a nodal culture system to maximize shoot production of a cottonwood hybrid (*P.* x *euramericana*). They used 4 explants: node, internode, node with the axillary bud excised, and reutilized stem. The shoot proliferation capacity of nodal explants and modified explants was compared, and the origin of multiple shoots was investigated. For these explants, most shoots were derived from the axillary meristems. Shoots also originated from the vascular cambium and occasionally from the lenticels (figures 1-4 to 1-6). A conventional method of directly using nodal explants produced a mean (± standard error) of 5.7 ± 0.6 shoots by treatment with 2.0 mg/l zeatin. However, shoot production from internodes and modified nodes was better using 0.5 mg/l BA with 2.0 mg/l zeatin, which produced averages of 21.7 ± 1.1 and 29.0 ± 0.9 shoots, respectively (figures 1-1 to 1-3). Shoot formation from lenticels was examined in thin sections using bright-field microscopy, and intact specimens were observed with a stereo microscope (figures 1-8 and 1-9).

For adventitious shoot production from *in vitro* cultured leaves of hybrid aspen clones (*P. alba* x *P. grandidentata* cv. 'Crandon' and cv. 'Hansen'), thidiazuron (TDZ) and NAA test combinations were more effective than BA and NAA test combinations or TDZ test concentrations without NAA (Kim et al. 1994b). Among 40 combinations of TDZ and NAA tested, 0.05 μM TDZ with 1.0 μM NAA produced the most adventitious shoots; 18 shoots per leaf explant for 'Crandon' and 15 shoots per leaf explant for 'Hansen.' Increasing TDZ concentrations (0.1, 0.2, 0.5, 1.0, and 2.0 μM) had varying effects on the frequency of regeneration and vitrification of the 2 hybrid aspen clones (Kim et al. 1994b). Significant clonal differences were observed for the number of adventitious shoot regenerated. After 2 to 3 weeks on TDZ/NAA regeneration media, adventitious shoots initiated on the proximal cut surface of the petiole and near punctured areas on the leaf surface. On BA/NAA regeneration media, most adventitious shoots formed at the proximal cut surface of the petiole, while only a few formed on the cultured leaf surface.

Shoot proliferation occurred on cultured root segments of quaking aspen, hybrid aspen (*P. alba* x *P. grandidentata*), and hybrid cottonwood (*P.* x *euramericana*) (Ahuja 1983; Chun 1990; Kang 1991; Son and Hall 1990). Using 'Crandon' and 'Hansen' clones of *P. alba* x *P. grandidentata*, Son and Hall (1990) tested root segments of various ages for shoot regeneration. They reported that root tip containing explants from 60-day-old roots produced the most shoots (an average of 111 shoots per root segment for 'Crandon' and 98 for 'Hansen') on WPM supplemented with 4.8 mg/l or 3.0 mg/l zeatin, respectively. Clonal propagation was used with root segments of hybrid cottonwood (*P.* x *euramericana*) clones (Kang 1991). Among 4 clones ('Canada Blanc,' 'Eugenei,' 'I-45/51,' and 'Wisconsin #5'), the most shoots (17.60 ± 8.96) were induced from 'Canada Blanc' explants on WPM with either a low concentration (0.2 mg/l) of BA or a high concentration (5.0 mg/l) of zeatin.

Generally, morphogenetic responses of *in vitro* cultured explants depend on the species, explant source, and combination of exogenously applied plant growth regulators. Research efforts to develop more generalized organogenesis techniques for explant culture of poplar species and hybrids include: 1) applications of physical treatment, such as the leaf-surface puncturing technique of Park and Son (1988); 2) the effects of various plant growth regulator combinations, such as 2,4-dichlorophenoxyacetic acid (2,4-D) vs. NAA; and 3) optimization of culture conditions for explant source material.

Adventitious Shoot Induction From Anther, Ovule, Embryo, and Catkin Culture

Haploid plantlets were produced from anther cultures of *P. glandulosa*, *P. deltoides*, and *P. maximowiczii* (Kim et al. 1983; Stoehr and Zsuffa 1990; Uddin et al. 1988). In a breeding program, embryo cultures can potentially rescue aborting embryos of interspecific hybrids. Several cultures were reported with ovules or embryos of *Populus* species (Kouider et al. 1984; Raquin et al. 1993; Savka et al. 1987). Recently, Kang and Hall (1996a) reported shoot formation

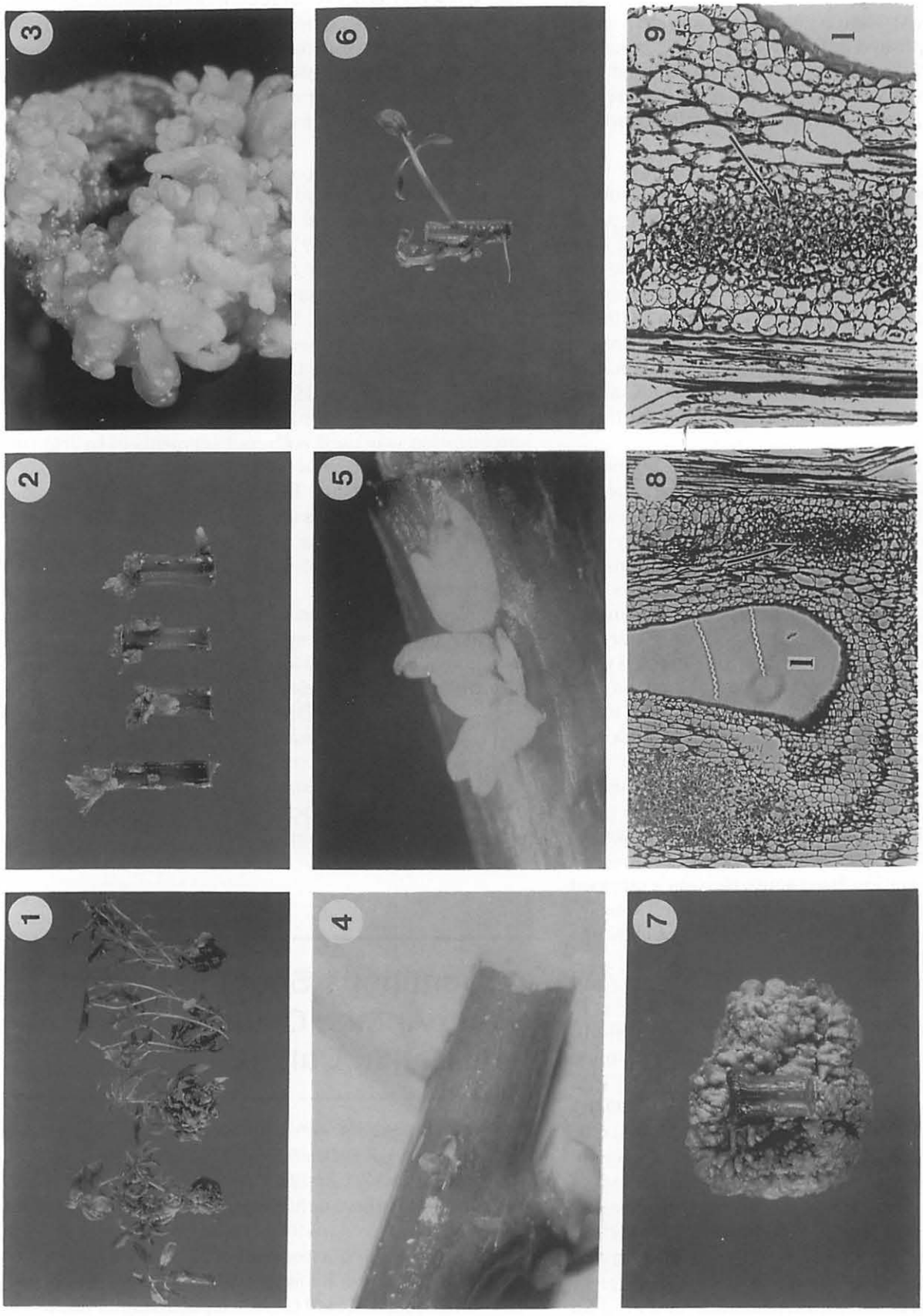


Figure 1. Shoot proliferation from different explants of the 'Ogy' clone (*Populus* x *euramericana*). 1-1) Multiple shoots from the explants of a control node, modified node with the axillary bud excised (2.0 mg/l zeatin), reutilized stem (0.5 mg/l BA), internode (0.5 mg/l BA), and node (2.0 mg/l zeatin). 1-2) Shoot formation from the internodal explants; 1-3) Shoot initiation from the vascular cambium of a stem internode. 1-4 to 1-6) Shoot initiation and elongation from lenticel. 1-7) Shoot induction with a high concentration of zeatin (5.0 mg/l) from a modified node axillary bud. 1-8 and 1-9) Longitudinal section of a partial lenticel with a shoot. l=lenticel (Kang and Hall 1996a).

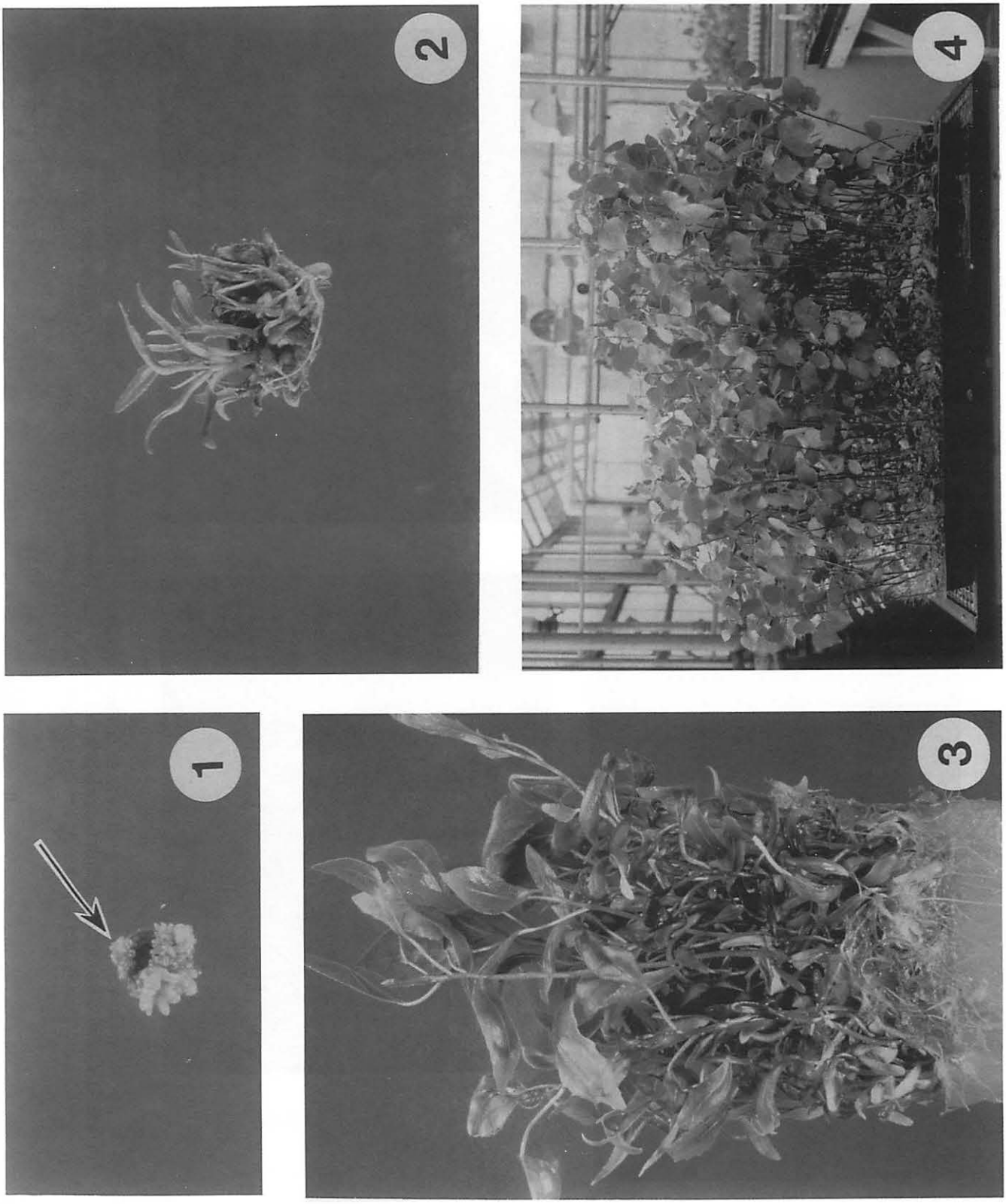


Figure 2. Shoot formation from immature ovules of *Populus deltoides*. 2-1) Shoot initiation from immature embryo and expanded cotyledon; arrow indicates the multiple shoots from the cotyledon. 2-2 and 2-3) Shoot elongation of multiple shoots in half-strength medium containing 0.02 mg/l IBA. 2-4) Plants after transplanting to soil mix and 6-weeks growth in a greenhouse (Kang and Hall 1996b)

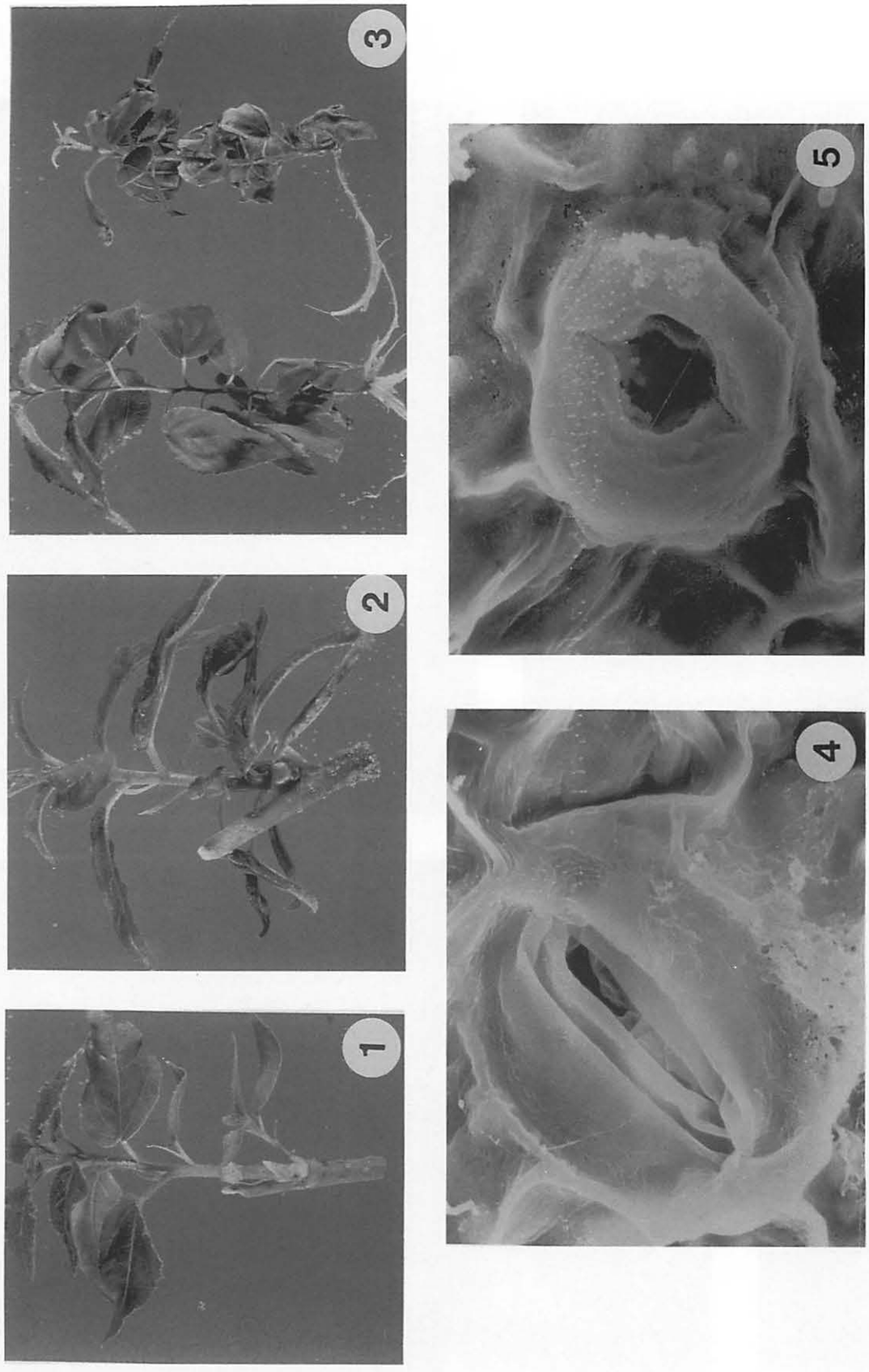


Figure 3. Shoot vitrification from stem nodal cultures of *Populus alba* x *P. grandidentata*, 'Crandon' clone. 3-1) Normal shoot induced from an axillary bud. 3-2) Vitrified shoots induced from an axillary bud. 3-3) Shoot elongation on the half-strength Murashige and Skoog (MS) medium. 3-4) Normal stomate of nonvitrified leaf. 3-5) Abnormal stomate of vitrified leaf (Kang and Hall 1996c).

from developing ovules cultured on vitamin-supplemented WPM containing BA and zeatin as cytokinins. Multiple shoots were obtained from immature ovules collected 20 days after pollination. Generally, zeatin was better than BA for inducing multiple shoots. The highest mean number (56.1 ± 7.4) of shoots was from immature ovules on WPM supplemented with 5.0 mg/l zeatin (figures 2-1 and 2-2). Roots formed after small clumps of shoots were transferred to half-strength WPM containing 0.02 mg/l indole-3-butyric acid (IBA) (figure 2-3). A survival rate of 97 percent was achieved by transferring plantlets to a greenhouse environment (figure 2-4).

Development of shoots from excised flower buds is extremely rare in tree species. Bawa and Stettler (1972) cultured female catkin primordia of *P. trichocarpa*. Although unsuccessful in developing whole plants, they found a general tendency for callus formation with increasing culture age, occasionally followed by a reversal to vegetative growth. Chung et al. (1993) successfully established a flower-bud culture system to maximize shoot regeneration of *P. deltoides* and *P. maximowiczii*. They used small slices (5 to 6 mm thick) of catkin prepared from well-developed (3 to 4 cm long) flower buds that were cultured on MS medium. An average of 3.8 shoots from *P. deltoides* and 4.1 shoots from *P. maximowiczii* were regenerated from catkin after culturing on MS medium containing 1.0 mg/l BA with 0.05 mg/l NAA or 0.5 mg/l BA with 0.05 mg/l NAA, respectively. More than 20 shoots can be regenerated from a single mature catkin of poplar such as *P. deltoides*, which is typically recalcitrant to shoot regeneration. Adventitious shoots were initiated from 3 locations on the explant; the outer and the cut surface of stalks, and the marginal area of small bracts.

Callus Culture

Calli, groups of undifferentiated cells, are induced from various explants, such as stem, leaf, root, embryo, pollen, or protoplast. Plants regenerate in 2 ways from calli manipulation: 1) organogenesis in which shoots form directly from calli; and 2) somatic embryogenesis from embryogenic development. Pioneering studies for *in vitro* shoot regeneration were conducted on callus cultures of triploid quaking aspen (Winton 1968; Wolter 1968) and quaking aspen (Winton 1970). Wolter (1968) suggested that shoots were induced on callus explants of *P. tremuloides* by supplementing the basal medium with 0.2 to 0.5 mg/l BA, and that fresh shoots were rooted on the same medium supplemented with 0.04 mg/l 2,4-D and 1.0 mg/l kinetin. *In vitro* plantlets were regenerated through organogenesis in *Populus* species (Coleman and Ernst 1990; Noh and Minocha 1986; Park and Son 1988). Cytokinin treatments of 0.2 to 1.0 mg/l BA or 2.0 to 5.0 mg/l zeatin were used for shoot regeneration.

McCown et al. (1988) defined nodules from cell suspension culture as independent, spherical, dense cell clusters that form a cohesive unit and display a consistent internal cell/tissue differentiation. They demonstrated that nodule culture has a high potential for organogenesis. Chung and Chun (1991) obtained fine nodules from cell suspension cultures of *P.* x *euramericana* and *P. nigra* x *P. maximowiczii* on MS medium with an optimal combination of BA and NAA. When these fine nodules were transferred to liquid regeneration medium with BA and NAA, an average of 27 shoots per nodule was induced after 8-weeks culture.

Overall, plant morphogenesis through *in vitro* culture depends on species, explant, type and concentration of plant growth regulators, culture medium composition, and culture environment. For further application, systems for successful plant regeneration need careful study to optimize these parameters.

Physiological Changes of *In Vitro* Cultured Plantlets

Vitrification is a physiological disorder affecting *in vitro* plantlets (Kevers et al. 1984). Although most vitrification studies have focused on herbaceous species, some woody species have been studied (Letouze and Daguin 1987; Marin et al. 1988; McLaughlin and Karnosky 1989). Numerous studies have reported on the abnormalities caused by factors such as medium type (Earle and Langhans 1975), gelling agents (Debergh 1983), organics (Zimmerman and Cobb 1989; Ziv et al. 1983), inorganics (Vieitez et al. 1987), and plant growth regulators (Leshem et al. 1988; Paques and Boxus 1987). In comparison with normal leaves of *in vitro* plantlets, vitrified plantlets have malfunctional stomata (Brainerd et al. 1981; Marin et al. 1988; Miguens et al. 1993; Ziv et al. 1987); unorganized vacuolated, spongy mesophyll cells (Earle and Langhans 1975; Vieitez et al. 1987); large intercellular spaces (Brainerd et al. 1981); reduced epicuticular waxes (Sutter 1985); and low chlorophyll content (Phan and Letouze 1983; Ziv et al. 1983). Vitrified *in vitro* plantlets also poorly acclimatize after transfer to soil, a problem for commercial production (Debergh et al. 1981). Vitrified plantlets demonstrated an increased cellular volume (Paques and Boxus 1987; Vieitez et al. 1985; von Arnold and Ericksson 1984), and vascular bundles were less developed in vitrified leaves (Leshem 1983; Ziv et al. 1983). Several rescue methods using modifications of media

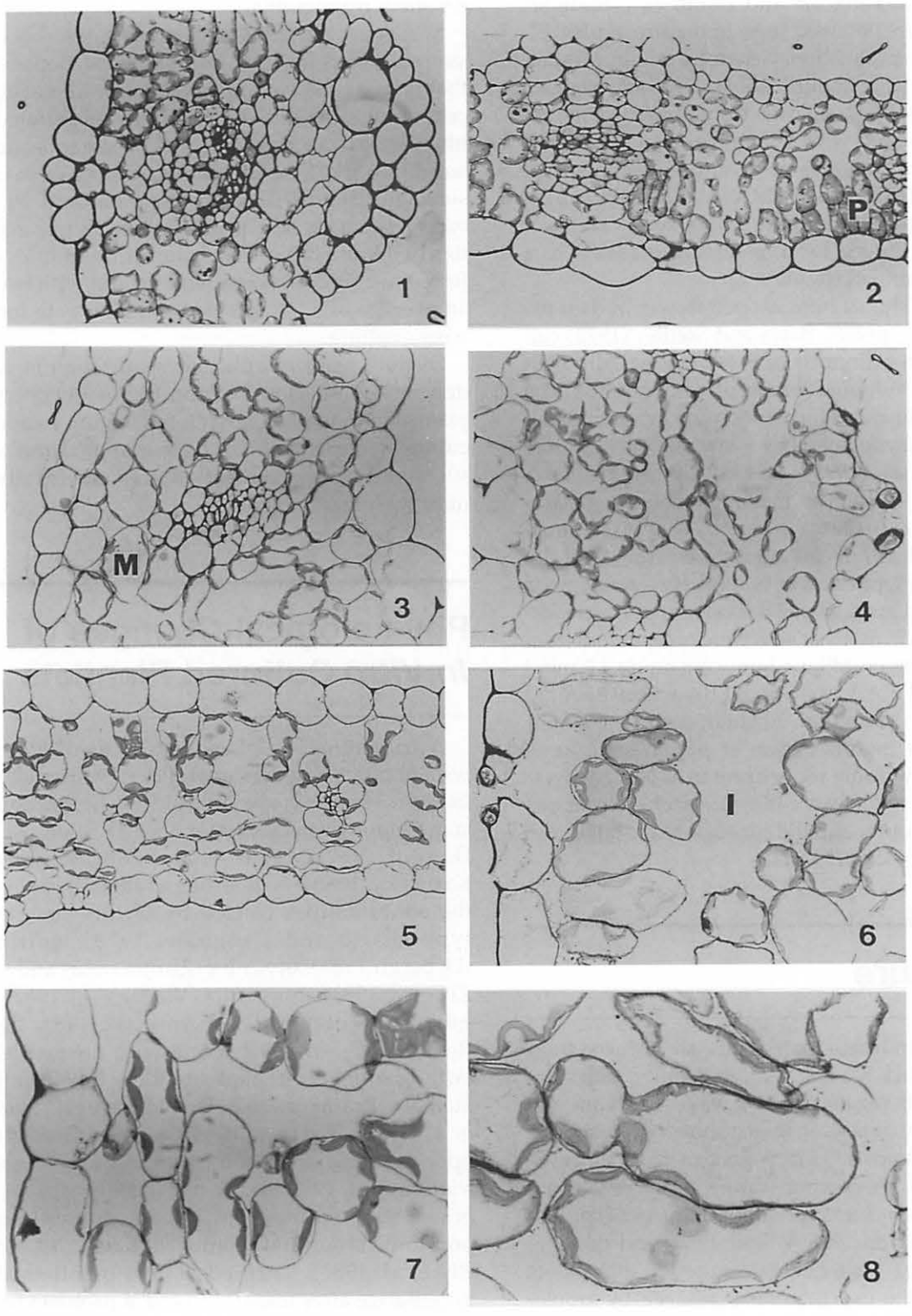


Figure 4. Morphological characteristics of leaf vitrification in the culture of *Populus alba* x *P. grandidentata*. 4-1) Normal leaf of 'Crandon' clone. 4-2) Adaxial orientation of normal 'Crandon' clone, P=palisade parenchyma. 4-3 and 4-4) Abnormal leaf of 'Crandon' clone; M=spongy mesophyll. 4-5) Normal leaf of 'Hansen' clone. 4-6) Abnormal leaf of 'Hansen' clone; I=enlarged intercellular space. 4-7) Normal leaf of 'Hansen' clone. 4-8) Abnormal leaf of 'Hansen' clone (Kang and Hall 1996c).

components have been reported (McLaughlin and Karnosky 1989; Sato et al. 1993).

In liquid culture, vitrified plantlets produce more ethylene, suggesting that increased 1-aminocyclo-propane-1-carboxylic acid (ACC) and ethylene production is a nonwound response to stress (Kevers et al. 1984). Subsequently, ACC and ethylene cause metabolic changes that activate peroxidases such as indole-3-acetic acid (IAA) and ACC oxidases. Increased peroxidase activities were detected in vitrified tissue of herbaceous and woody plantlets (Kervers et al. 1984).

Kang and Hall (1996c) studied *in vitro* morphological abnormalities in hybrid aspen (*P. alba* x *P. grandidentata* cv. 'Crandon' and cv. 'Hansen'). Morphological differences in stomata and other cell types were investigated through scanning electron microscopy. Phenotypic differences are shown in figures 3-1 to 3-3. In vitrified leaves of 'Crandon' and 'Hansen,' stomatal shape was circular and inflated with larger stomatal apertures (figures 3-4 and 3-5); however, stomatal densities were similar between normal and vitrified leaves. Vitrified leaves were thicker than those of normal leaves (figure 4). Mesophyll cells were arranged irregularly and palisade parenchyma was less developed in vitrified leaves (figures 4-1 to 4-4). Chloroplasts of vitrified leaves were arranged sparsely within the larger mesophyll cells, whereas they were highly condensed in normal leaves (figures 4-5 to 4-8).

Although many reports relate to vitrification studies in herbaceous horticultural species, only a few papers have been published for woody species. The morphological study of Kang and Hall (1996c) is the only report on the physiological variations related to vitrification in *Populus* species. Studies on such physiological phenomena provide a basis for developing successful *in vitro* culture systems.

Summary

Organogenesis has become a basic technique for advancing studies such as genetic manipulation and can provide a primary plant regeneration system to obtain "true-to-type" plantlets. Fundamental studies on *in vitro* physiology are prerequisites for developing and optimizing a successful tissue culture system.

Biotechnology can support future industrial applications to produce synchronized plantlets for tree-breeding programs and secondary metabolites for pharmaceutical programs. Further applications of biotechnology require more studies on: 1) *in vitro* physiological phenomena; 2) development of generalized regeneration systems for poplar species and hybrids; 3) variation among plantlets from different explants; and 4) transplanting for greenhouse growth.

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Chapter 4

Protoplast Isolation and Culture[1]

Julie Russell Kikkert

Introduction

Protoplasts are cells without cell walls. In plants protoplasts are usually liberated by tissue digestion with cellulases and pectinases purified from wood-rotting fungi. To keep the plasma membrane from bursting during protoplast isolation, an isotonic or slightly hypertonic medium is used. Because of the lack of external constraints on the membrane, protoplasts are spherical (figure 1B). One use of plant protoplasts is to isolate single cells for physiological studies. In *Populus* (poplar), protoplasts have been used to study reactions of the photosynthetic pathway (Dalakishvili et al. 1989; Mgaloblishvili et al. 1988; Sanadze et al. 1986, 1990).

After the demonstration of totipotency of tobacco protoplasts by Takebe et al. (1971), many laboratories began developing protocols to regenerate plants from herbaceous and woody plant protoplasts to use in genetic engineering. Protoplasts were considered essential for gene transfer because the cell wall is usually impermeable to DNA. Several protoplast-based genetic engineering techniques emerged, including protoplast fusion, DNA microinjection, electroporation, and polyethylene glycol (PEG)-mediated gene transfer. Today, other methods, such as *Agrobacterium*-mediated gene transfer and biolistics, are available to transform cells within intact tissues. Still, protoplasts are 1 method that has proven valuable for genetic engineering research.

To obtain transgenic plants, the genetically altered protoplasts must regrow their cell walls, divide, and regenerate into whole plants. Figure 1 shows the steps involved and table 1 lists reports of poplar protoplast isolation and culture. Woody plants were once considered difficult to regenerate from protoplasts, but breakthroughs occurred in the mid-1980s with *Populus* spp. protoplasts as one of the first succesful genera (McCown 1988; McCown and Russell 1987; Russell and McCown 1986a,1986b). Critical to this success was the use of juvenile or juvenile-like source tissues, such as *in vitro* grown plants (shoot cultures) or embryogenic cell cultures (McCown 1988), and optimum protoplast isolation and culture conditions (Russell 1993). Currently, whole plants have been regenerated from a wide range of poplar species and hybrids (table 1, section D).

This chapter compares reports of plant regeneration from poplar protoplasts and highlights the important common success factors. For a more thorough description of general protoplast culture, refer to reviews by Kirby et al. (1989); Rashid (1988); and Russell (1993).

Key Factors

Numerous factors affect the isolation and culture of protoplasts. A variety of poplar genotypes have been successfully cultured by several laboratories. When the procedures are compared, the following common features emerge.

Protoplast Isolation

The source tissue is a common and important factor in successful poplar protoplast culture. In 10 of the 12 reports listed in table 1, section D, the protoplast source was leaves from shoot cultures. Further, most of the shoot cultures were from nonseedling trees, which allowed researchers to work with already proven clone varieties. In woody species, shoot cultures yielded higher numbers of protoplasts with better viability than did leaves from greenhouse- or field-grown plants (Smith and McCown 1982). In addition to being juvenile, shoot cultures are aseptic, adapted to tissue culture conditions, and maintain genetic and physiological tissue uniformity (McCown 1988). To

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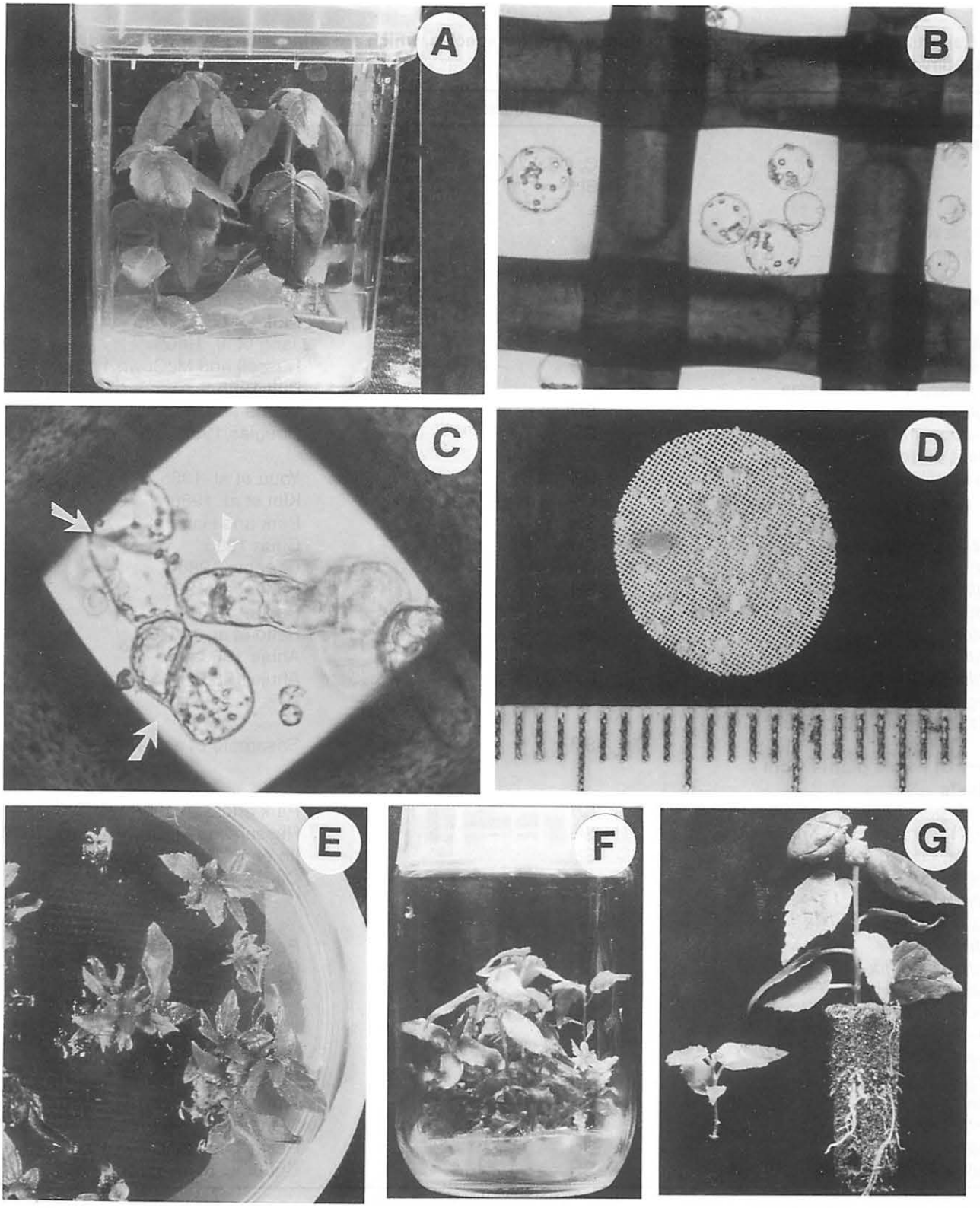


Figure 1. Stages in the culture of protoplasts through plant regeneration for *Populus alba* x *P. grandidentata*. A) Leaves from shoot cultures are the source tissue. B) Newly isolated protoplasts plated in liquid medium with a polyester screen disc for support (openings in the screen are 150 μm). C) Cell divisions approximately 2 weeks after protoplast isolation. D) Protoplast-derived calli developing on the screen disc (ruler markings represent 1 mm). E) Shoot regeneration. F) Shoot multiplication. G) Harvest and rooting of micro cuttings that are ready for transfer to the greenhouse. For detailed procedures, see Russell and McCown (1988).

Table 1. Reports of protoplast isolation and culture in *Populus* species, which are categorized by the most advanced protoplast development for a given report.

Species	Source[1] tissue	Reference
Protoplast isolation		
P. alba	C, CS	Park and Son 1987
	SH	Park et al. 1987
P. nigra var. *charkowiensis* x *P. nigra* var. *caudina*	L	Ito et al. 1986
P. deltoides	L	Sanadze et al. 1986
		Xin et al. 1991
P. x *euramericana*	L-SDL	Saito, 1976, 1980a
	SH	Park and Son 1986
P. glandulosa		Jang et al. 1987
P. nigra x *P. laurifolia*	SH	Russell and McCown 1986a
P. tacamahaca x *P. trichocarpa*	L	Butt 1985
P. tremuloides	SDL	Verma and Wann 1983
P. trichocarpa x *P. tacamahaca*	CS	Douglas 1982
Protoplast division		
P. alba x *P. glandulosa*	CS	Youn et al. 1985
	L	Kim et al. 1988
	SH	Park and Han 1986
P. alba x *P. grandidentata*	SH	Chun 1985
P. ciliata	C	Cheema 1988
P. davidiana	SH	Park et al. 1988
P. glandulosa	SH	Park et al. 1988
P. sieboldii	SH	Saito et al. 1987
P. tremula	L	Ahuja 1983a,b
P. tremuloides	L	Ahuja 1983b
Organogenesis from protoplast-derived calli		
P. alba	SH	Sasamoto et al. 1989
Recovery of whole plants from protoplasts		
P. alba x *P. glandulosa*	SH	Park and Son 1988
P. alba x *P. grandidentata*	SH	Russell and McCown 1986b, 1988
P. nigra var. *charkowiensis* x *P. nigra* var. *caudina* somatic hybrid with *Hibiscus sabdariffa*	SH	Ito et al. 1986; Oji-Paper 1989
P. glandulosa	SH	Park et al. 1990
P. koreana x *P. nigra* somatic hybrid with *P.* x *euramericana*	SH	Park et al. 1992
P. nigra	C	Lee et al. 1987
P. nigra x *P. maximowiczii*	SH	Park and Son 1992
P. nigra x *P. trichocarpa*	SH	Russell and McCown 1988
P. simonii	CS	Wang et al. 1995
P. tomentosa	SH	Wang et al. 1991
P. tremula	SH	Russell and McCown 1988
P. tremula x *P. alba*	SH	Chupeau et al. 1993

[1] C=callus; CS=cell suspensions; L=leaf; SDL=seedlings; SH=shoot cultures

ensure high yields of viable protoplasts, growth conditions of shoot cultures must be optimum. Lighting conditions (Sasamoto et al. 1989) and the growth medium (Russell and McCown 1988) are especially important.

A second component of protoplast isolation is tissue digestion. Table 2 lists the enzymes and treatment times that have been used for poplars. Cellulase R10 and Macerozyme R10 were the most commonly used, often in

Table 2. Enzyme treatments used to liberate protoplasts from *Populus* shoot culture-derived leaves. Only reports in which whole plants were regenerated from protoplasts are listed.

	Enzyme concentration (percent w/v)								
Species	Cellulase (Cooper)	Cellulase R10	Cellulase RS	Macerase	Macerozyme R10	Hemi-cellulase	Driselase	Pecto-lyase	Time
P. alba (Sasamoto et al. 1989)	---	---	1.0	---	---	---	---	0.25	1.5 h
P. alba x *P. glandulosa* (Park and Son 1988) *P. nigra* x *P. maximowiczii* (Park and Son 1992)	---	2.0	---	---	0.8	1.2	2.0	0.05	1.8 h
P. alba x *P. grandidentata* *P. nigra* x *P. trichocarpa* *P. tremula* (Russell and McCown 1988)	0.5	---	---	0.1	---	---	---	---	4 h
P. x *euramericana* (Park et al. 1992)	---	1.0	---	---	0.4	1.2	2.0	0.05	10 h
P. glandulosa (Park et al. 1990)	---	1.5	---	---	0.5	0.5	0.5	0.05	12 h
P. koreana x *P. nigra* (Park et al. 1992)	---	2.0	---	---	0.4	1.2	2.0	0.05	10 h
P. tomentosa (Wang et al. 1991)	---	2.0	---	---	0.5	---	---	---	14 h
P. tremula x *P. alba* (Chupeau et al. 1993)	---	0.1	---	---	0.02	---	0.05	---	16 h

combination with Hemicellulase, Driselase, or Pectolyase. However, the exact concentrations and time of treatment vary with the genotype and the researcher preferences. The digestion environment may contain many toxic compounds, such as enzyme impurities and components released after tissue wounding. Thus, digestive time is a critical factor for good protoplast viability (table 2). Because enzymes are relatively large molecules, they penetrate tissues slowly. To increase penetration, leaf tissue is usually sliced or chopped before enzyme treatment. However, cell exposure to the enzymes is still not uniform, which results in over digested cells near the edges of the cuts and under digested cells in the interior of the leaves. Russell and McCown (1986a) demonstrated that when leaves of poplar shoot cultures were processed in an Omni-mixer, the epidermis was stripped away and the cells were more uniformly exposed to the enzymes. The results were higher protoplast yields and better viability. Leaves processed with an Omni-mixer required only a 4-h digestion, whereas other labs used a 10- to 16-h digestion (table 2). Park and Son (1988, 1992) digested poplar leaf tissue for only 1.8 h, even though the leaves were sliced with a scalpel. The shorter digestion may have been possible because the leaves were placed in fresh enzyme solution 4 times. Thus, toxic components were removed along with the old enzyme solution.

Osmoticum, salts, growth regulators, and buffers in the isolation medium are also important for long-term viability of poplar protoplasts (Park and Son 1992; Chupeau et al. 1993). However, no general recommendations are made here.

Protoplast Culture and Regeneration

Key factors for poplar protoplast culture through sustained division and plant regeneration are listed on tables 3 and 4. One of the most common factors is plating in liquid medium (10 of the 12 reports) (table 3). Agar or agarose plating generally produces few or no protoplast-derived calli (Chupeau et al. 1993; Park and Son 1988, 1992; Russell and McCown 1986b). One reason for using liquid plating is to avoid buildup of toxic exudates around the

Table 3. Conditions for the culture of *Populus* protoplasts that led to sustained cell division and eventual plant recovery.

Species	Culture method	Plating density[1]	Basal medium[2]	NH_4^+ (ammonium nitrate)	Osmoticum	Growth regulators
P. alba (Sasamoto et al. 1989)	Liquid	$2.5x10^4$	MS	No	0.08 M sucrose 0.6 M mannitol	1.0 µM 2,4-D 0.1 µM BA
P. alba x *P. glandulosa* (Park and Son 1988)	Agar/ Gauze	$2.4x10^5$	MS	No	0.2 M mannitol 0.4 M glucose	9.0 µM 2,4-D 22.2 µM BA
P. alba x *P. grandidentata* (Russell and McCown 1988)	Liquid Floating Disc	$5.0x10^4$	WPM	No	0.366 M sucrose 0.046 M mannitol 0.046 M sorbitol 0.046 M xylitol 0.046 M inositol	1.0 µM NAA 0.1 µM BA
P. glandulosa (Park et al. 1990)	Liquid	$1.0x10^5$	KM8P	Yes	0.6 M sucrose	5.0 µM 2,4-D 0.5 µM BA
P. koreana x *P. nigra* somatic hybrid with *P.* x *euramericana* (Park et al. 1992)	Liquid	un-available	KM8P	Yes	0.6 M sucrose	0.4 µM 2,4-D 0.5 µM BA
P. nigra (Lee et al. 1987)	Liquid	$3.7x10^4$	WPM	No	0.366 M sucrose 0.046 M mannitol 0.046 M sorbitol 0.046 M xylitol 0.046 M inositol	1.0 µM NAA 0.1 µM BA
P. nigra x *P. maximowiczii* (Park and Son 1992)	Agar/ Gauze	$2.4x10^5$	MS	No	0.2 M mannitol 0.4 M glucose	9.0 µM 2,4-D 0.4 µM BA
P. nigra x *P. trichocarpa* (Russell and McCown 1988)	Liquid Floating Disc	$0.5x10^4$	WPM	No	0.366 M sucrose 0.046 M mannitol 0.046 M sorbitol 0.046 M xylitol 0.046 M inositol	1.0 µM NAA 0.1 µM BA
P. simonii (Wang et al. 1995)	Liquid	$3.0x10^6$	KM8P	Yes	0.4 M glucose	13.6 µM 2,4-D 1.1 µM NAA 0.9 µM KT
P. tomentosa (Wang et al. 1991)	Liquid	$2.5x10^5$	KM8P	Yes	glucose	2.2 µM 2,4-D 2.5 µM NAA 2.2µM BA
P. tremula (Russell and McCown 1988)	Liquid Floating Disc	$1.0x10^5$	WPM	No	0.366 M sucrose 0.046 M mannitol 0.046 M sorbitol 0.046 M xylitol 0.046 M inositol	1.0 µM NAA 0.1 µM BA
P. tremula x *P. alba* (Chupeau et al. 1993)	Liquid	$5\text{-}8x10^4$	Chupeau	Yes	0.55 M glucose	14.0 µM 2,4-D 0.05 µM TDZ

[1] Protoplasts per ml.

[2] Three basal media formulations have been used for protoplast culture of poplars in an equal number of cases. WPM is a lower salt and lower chloride medium than MS. KM8P is a complex medium containing numerous vitamins, sugars and sugar alcohols, organic acids, casamino acids, and coconut water. WPM=Lloyd and McCown (1980); MS=Murashige and Skoog (1962); KM8P=Kao and Michayluk (1975).

cells (Chupeau et al. 1993; Russell and McCown 1986b). In liquid culture, the medium can be refreshed periodically, thus reducing the concentration of toxic exudates. Russell and McCown (1986b, 1988) used a floating disc plating method, in which the protoplasts were cultured in contact with a polyester screen at the surface of the medium (figure 1). The protoplasts adhered to the screen, which aided in medium replenishment and observation.

Table 4. Plant growth regulators used for shoot regeneration from protoplast-derived calli of *Populus*.

Species	Concentration in Regeneration Medium (μM)					
	BA[1]	KT[2]	TDZ[3]	ZT[4]	2,4-D[5]	NAA[6]
P. alba (Sasamoto et al. 1989)	0.1	---	---	---	1.0	---
P. alba x *P. glandulosa* (Park and Son 1988)	---	---	---	4.6	---	---
P. alba x *P. grandidentata* (Russell and McCown 1988)	---	---	0.1	---	---	---
P. glandulosa (Park et al. 1990)	---	---	---	7.5	---	---
P. koreana x *P. nigra* somatic hybrid with *P.* x *euramericana* (Park et al. 1992)	---	---	---	5.0	---	---
P. nigra (Lee et al. 1987)	0.1	---	---	---	---	1.0
P. nigra x *P. maximowiczii* (Park and Son 1992)	---	---	---	6.8	---	---
P. nigra x *P. trichocarpa* (Russell and McCown 1988)	---	---	0.1	---	---	---
P. simonii (Wang et al. 1995)	4.44	2.32	---	2.28	---	0.54
P. tomentosa (Wang et al. 1991)	2.2	---	---	---	---	1.0
P. tremula (Russell and McCown 1988)	0.4	---	0.01	---	---	---
P. tremula x *P. alba* (Chupeau et al. 1993)	---	---	0.1	---	---	---

[1] benzyladenine
[2] kinetin
[3] thidiazuron
[4] zeatin
[5] 2,4-dichlorophenoxyacetic acid
[6] α-naphthaleneacetic acid

Protoplasts will float only when sucrose is used as the osmoticum; however, sucrose is toxic (Chupeau et al. 1993) or not optimal for some genotypes (table 3). Sucrose, glucose, and mannitol were the most commonly used osmotica.

Another apparent requirement for poplar protoplasts, though not listed on table 3, is culturing under dark conditions until colonies have formed. Poplar protoplasts produced anthocyanins and did not divide even when cultured in dim light (Chupeau et al. 1993; Russell and McCown 1986b). Plating density also affects protoplast development. With poplars, the optimal densities range from 0.5 x 10^4 to 3.0 x 10^6 protoplasts per ml of culture

medium (table 3). Optimal plating densities vary with the genotype, culture medium (Russell and McCown 1988), and culture method.

Protoplasts can be sensitive to the types and concentrations of nitrogen in the culture medium. The usual concentrations of ammonium nitrate in the basal formulations (4.94 mM in WPM; 20.6 mM in MS; 7.4 mM in KM8P) are sometimes toxic and thus, are eliminated or reduced in the protoplast culture medium (Russell and McCown 1988; Russell 1993). In poplars, 7 of the 10 reports of successful culture were without ammonium ions (NH_4^+) in the medium (table 3). Similarly, the response to organic nitrogen sources is genotype dependent. When Russell and McCown (1988) added casein hydrolysate and coconut water to their WPM-based protoplast medium, the supplements were required for 1 genotype, toxic to another, and neither required nor toxic to yet another. Organics in the medium lowered the optimal plating density. Wang et al. (1995) found that either ammonium nitrate or glutamine plus aspartic acid supported low rates of colony formation, but that a combination doubled the rates of colony formation. With *P. tremula* x *P. alba*, organic nitrogen produced no apparent benefits. Rather, optimal protoplast culture occurred when a low initial concentration of mineral nitrogen was successively increased over time (Chupeau et al. 1993).

Plant growth regulators are another important component of the protoplast culture medium. However, the optimal type and concentration varies (table 3). The most commonly used auxin and cytokinin are 2,4-D (2,4-dichlorophenoxyacetic acid) and BA (benzyladenine), respectively. Chupeau et al. (1993) were the only group to test thidiazuron (TDZ) for poplar protoplast culture. TDZ is a substituted phenylurea with cytokinin-like activity, and in combination with 2,4-D, was required for sustained development of protoplast-derived cells of *P. tremula* x *P. alba*. TDZ also reduced the release of exudates from the cells. In another study, Sasamoto et al. (1989) found that the optimal growth regulator combination of 1 µM 2,4-D with 0.1 µM BA could overcome inhibitory conditions of osmoticum or ammonium ions to promote cell division and colony formation.

Plant growth regulators are also important in the medium for organogenesis from the protoplast-derived calli. The requirements appear to be highly genotype dependent (table 4). Zeatin and BA were commonly used, with TDZ sometimes required. Standard micropropagation techniques were used to propagate and move regenerated plants to the greenhouse and field.

Protoplast-Based Genetic Engineering in Poplars

There are few reports of protoplast-based genetic engineering in poplars. This is because of the availability of other gene transfer techniques and because regeneration systems have only recently been accomplished for many genotypes. Somatic hybridization, which requires protoplasts, has been reported in 3 cases. Saito (1980b) fused protoplasts of *P.* x *euramericana* with those of either the same species or of *Paulownia taiwaniana*. Heterokaryons were identified by differential staining, but were not cultured. In a second report, leaf protoplasts of *P. nigra* var. *charkowiensis* x *P. nigra* var. *caudina* were fused with callus-derived protoplasts of *Hibiscus sabdariffa* (Ito et al. 1986; Oji-Paper 1989). Plants were regenerated that were poplar-like. Thirdly, protoplasts of *P. koreana* x *P. nigra* were fused with protoplasts of *P.* x *euramericana* (Park et al. 1992; Park and Son 1994). Two of the regenerated plants showed intermediate protein band patterns in SDS-PAGE analysis.

Chupeau et al. (1994) recovered plants after electroporation of protoplasts with genes encoding resistance to 3 selective agents; paromomycin, chlorsulfuron, and phosphinothricin. Standard electroporation techniques developed for herbaceous crops were used. Transgenic plants that expressed the genes were obtained with relatively high frequency. The patterns of gene integration were single and clear.

Conclusions

Several poplar species and hybrids have been cultured from protoplasts to whole plants. Common requirements for protoplast culture are the use of shoot cultures as a source tissue, liquid plating medium, and culture in the dark. Other important factors for each genotype include the enzyme concentration and incubation time, the isolation medium, and the protoplast culture medium, including osmoticum, nitrogen, and plant growth regulators. Growth regulators for plant regeneration from protoplast-derived calli are also genotype dependent. Though protoplast isolation and culture must be optimized for each new genotype, the parameter ranges for poplars are better defined than in the past. The ability to successfully isolate and culture poplar protoplasts allows for their use in physiological and genetic engineering studies.

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Chapter 5

Somaclonal Variation in *Populus*: An Evaluation[1]

D. John Fry, Gerry C. Douglas, and N. Th. Saieed

Introduction

Substantial genetic and phenotypic variation occurs within plant cell, protoplast, anther, or tissue cultures. This variation also occurs among plantlets regenerated from these cultures, and it is assumed that this variation is genetically based (Michler 1993). Larkin and Scowcroft (1981) proposed that the variation occurring within cultures, or recoverable from cultures following plantlet regeneration, be termed somaclonal variation. They believed that the term described the variation among calliclones and protoclones from stem callus material and protoplast culture, respectively (Ahuja 1987a). However, some still consider protoclonal variation a separate category (Serres et al. 1991) and use the word gametoclonal to denote variation recoverable from haploid cultures. Ahuja (1988) proposed that somatoclonal variation encompass variability among genetically transformed plant materials.

Demonstrating this capacity for variation is dependent upon developing culture protocols for the species in question, but some genera, species, or varieties are recalcitrant to micropropagation techniques and have yet to show genetic variation within culture. Most micropropagation protocols were developed for herbaceous plants, but successful culture and regeneration was achieved in diverse woody perennial species (Dhawan 1993; Thorpe et al. 1991). Dhawan (1993) lists 7 culture systems for *Populus* and 3 for *Salix;* although, cell, protoplast, and anther culture systems exist for many species of the Salicaceae, including some poplars that are difficult to propagate (Ahuja 1987b; Cheema 1989; Chen 1987; Chun 1993; Douglas 1989; Gebhardt 1989; Lu and Liu 1990; Rutledge and Douglas 1988). The first clonally propagated forest trees were from juvenile aspen cultures (Winton 1970). *Populus* was the first genus used for *in vitro* production of haploid plants (Lu and Liu 1990). Ahuja (1987b) and Chun (1993) recorded plantlet regeneration from tissue cultures of at least 29 *Populus* species, hybrids, or clones.

One purpose of micropropagation was to obtain genetic and morphological stability in culture and fidelity of the regenerants to the parental phenotype. Therefore, initially, acknowledgment of somaclonal variation in the literature probably depended upon the researchers' paradigm; particularly whether they viewed variation as an experimental failure. *Populus* and *Salix* species are generally easy to stabilize in culture (Thorpe et al. 1991) and were used for early experimental tissue culture systems. However, the genetic stability of regenerants was frequently doubted and callus culture was usually replaced by organ culture for propagation work (Ahuja 1987a, 1987b, 1993).

Somaclonal variation is recorded among regenerants of widely ranging species and is particularly well documented for herbaceous plants; reflecting their ability for adventitious shoot regeneration and micropropagation, and their pre-eminence among species of economic interest. Frequently, variation was a concern, but it is not universal among regenerants of successfully cultured species. Diverse semi-woody and woody perennials also exhibited somaclonal variation (table 1). Gymnosperms (especially the genus *Pinus*) are considered more genetically stable in culture than angiosperms (Diner and Karnosky 1987), but somaclonal variants were recovered in *Pinus, Abies,* and *Picea* (table 1). Among perennial angiosperms, somaclonal variants were recovered in woody monocots including *Musa* (Skirvin et al. 1994), oil palm, and bamboo; in dicot fruit crops, it was observed in semi-woody *Rubus* species, *Olea,* and various species of *Citrus, Malus, Prunus, Pyrus,* and *Carica* (Hammerschlag 1992; Manshardt 1992); in timber yielding hardwoods, excluding the Salicaceae, it was observed in *Ulmus* sp., *Eucalyptus* sp., *Albizia lebbeck,* and *Santalum album,* and in ornamentals such as *Paulownia tomentosa* (Marcotrigiano and Jagannathan 1988) (table 1).

[1] Klopfenstein, N.B.; Chun, Y. W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

Table 1. Range of woody perennial species that showed somaclonal, protoclonal or gametoclonal variation.

Genus/species	Reference
Abies	Gajdosova and Vookova 1991
Picea	Isabel et al. 1995
Pinus	Berlyn et al. 1986
Musa	Hammerschlag 1992
Elaeis guineensis (oil palm)	Benbadis 1992
Bambusa	Huang and Hunag 1993
Rubus	Swartz et al. 1983
	Skirvin et al. 1994
Olea	Hammerschlag 1992
Citrus	Gmitter et al. 1992
Malus	Hammerschlag 1992
Prunus	Hammerschlag 1992
Pyrus	Hammerschlag 1992
Carica	Manshardt 1992
Coffea arabica	Söndahl and Lauritius 1992
Santalum album (sandal wood)	Sita 1992
Eucalyptus	Haines 1994b
Albizzia lebbeck	Varghese and Kaur 1991
Ulmus	Lineberger et al. 1990
	Domir and Schreiber 1993
Paulownia tomentosa (foxglove tree)	Marcotrigiano and Jagannathan 1988

Poplar Somaclonal Variation

In the Salicaceae, somaclonal variation is well documented among *Populus* species, but apparently has not been recorded for *Salix*. This is probably due to the relative ease in culturing *Populus*, the general recalcitrance of *Salix* spp. *in vitro*, and the economic encouragement for working with *Populus*. Retrospectively, one of the earliest demonstrations of recoverable somatic variation in poplar was a semi-evergreen mutation on a branch of *P. nigra* cv. 'Italic' in Chile before 1900 (Pryor and Willing 1965). Somaclonal variation was also apparently observed in the 1960s when concern was expressed that regenerants from poplar cultures might be abnormal or genetically unstable (Ahuja 1987b), but no significant survey was initiated. Lester and Berbee (1977) first published the systematic demonstration of within-clone variation (somaclonal variation) in *P. nigra* cv. 'Italica' (*P. nigra* var. *italica*) and *P.* x *euramericana*. Since then, somaclonal variation has been repeatedly demonstrated within the genus (table 2), as have protoclonal (Serres et al. 1991) and gametoclonal variation (Chen et al. 1981; Chen 1987; Liu et al. 1992; Wu et al. 1981). Ignoring duplications at the clonal level, somaclonal variation was demonstrated in cultures of at least 21 poplar species or hybrids (table 2; table 3). Furthermore, DNA analyses by Keim et al. (1989) demonstrated natural intra specific polymorphism in overlapping populations of *P. angustifolia, P. fremontii*, and their F_1 (first generation) hybrid progeny. Like the semi-evergreen *Populus nigra* cv. 'Italica', this might reflect *in vivo* somatic mutation, particularly since the hybrid genome was apparently perpetuated by natural vegetative propagation.

The use of callus culture (especially repeated subculturing) to rejuvenate hardwood tissues might be similar to somaclonal variation since it attempts to reverse the physiological change accompanied by dramatic alterations in cell DNA (Berlyn et al. 1986; Bonga and von Aderkas 1993; Franclet et al. 1987). Rejuvenation by repeated subculturing also was achieved with *Salix babylonica* (Franclet et al. 1987); but the technique is not widely applied to the Salicaceae because juvenile tissues from these species are regenerated by other means.

From the research perspective, somaclonal variation is considered highly undesirable since it may complicate studies on genetic transformation and transmission. Horticultural or silvicultural applications are of further concern because variation raises doubts about the stability of any micropropagated perennial plant over a long cropping cycle or rotation (Berlyn et al. 1986; Hammerschlag 1992; Skirvin et al. 1994). Alternatively, it has long been argued that somaclone production is useful to uncover variants that could combine new desirable traits with the favorable characteristics of the existing variety or vegetative clone (Larkin and Scowcroft 1981; Phillips et al. 1990). The potential application of biotechnology to selective tree improvement and the drawbacks and potential benefits of somaclonal variation was generally reviewed (Ahuja and Libby 1993; Haines 1994a, 1994b; Huang et al. 1993; Zimmerman 1988); in relation to selection and improved disease resistance (Michler 1993; Ostry 1994); and for individual groups or species including conifers (Berlyn et al. 1986; Dunstan 1988) and poplars (Nelson et al. 1984; Ostry and Michler 1993).

Cultures or regenerants within the genus *Populus* exhibit the full range of genetic, physiological, anatomical, and morphological variation suggested by Parfitt and Arulsekar (1987). Lester and Berbee (1977) observed variation in root cell chromosome counts; Noh and Minocha (1990) detected isozyme variation in 4 of 5 enzymes tested; and Son et al. (1993) recorded correlations between growth rates and specific SDS-PAGE protein banding patterns. At the physiological level, differences were recorded and/or selected for disease resistance (Antonetti and Pinon 1993; Ostry and Skilling 1988; Prakash and Thielges 1989; Valentine et al. 1988); herbicide tolerance (Michler and Haissig 1988); and gas exchange characteristics (Saieed et al. 1994a, 1994b). In some cases, variability is only detectable at the physiological level because regenerants are morphologi-

Table 2. Taxonomic distribution of *Populus* species, hybrids, or clones that exhibited somaclonal (calliclone or protoclonal) variation for one or more features.

Species/hybrid	Section[1]	Author(s)
Populus alba	L	Iordan-Costache et al. 1991
P. alba; cl. '5972-S38'	L	Antonnetti and Pinion 1993
P. alba x *P. grandidentata*	L	Son et al. 1993
P. alba x *P. grandidentata*; cv. 'Favorit'	L	Iordan-Costache et al. 1991
P. alba x *P. grandidentata*; cv. 'Crandon' ('NC5339')	LxL	Michler and Haissig 1988
P. alba x *P. grandidentata*; cv. 'Crandon' ('NC5339')	LxL	Serres et al. 1991
P. alba x *P. tremuloides*; cl. '802-36-7'	LxL	Antonnetti and Pinion 1993
P. angustifolia	T	Keim et al. 1989[2]
P. angustifolia x *P. fremontii*	TxA	Keim et al. 1989[2]
P. deltoides cl. 'K417'	A	Prakash and Thielges 1989
P. x *euramericana*	AxA	Lester and Berbee 1977
P. x *euramericana* cv. 'Ogy'	AxA	Antonnetti and Pinion 1993
P. x *euramericana* cv. 'Robusta'	AxA	Antonnetti and Pinion 1993
P. fremontii	A	Keim et al. 1989[2]
P. x *interamericana* cv. 'Barn'	AxT	Antonnetti and Pinion 1993
P. x *interamericana* cv. 'Rap'	AxT	Antonnetti and Pinion 1993
P. maximowiczii x *P. trichocarpa*; cv. 'Androscoggin'	TxT	Ostry and Skilling 1988
P. maximowiczii x *P. trichocarpa*; cl. 'NC11390'	TxT	Michler and Haissig 1988
P. nigra var. *italica*	A	Lester and Berbee 1977
P. nigra cl. 'R1029'	A	Iordan-Costache et al. 1991
P. nigra x *P. laurifolia*; cl. 'NC5272'	AxT	Michler and Haissig 1988
P. nigra var. *betulifolia* x *P. nigra*; cv. 'Volga'	A	Ostry and Skilling 1988
P. nigra var. *betulifolia* x *P. trichocarpa*	AxT	Ostry and Skilling 1988
P. nigra var. *betulifolia* x *P. trichocarpa*; cl. 'NE299'	AxT	Ostry et al. 1994
P. nigra var. *betulifolia* x *P. trichocarpa*; cl. 'NC5331'	AxT	Michler and Haissig 1988
P. nigra var. *charkowiensis* x *P. deltoides*	AxA	Ostry and Skilling 1988
P. nigra var. *charkowiensis* x *P. nigra* var. *caudina*	A	Ostry and Skilling 1988
P. tremula	L	Ahuja 1983
P. tremula x *P. alba*; cl. '717-1-B4'	LxL	Antonnetti and Pinion 1993
P. tremula x *P. alba*; cl. '706-8'	LxL	Antonnetti and Pinion 1993
P. tremuloides	L	Valentine et al. 1988
P. tremuloides	L	Noh and Minocha 1990
P. trichocarpa x *P. deltoides*; cv. 'Hunnegem'	TxA	Jehan et al. 1994
P. trichocarpa x *P. balsamifera* cl. 'TT32'	TxT	Saieed et al. 1994a/b
P. hybrid?-protoclone	?	McCown (In: Michler 1993)
P. hybrid?-shoot culture	?	Michler (In: Michler 1993)

[1]A = Aigeiros
L = Leuce (currently termed Populus)
T = Tacamahaca
[2]natural intra specific polymorphism
? = unavailable

cally similar to the parental lines (Michler and Haissig 1988; Ostry and Skilling 1988; Prakash and Thielges 1989). Differences also were noted in various leaf characteristics including albinism, variegation, color, and chlorophyll content (Ahuja 1983; Douglas 1989; Michler 1993; Noh and Minocha 1990; Saieed et al. 1994a, 1994b; Serres et al. 1991; Son et al. 1993); and marginal serration and pubescence (Michler and Haissig 1988; Serres et al. 1991). Leaf morphotype and thickness also vary (Antonetti and Pinon 1993; Lester and Berbee 1977; Saieed et al. 1994a, 1994b; Serres et al. 1991; Son et al. 1993) with the morphotype and its ratio of leaf length to width, showing apparent correlation with other characteristics (Lester and Berbee 1977; Saieed et al. 1994a, 1994b; Serres et al. 1991). In addition, gross morphological variation is recorded for root form, stem girth, stem height, degree of branching, and overall

Table 3. Taxonomic distribution of *Populus* species, hybrids, or clones that exhibited gametoclonal or somatoclonal variation for one or more features.

Species/hybrid	Section[1]	Author(s)
Gametoclonal variation		
P. x *berolinensis*	TxA	Lu et al. (In: Chen 1987)
P. simonii x *P. nigra*	TxA	Wu et al. 1981
P. xiaohei	T	Liu et al. 1992
Populus sp.	?	Chen et al. 1981
Somatoclonal variation		
P. tremula x *P. alba*	LxL	Michel et al. 1988

[1]A = Aigeiros
L = Leuce (currently termed *Populus*)
T = Tacamahaca
? = unavailable

growth rates (Ahuja 1983; Antonetti and Pinon 1993; Lester and Berbee 1977; Saieed et al. 1994a, 1994b; Serres et al. 1991).

Somaclonal Variation Basis and Frequency

Having demonstrated somaclonal variation, scientific and practical questions remain. Where does the variation come from, how is it evoked, and can it be avoided? Does somaclonal variation have practical use, and if so, how can it be evaluated and selected for? Once selected, will the variation remain stable throughout the life of a tree crop?

Ensuring culture stability is a crucial first step to maintaining genetic and phenotypic fidelity of regenerants. This applies to mass-propagation protocols and to culture systems used for germ plasm preservation (Ahuja 1993; Chun 1993; Wang et al. 1993). In the latter case, it is reassuring that prolonged below- or above-zero tissue cold storage apparently does not induce somaclonal variation (Cyr et al. 1994; Hausman et al. 1994). However, culture systems that produce somaclonal variation or rejuvenation involve presumed genetic changes, and these are occasionally observed at a physical level. With somaclonal variation, separate studies on the same parental material indicate that specific culture conditions can induce genetic and somaclonal variation that was not produced by gamma radiation (Douglas 1986; Saieed 1994a). Suggested causes of variation include minor point mutations in nuclear or organelle DNA, activation of transposable elements, polyploidy, aneuploidy, and epigenetic change (Michler 1993; Ostry and Michler 1993). Point mutations could have caused some albino variants that Son et al. (1993) correlated with the loss of 2 proteins, and other minor rearrangements may have caused the isozyme variation reported by Noh and Minocha (1990). Nevertheless, genetic analysis suggests that herbaceous somaclones principally change at the chromosome level rather than single genes (Michler 1993). Poplar somaclonal variation also has been correlated with changes at the chromosome level, but this is not universal. Native poplars are generally diploid (2n = 38), although some naturally occurring triploids and tetraploids exist (Ahuja 1993; Chen and Ahuja 1993; Särkilahti et al. 1988).

In contrast, poplar callus cultures often demonstrate variable ploidy levels (Son et al. 1993), and several poplar somaclones were fully or partially tetraploid or exhibited a leaf morphology characteristic of polyploid plants (Antonetti and Pinon 1993; Jehan et al. 1994; Lester and Berbee 1977; Saieed et al. 1994b; Serres et al. 1991). However, some desirable variants from the parental type were either diploid or similar to the parental leaf phenotype, suggesting that ploidy levels were unchanged (Antonetti and Pinon 1993; Lester and Berbee 1977; Saieed et al. 1994a, 1994b). Again, this reinforces evidence from herbaceous culture systems where most beneficial mutations are single trait characteristics (Michler 1993).

Somaclonal variation occurs inconsistently; a variability that presumably relates to tissue source and culture conditions. In most systems where somaclonal variation is undesirable and detectable by gross morphological changes, the variant poplars constitute less than 25 percent of the total regenerant population (Ahuja 1983; Antonetti and Pinon 1993; Noh and Minocha 1990; Son et al. 1993). Nevertheless, after observing the distribution of inter- and intra-treatment variation within 166 regenerants from 18 differing culture regimes that were subsequently aggregated into 4 morphotype groups, Saieed et al. (1994a, 1994b) concluded that each regenerant should be regarded as a separate somaclone. Further evidence for a significantly higher and subtler level of variation is provided by Noh and Minocha (1990) who showed a 25 to 34 percent variation for isozyme changes among tested regenerants. A considerably higher variation percentage among successful regenerants is expected when somaclonal selection is employed (Michler 1993) and variants are actively selected in culture (e.g., in systems to produce disease resistance or herbicide tolerance) (Michler and Haissig 1988; Ostry et al. 1990; Prakash and Thielges 1989; Riemenschneider et al. 1988; Valentine et al. 1988). The frequency of variation also is high among haploid plants, because some anther culture regenerants originate somatically and because of late chromosomal adjustments; the percentage of haploid cells decreases with age (Lu and Liu 1990).

Induction of Somaclonal Variation in Culture

Variability in the apparent level of induction suggests certain predisposing conditions. These conditions include differences in the inherent genetic variability within the parental material, variation with the type of culture, age-related changes amplified by the duration of culture or repeated subculturing, and dosage-related effects associated with the use of potentially mutagenic chemicals (Parfitt and Arulsekar 1987; Skirvin et al. 1994).

Genotype significantly influences the ability to micropropagate poplars (Rutledge and Douglas 1988), and evidence from herbaceous systems and *Musa* (Hwang and Ko 1986) suggests that intra generic differences affect culture stability and the level of somaclonal induction. In a broad survey of poplar regenerants, Antonetti and Pinon (1993) examined 1,092 regenerants from 13 clones and identified 44 morphological and disease-resistant somaclones representing 8 parental lines. They suggested that somaclonal induction might occur more frequently in species from the Leuce (currently termed Populus) section than from Aigeiros or Aigeiros x Tacamahaca hybrids. Circumstantial support for this hypothesis is that Leuce calli can be grown on modified MS (Murashige and Skoog 1962) medium without potentially mutagenic growth regulators, and that only 1 of their 5 stable clones was from Leuce (table 4). Furthermore, although the calli were heteroploid or tetraploid and most somaclones were tetraploid, the more pronounced Leuce variants were diploid. This suggests that Leuce somaclones might be induced without undergoing major chromosomal change.

In contrast, Ostry et al. (1994) suggested that the Aigeiros x Tacamahaca (cl. 'NE299') (*P. nigra* var. *betufolia* x *P. trichocarpa*) clone is particularly unstable in culture. In addition, in a limited study of herbicide tolerance in 4 different clones, 2 Aigeiros x Tacamahaca clones produced the lowest numbers of tolerant microplants, although they yielded more microshoots than either the Leuce or Tacamahaca lines (Michler and Haissig 1988). Unfortunately, the separate studies cannot be fully compared since culture and regeneration conditions were not identical for all lines.

Ignoring duplications, a survey of published reports on somaclonal variation at the species or hybrid level (table 2) indicates the following: Leuce (6), Aigeiros (6), Tacamahaca (2), Aigeiros x Tacamahaca hybrids (4). Given that Tacamahaca is the largest section of the genus and that most cultivated poplars are in section Aigeiros, Leuce does seem to dominate. However, Leuce species may be over represented because they are prime candidates for micropropagation due to difficulties in stem cutting propagation (Ahuja 1987b; Ernst 1993; Zsuffa 1992).

As in herbaceous systems, studies of poplar link somaclonal induction to aspects of culture history (Ahuja 1983; Iordan-Costache et al. 1991; Jehan et al. 1994; Ostry et al. 1994). Lester and Berbee (1977) were concerned that their observed variation was associated with incomplete elimination of culture viruses. This was not observed in subsequent poplar studies, but it has been suggested that the explant source may be a contributing factor. Ostry et al. (1994) produced calli from several tissue sources and showed that only regenerants from hardwood cuttings were resistant to somaclone formation. The cultural stability of tissue derived from hardwood cuttings may correlate with the general clonal fidelity of poplars propagated by rooted stem cuttings. While studying somaclonal variation, Saieed et al. (1994a) demonstrated that propagation by stem cuttings ensured phenotypic stability among individual somaclones and the parental ('TT32') clone from which the original callus was derived.

Evidence from herbaceous plants suggests that somaclones are more likely when protocols involve callus formation. Poplar micropropagation systems designed to ensure clonal stability generally involve little or no callus formation because it is assumed that callus or suspension

Table 4. Taxonomic distribution of *Populus* species, hybrids, or clones that seemed stable in culture.

Species/hybrid	Section[1]	Author(s)
Populus ciliata	T	Cheema 1989
P. x *euramericana* cv. 'Guariento'	AxA	Antonnetti and Pinion 1993
P. x *euramericana* cv. 'L Avanzo'	AxA	Antonnetti and Pinion 1993
P. x *euramericana* cv. 'Carpaccio'	AxA	Antonnetti and Pinion 1993
P. x *interamericana* cv. 'Beaupré'	AxT	Antonnetti and Pinion 1993
P. tremula x *P. tremuloides*; cl. '333-4'	LxL	Antonnetti and Pinion 1993

[1] A = Aigeiros
L = Leuce (currently termed Populus)
T = Tacamahaca

cultures have an inherent risk of somaclonal induction (Ahuja 1987a, 1987b; Ahuja et al. 1988; Jehan et al. 1994; Ostry et al. 1994; Park and Son 1988). Saieed et al. (1994a) compared S_2 plants (stem cuttings from primary regenerants) from suspension culture with those from short- and long-term callus culture. They suggested that factors related to callus culture duration or repeated subculturing, rather than callus formation, contribute to instability. Degenerative changes (i.e., the ability of cells to proliferate or regenerate) were shown to increase with poplar callus age. For example, 2,4-D/BA (2,4-dichlorophenoxyacetic acid/benzyladenine) combinations induced regeneration or protoplast division in six-month old *P. alba* suspension cultures, but not in cultures over one-year old (Sasamoto et al. 1995). Other poplar studies were similarly explained or interpreted (Chen 1987; Jehan et al. 1994; Ostry and Skilling 1988), and studies on other diverse woody species offer further support (Ahuja 1987a; Domir and Schreiber 1993; Donovan et al. 1994; Gmitter et al. 1992; Hammerschlag 1992).

Conversely, Cheema (1989) demonstrated that plants can be regenerated by either organogenesis or somatogenesis from long-term subculture systems with no immediately obvious somaclonal variation. Similarly, micropropagated apple shoots were repeatedly subcultured for up to 9 years without inducing discernible changes in the bark or leaf characteristics of regenerants (Webster and Jones 1992). This suggests that other factors, such as specific concentrations of certain growth regulators, may have roles as mutagenic agents (Ahuja 1987a; Bayliss 1973).

Some Leuce cultures were maintained without regulators (Antonetti and Pinon 1993), but controlling culture maintenance and regeneration usually required concentrations of potentially mutagenic regulators such as BA, NAA (α-naphthaleneacetic acid), and 2,4-D (Cheema 1989; Douglas 1982) that were greater than endogenous amounts. Auxins in particular are known to enhance DNA methylation and their elimination from culture systems might reduce the incidence of somaclonal variation (Phillips et al. 1990). In attempts to reverse tissue maturation changes, rejuvenation in shoot apices of diverse tree species was achieved by repeated subculturing in the presence of cytokinins (Thorpe et al. 1991).

Some studies attempted to assess the long-term implications of using particular regulators or regulator combinations. Following a three-year study of regenerants from calli exposed to varying combinations of 2,4-D and BA, Saieed et al. (1994a) observed a simple negative relationship between final growth performance and the applied BA concentration range (0.25, 0.50, or 1.00 mg/l). The response to the 2,4-D concentration range (0.1 to 0.6 mg/l) in callus culture was more complex, but superior lines were associated with either 0.1 or 0.4 mg/l 2,4-D. These results suggest that the chemical environment of final calli stages may have amplified or exerted a secondary screening affect on potential variation. Comparable screening of aneuploid cells by cytokinins was demonstrated in polymorphic, herbaceous callus cultures (Dolezel and Novak 1985; Lavania and Srivastava 1988).

Poplar Somaclonal Stability

Many somaclones are nonadaptive and should probably be screened out during post-regeneration steps. Some recorded variation would be beneficial if it becomes stably fixed in somaclones exhibiting otherwise normal characteristics (Michler 1993; Ostry and Michler 1993). Heritable somaclonal stability must be confirmed by genetic analysis following sexual reproduction. Nevertheless, in an asexually propagated genus such as *Populus*, the demonstration of stability following vegetative propagation may allow practical exploitation of beneficial traits (Ostry and Skilling 1987; Skirvin et al. 1994).

Several authors report a variation in disease resistance or growth potential in somaclones that are superior or inferior to the parental line (Liu et al. 1992; Ostry and Skilling 1988; Prakash and Thielges 1989; Riemenschneider et al. 1988; Saieed et al. 1994a, 1994b; Son et al. 1993). These individual traits are often identified or selected in culture and are clearly sustained through the S_1 (primary regeneration) phase. However, questions of long-term stability arise; whether the desirable S_1 features will be continually expressed throughout the plant's life span under varying environmental conditions, and whether they persist into the S_2 stage following further vegetative or sexual propagation. A degree of instability might be expected in haploid regenerants as the growing points change to approach the more normal diploid condition (Chen and Ahuja 1993). Wu et al. (1981) observed a mixture of stability and late developing changes in leaf characteristics of haploid *P. simonii*. Such random changes might produce further material for screening and selection purposes (Chen 1987; Ernst 1993; Lu and Liu 1990).

In contrast, diploid or tetraploid plants are expected to exhibit greater levels of long-term stability. Some protoclones identified by Serres et al. (1991) expressed morphological variances in the greenhouse but not under field conditions, and vice versa. Variation in resistance to *Melampsora* rust identified by Antonetti and Pinon (1993) was not immediately detectable, but appeared in 2 somaclones after a growing season in the field. Alternatively, the variant leaf morphology of many individual somaclones appeared stable over periods ranging from 4 months to 3 years and persisted through a secondary propagation phase (Lester and Berbee 1977; Michler and Haissig 1988; Saieed et al. 1994a). These observations are reconcilable and the differences are probably due to nor-

mal adjustment to environmental change or internal maturation processes. For example, the leaf length to width ratio appears to show an age-related decline even in a stable poplar clone (Pieters 1974).

Long-Term Field Stability

Silvicultural applications of clonal forestry indicate that field trials are required for several years to fully evaluate the relative performances of nonsomaclonal poplar lines (Zsuffa 1992). Somaclones are a strong argument for long-term trials due to the need to prove field stability. Some studies have been promised (Iordan-Costache et al. 1991), and our own study of stability among poplar somaclones was extended to 8 years from regeneration.

Saieed (1990) investigated 166 ('TT32') poplars from calli after 16-months culture with 6 different 2,4-D concentrations, then regenerated in 1985 with 3 different BA concentrations. Regenerated plantlets from 15 of these 18 treatment combinations (designated as lines A - O) survived to potting. The variation in several morphological, physiological, and leaf gas exchange traits was evaluated among the primary regenerants in 1986, and in their S_2 vegetative propagules in 1987 and 1988. Qualitative differences among lines were detected by the end of 1986, most of which were statistically confirmed within the three-year period. Significant correlations were detected between gas exchange parameters and morphological characters, with some lines (especially C, F, and G) performing better than the parental reference clone over a large range of parameters, while others (especially A, H, and M) performed worse. The relative ranking among the experimental lines remained consistent in successive years despite significant changes in individual parameters induced by annual growth changes (Saieed et al. 1994a).

In 1989, the 105 surviving S_2 plants (3 to 5 per somaclone) were planted outdoors in a semi-randomized block at Kinsealy Research Centre, Dublin, and investigated for morphological growth characteristics between August 1992 and January 1993. Some previously investigated parameters were reassessed, such as height, stem diameter, and characteristics of fully-expanded, primary sun leaves. Leaf thickness was investigated for the first time and displayed an inverse correlation with leaf length or area. The comparative performances of these lines are in table 5, where the accumulated data (except leaf thickness) are ranked in descending numerical order. The ranking among

Table 5. Ranked tabulation in descending numerical order (except leaf thickness) of mean values[1] for morphological measurements on field-grown plants of 15 poplar somaclonal lines A-O[2] and the ('TT32') parental reference line[3].

Stem characters		Leaf characters						
Height	Diameter	FW	DW	$\%H_2O$[4]	Length	Width	Area	Thickness[5]
F	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref
G	G	G	G	O	F	F	F	F
Ref	F	B	F	K	C	G	G	C
C	C	F	C	F	G	C	C	G
B	B	A	H	A	O	E	E	E
E	E	H	A	G	E	H	O	B
O	O	O	E	H	B	A	B	O
N	N	C	B	B	M	B	H	N
A	A	M	O	E	H	O	A	H
H	H	E	M	C	A	K	M	I
K	K	N	I	M	K	M	K	M
M	J	L	L	N	L	N	L	J
L	I	I	N	L	N	L	N	L
I	D	K	K	I	D	I	I	A
J	L	J	D	J	I	J	J	D
D	M	D	J	D	J	D	D	K

[1] Parameters measured August 1992 - January 1993 (3-5 plants per line).

[2] Lines regenerated from fifteen 2,4-D/BA regimes, designated as treatments A - O.

[3] Reference line produced by vegetative propagation from the parental 'TT32' clone.

[4] $\%H_2O$ = % leaf water content

[5] Leaf thickness ranked in ascending numerical order.

lines is fairly consistent across the range of parameters, with the reference and somaclonal lines C, G, and F dominating. No significant differences were apparent in stem height or diameter among lines F, G, and the reference; these lines significantly outperformed line A and all lower ranking lines for these parameters ($P > 0.999$). This overall performance pattern is comparable to that obtained by Saieed (1990) and further demonstrates the long-term stability of the somaclones. Lineberger et al. (1990) used a comparable ranking technique as evidence of the fixation of somaclonal variation following a secondary propagation phase in elm somaclones. The original growth advantage of lines F and G over the reference appears to have eroded and it is unknown whether these somaclones will retain superior performance in respect to gas exchange or total biomass. No gas exchange measurements were conducted in 1992, but estimates of abaxial stomatal frequency were obtained for 5 lines. Frequencies for the reference and somaclonal lines C and G were essentially comparable, and were twice as high as those of lines K and H. This corresponds with a previous demonstration of high photosynthetic performances in lines C and G, and poor gas exchange by lines K and H (Saieed et al. 1994a). These results provide continuing evidence of stability within the somaclonal lines and reinforce the earlier belief that morphological and gas exchange measurements of newly regenerated plants are useful to predict final growth and yield.

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Chapter 6

Germplasm Preservation of *Populus* Through *In Vitro* Culture Systems[1]

Sung Ho Son, Young Goo Park, Young Woo Chun, and Richard B. Hall

Introduction

Demand for germplasm preservation of various plant species has recently increased due to air pollution, climate change, acid rain, natural destruction, illegal collection, and human impact to ecosystem biodiversity. Forested land in the world is rapidly declining at an annual rate of 9.9 million ha (Singh and Janz 1995). As a result, there has been a decrease in the genetic pool of forest tree species, including wild genotypes that could serve as future breeding sources.

Seed storage is common for germplasm preservation of most plant species. For *Populus* species, seed longevity under natural conditions is from 2 weeks to 1 month, depending on the species and/or clone, time of collection, and environmental conditions (Graham et al. 1964; Trappe 1964). The longevity of seed storage can be greater than 2 years by drying the seeds soon after collection, adjusting seed moisture content to 8 percent, using sealed containers in cold storage, or storing vacuum-packed seeds under freezing conditions (Trappe 1964). Even then, seed propagation of selected tree genotypes may not guarantee the preservation of genetic traits such as fast growth and . Because of the vast land area requirement and the difficulty in controlling pests and/or disease, *in situ* and *ex situ* conservation of vegetatively propagated plants is challenging.

In this review we: 1) suggest an alternative approach for germplasm preservation through an *in vitro* culture system; 2) discuss methods of germplasm preservation; 3) discuss the currently available technology; and 4) suggest future strategies.

Poplars as a Model System for Germplasm Preservation *In Vitro*

The genus *Populus* includes about 30 species that are widely distributed throughout the North Temperate Zone (between the Arctic Circle and the Tropic of Cancer). This wide distribution represents considerable species' adaptability and demonstrates its potential over a wide geographic region. Among the woody plants, poplar is one of the most intensively studied species for breeding because of the enormous genetic variation that exists. Thousands of poplar clones are being tested and hundreds of these are commercially propagated throughout the world (Sato 1959). *Populus* spp. are fast growers and have modest nutritional requirements, therefore, they are an economically important source of pulp for paper industries and are considered an energy crop. Also, cryopreservation of cells/tissues requires relatively little space because regeneration of *Populus* plantlets from single-cell/protoplast cultures is possible. Commercial production of selected individuals does not depend on seed reproduction because vegetative macro and micropropagation methods without adulteration or dilution of their genetic potential have been intensively studied (Ahuja 1987; Park and Son 1988). Problems controlling open pollination under natural conditions makes conservation of taxonomically diverse and newly created clones by *in situ* or *ex situ* preservation programs for poplar germplasm difficult.

Development of reliable micropropagation systems is essential for germplasm preservation *in vitro*. Many reports of *in vitro* propagation technology of poplars have further developed this species as a model system for germplasm preservation (Aitken-Christie and Singh 1987; Ahuja 1987; Moon et al. 1987; Park and Son 1988; Son et al. 1991; Stoehr

[1] Klopfenstein, N.B.; Chun, Y. W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

and Zsuffa 1990). In many clones with a high regeneration capacity in a microenvironment, plantlets from shoot-tip cultures were used in large-scale production and their productivity was found superior to conventionally propagated plants (Chen and Huang 1980; Lester and Berbee 1977; Mehra and Cheema 1980). Most tissues and organs can serve as initial explant materials for *in vitro* culture of *Populus* spp. Germplasm preservation through *in vitro* culture is advantageous because: 1) entire sets of genetic materials can be copied through regeneration schemes; 2) rapid proliferation is achievable; 3) minimal space is required; 4) disease-free plants can be produced and maintained; and 5) risks related to environmental changes are avoided.

Low-Temperature Storage

Low temperature (about 4 °C) has been widely applied for short-term, minimal-growth storage of cultured plant cells, tissues, and organs. To prepare for nursery planting during the optimal growing season, low-temperature storage was used successfully for large-scale synchronization of transplanting propagules (Son et al. 1991). If suitable methods are developed, valuable genotypes can be selected at the *in vitro* level.

For low-temperature storage *in vitro*, many techniques can be applied individually or in combination. For example, methods related to maintenance of plant materials under conditions of a mineral oil overlay (Caplin 1959), low-level illumination (Preil and Hoffmann 1985), complete darkness (Marino et al. 1985), reduced temperature (Bhojwani 1981), various osmotica or plant growth regulators (Henshaw et al. 1978), modified culture atmosphere (Moon and Kim 1987), and modified subculturing schedules (Withers 1985) have been described.

Although nonfrozen storage systems for germplasm preservation have been reported, such studies focus on herbaceous and/or crop species and may not apply directly to woody plants. During low-temperature poplar storage, the duration, previous subculturing, and various culture media affect the survival rate (Son et al. 1991). With hybrid aspen (*Populus alba* x *P. grandidentata*), the previous subculturing period and medium composition are critical variables for the success of germplasm preservation under low-temperature storage (Son et al. 1991). In our experiment, after 9-months of storage at low temperature, hybrid aspen shoots exhibited slow growth, yet continued to possess some healthy, green leaves and stems (figure 1b). After 20-months of cold storage, shoot cultures had nearly stopped incremental height growth and had thin stems and small, chlorotic leaves. According to one report, explant size can greatly influence survival during cold storage (Aitken-Christie and Singh 1987). In our experiment, no significant difference in cold-storage survival was observed among the five shoot sizes (1, 3, 5, 7, and 9 cm) tested; although 3 cm shoots did exhibit a slightly higher survival rate. Under low-temperature storage, survival rates after 2-years and 5-years cold storage were 75 percent and 25 percent, respectively. From 2,000 plantlets transplanted in the greenhouse and/or nursery, less than 1 percent displayed phenotypic variation related to growth performance or pigment accumulation.

Cryopreservation

Cryopreserved plant cells, tissues, and organs stopped their metabolic functions when exposed to ultra-low temperature (-196 °C); yet they retained viability. In this condition, plant cells, tissues, and organs are preserved for a long period of time. Cryopreservation systems can reduce the cost of conventional labor-intensive nursery practices and minimize genetic changes, such as point mutation and/or ploidy level change, which may occur during long-term subculture. However, developing a reliable cryopreservation system involves several complicated steps. In many examples of cryopreservation, only a few species were successfully preserved (Bajaj 1977; Kartha et al. 1979; Kartha et al. 1980; Uemura and Sakai 1980). In other examples, further investigations are needed to optimize treatments at general stages of pregrowth, cryoprotection, freezing, thawing, and recovery (Chen and Kartha 1987). Preconditioning, such as cold treatment during culture or applying osmotically active chemicals to the medium before cryopreservation, was beneficial for viability (Fuchigami et al. 1981). Plant samples require sufficient desiccation before cooling in liquid nitrogen. Without such care, most samples produce intracellular ice crystals that eventually cause cell mortality.

When poplar callus was used for ultra low-temperature storage, optimal results were obtained with the cells from the end of lag phase or exponential growth phase, which occurs approximately 1 week after subculture (data is not shown). This may relate to lower water content and smaller vacuole size of optimal source cells. Pretreatment with cryoprotective chemicals, dimethyl sulfoxide (DMSO), glycerol, polyvinylpyrrolidone (PVP), and dextran were useful separately or in combination. Although its mode of action is not clearly understood, DMSO may form multiple hydrogen bonds that prevent water from crystallizing at intracellular levels. Although cryoprotective chemicals were harmful to plant cells in our experiment, DMSO treatments produced 62 percent survival after long-term preservation; until now, calli were stored for 5 years and survival tests were conducted annually.

Protocols

Species used for low-temperature storage and cryopreservation were *Populus alba* x *P. grandidentata* and *P. glandulosa* (Suwon poplar), respectively. Protocols described here are based on preliminary experiments; supporting data are not included.

Low-Temperature Storage

a) Shoot cultures were established by bud cultures from greenhouse-grown stock plants. Shoot multiplication, elongation, and root induction media were Murashige and Skoog (MS) (Murashige and Skoog 1962) medium containing 1.33 μM 6-benzyladenine (BA), no plant growth regulators, and 0.98 μM indole-3-butyric acid (IBA), respectively. Cultures were maintained under a 16-h light regime (photosynthetic photon flux rate of 40 to 60 $\mu M\ m^{-2}\ s^{-1}$ from cool-white, fluorescent tubes) at 26±1 °C.

b) Shoots approximately 3 cm long were used (figure 1a).

c) Shoots were maintained on shoot multiplication medium for 1 month before cold storage.

d) Magenta GA-7 culture vessels (7.6 x 7.6 x 10.2 cm, Magenta Corp., Chicago, IL) containing 50 ml medium and test tubes containing 15 ml of medium were used for cold storage.

e) To prevent drying, culture vessels were double sealed with Nesco film (Bando Chemical Ind., Ltd., Kobe, Japan) and stored within two transparent plastic bags.

f) During cold storage, cultures were maintained at 4 °C without light.

g) After 4-weeks subculture of cold-stored plants on fresh MS medium, each shoot was excised from its root system and transferred to a Polyterra™ plug styrofoam tray (M40045, Techniculture Co., Salinas, CA) and eventually to pots containing artificial soil mix (vermiculite : perlite : peat at 1:1:1) (figure 1f).

Cryopreservation

a) Callus was induced from cambial tissues isolated from *in vitro*-, greenhouse-, and field-grown donor plants.

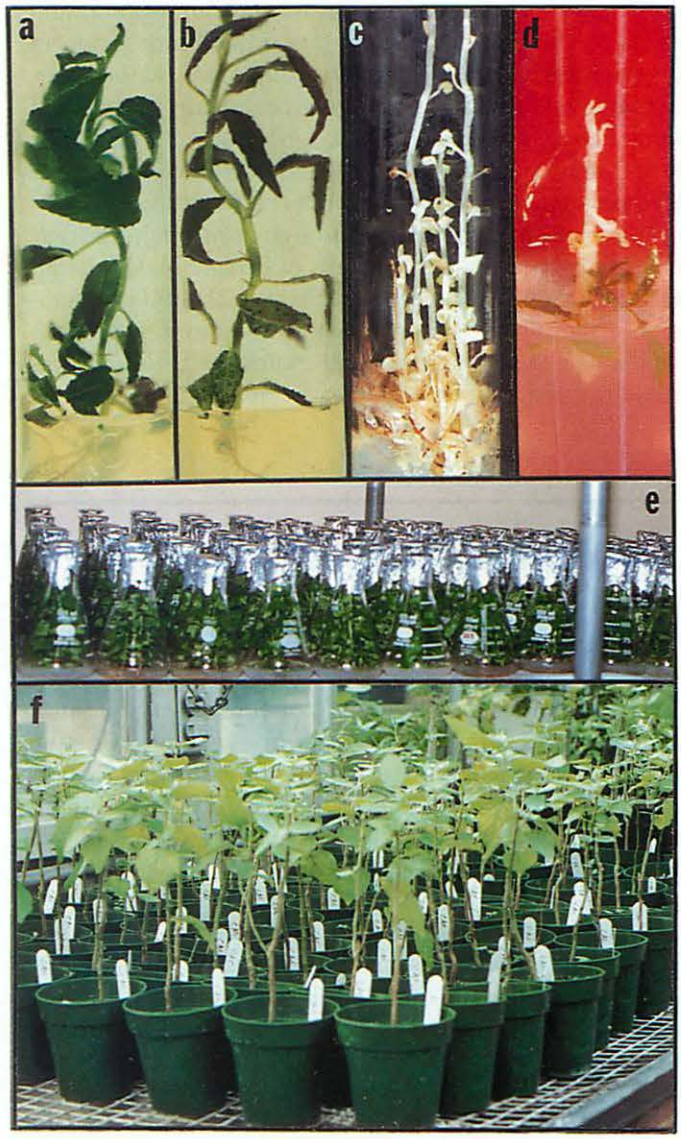


Figure 1. Cold storage of *in vitro* shoot cultures of hybrid poplar (*Populus alba* x *P. grandidentata*). a) Shoot cultures before storage. b) Shoot cultures after 9-months storage at 4 °C. c) Multiplied shoot cultures on shoot proliferation medium after 5-years storage. d) Shoot regeneration from the cold-stored materials. e) Regenerated plantlets derived from cold-stored shoots. f) Plants established in pots containing artificial soil mix.

b) Callus proliferation was conducted on MS medium with 2.26 μM 2,4-dichlorophenoxyacetic acid (2,4-D).

c) Cold hardening was performed at 10 °C for 1 week before placing the callus into cryogenic plastic vessels and adding DMSO (callus : DMSO at 1:5 weight/volume) as a cryoprotectant solution.

d) Further cooling was initiated by incubating the callus on ice for 1 to 2 h.

e) A freezing temperature ranging from -25 to -45 °C was obtained using a programmable freezer controller to drop the cooling temperature by 0.5 to 1 °C per minute, followed by liquid nitrogen immersion.

f) For regrowth of stored callus, temperature was increased in reverse of the freezing procedures but quicker. Before culturing, calli were rinsed several times with MS liquid medium containing no plant growth regulators.

g) After subculturing on MS basal medium supplemented with auxin at the appropriate levels, newly grown callus was collected for use as shoot induction material.

h) For regeneration, selected callus was subdivided into small pieces (5 to 10 mm in diameter) and cultured on a shoot induction medium supplemented with optimal levels of zeatin (figure 2c).

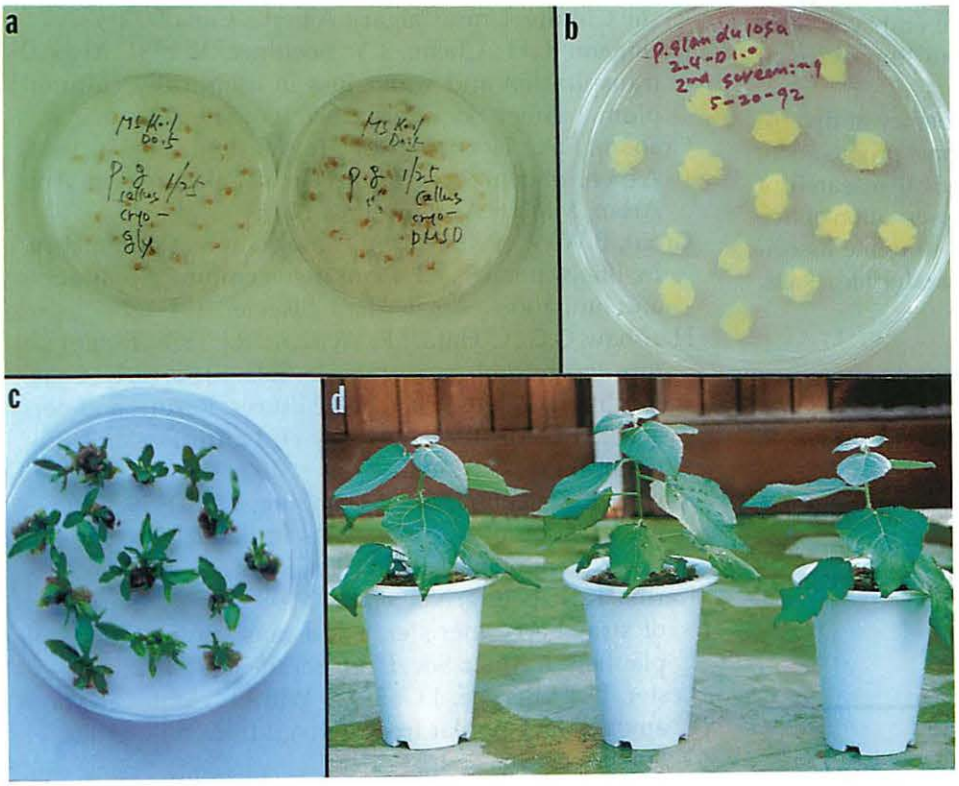


Figure 2. Cryopreservation of *in vitro* shoot cultures of poplar (*Populus glandulosa*). a) Cryopreserved callus recently subcultured to proliferation medium. b) Regrowth of selected callus. c) Shoot regeneration from callus. d) Plants established in pots containing artificial soil mix.

Limitations

Plant cell and tissue culture techniques have developed rapidly and the knowledge associated with culturing systems has increased significantly in comparison to other plant science fields. Based on the theory of totipotency, any cells, tissues, and organs should be regenerable; however, successful establishment of reliable regeneration systems is limited, especially for woody plants. This restriction frequently confines the application of tissue culture methods and restricts plant biotechnological applications for many valuable species.

A basic premise of *in vitro* conservation techniques for plant germplasm is that genetic materials must be preserved without genetic alteration at any level. Most tissue culture work is based on adventitious shoot regeneration systems. These systems are typically associated with a prolonged undifferentiated phase before regeneration. However, such culture systems have generated phenotypic, genotypic, and/or biochemical variation (Bebeli and Kaltsikes 1990; D'Amato 1978; Whelan 1990). Although this variation frequently occurs at negligible levels, the potential for genetic variation should be addressed.

There is also potential for genetic alteration associated with low and ultra-low temperature storage methods. In a nonfrozen storage system, for example, phenotypic variation was reported for traits such as color accumulation in leaves (Son et al. 1991), organ shape, seed yield, and photoperiodic response (Grout 1990). During cryopreservation at -196 °C, most cellular chemical reactions cannot occur because the energy levels are too low to allow sufficient molecular motion to complete enzymatic reactions. Nevertheless, certain chemical reactions that affect genetic alteration by damaging nucleic acids via ionizing radiation can accrue at unacceptable levels. Although this kind of chromosome damage is observed in plants and microorganisms under storage conditions, DNA repair mechanisms to overcome damage from freezing or low temperature conditions are not fully understood.

Most storage systems have not provided 100 percent survival and are not adaptable for diverse species. Intensive studies are needed to develop

methodology and accumulate relevant knowledge for successful germplasm storage of target plant materials.

Conclusions and Prospects

Due to rapid environmental destruction and associated declines in biological diversity, genetic resource conservation has become critical. Recently, major approaches to genetic resource conservation such as *ex situ* and *in situ* preservation programs were established for forestry. Although these approaches have merit, a successful genetic conservation program will require modification and/or a combination of existing methods with new technology to meet current needs.

Advances in plant tissue culture systems are widely applicable throughout plant science. This technology is also a valuable tool for tree germplasm conservation through low- or ultra-low temperature storage. Recent progress in somatic embryogenesis may provide other opportunities for storage material. In particular, somatic embryos exhibit high genetic stability and are easily manipulated. Above all, relatively simple desiccation methods that reflect the physiology and development of zygotic embryos offer additional potential for extending the longevity of germplasm storage *in vitro*.

Because poplar has demonstrated totipotency at diverse cell levels, it is a model species for mass clonal propagation, genetic transformation, and other biological research of woody plants. For the conservation and preservation of tree germplasm, intensive studies are needed to refine associated methods and to provide reliable and reproducible results.

Acknowledgments

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Section II
Transformation and Foreign Gene Expression

Chapter 7

Agrobacterium-mediated Transformation of *Populus* Species[1]

Mee-Sook Kim, Ned B. Klopfenstein, and Young Woo Chun

Introduction

Although molecular biology of woody plants is a relatively young field, it offers considerable potential for breeding and selecting improved trees for multiple purposes. Conventional breeding programs have produced improved growth rates, adaptability, and pest resistance; however, tree improvement processes are time consuming because of the long generation and rotation cycles of trees (Dinus and Tuskan this volume; Leplé et al. 1992). Genetic engineering of trees helps to compensate for conventional breeding disadvantages by incorporating known genes into specific genetic backgrounds. Since the first successful plant transformation was reported in 1983 (Herrera-Estrella et al. 1983; Murai et al. 1983), several nonsexual gene transfer methods were developed for important agronomic crops and forest tree species. These methods include biolistics (microprojectile bombardment), electroporation, and *Agrobacterium*-mediated transformation. Biolistics and electroporation are discussed by Charest et al. (this volume). This chapter focuses on *Agrobacterium*-mediated gene transfer methods, which are widely-used for plant transformation of broad-leaved, woody plants because of their versatility and efficient application (Brasileiro et al. 1991; Chun 1994; Han et al. 1996; Leplé et al. 1992).

Agrobacterium spp. are soil bacteria that naturally infect many dicotyledonous and gymnospermous plants predisposed by wounding (Perani et al. 1986). Infection by *A. tumefaciens* causes crown gall disease (figure 1), whereas *A. rhizogenes* causes hairy root disease. In addition to its chromosomal DNA, *Agrobacterium* contains 2 other genetic components that are required for plant cell transformation; T-DNA (transferred DNA) and the virulence (*vir*) region, which are both located on the Ti (tumor-inducing) or Ri (root-inducing) plasmid (Zambryoski et al. 1989). The T-DNA portion of the *A. tumefaciens* Ti plasmid or the *A. rhizogenes* Ri plasmid is transferred to the nucleus of a host plant where it integrates into the nuclear DNA genetically transforming the recipient plant. A region of the Ti plasmid outside the T-DNA, referred to as the virulence region, carries the *vir* genes. Expression of *vir* genes occurs during plant cell infection and is a prerequisite for the subsequent transfer of the T-DNA. *Agrobacterium* chromosomal regions are involved in attachment of *Agrobacterium* to plant cells. The T-DNA of *A. tumefaciens* contains auxin (*iaaH, iaaM*) and cytokinin (*IPT*) synthesis genes (Zambryoski et al. 1989). These genes are referred to as oncogenes and are responsible for tumor induction. In *A. rhizogenes*, T-DNA contains multiple *rol* genes that induce root formation (Zambryoski et al. 1989). The T-DNA also encodes several genes responsible for the synthesis of compounds called opines, which are metabolic substrates for the bacteria (Nester et al. 1984). Efficient transfer of T-DNA is facilitated by 24-base pair direct repeats at the T-DNA borders. Genes within the T-DNA can be replaced with genes of interest without affecting transfer efficiency (Han et al. 1996; Jouanin et al. 1993).

Members of the genus *Populus* have a small genome size, short rotation cycle, fast growth rate, and the capacity for vegetative propagation. In addition, *Populus* spp. demonstrate developmental plasticity to tissue culture manipulations. These traits and susceptibility to *Agrobacterium*-mediated transformation and techniques to regenerate transgenic trees make *Populus* a suitable model system for genetic engineering of deciduous trees. In this chapter, we describe the main *Agrobacterium*-mediated transformation procedures developed for *Populus* and review the results obtained using several *Populus* species.

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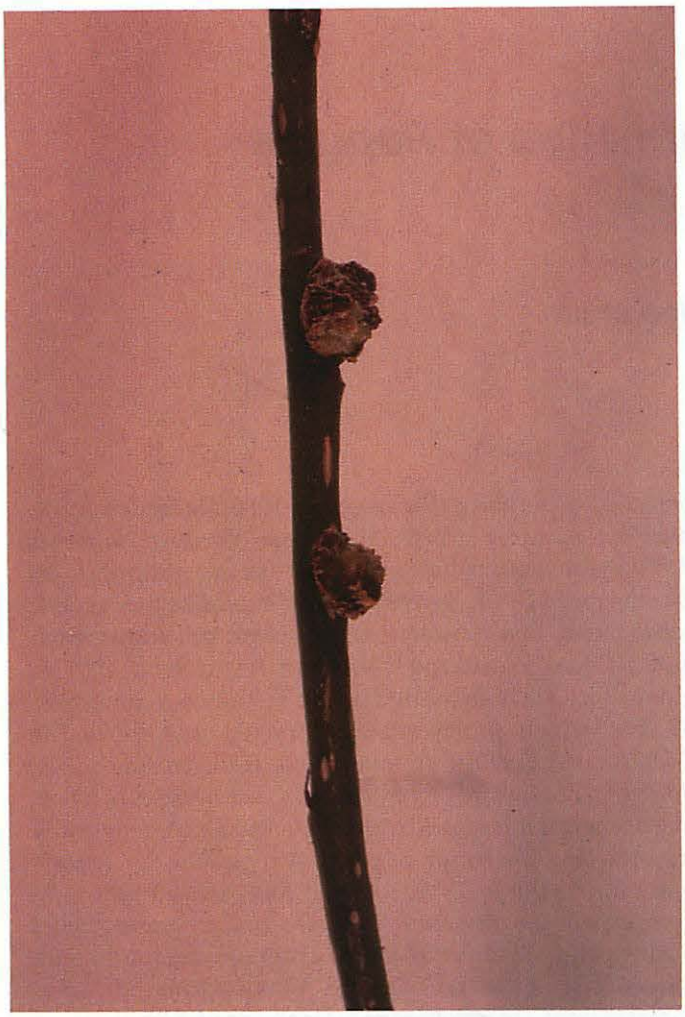


Figure 1. Crown gall produced by *Agrobacterium tumefaciens* strain A281 infection of hybrid poplar (*Populus alba* x *P. grandidentata*) stem after approximately 10 weeks.

Gene Transfer to *Populus* Species

Populus has been known as a natural host for *Agrobacterium* for many years. DeCleene and DeLey (1976) cite early literature that suggests the susceptibility of 3 *Populus* species to infection by *A. tumefaciens*. The presence of T-DNA sequences in gall and root tissue confirmed *Populus* as a host for *A. tumefaciens* and *A. rhizogenes* (Parsons et al. 1986; Pythoud et al. 1987). These early pathogenicity studies of *Agrobacterium* provided the basis for its use as a tool to transfer foreign genes into the poplar genome.

The process for producing transgenic poplar plants includes 5 main components (figure 2): 1) initiation: starting explants (host species/genotype/tissue type) are selected for infection and transformation; 2) infection: wounded starting explants are co-cultivated with an *Agrobacterium* strain containing co-integrate or binary vectors; 3) selection: after removal of residual *Agrobacterium*, transformed cells are selected for subsequent regeneration into transgenic plants (figure 3); 4) regeneration: transformed cells are regenerated during or after the selection period (figures 3 and 4); and 5) confirmation: the presence or function of transgenes in the genome of transgenic plants is confirmed using molecular techniques such as polymerase chain reaction, Southern hybridization, northern hybridization, western blotting, enzyme-linked immunosorbent assay (ELISA), or enzyme activity assays.

Transgenes

Several silviculturally useful genes have been isolated and used for *Agrobacterium*-mediated transformation of *Populus*. A table listing genes used in *Populus* transformation (Chun 1994) was updated for this chapter (table 1). These genes include the: 1) mutant *aroA* gene, which encodes glyphosate tolerance via a 5-enolpyruylshikimate-3-phosphate synthase (EPSP) that is less sensitive to the herbicide glyphosate (Donahue et al. 1994; Fillatti et al. 1987); 2) *bar* gene encoding the enzyme phosphinotricin acetytransferase (PAT) that inactivates the herbicide phosphinotricin (glufosinate) (De Block 1990; Devillard 1992); 3) mutant *crs1-1* gene from a chlorsulfuron-herbicide-resistant line of *Arabidopsis thaliana* (Brasileiro et al. 1992); 4) *OCI* (oryzastatin), a cystein proteinase inhibitor, and *PIN2* (proteinase inhibitor II), a trypsin/chymotrypsin inhibitor gene for pest resistance (Heuchelin et al. 1997 this volume; Klopfenstein et al.1991, 1993, 1997; Leplé et al. 1995); and 5) insecticidal protein genes from *Bacillus thuringiensis* (*Bt*) (Howe et al. 1994). Other studies have focused on transgene regulation (Chun and Klopfenstein 1995; Confalonieri et al. 1994; Kajita et al. 1994; Klopfenstein et al. 1991; Leplé et al. 1995; Nilsson et al. 1992) and developmental influences (Ahuja and Fladung 1996; Charest et al. 1992; Ebinuma et al. 1992; Nilsson et al. 1996a, 1996b; Schwartzenberg et al. 1994; Sundberg et al. this volume; Tuominen et al. 1995; Weigel and Nilsson 1995).

Transgene Copy Number

Few studies have reported the copy number of inserted transgenes by *Agrobacterium*-mediated transformation on *Populus* species. Transgenic microshoots of hybrid aspen (*P. alba* x *P. tremula*) contained from 1 to 3 copies of the inserted foreign *bar* genes (De Block 1990); whereas, *in vitro* plants (*P. tremula* x *P. alba*) regenerated from transformed roots contained 1 copy of the *bar* gene (Devillard 1992). Only a single copy of the chloramphenicol acetyltransferase (*CAT*) gene was inserted into the genome of transgenic hybrid poplar (*P. alba* x *P. grandidentata*) (Klopfenstein et al. 1991). In addition, 1 to 4 copies of *crs1-1* gene had been inserted per hy-

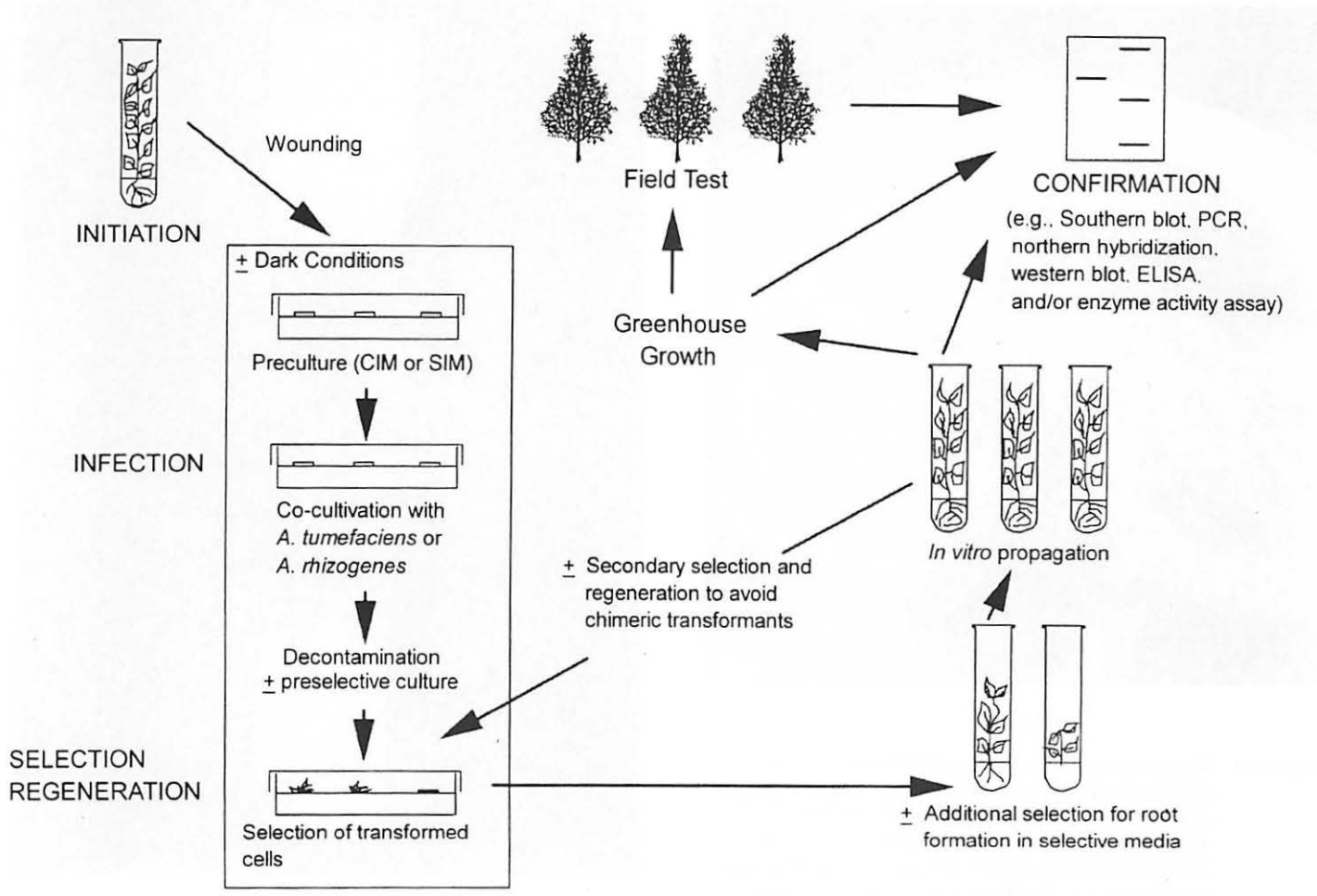


Figure 2. The primary steps for *Agrobacterium*-mediated transformation of *Populus* species. CIM=callus inducing medium; SIM=shoot inducing medium.

brid aspen (*P. tremula* x *P. alba*) genome (Brasileiro et al. 1992). Also, Howe et al. (1991) showed that the number of inserted DNA copies ranged from 1 to 10 after the maize transposable element *Ac* (Activator) was transferred into hybrid poplar (*P. alba* x *P. grandidentata*). However, it is unknown if all inserted gene copies were expressed (Chun 1994; Leplé et al. 1992).

Agrobacterium-mediated Transformation

Host Species/Genotype/Tissue Type

A prerequisite for any genetic transformation work using *Agrobacterium* is the ability of the bacterium to infect the plant of interest. The effect of 2 *Agrobacterium tumefaciens* strains, A281 and A348, on infection of *P. trichocarpa* x *P. deltoides* (Parsons et al. 1986) was studied and additional information was gathered on the effect of poplar genotypes (Charest et al. 1992). Previous studies showed significant differences among the genotypes within species and the clones within genotype (Confalonieri et al. 1994; De Block 1990; Riemenschneider 1990). A differential response of Leuce (currently termed Populus) section cultivars to infection by *A. tumefaciens* was described by Nesme et al. (1987), and susceptibility of aspen cultivars to *A. tumefaciens* was correlated to cytokinin sensitivity by Beneddra et al. (1996). In addition, intra- and inter-specific hybrid poplars coming from Aigeiros or Tacamahaca sections differed in susceptibility to *A. tumefaciens* C58 strain (Riemenschneider 1990).

It is critical to select appropriate starting materials (or explants) for *Agrobacterium*-mediated transformation. Potentially, explant material can be derived from seedling, leaf, internode, petiole, root, callus, or other cells, tissues, and organs. *In vitro* cultured leaves and internodes (stems) have been used most often to trans-

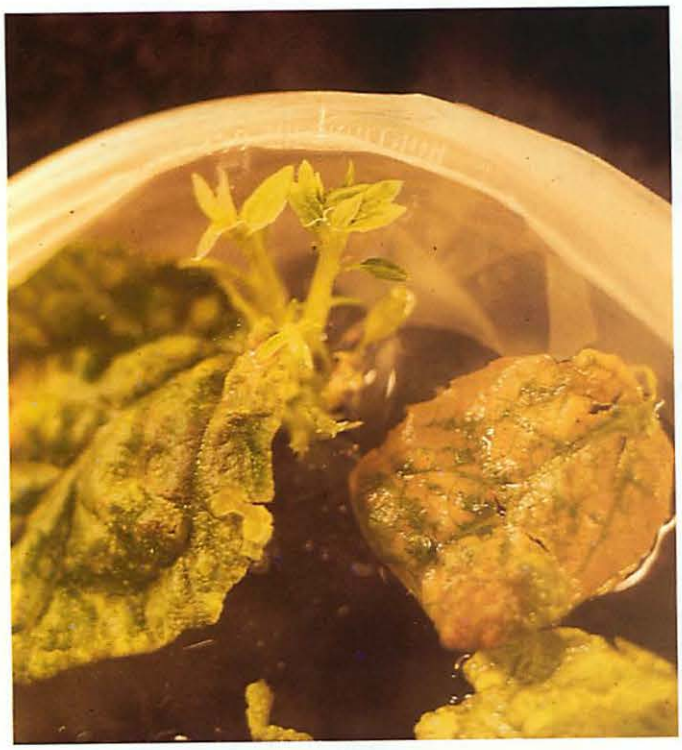


Figure 3. Regeneration of a transformed shoot on selective medium. After co-cultivation of hybrid poplar (*Populus alba* x *P. grandidentata*) leaf pieces with *Agrobacterium tumefaciens* containing *NOS-NPTII* and *PIN2-CAT* genes, transformants were selected on Murashige and Skoog (MS) (1962) regeneration medium supplemented with 40 μg/ml kanamycin.

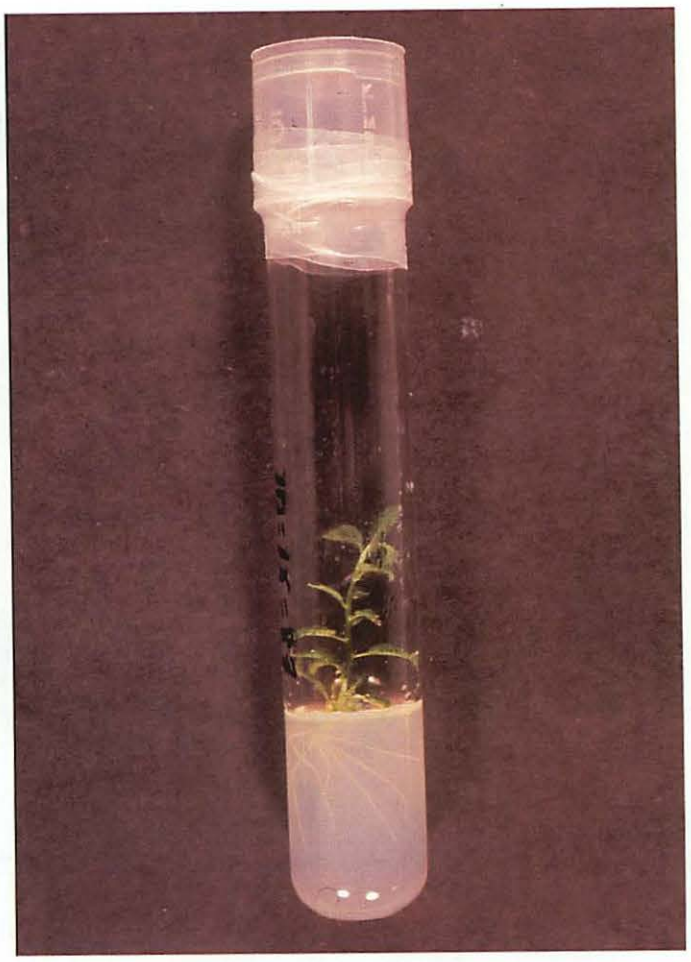


Figure 4. Secondary selection of transformants occurred on Murashige and Skoog (MS) rooting medium containing 20 μg/ml kanamycin. Rooted plantlets of transgenic hybrid poplar (*Populus alba* x *P. grandidentata*) were propagated *in vitro* (Klopfenstein et al. 1991).

form many *Populus* species. Greenwood stem internode sections of *P. tremuloides* are the most susceptible to tumor formation and leaf disks are the least susceptible (Kubisiak et al. 1993). Leplé et al. (1992) showed that internode explants of *P. tremula* x *P. alba* produced more transformed calli than leaf explants.

A suspension culture transformation system for inserting genes into poplar might offer several advantages including: 1) the ability to screen large numbers of potentially transformed cells; 2) effective inhibition of residual *Agrobacterium* following co-cultivation; and 3) high transformation frequencies due to rapidly dividing suspension cultures that may be amenable to stable integration of foreign DNA (Howe et al. 1994). However, it is frequently unknown which cell type within an explant is the most transformable or the most capable of regenerating into a fertile plant. The small amount of available data indicates that the most regenerable cells do not necessarily correspond with the most transformable cells (De Block 1993).

Agrobacterium Strain

To assure high infectivity levels for effective transformation, the most suitable *Agrobacterium* strain should be determined for each host species/genotype/tissue. Generally, tree species respond better to the nopaline strains than octopine strains of *A. tumefaciens* (Ahuja 1987). Most transgenic poplars have been produced using nopaline strains of *Agrobacterium* (Han et al. 1996). The plasmid rather than the chromosomal background was the most critical determinant for infection (Kubisiak et al. 1993). However, influence of plasmid type on infection levels has varied with host species/genotype/tissue type (Kubisiak et al. 1993).

Two designed vector systems are used in *Agrobacterium*-mediated transformation: 1) co-integrate: T-DNA includes

Table 1. Transformation research using *Agrobacterium*-mediated transformation systems with *Populus* species.

Species	Transgenes[1]	Bacterial spp.[3]	Reference
P. trichocarpa x *P. deltoides*	T-DNA[2]	*A.t.*	Parsons et al. 1986
P. trichocarpa x *P. deltoides*	T-DNA	*A.r.*	Pythoud et al. 1987
P. trichocarpa x *P. deltoides*	*bar, NPTII*	*A.t.*	De Block 1990
P. trichocarpa x *P. deltoides*	*GUS, NPTII*	*A.t.*	Wang et al. 1994
P. alba x *P. grandidentata*	*aroA, NPTII*	*A.t.*	Fillatti et al. 1987
P. alba x *P. grandidentata*	*CAT, NPTII*	*A.t.*	Klopfenstein et al. 1991
P. alba x *P. grandidentata*	*aroA, NPTII*	*A.t.*	Donahue et al. 1994
P. alba x *P. grandidentata*	*Ac, Bt, HPT, NPTII*	*A.t.*	Howe et al. 1994
P. alba x *P. grandidentata*	*PIN2, NPTII*	*A.t.*	Klopfenstein et al. 1997
P. alba x *P. glandulosa*	T-DNA	*A.r.*	Chung et al. 1989
P. davidiana	T-DNA	*A.r.*	Lee et al. 1989
P. tomentosa	*CAT, NPTII*	*A.t.*	Wang et al. 1990
P. alba x *P. tremula*	*bar, NPTII*	*A.t.*	De Block 1990
P. tremula x *P. alba*	*GUS, NPTII*, T-DNA	*A.t.*	Brasileiro et al. 1991
P. tremula x *P. alba*	*crs1-1, NPTII*	*A.t.*	Brasileiro et al. 1992
P. tremula x *P. alba*	*bar, NPTII*	*A.r.*	Devillard 1992
P. tremula x *P. alba*	*GUS, NPTII*	*A.t.*	Leplé et al. 1992
P. tremula x *P. alba*	*IPT, NPTII*	*A.t.*	Schwartzenberg et al. 1994
P. deltoides x *P. nigra*	T-DNA	*A.t./A.r.*	Charest et al. 1992
P. deltoides x *P. nigra*	*PIN2, NPTII*	*A.t.*	Heuchelin et al. 1997
P. nigra x *P. maximowiczii*	T-DNA	*A.t./A.r.*	Charest et al. 1992
P. sieboldii x *P. grandidentata*	*iaaM, GUS, MPTII*	*A.t.*	Ebinuma et al. 1992
P. sieboldii x *P. grandidentata*	*prxA1, GUS, NPTII*	*A.t.*	Kajita et al. 1994
P. sieboldii x *P. grandidentata*	*GR, NPTII*	*A.t.*	Endo et al., this volume
P. tremula x *P. tremuloides*	*luxF2, HPT, NPTII*	*A.t.*	Nilsson et al. 1992
P. tremula x *P. tremuloides*	*OCI, NPTII*	*A.t.*	Leplé et al. 1995
P. tremula x *P. tremuloides*	*OCI, NPTII*	*A.t.*	Leplé et al. 1995
P. tremula x *P. tremuloides*	*iaaH, iaaM, HPT, NPTII*	*A.t.*	Tuominen et al. 1995
P. tremula x *P. tremuloides*	*LFY, NPTII*	*A.t.*	Weigel and Nilsson 1995
P. tremula x *P. tremuloides*	*Ac, rolC, NPTII*	*A.t.*	Ahuja and Fladung 1996
P. tremula x *P. tremuloides*	*GUS, HPT*	*A.t.*	Nilsson et al. 1996a
P. tremula x *P. tremuloides*	*rolC, NPTII*	*A.t.*	Nilsson et al. 1996b
P. tremula x *P. tremuloides*	*phyA, phyB, NPTII*	*A.t.*	Sundberg et al., this volume
P. tremuloides	T-DNA	*A.t.*	Kubisiak et al. 1994
P. tremuloides	*GUS, NPTII*	*A.t.*	Tsai et al. 1994
P. nigra	*GUS, HPT, NPTII*, T-DNA	*A.t.*	Confalonieri et al. 1994
P. nigra	*GUS, NPTII*, T-DNA	*A.t.*	Confalonieri et al. 1995
P. tremula	*Ac, rolC, NPTII*	*A.t.*	Ahuja and Fladung 1996
P. deltoides	T-DNA	*A.t.*	Riemenschneider 1990
P. deltoides	*GUS, NPTII*	*A.t.*	Dinus et al. 1995

[1] Ac (*Activator*)=transposable element from maize; *aroA*=bacterial 5-enolpyruvylshikimate-3-phosphate synthase chimeric gene; *bar*=phosphinotricin acetyltransferase gene; *Bt*=endotoxin gene from *Bacillus thuringiensis*; *CAT*=chloramphenicol acetyltransferase gene; *crs1-1*=mutant acetolactate synthase gene; *GR*=glutathione reductase gene; *GUS*=β-glucuronidase gene; *HPT*=hygromycin phosphotransferase gene; *iaaH*=agrobacterial indoleacetamide hydrolase gene; *iaaM*=agrobacterial tryptophan monooxygenase gene; *IPT*=agrobacterial isopentenyltransferase gene; *LFY*=flower-meristem-identity gene; *luxF2*=luciferase gene; *NPTII*=neomycin phosphotransferase gene; *OCI*=cystein proteinase inhibitor gene; *phyA, phyB*=phytochrome genes; *PIN2*=wound-inducible potato proteinase inhibitor II gene; *prxA1*=peroxidase gene; and *rolC*=one of the genes responsible for hairy root disease, caused by the *Agrobacterium rhizogenes*

[2] Transferred DNA

[3] *A.t.=Agrobacterium tumefaciens; A.r.=Agrobacterium rhizogenes*

gene(s) of interest with a selectable marker gene instead of oncogenes on the Ti-plasmid; and 2) binary: T-DNA is located on a separate vector plasmid instead of the Ti-plasmid. T-DNA also includes the gene(s) of interest and selectable marker gene (Walkerpeach and Velten 1994). No recombination event is necessary for the binary vector system, unlike the co-integrate vector system. Overall, *A. tumefaciens* strains C58, A281, EHA101, and LBA4404 were commonly used with binary vectors for transformation of many poplars and seem to generate suitable transformation efficiencies (Brasileiro et al. 1991, 1992; Confalonieri et al. 1994, 1995; De Block 1990; Ebinuma et al. this volume; Howe et al. 1994; Kajita et al. 1994; Klopfenstein et al. 1991, 1993, 1997; Leplé et al. 1992, 1995; Nilsson et al. 1992; Schwartzenberg et al. 1994; Sundberg et al. this volume; Tuominen et al. 1995).

Transformation Procedures

Several factors should be considered to improve transformation efficiency such as the *Agrobacterium* inoculum titer, *vir* inducer, selectable marker system, and *in vitro* tissue culture manipulation techniques. Optimal results were obtained by dipping initial host explants into a bacterial suspension (5 to 6 x 10^8 cells/ml) for 20 min to 4 h, then co-cultivating them for 24 to 72 h on a liquid or semisolid regeneration medium that contained plant growth regulators such as benzyladenine (BA), 2,4-dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA), or thidiazuron (TDZ) (Confaloniei et al. 1994; Wang et al. 1994).

Acetosyringone (AS) and hydroxy-acetosyringone (OH-AS) elicited the expression of *Agrobacterium vir* region genes (Stachel et al. 1985). AS and OH-AS occur specifically in exudates of wounded and metabolically active plant cells and perhaps allow *Agrobacterium* to recognize susceptible cells (Stachel et al. 1985). Transformation efficiency could be increased during co-cultivation by using a *vir* region inducer such as AS (10 to 200 μM) (Confalonieri et al. 1995; Howe et al. 1994; Kubisiak et al. 1993; Nilsson et al. 1992; Weigel and Nilsson 1995).

A practical selectable marker system is essential to obtain high efficiency transformations while avoiding nontransformed plants that escape selection (Leplé et al. 1992). Selectable marker genes used for *Populus* transformation have encoded traits such as hygromycin resistance (hygromycin phosphotransferase; *HPT*), neomycin resistance (neomycin phosphotransferase II; *NPTII*), phosphinotricin (glufosinate) resistance (phosphinotricin acetyltransferase; *bar*), and chlorsulfuron resistance (mutant acetolactate synthase; *crs1-1*). Because the *NPTII* gene has been frequently employed in several woody plants including *Populus* species to select transformants (table 1), kanamycin is one of the most commonly used antibiotics for a transformation selection system. Even modest kanamycin concentrations (10 mg/l) can inhibit regeneration of untransformed hybrid poplar (*P. alba* x *P. grandidentata*) (Chun et al. 1988). Culture on nonselective medium (without selective antibiotics) for 2 days to 2 weeks before transfer to a selective medium (with selective antibiotics) has been used to obtain higher transformation frequencies (Charest et al. 1992; Dinus et al. 1995; Tuominen et al. 1995; Wang et al. 1994).

The transfer of explants to light conditions after decontamination using cefotaxime (250 to 500 mg/l) and/or carbenicillin (250 to 500 mg/l), a preculture (shoot-inducing or callus-inducing medium including BA, 2,4-D, NAA, or TDZ) period before *Agrobacterium*-mediated infection, or a prolonged infection period can enhance transformation frequencies dramatically (Confalonieri et al. 1994, 1995; De Block 1993; Leplé et al. 1992; Schwartzenberg et al. 1994; Tsai et al. 1994). Several studies demonstrate that the *Agrobacterium* plasmid, explant type, *in vitro* techniques, and use of a *vir* region inducing compound can substantially influence stable transformation frequency (Confalonieri et al. 1994, 1995; De Block 1990; Kubisiak et al.1993).

Reporter genes used to detect transgene expression have included *CAT*, β-glucuronidase (*GUS*), and luciferase (*luxF2*) genes (table 1). To date, *GUS* has been used most often and has been effective as a reporter gene in poplar (Jouanin and Pilate this volume; Pilate et al. this volume). Use of *luxF2* as a reporter allows *in vivo* monitoring of gene expression by nondestructive imaging (Nilsson et al. 1992; Schneider et al. 1990). Inhibitors present in poplar leaf extracts can interfere with CAT-activity assays reducing the advantage of *CAT* as a reporter gene in poplar (Klopfenstein et al. 1991).

Limitations and Prospects

Although transformation technology has reached a relatively advanced level, many variables exist that can interfere with the generation of stable transformed plants that express transgenes in a predictable manner (Ahuja this volume; De Block 1993). Recently, there have been several papers about the quantitative and qualitative instability of transgenes in primary transformed plants and subsequent generations (reviewed by De Block 1993; Ahuja this volume). *Agrobacterium*-mediated transformation is believed to result in random integration of transgenes into the genome causing high variation in quantitative and qualitative expression levels of transgenes in primary transformants and/or subsequent generations. However, an *Agrobacterium*-mediated system is a desirable method

to transform *Populus* because it is relatively inexpensive, easy to use, can produce an acceptable transformation rate, and transfers a limited copy number of transgenes.

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Chapter 8

Direct Gene Transfer in Poplar[1]

Pierre J. Charest, Yvonne Devantier, Cathy Jones, Jim C. Sellmer, Brent H. McCown, and David D. Ellis

Introduction

Agrobacterium-mediated genetic transformation and regeneration of transgenic *Populus* has been achieved with selected genotypes (Kim et al. this volume). Direct gene transfer using electroporation and microprojectile-mediated DNA delivery has also been accomplished (Chupeau et al. 1994; Devantier et al. 1993; McCown et al. 1991), with successful regeneration of transgenic *Populus* from a few genotypes (Chupeau et al. 1994; McCown et al. 1991). Microprojectile DNA delivery has been also used to study transient expression of chimeric genes (Devantier et al. 1993).

Several differences exist between *Agrobacterium*-mediated DNA delivery and direct gene transfer. Using *Agrobacterium*, only a fragment of the transformation vector within the T-DNA border of the Ti plasmid is transferred to the host cell and, in most cases, only 1 copy or a few copies of this fragment are integrated into the host genome. With direct DNA delivery, the whole transformation vector is introduced into the host cell and often several copies of the delivered DNA are integrated into the host genome. Although *Agrobacterium*-mediated DNA delivery is a more precise transformation method, direct DNA delivery has two advantages; it is genotype independent, and it bypasses the long life cycle of tree species by delivering DNA into a variety of plant tissues for gene expression studies (Charest et al. 1993). For instance, somatic and zygotic embryos, embryonal masses, pollen, germinated seedlings, flower organs, differentiating wood, needles, and vegetative buds of coniferous trees were used for transient gene expression using microprojectile-mediated DNA delivery (reviewed by Aronen et al. 1994, 1995; Charest et al. 1993; Ellis 1995). Electroporation is less flexible because, as with protoplast culture, it is often genotype dependent and has a more restricted range of tissues that respond to the procedure (Adbudl-Baki et al. 1990; Dekeyser et al. 1990; D'Halluin et al. 1992; Laursen et al. 1994; Luong et al. 1995; Songstad et al. 1993; Xu and Li 1994; Yang et al. 1993). Both microprojectile-mediated DNA delivery and electroporation could be useful alternatives to *Agrobacterium*-mediated transformation for recalcitrant genotypes or transient gene expression studies. Regeneration of transgenic poplar trees following *Agrobacterium*-mediated transformation, which required several months to obtain the transgenic plant material, was used for gene expression studies (Leplé et al. 1992; Miranda Brasileiro et al. 1992; Strohm et al. 1995). Similar results to evaluate the relative strength of promoters could have been obtained with microprojectile-mediated DNA delivery within a few days.

The most limiting factors to enhance foreign DNA integration and/or the recovery of stable transformants for direct DNA delivery are the host cell biology and defining cellular factors that interact with the introduced DNA. Experimental protoplast systems can be used for genetic transformation but assessing cellular changes that interact with the integration of the introduced DNA is difficult. Problems associated with protoplast systems include the enzymatic cell treatment, gene transfer environment, and need to transfer cells through a sequential series of different media. This difficulty is illustrated by the high mortality (50 percent) of poplar protoplasts following electroporation (Sellmer 1991). In contrast, microprojectile bombardment offers more potential for examining cellular factors involved in DNA incorporation because DNA can be delivered into any plant cell or tissue. One drawback of this approach is the heterogeneity of the cell population used for bombardment; changes in 1 tissue may alter a response by another tissue within an explant. For both methods, regulation of the transiently expressed genes may be less stringent than with transgenic plants because multiple copies of the gene can occur transiently, thus gene expression is not necessarily controlled through chromo-

[1] Klopfenstein, N.B.; Chun, Y. W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

some structure (Katagari and Chua 1992; Quail et al. 1987; Vernet et al. 1982).

Electroporation

DNA transfer using electroporation is due to increased cell wall and membrane permeability caused by a short electrical pulse applied to the target plant tissue, whether protoplasts or whole tissue explants (Van Wert and Saunders 1992). This method has been used to study transient gene expression and produce transgenic tissues and plants (Chupeau et al. 1993; Russell and McCown 1986; Wang et al. 1991). A prerequisite is the availability of tissue cultures amenable to electroporation. For poplar, protoplast culture and tree regeneration were achieved (Chupeau et al. 1993; Russell and McCown 1986; Wang et al. 1991) that provided a basis for gene transfer via electroporation. Transgenic *P. tremula* x *P. alba* trees were produced following electroporation of protoplasts with a decay-wave electroporation device (Chupeau et al. 1994). Transformation selection was achieved using phosphinotricin with the phosphinotricin acetyl transferase (*PAT*) gene, and chlorsulfuron with the acetolactate synthase (*ALS*) gene. The transformation frequency was from 1.3 to 7.5×10^{-6}, and 56 independent transgenic trees were regenerated. An interesting finding of this study was that the introduced genes were integrated in a relatively simple and straightforward pattern.

Microprojectile-mediated DNA Delivery

In poplar, the propulsion of microprojectiles coated with DNA was accomplished using 3 different mechanisms: electric discharge, gunpowder, and helium gas (Devantier 1992; Devantier et al. 1993; McCown et al. 1991; Sellmer 1991). These methods were optimized primarily using cell suspensions and were not directly compared. Transgenic trees were obtained using the electric discharge method (McCown et al. 1991), while only transgenic calli were obtained with the gunpowder method (Devantier 1992; Devantier et al. 1993).

Electric Discharge Propulsion

Optimization with Cell Suspension

With the electric discharge method, optimization for transient expression was performed using a cell suspension of *P. alba* x *P. grandidentata* cl. 'NC5339' (cv. 'Crandon') from leaf callus. The experiment included an evaluation of the effects of particle size, discharge voltage, and particle load (Sellmer 1991). Although no strong correlation between transient expression and stable expression of transgenes has been reported, maximizing transient expression in the cell suspension provides a uniform, reproducible system to evaluate biological parameters that affect transgene expression.

The first step toward developing a reproducible method for gene transfer is characterization of the target cells. Cell density (initial volume of cells/volume of tissue culture medium) on the target plate did not affect transient gene expression 2 days after bombardment for cell concentrations of 5 percent, 10 percent, and 15 percent; however, 50 percent yielded a significant increase in transient expression. Interestingly, no differences in β-glucuronidase (*GUS*) gene expression were observed among the 4 concentrations 1 week after bombardment. Cell culture age significantly affected transient expression levels of the *GUS* gene. Based on weekly subculturing (5 ml packed cells into 50 ml fresh medium), 3-day-old cell suspensions had significantly higher *GUS* gene expression compared to cells harvested at other times in the culture cycle. Based on dry weight, the cell suspension culture was in a lag phase through day 4, underwent a rapid growth phase during the next 4 days, and were in a stationary phase at day 9. Maximum transient gene expression corresponded to the mid-lag phase. During this 3-day period, growth was not noticeable, but a profound change in cellular morphology and the initiation of active cell division occurred. Cytologically, 32 percent of the cells on day 3 contained a division plate, in contrast with only 13 percent on day 1 and 9 percent on day 5. Furthermore, cells from 3-day-old cultures had almost twice as much ^{3}H-thymidine incorporation compared with cells from other stages. Cell size was also the smallest (less than 100 μm) from 3-day-old cultures. Because of this smaller size, more cells per volume were used causing a bias in transient *GUS* expression. When adjusted for cell number, cells from 1-day-old and 3-day-old cultures exhibited similar expression levels, but both produced higher *GUS* expression than those at other stages. However, this difference was diminished in *GUS* expression assays 21 days after bombardment, and no difference was detectable 56 days after bombardment. This long-term pattern of gene expression was independent of transformation vector size (Sellmer 1991).

Later experiments focused on factors that could promote cell competence for transient gene expression and stable transgene incorporation. These factors were associated with diverse approaches including preculturing cells with growth regulators, cell synchronization, and DNA breakage experiments. To test the effect of cell division (rate and synchronization) on transient *GUS* gene expression, cultures were grown on various hormone regimes. Experiments were inspired by previous cell-age experiments and

other reports that preculturing of tissue culture materials can enhance gene transfer and transient gene expression by promoting axillary and adventitious meristem development before bombardment (Ellis et al. 1991; McCown et al. 1991; Serres et al. 1992). In 1 set of experiments, 2,4-dichlorophenoxyacetic acid (2,4-D) was required for cell growth. Higher cytokinin levels or the inclusion of thidiazuron (TDZ) did not significantly change the growth rate of the cultures. Although growth rates were unchanged, incorporation of ^{3}H-thymidine was the highest with the highest concentration of cytokinin tested (1.0 μM benzyladenine). However, transient *GUS* gene expression did not change (Sellmer 1991). These results suggest that tissue cultures responded toward differentiation and meristematic development, but transient gene expression was unaffected.

Continuing this rationale of enhancing cell competence and *GUS* gene expression, experiments were conducted to synchronize poplar cell suspension to potentially increase transient gene expression and stable incorporation of genes. Some success was achieved with aphidicolin, a tetracyclic diterpenoid that reversibly blocks cell division before DNA strand synthesis. Aphidicolin was used to halt cell division and was effective at blocking DNA synthesis based on ^{3}H-thymidine incorporation in early exponential (3-day-old) and stationary (8-day-old) cultures. Effects of the block apparently remained for 12 to 24 h after release from the aphidicolin because little ^{3}H-thymidine incorporation was observed. In both cell ages, a sharp peak of ^{3}H-thymidine incorporation was observed after 24 h. In the cells from 3-day-old cultures, this peak dropped back to the baseline level suggesting synchronization of DNA synthesis. The highest level of transient *GUS* gene expression was observed at the 3-day-old stage although the level was not significantly different from cell suspensions at other times following aphidicolin release. Differences were not apparent in *GUS* gene expression, nor in the level of stably transformed calli recovered from the different time points (Sellmer 1991).

In a complementary study, ionizing radiation (gamma rays) was tested alone and in combination with aphidicolin to evaluate if DNA repair in conjunction with synchronized division could increase stable transformation. Radiation levels up to 3,000 rads did not affect cell growth, although no difference on the transient or long-term expression of *GUS* was observed, even at higher radiation levels. Also, no difference in *GUS* gene expression was evident when this treatment was combined with the aphidicolin treatment (Sellmer 1991).

Gene Delivery into Protoplast-Derived Cells and Nodules

Electric discharge propulsion was used to deliver microprojectiles in cells from protoplasts and nodule cultures of *P. alba* x *P. grandidentata* cl. 'NC5339' and *P. nigra* x *P. trichocarpa* cl. 'NC5331,' and to obtain transgenic trees containing a chimeric *Bacillus thuringiensis* δ-endotoxin gene conferring resistance to some lepidopteran pests (McCown et al. 1991). Transient *GUS* gene expression was obtained with 2 cell types (cell microcalli and nodules) and in stem sections. Bombardment intensity, particle load, and target tissue type were studied. Transgenic nodules resistant to kanamycin were obtained with both genotypes, but only nodules of *P. alba* x *P. grandidentata* regenerated transgenic trees. Transgenic tree tissues were tested for feeding toxicity against forest tent caterpillar and gypsy moth larvae. Reduced survival was observed for the forest tent caterpillar only, but both insect species manifested reduced growth.

Gene Delivery into Plantlet Tissues

As with poplar cell suspensions, the developmental and physiological stage of the poplar explant influences transient expression following particle bombardment. With poplar hybrid clones 'NC5339' (*P. alba* x *P. grandidentata* cv. 'Crandon') and 'NM6' (*P. nigra* x *P. maximowiczii*), explant pretreatment on shoot inducing media significantly increased transient *GUS* gene expression, driven by an enhanced CaMV *35S* promoter, in nodules, petioles, internodes, and leaf explants (Sellmer 1991). Pretreatment of 0.5 to 1 cm petiole segments in a liquid Woody Plant Medium (WPM) (Lloyd and McCowm 1980) supplemented with 0.1 μM each benzyladenine (BA), naphthaleneacetic acid (NAA), and thidiazuron (TDZ) progressively increased the number of cells histochemically staining for GUS activity for up to 7 days (Wraith et al. 1994). Although proliferation at the cut end of the petiole explant had initiated, cells were not yet sloughing off. Interestingly, pretreatment on callus induction media did not markedly increase transient gene expression in petiole segments, despite the similar morphological appearance of the explants (Wraith et al. 1994).

To increase the number of *GUS*-expressing cells, pretreatment must either increase the number of cells impacted or alter cell competency for transiently expressing inserted DNA. Explant pretreatment with a bud-induction medium may induce differentiation of cells capable of forming shoots or meristematic cells. These pretreated cells may be similar to the cells of the late-lag-phase cell suspensions.

For stable integration of delivered genes, a callus induction phase before culture on a bud-induction medium may improve the recovery of transgenic tissues after bombardment. Although this phase is not always required when no selection is applied, it is necessary when kanamycin selection is performed after bombardment of the hybrid poplar line 'NM6' (Wraith et al. 1994). There was no strong correlation between level of transient gene expression and number of stably transformed tissues recovered. Eight weeks after particle bombardment, the low number of *GUS*-expressing cells was constant regardless of gene construct, level of transient gene expression, and prebombardment treatments.

Gunpowder and Helium Propulsions

Optimization With Cell Suspension

Similar to work with the electric discharge method, optimization of gene delivery conditions with the gunpowder and the helium propulsion methods (Biolistic PDS-1000, Biolistic PDS-1000/He) was done with a cell suspension of *P. nigra* x *P. maximowiczii* cl. 'NM6' (Devantier 1992; Devantier et al. 1993). Evaluations were conducted on the effects of propulsion mechanism type, DNA precipitation method, DNA amount, bombardment number, microprojectile type and size, use of screens on top of the target tissues, target sample position in the bombardment chamber, and transient gene expression assay time. All these factors influenced gene expression levels. In addition, the helium propulsion device, using gold particles on which DNA was precipitated with $CaCl_2$ (calcium chloride), produced a higher level of transient gene expression. The optimum cell suspension age was between 7 and 9 days after subculturing, and the maximum transient gene expression was 1 day after bombardment. No study of growth rate or cell division was conducted. Following cell suspension bombardment, subcellular localization of microprojectiles revealed that most *GUS*-expressing cells contained microprojectiles in their cytoplasm suggesting that a mechanism may be available to transport the DNA to the nucleus.

In this study, microprojectile-mediated DNA delivery in a cell suspension of 4 different poplar genotypes (*P. nigra* x *P. maximowiczii* cl. 'NM1' and cl. 'NM6,' *P. deltoides* x *P. nigra* cl. 'DN106,' and *P. tremula* x *P. alba* cl. '7171-B4') was used to evaluate the strength of different chimeric promoters (Devantier et al. 1993). A genotype effect was observed, with 'NM1' yielding the highest level of transient gene expression by a vector containing the *GUS* gene linked to a double *35S* promoter with an alfalfa mosaic virus translational enhancer. This effect was perhaps associated with different morphologies of the cell suspensions for each genotype. Lower expression levels were obtained with chimeric promoters containing only the single *35S* promoter with the alfalfa mosaic virus promoter, the double *35S* promoter, and the single *35S* promoter (in order of decreasing strength). Similar results were obtained with the single and double *35S* promoter in transgenic poplar (Leplé et al. 1992).

Stable integration of the introduced genes was obtained following selection on 500 mg/l kanamycin. Expression of the introduced neomycin phosphotransferase II (*NPTII*) gene was detected using a radioactive assay. However, because the cell suspension was over 5 years old, no transgenic plants were regenerated (Devantier 1992; Devantier et al. 1993).

Gene Delivery Into Plantlet Tissues

As with the electric discharge method, explants were used to attempt recovery of transgenic poplar trees (Devantier 1992; Devantier et al. 1993). Three genotypes (*P. nigra* x *P. maximowiczii* cl.'NM1' and cl. 'NM6,' and *P. tremula* x *P. alba* cl. '7141-B4') were tested to optimize transient gene expression. The Biolistic Helium device using gold particles was superior, and 6-day-old explants gave higher transient gene expression. Different hormone treatments, a callusing phase, bombardment time after excision, different chimeric promoter strengths, and the timing of kanamycin selection were evaluated for stable genetic transformation; however, no transgenic tissues were obtained (Jones and Charest, unpublished). Furthermore, selection with hygromycin and methotrexate was attempted without success. A current approach is to obtain cell suspensions from *in vitro* grown plantlets and use these cells to produce transgenic calli from which trees will be regenerated.

Conclusions

Direct gene transfer by electroporation or microprojectile-mediated DNA delivery was achieved in poplar, and transgenic trees were obtained. These methods can also be used for fast experiments on transient gene expression. However, only a few genotypes were useful for regenerating transgenic trees, despite several attempts to extend these methods to a wider range of genotypes. Several biological parameters need exploring, and a better understanding of the mechanisms of DNA integration into plant cell genomes is required to improve currently used protocols. Once DNA delivery parameters are established, careful consideration must be given to the: 1) target cell response to the delivery process; 2) cell competence for DNA uptake, transient gene expression, and stable expression of the delivered DNA; and 3) interactions between the delivered DNA and the target cell machinery.

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Chapter 9

Gene Expression Studies[1]

Lise Jouanin and Gilles Pilate

Introduction

In this chapter, we discuss published research on the isolation and expression of poplar genes, including complementary cDNA (cDNA) and genomic DNA of several characterized genes. The over and under expression of introduced genes in homologous or heterologous host plants is discussed and published results are presented. Gene expression studies in poplar are now possible because procedures to transform and regenerate different genotypes are commonly available (reviewed in Jouanin et al. 1993 and this volume). Genes of plant origin are the focus of this chapter. Sequences of nonplant origin are discussed elsewhere in this book.

Poplar Gene Isolation

The list of genes from woody plants that have been isolated, sequenced, and published is relatively small. Continued effort is directed toward understanding the metabolic aspects of trees. Poplar is particularly amenable to gene isolation because of its small genome size (1.12 picograms DNA/diploid genome), which promotes the production of genomic libraries representative of the complete genome.

Among trees, most of the available gene sequences are from poplar (mainly cDNA sequences). This information is summarized in table 1. Research efforts have focused on genes: 1) involved in the wounding response (Bradshaw et al. 1989; Davis et al. 1991; Parsons et al. 1989); 2) involved in lignification (Bugos et al. 1991; Dumas et al. 1992; Kawai et al. 1993; Osakabe et al. 1994, 1995a, b, c; Subramanian et al. 1993; Tsai et al. 1995; van Doorsselaere et al. 1995a); and 3) encoding storage proteins (Clausen and Apel 1991; Coleman et al. 1992). In most studies, gene sequences from annual dicotyledonous plants were the basis of probes or polymerase chain reaction primers used to isolate poplar cDNAs (potato *PAL* cDNA: Subramanian et al. 1993; tobacco *CAD* cDNA: van Doorsselaere et al. 1995a; aspen *OMT* primers: Tsai et al. 1995).

Where possible, the poplar cDNA sequences that were obtained were compared with those of the corresponding genes in annual dicot plants (*WIN*: Davis et al. 1991; *OMT*: Dumas et al. 1992; *PAL*: Osakabe et al. 1995a). A high degree of homology was observed between poplar cDNA and annual dicot plant cDNA; frequently more than 80 percent at the amino acid level. However, the highest homology was found with sequences originating from other deciduous trees; for example, comparison of *CAD* and *OMT* sequences between poplar and eucalyptus (*CAD* 80.9 percent, van Doorsselaere et al. 1995a; *OMT* 84 and 80 percent, Hayakawa et al. 1996). When present, intron sequences were found in the expected locations, as demonstrated by genes encoding chitinases (Davis et al. 1991) and anionic peroxidases (Osakabe et al. 1995b). Overall, poplar genes showed a closer relationship to dicotyledonous angiosperms than to gymnosperms or monocotyledons as shown in table 2 (*PAL*: Osakabe et al. 1995a; *CAD*: van Doorsselaere et al. 1995a; *OMT*: Hayakawa et al. 1996).

Most of the studied genes are members of small multigene families (*WIN*: Davis et al. 1991; *OMT*: Hayakawa et al. 1996; *PRX*: Osakabe et al. 1995b; *PAL*: Osakabe et al. 1995c, *CHS*: Lurin and Jouanin 1995). In some cases, they are clustered in the genome (*WIN*: Davis et al. 1991; *PRX*: Osakabe et al. 1995b; *PAL*: Osakabe et al. 1995c).

[1] Klopfenstein, N.B.; Chun, Y.W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

Table 1. cDNA and genomic sequences cloned in poplar.

Name	Enzyme	Origin	Poplar type	References
Ptom1	*O*-methyl transferase (OMT)	cDNA	*P. tremuloides*	Bugos et al. 1991
PTOMT	*O*-methyl transferase (OMT)	genomic	*P. tremuloides*	Tsai et al. 1995
pPCL4	*O*-methyl transferase (OMT)	cDNA	*P. deltoides* x *P. trichocarpa*	Dumas et al. 1992
HOMT1 & HOMT2	*O*-methyl transferase (OMT)	genomic	*P. sieboldii* x *P. grandidentata*	Hayakawa et al. 1996
PAL	Phenylalanine ammonia-lyase (PAL)	cDNA	*P. deltoides* x *P. trichocarpa*	Subramaniam et al. 1993
cDNA1	Phenylalanine ammonia-lyase (PAL)	cDNA	*P. sieboldii* x *P. grandidentata*	Osakabe et al. 1995a
PAL G1 & G2A	Phenylalanine ammonia-lyase (PAL)	genomic	*P. sieboldii* x *P. grandidentata*	Osakabe et al. 1995a
PALG2B & PALG4	Phenylalanine ammonia-lyase (PAL)	genomic	*P. sieboldii* x *P. grandidentata*	Osakabe et al. 1995c
POPCAD (CAD)	Cinnamyl alcohol dehydrogenase	cDNA	*P. deltoides* x *P. trichocarpa*	van Doorsselaere et al. 1995a
WIN3	Wound-inducible (similar to sporamin	cDNA	*P. trichocarpa* x *P. deltoides*	Hollick and Gordon 1993
g*WIN3*	and Kunitz proteinase inhibitor)	genomic	*P. trichocarpa* x *P. deltoides*	Bradshaw et al. 1989
WIN6 & 8	Chitinase	cDNA	*P. trichocarpa* x *P. deltoides*	Parsons et al. 1989
g*WIN6,8 & X*	Chitinases	genomic	*P. trichocarpa* x *P. deltoides*	Davis et al. 1991
pPOP1	Malic enzyme	cDNA	*P. deltoides* x *P. trichocarpa*	van Doorsselaere et al. 1991
samPdxPt	S-adenosyl-L-Methionine synthetase	cDNA	*P. deltoides* x *P. trichocarpa*	van Doorsselaere et al. 1993
DHDPS	Dihydrodipicolinate synthase	cDNA	*P. deltoides* x *P. trichocarpa*	Vauterin and Jacobs 1994
BSP	Bark storage protein	cDNA	*P. deltoides*	Coleman et al. 1992
bspA	Bark storage protein	genomic	*P. deltoides*	Coleman and Chen 1993
WS1	Xylem ray cell storage protein	cDNA	*P. nigra* x *P. deltoides*	Clausen and Apel 1991
pA2, pA3	Anionic peroxidases	cDNA	*P. sieboldii* x *P. grandidentata*	Osakabe et al. 1994
PRXA1	Anionic peroxidase	genomic	*P. sieboldii* x *P. grandidentata*	Kawai et al. 1993
PRXA3A				
PRXA4A	Anionic peroxidases	genomic	*P. sieboldii* x *P. grandidentata*	Osakabe et al. 1995b

Table 2. Identity comparison at the amino acid level (%) among the *PAL*, *OMT*, and *CAD* genes of poplar and other plants.

	Dicot		Monocot		Conifer		
Gene	Plant	(%)	Plant	(%)	Plant	(%)	References
PAL G2	bean	(85.9)			pine	(66.1)	Osakabe et al. 1995a
	parsley	(84.7)					
PAL G2	bean	(81.5)			pine	(64.2)	Osakabe et al. 1995a
	parsley	(82.8)					
HOMT1	tobacco *OMT1*	(77)	maize	(28)			Hayakawa et al. 1996
	OMT2	(56)					
	eucalyptus	(84)					
HOMT2	tobacco *OMT1*	(76)	maize	(26)			Hayakawa et al. 1996
	OMT2	(54)					
	eucalyptus	(80)					
CAD	tobacco	(79.8)			spruce	(69.2)	van Doorsselaere et al. 1995a
	alfalfa	(84)					

Gene Expression at the RNA Level

Isolated cDNAs have been used as probes to monitor gene expression in different tissues and under various environmental conditions. The most detailed studies have focused on the systemic accumulation of specific messenger RNA (mRNAs) in response to wounding (Parsons et al. 1989; Bradshaw et al. 1989), seasonal changes and tissue-specific expression of mRNAs encoding bark and xylem ray cell storage proteins (Clausen and Apel 1991; Coleman et al. 1992), and genes involved in the phenylpropanoid pathway (*PAL*: Subramanian et al. 1993; Osakabe et al. 1995a, c; *OMT*: Bugos et al. 1991, Hayakawa et al. 1996; anionic peroxidases: Osakabe et al. 1995b; *CHS*:

Lurin and Jouanin 1995). In these studies, mRNA abundance was measured either by northern hybridization or by reverse transcriptase-polymerase chain reaction. From these studies, we can infer that gene regulation often occurs at the transcriptional level.

Poplar Heterologous Promoters

When establishing a genetic transformation procedure for a specific poplar hybrid, vectors frequently contain a neomycin phosphotransferase gene (*NPTII*), which is a selectable marker that confers resistance to kanamycin, and a β-glucuronidase gene (*GUS*), which is used as a reporter of gene expression (Jouanin et al. 1993). Binary vectors such as pBI121 (Jefferson et al. 1987) or its derivatives, in which the *NPTII* and the *GUS* genes are placed under control of the *Agrobacterium tumefaciens* nopaline synthase (*NOS*) promoter and either the *35S* or *70S* (double enhancer sequences) CaMV promoters, function efficiently in poplar (Leplé et al. 1992). Thus, vectors used for dicot transformation are frequently suitable for poplar use.

Reports that illustrate the function of heterologous plant promoters in poplar include promoters from the potato proteinase inhibitor II (*PIN2*) gene (Klopfenstein et al. 1991), the *Arabidopsis thaliana* acetolactate synthase (*AtALS*) gene (Brasileiro et al. 1992), and the Eucalyptus cinnamyl alcohol dehydrogenase (*EuCAD*) gene (Feuillet et al. 1995). Expression of an associated reporter gene (*CAT* or *GUS*) indicates that regulation is conserved. This is represented by the wound induction of the *PIN2-CAT* construct (Klopfenstein et al. 1991), and the tissue-specificity of the *EuCAD-GUS* construct in relation to lignification (Feuillet et al. 1995). These studies illustrate the basis of considerable interest in poplar, an easily transformable woody plant, in which sequences originating from other plant species have conserved patterns of expression.

Poplar Promoters in Heterologous Plants

In some cases, the characterization of poplar genomic sequences has involved promoter isolation. These promoters have been linked to a reporter gene such as *GUS* to study their expression pattern in transgenic plants. Two wound-inducible poplar promoters, *WIN3* (Hollick and Gordon 1993) and *WIN6* (Clarke et al. 1994), are also induced upon wounding in tobacco. This demonstrates conservation of expression mechanisms between an annual dicot and a woody angiosperm. It may be feasible to study woody plant gene promoters in a herbaceous plant model such as tobacco. However, reintroduction of sequences into the same species is required to study promoter function in relation to unique structural, metabolic, and developmental features. Currently, there are no reports of poplar promoters reintroduced into poplar. This research field is expected to develop rapidly.

Over and Under Expression of Poplar Genes in Plants

Of major interest, at the fundamental and applied levels, is the manipulation of gene expression to alter levels of specific proteins and shift metabolic processes. Such work in poplar is just emerging. As of this writing, the only report of over expression of a gene concerns a peroxidase gene in poplar callus (Kajita et al. 1994). The only report of under expression of a gene is that of a caffeic acid/5-hydroxyferulic acid *O*-methyltransferase (*OMT*) gene in poplar stems (van Doorseelaere et al. 1995b).

A chimeric gene containing the CaMV *35S* promoter and the 5' end of the poplar *OMT* gene in antisense orientation was introduced into tobacco by Dwivedi et al. (1994). In some transgenic tobacco plants, the level of OMT activity was decreased and modification of the lignin composition was observed. This demonstrates that homology between the tobacco and poplar *OMT* genes is sufficient for a functional antisense strategy. Another study reported under expression of *OMT* in transgenic poplars expressing a poplar antisense RNA (van Doorsselaere et al. 1995b). OMT activity was reduced up to 95 percent and critical modification of the lignin composition was observed. Reducing the activity of enzymes involved in lignin monomer biosynthesis is potentially important for the paper pulp industry and can be effectively tested in woody plants. Detailed information on this subject is included in the chapter by Boerjan et al. in this volume.

Summary

Poplar is considered a model plant for gene expression studies in woody angiosperm species. The advantages of the poplar model system are the availability of transformation procedures and its small genome size, which allows for relatively easy gene isolation. High levels of homology and conservation of the regulatory systems have been observed between poplar (a dicot) genes and other

dicots. Poplar promoters and genes potentially can be studied in other model plant species such as tobacco. However, for specific metabolic pathways, such as lignification, more representative information will result if gene expression is tested in poplar. The ability to transform many poplar genotypes affords diverse opportunities to study expression of genes from other woody species, including conifers. In the future, the development of such studies could provide critical information on gene expression in woody plants.

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Chapter 10

Differential Expression of *aro*A Gene in Transgenic Poplar: Influence of Promoter and Ozone Stress[1]

David F. Karnosky, Gopi K. Podila, Dongill Shin, and Don E. Riemenschneider

Introduction

Fillatti et al. (1986, 1987a) first demonstrated *Agrobacterium*-mediated transformation and regeneration of whole plants with hybrid poplar clone 'NC5339' (*Populus alba* x *P. grandidentata* cv. 'Crandon'). They inserted a mutant bacterial *aro*A gene with a mannopine synthetase (*MAS*) promoter using a binary vector plasmid (pPMG 85/587) of *Agrobacterium tumefaciens*. Riemenschneider et al. (1988) conducted glyphosate spray trials that suggested that 5 of 6 transgenic hybrid poplars had improved glyphosate tolerance compared with nontransformed controls. However, the maximum glyphosate concentration tolerated (0.28 kg/ha) was still much less than that used in commercial applications. Riemenschneider et al. (1988) attributed the low glyphosate tolerance to an ineffective *MAS* promoter and the lack of transit peptide coding sequences that would target the modified EPSP synthase to the chloroplasts where native EPSP synthase is localized (Mousdale and Coggins 1985).

To determine if herbicide tolerance could be increased in hybrid poplar by a more efficient promoter and to target *aro*A gene product activity to the chloroplasts, Riemenschneider and Haissig (1991) cocultivated hybrid poplar 'NC5339' with a second binary vector (pCGN 1107). The second binary vector (pCGN 1107) contained the mutant *aro*A gene, which was fused to the cauliflower mosaic virus promoter (CaMV *35S*), and RUBISCO small subunit transit peptide coding sequences from *Pisum sativum* and *Glycine max* (Fillatti et al. 1987b). Transformed plants from this experiment were tested for their growth, photosynthetic physiology, and herbicide tolerance (Donahue et al. 1994). As expected, glyphosate tolerance was markedly higher for transgenic plants containing the *35S* promoter and the RUBISCO transit peptide sequences.

In this study, we sought a molecular explanation of the Donahue et al. (1994) report that suggested that the *35S* promoter and the RUBISCO transit peptide sequences had conveyed increased glyphosate tolerance. We examined: 1) transcription and translation of the *aro*A gene by transformed hybrid poplar 'NC5339' using both *MAS* and *35S* promoters; and 2) intracellular translocation of modified EPSP synthase (via cell fractionization). In addition, we examined the stability of transgene expression under stress by assessing the effects of acute oxidative stress (200 ppb ozone [O_3] for 4 h per day for 1 to 5 days) on *aro*A gene expression.

Methods

We used *Agrobacterium tumefaciens* strain C58-based binary vectors. One strain contained the engineered plasmid pPMG 85/587 with the *aro*A gene under control of the mannopine synthase (*MAS*) promoter. A second strain contained plasmid pCGN 1107 with the *aro*A gene under control of the *35S* (CaMV *35S*) promoter and also ligated to RUBISCO small subunit transit peptide leader sequences. The leader sequences should target intracellular translocation of EPSP synthase from the cytosol to chloroplasts. T-DNA vectors were separately inserted into hybrid poplar 'NC5339' using the tobacco feeder layer, leaf disc transformation method (Fillatti et al. 1986, 1987a, 1987b).

Shoots were regenerated via organogenesis from cocultivated leaf discs on selective media (Riemenschneider and Haissig 1991). Shoot cuttings from stabilized shoot cultures were collected from 3 different transgenic hybrid poplar clones and 1 untransformed control and rooted *ex vitro*

[1] Klopfenstein, N.B.; Chun, Y.W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

(Riemenschneider and Haissig 1991). Clone PT-1 and PT-7 contained the *aro*A gene controlled by the *35S* promoter fused to the RUBISCO leader sequence for chloroplast targeting. Clone 12182 contained the *aro*A gene controlled by the *MAS* promoter.

RNA was isolated (Bugos et al. 1995) and analyzed via northern blots using an *aro*A gene probe (Shin et al. 1994). Protein extraction and western blot analyses of *aro*A protein (EPSP synthase) were performed (Shin et al. 1994). Cell fractionization and isolation of chloroplasts were performed (Shah et al. 1986). Protein quantification was performed (Bradford 1976). Scanning densitometry of northern and western blot autoradiograms was done using a mirror scanner and scananalysis software.

Ozone (O_3) fumigations were conducted in a continuously stirred tank reactor (CSTR) in a greenhouse at a concentration of 200 ppb for 4 h per day from 10 AM to 2 PM for 1, 3, or 5 days. Ozone was generated and monitored (Karnosky et al. 1996). Four-month-old plants (40 to 60 cm in height) were maintained under standard greenhouse conditions before and during fumigations. Immediately after the 1-, 3-, or 5-day exposures, all leaves were removed and stored in -80 °C after being frozen in liquid nitrogen.

Results and Discussion

The *35S* promoter was more efficient than the *MAS* promoter for transcription of the *aro*A gene (figure 1a) and translation of the *aro*A gene to EPSP synthase (figure 1b). Densitometer readings (data not shown) suggest that about 5 times more messenger RNA (mRNA) and more protein was produced in transgenic hybrid poplar plants with the *35S* promoter than with the *MAS* promoter. These results are consistent with those of Donahue et al. (1994) and explain why the *35S* promoter conveyed a higher level of glyphosate tolerance than the *MAS* promoter in previous studies (Donahue et al. 1994).

EPSP synthase was found in chloroplasts of poplars transformed with construct pCGN 1107 containing the *35S* promoter and transit peptide coding sequences (figure 1c). In contrast, EPSP synthase produced by poplars transformed with construct pPMG 85/587 was not found in chloroplasts (figure 1c). We conclude that heterologous RUBISCO small subunit leader sequences were effective in targeting the translocation of the modified EPSP synthase from the cytosol to chloroplasts. Size differences between the chloroplastic and cytosolic fractions (figure 1c) suggest that the cytosolic EPSP synthase possessed an attached leader sequence, whereas the chloroplastic EPSP synthase is smaller, indicating effective post-translocation excision of the leader sequences.

For genetic engineering to be successful in the long term, the transgenes must be stably expressed, even in the presence of commonly occurring plant stresses. To examine the effects of acute oxidative stress on *aro*A gene expression in transgenic hybrid poplar plants, we exposed plants to O_3 for 4 h per day for either 1, 3, or 5 days in a controlled environment chamber. As is shown in figure 2a, O_3 stress resulted in a down regulation of the *aro*A gene under control of the *MAS* promoter. However, transcription of the *aro*A gene under the influence of the *35S* pro-

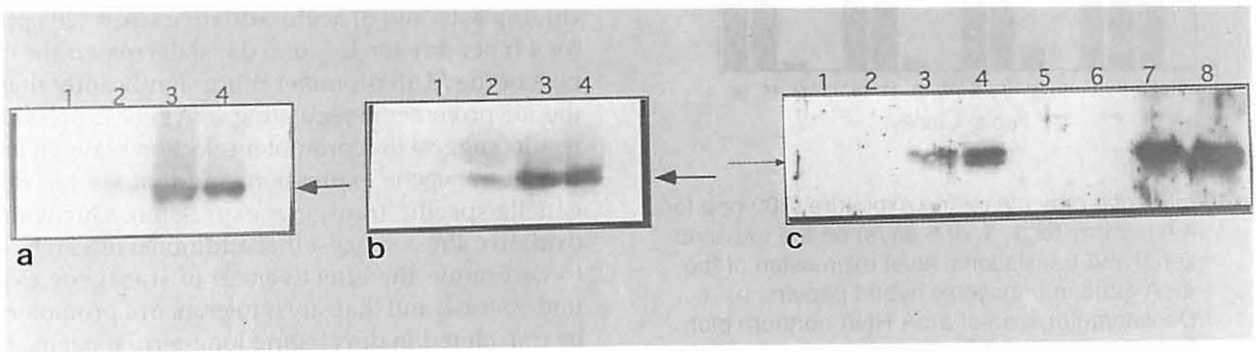


Figure 1. Transcriptional and translational expression of the *aro*A gene in transgenic hybrid poplar and subcellular localization of the EPSP synthase protein. a) Northern blot analysis was performed using 10 μg each of total RNA from untransformed control (lane 1), clone 12182 (lane 2), clone PT-1 (lane 3), and clone PT-7 (lane 4). Blots were probed with ^{32}P labeled *aro*A gene probe. Arrow points to *aro*A RNA. b) Western blot analysis was performed using 20 μg each of total cellular proteins from untransformed control (lane 1), clone 12182 (lane 2), clone PT-1 (lane 3), and clone PT-7 (lane 4). Blots were probed with EPSP synthase antibodies. Detection of EPSP synthase protein was done using HRP-conjugated, secondary antibodies and ECL detection method (Amersham Corp., Arlington Heights, IL). c) Western blot analysis of EPSP synthase was performed using 20 μg each from cytosolic and chloroplastic protein fractions. Lanes 1 through 4 are chloroplastic EPSP synthase from control, clone 12182, clone PT-1, and clone PT-7, respectively. Lanes 5 through 8 are cytoplasmic EPSP synthase from control, clone 12182, clone PT-1, and clone PT-7. Arrow points to mature chloroplastic form of EPSP synthase and arrowhead points to cytosolic precursor form of EPSP synthase.

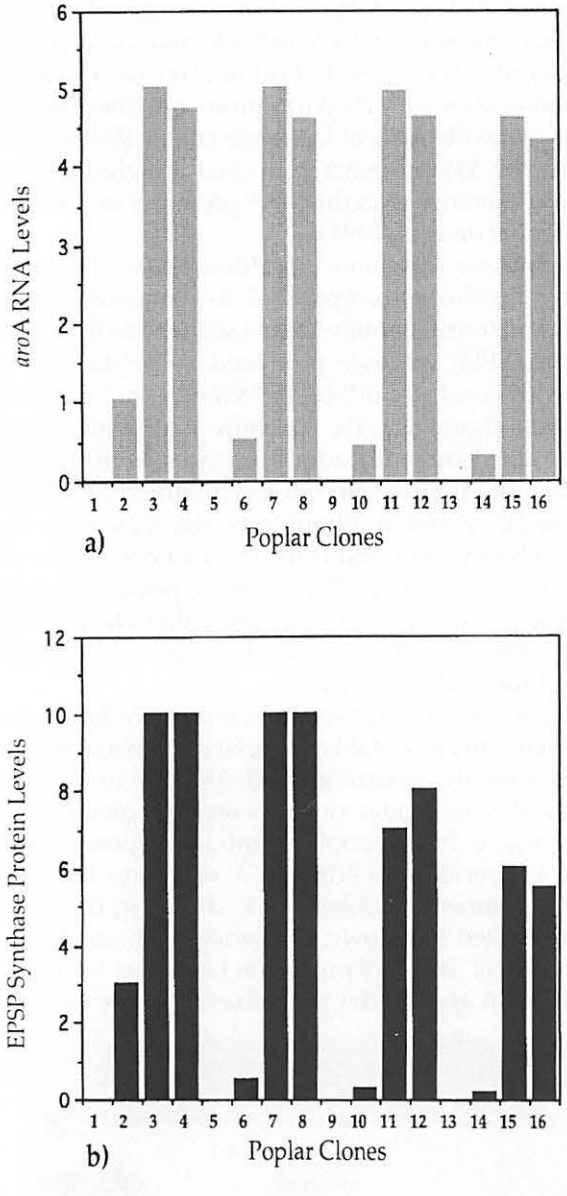


Figure 2. Influence of acute ozone exposure (200 ppb for 4 h per day for 1, 3, or 5 days) on the transcriptional and translational level expression of the *aro*A gene in transgenic hybrid poplars. a) Densitometer scan of *aro*A RNA northern blot from untreated and O_3 treated untransformed and transformed plants. Lanes 1, 5, 9, and 13 are untransformed control plants treated with O_3 for 0, 1, 3, or 5 days. Lanes 2, 6, 10, and 14 are transformed clone 12182 treated with O_3 for 0, 1, 3, or 5 days. Lanes 3, 7, 11, and 15 are transformed clone PT-1 treated with O_3 for 0, 1, 3, and 5 days. Lanes 4, 8, 12, and 16 are transformed clone PT-7 treated with O_3 for 0, 1, 3, and 5 days. b) Densitometer scan of EPSP synthase western blot from untreated and O_3 treated untransformed and transformed plants. Lanes are as described for figure 2a.

moter was not detectably different from control conditions after 1, 3, or 5 days of O_3 (figure 2a). Concentration of modified EPSP synthase in transgenic poplars containing the *35S* promoter was affected by O_3 exposure with both promoters (figure 2b). We do not know if this was due to reduced EPSP synthase biosynthesis or an increased protein degradation rate. However, if degradation was a major factor, we would expect to see an increase in degradation products resulting in new bands in figure 2b. Since this did not occur, we conclude that the main influence of O_3 was in decreasing the translation of mRNA into protein.

Summary

The herbicide glyphosate [N-(phosphonomethyl) glycine] kills plants primarily by inhibiting the enzyme EPSP synthase (5-enolpyruvylshikimate-3-phosphate synthase). A mutant *aro*A gene encoding a modified EPSP synthase was used to convey increased glyphosate tolerance to hybrid poplar cl. 'NC5339' and other plants. While earlier studies showed that tolerance of transgenic 'NC5339' to glyphosate was increased, transgenic 'NC5339' was not immune to recommended commercial application rates. In this study, we showed that: 1) the *35S* promoter was more efficient than the *MAS* promoter in eliciting both transcriptional and translational expression of the *aro*A gene; 2) the RUBISCO transit peptide coding sequees successfully mediated translocation of the *aro*A gene product to chloroplasts, and 3) acute oxidative stress (200 ppb ozone for 4 h per day for 1, 3, or 5 days) decreased the effectiveness of the *MAS* promoter more significantly than that of the *35S* promoter in regulating *aro*A gene expression. These results suggest that promoter selection plays an important role in transgene expression, and that we can design organelle-specific transgene expression. Our results with oxidative stress suggest that additional research is needed to determine the effectiveness of transgene expression under stress, and that stress tolerance of promoters should be considered in developing long-term biogenic products in forest trees.

Acknowledgments

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Chapter 11

Growth and Development Alteration in Transgenic *Populus*: Status and Potential Applications[1]

Björn Sundberg, Hannele Tuominen, Ove Nilsson, Thomas Moritz, C. H. Anthony Little, Göran Sandberg, and Olof Olsson

Introduction

With the development of gene-transfer techniques applicable to forest tree species, genetic engineering is becoming an alternative to traditional tree breeding. To date, routine transformation methods for several hardwood species, particularly *Populus* and *Eucalyptus*, have been established and promising advances have occurred in the development of transformation protocols for conifers (Charest et al. 1996; Ellis et al. 1996; Jouanin et al. 1993; Walters et al. 1995). Rapid progress in transformation technology makes it possible to develop genetic engineering tools that modify economically tractable parameters related to growth and yield in tree species. Such work will also increase our understanding of genetic and physiological regulation of growth and development in woody species.

Several hybrid aspen lines with phenotypes modified by genetic engineering were produced and characterized in our laboratory. Their existence shows, unequivocally, that tree growth and development are alterable by genetic engineering. Some modified phenotypes are described in this chapter. We also briefly discuss how genetic engineering might be used to generate trees with properties, such as modified wood structure, which are desirable to the forest industry.

Using Transgenic *Populus* to Study Growth and Development in Woody Species

The best model system for understanding the genetic and physiological control of tree growth and wood formation is a perennial species containing a vascular cambium, which is the meristem that produces secondary xylem and phloem. Presently, *Populus* is the preferred tree-model system because it has several useful features. *Populus* has a small genome, approximately 5×10^8 base pairs (bp), which encourages molecular mapping, library screening, and rescue cloning. Saturated genetic maps are already constructed for several *Populus* spp. (Bradshaw et al. 1994; Cervera et al. 1996). Moreover, *Populus* can be efficiently transformed and regenerated, and it grows rapidly. Thus, isolated genes inserted into proper vectors can easily be introduced into *Populus*, readily producing transgenic plants.

The major disadvantage of the *Populus* model system is that controlled crosses are time consuming and difficult to perform. Consequently, expression of introduced genes in the F_2 generation remains undetermined. To date, all physiological and phenotypic investigations involving transgenic *Populus* have been restricted to the primary transformant. However, as research on *Populus* increases, novel schemes will be developed to induce early flowering by physiological, biochemical, or genetic manipulation. As discussed below, the potential for genetic manipulation has been shown by the expression of the *Arabidopsis LEAFY* (*LFY*) gene in a hybrid aspen.

We used a hybrid aspen, *Populus tremula* x *P. tremuloides*, as a model system for genetic engineering experiments. Expression vectors containing the gene(s) and promoter(s) of interest were introduced by electroporation into the C58 *Agrobacterium tumefaciens* strain GV3101 (pMP90RK). To move the transfer DNA (T-DNA) from these vectors to the

[1] Klopfenstein, N.B.; Chun, Y.W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

Populus genome, sterile, internodal stem segments were cocultivated with the *Agrobacterium* for 2 days on solid Murashige and Skoog (MS) medium (Murashige and Skoog 1962). Cocultivated explants were transferred to new media containing plant growth substances to initiate shoot formation and antibiotics to select for transformants and eliminate remaining *Agrobacterium* cells (Nilsson et al. 1992). Using this protocol, transformation frequency was typically about 20 percent, regeneration success was higher than 95 percent, and rooted plants 7 to 10 cm tall were obtained after about 4 months in sterile culture. Transgenic plantlets were either placed in pots and cultured in a greenhouse or placed in a controlled environment chamber (figure 1). For the first 7 to 10 days of culture, plants are covered with a plastic bag for acclimation to decreased humidity.

Gene expression, and therefore phenotype, is significantly affected by environmental conditions such as light quality and quantity, nutrient availability, and temperature. To compare phenotypes from different transformation events, plants must be cultured under controlled and reproducible environmental conditions, especially when performing experiments at different times of the year. For critical evaluation of transgenic phenotypes, we used controlled environment chambers and potted the plants in mineral wool. This potting medium was used for a stable and defined nutrient supply through daily surplus watering with an optimal nutrient solution (Ingestad 1970). These culture conditions induced rapid growth; about 1 leaf primordia was produced every 2 days in wild-type plants, and a plant 1 m tall was produced after about 6 weeks in the growth chamber.

The *Populus* model system enables the function of genes isolated from any organism to be evaluated in a tree species by transgene expression. Alternatively, the corresponding endogenous *Populus* gene can be cloned and used for transformation in either sense (overproduction of the gene product) or antisense (suppression of the gene expression) orientation.

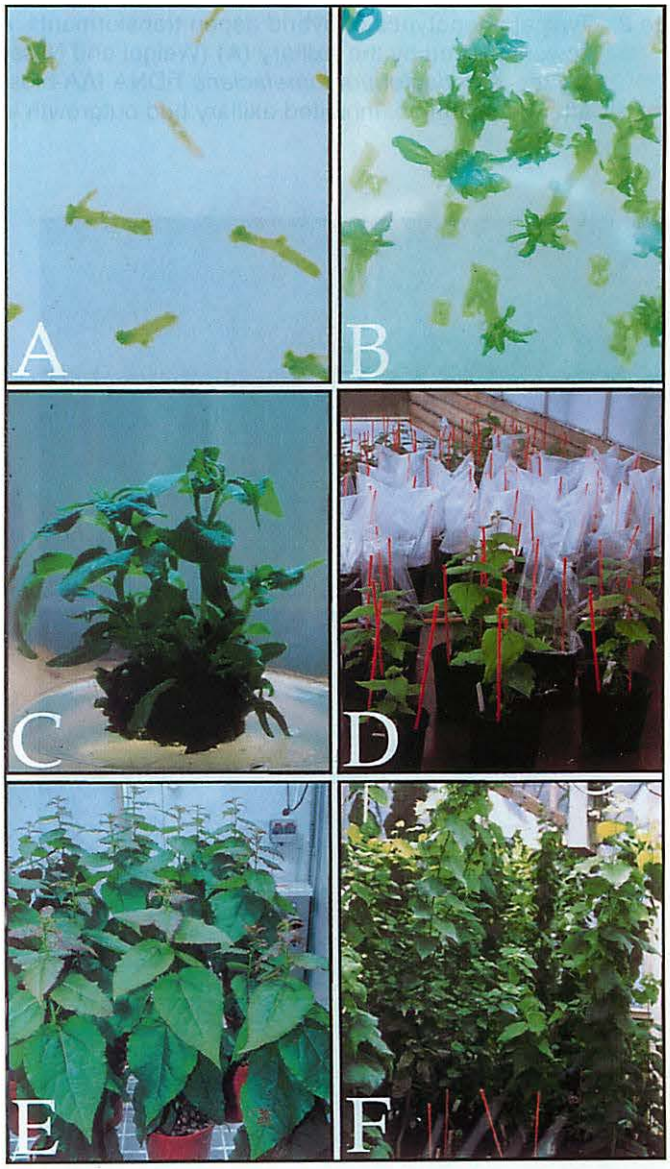


Figure 1. Regeneration and culture of hybrid aspen plants. A) Internodal stem segments. B) Shoots regenerated on stem segments. C) Shoot proliferation on cytokinin-rich medium. D) Initial culture of potted plants under plastic bags. E) Plants cultured under controlled environment conditions in a climate chamber. F) Plants cultured in a greenhouse.

Transgenic *Populus* With Altered Growth and Development

Transgenic Hybrid Aspen Expressing the *Arabidopsis LEAFY* Gene

Knowledge of gene function in a distantly related model system, such as *Arabidopsis*, can be directly applied to a tree species. This was recently shown by expression of the *Arabidopsis* gene *LEAFY* (*LFY*) in hybrid aspen to promote precocious flowering (Weigel and Nilsson 1995). *Arabidopsis* plants with loss-of-function mutations in *LFY* have shoots where typically flowers grow (Huala and Sussex 1992; Schultz and Haughn 1991; Weigel et al. 1992), which shows that *LFY* is necessary for normal flower initiation in that species. Furthermore, *LFY* is the first gene expressed in the flower primordium, and *LFY* transcription is detected in the predicted positions of the flower primordia before any other signs of primordium formation are evident (Weigel et al. 1992).

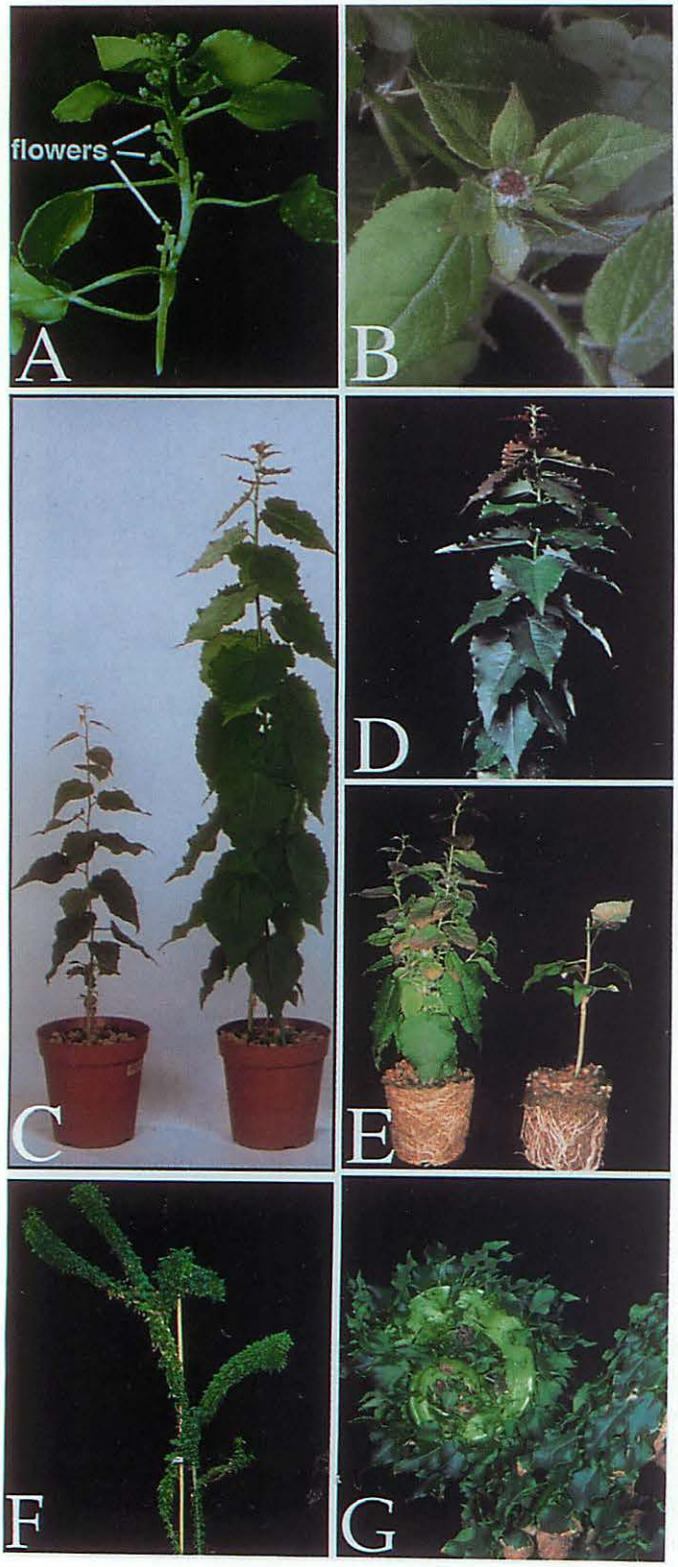


Figure 2. Typical phenotypes of hybrid aspen transformants. A) and B) Plants expressing the *Arabidopsis LFY* gene. Note flowers formed by the axillary (A) (Weigel and Nilsson 1995) and apical (B) meristems. C) and E) Plants expressing the *Agrobacterium tumefaciens* T-DNA IAA-biosynthesis genes. Note the slower growth, smaller leaves and, after decapitation, inhibited axillary bud outgrowth in transformants (C-left, E-right) compared with wild-type plants (C-right, E-left). D) Plants expressing the oat *phyA* gene. Note the short internodes. F) and G) Plants expressing the *Agrobacterium rhizogenes* T-DNA *rolC* gene. Note the bushy phenotype (F) and stem fasciation (G) (Nilsson et al. 1996b).

These findings suggest that *LFY* is the "main switch" for the flower formation program, and that it might be necessary and sufficient to induce flowering. This function of *LFY* was shown by fusing the *LFY* coding region to the strong cauliflower mosaic virus (CaMV) *35S* promoter and transforming the *35S-LFY* construct into *Arabidopsis* (Weigel and Nilsson 1995). The transgenic *35S-LFY* plants flowered earlier than their wild-type counterparts because the meristem in the leaf axil, which normally produces a secondary shoot, produced a single flower. Eventually, the apical meristem of the primary shoot formed a terminal flower. However, because *Arabidopsis* naturally flowers very early, after about 3 weeks under inductive long-day conditions, the induced difference in flowering time was not very dramatic.

A more stringent test of the flower-inducing capability of *LFY* and for the conservation of *LFY* function between species was the introduction of the same *35S-LFY* construct into a hybrid aspen whose parental species take 8 to 20 years to flower under natural conditions (Schreiner 1974). Remarkably, the *35S-LFY* hybrid aspen transformants flowered after only a few months in tissue culture (Weigel and Nilsson 1995) (figure 2A, 2B). Transformants with high *LFY* expression produced a solitary flower in the axil of a few leaves before the apical meristem of the shoot was consumed in the formation of an aberrant terminal flower. Transformants with low *LFY* expression could be rooted and subsequently were transferred to the greenhouse. Some of these plants also formed a single flower with normal appearance in the leaf axils, but it remains unclear whether these flowers are fertile.

Thus, the phenotype of hybrid aspen plants that strongly express *35S-LFY* is similar to analogous *Arabidopsis* transformants. This shows that the function of the LEAFY protein is highly conserved between unrelated dicot species and raises the possibility that the *Arabidopsis LFY* can be used to induce early flowering in a wide range of tree species. This range of tree species may include conifers as Douglas-fir contains a gene that shows high homology with *Arabidopsis LFY* (S. Strauss, personal communication).

Transgenic Hybrid Aspen Expressing *phyA* and *phyB* Genes From Oat and *Arabidopsis*

In temperate-zone forest tree species, adaptation to a climate is based primarily on the proper timing of growth and dormancy during the year. Photoperiod and its interaction with temperature are the main environmental factors that control the periodicity of growth and frost hardiness (Heide 1974; Junttila 1989). Phytochrome is generally believed to be the pigment system responsible for the perception of light (i.e., dawn and dusk) in the photoperiodic regulation of plant growth and development (Sharrock 1992). However, the molecular mechanism of phytochrome action and the components of its signaling pathway are largely unknown. Most studies have focused on 2 of the 5 identified types of phytochrome genes in plants, phytochrome A (*phyA*) and phytochrome B (*phyB*).

In collaboration with Drs. Junttila, Nilsen, and Olsen at the University of Tromsø, Norway, the *phyA* and *phyB* genes from oat and *Arabidopsis*, respectively, were expressed in our hybrid aspen under the control of the CaMV *35S* promoter. The most striking morphological alterations were shorter internodes in *phyA* transformants (figure 2D). Out of 22 *phyA*-expressing lines, as verified by RNA blot analysis, 6 lines with varying degree of phenotypic alteration were selected and characterized with respect to growth pattern and photoperiodic control of growth cessation. Preliminary data showed that lines with weak expression of *phyA* had internodes with normal elongation and that increased *phyA* expression was associated with reduced internode length. Morover, the lines with highest *phyA* expression lost their photoperiodic control almost completely. The normal, critical day length for wild-type plants is 15 h, but even exposure to a photoperiod of only 8 h did not induce bud set in these lines.

Transgenic Hybrid Aspen Expressing the *Agrobacterium tumefaciens* T-DNA IAA-Biosynthesis Genes

Plant hormones are important molecular regulators of growth and development. They are major signalers in environmentally induced growth responses and are involved in integrating patterns of growth and development within the plant. The hormone indole-3-acetic acid (IAA) is the most abundant endogenous auxin, and it plays a major role in the control of basic developmental processes such as cell division, expansion, and differentiation. IAA influences many aspects of plant anatomy and morphology; for example, leaf enlargement, internode elongation, abscission, shoot and root induction, apical dominance, and tropisms (Davies 1995). IAA also maintains the integrity of the vascular cambium, promotes cambial activity, and affects xylem cell type and anatomical structure (Little and Pharis 1995).

To investigate the effect of IAA on wood formation using the transgenic approach, we transformed a hybrid aspen with the IAA-biosynthesis genes *iaaM* and *iaaH*, originating from the soil bacterium *Agrobacterium tumefaciens* T-DNA (Guadin et al. 1994). Resulting transformants showed that both tree phenotype and wood properties can be altered using genetic engineering to modify the hormone balance (Tuominen et al. 1995). The most severe phenotypic change was in a line expressing the *iaaM* and *iaaH* genes under control of the mannopine synthase (*MAS*) 1' and 2' promoters, respectively (figure 2C, 2E). This line exhibited reduced stem growth, radially and longitudinally, had an altered leaf and root morphology, and maintained apical dominance over axillary buds after removal of the apical shoot. Wood formed in transgenic poplars expressing the IAA-biosynthesis genes was characterized in considerable detail. Not surprisingly, the xylem in these plants was altered with respect to composition, pattern, and size of the rays, fibers, and vessels (figure 3).

Hybrid aspen transformants expressing the IAA-biosynthesis genes were also characterized for free and conjugated IAA. Elevated concentrations of free and conjugated IAA were found in the basal leaves and root tips but not in the apical part of the shoot. Apparently these promoters increased the level of gene expression basipetally (Langridge et al. 1989). However, an elevated IAA concentration was not observed in the extraxylary tissues of the stem base, although RNA blot analysis showed that the *iaaM* and *iaaH* genes were both expressed in these tissues (unpublished data). This observation highlights the problem of separating the primary and secondary effects of ectopic gene expression on metabolic events and phenotypic expression. In this example, the ectopic expression of the IAA-biosynthesis genes resulted in a general growth inhibition. This growth reduction likely decreased the supply of endogenous IAA, which is produced predominantly in apical shoots and transported basipetally down the stem (Rinne et al. 1993; Sundberg and Little 1987). This is supported by more detailed IAA measurements in specific extraxylary tissues (see Uggla et al. 1996) that showed that transgenic plants had a lower concentration of IAA in the cambial cell division zone, which is the major pathway of polar transport (unpublished data). However, expression of the introduced IAA-biosynthesis genes seemed to increase IAA concentration in cambial derivatives, which resulted in an unchanged total concentration of IAA in the extraxylary tissues. Another possibility is that the transformants compensated for the induced increase in IAA biosynthesis either by down-regulating endogenous IAA biosynthesis through feedback inhibition or by enhancing IAA degradation. The important observation concerning the transformants described are that tree phenotype, and wood properties in particular, can be altered using the transgenic approach.

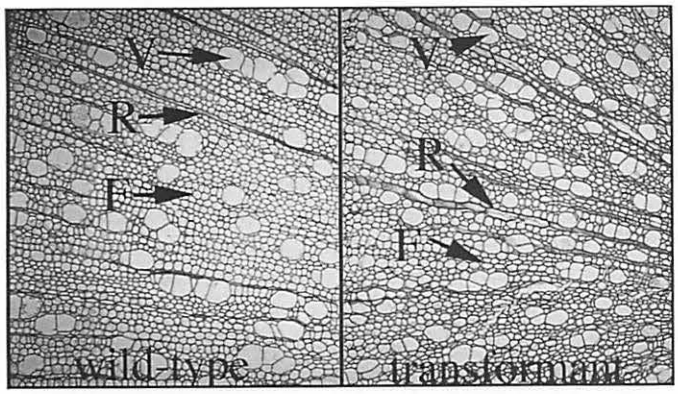


Figure 3. Transverse section of the xylem in hybrid aspen wild-type plants (left) and transformants expressing the *Agrobacterium tumefaciens* T-DNA IAA-biosynthesis genes (right). Note the distortion of radial files, the occurrence of intermediate-sized xylem elements, and the larger fibers and ray cells in transformants. V=vessel; F=fiber; R=ray.

Transgenic Hybrid Aspen Expressing the *Agrobacterium rhizogenes* T-DNA *rolC* Gene

The most compelling example of an altered phenotype in a hybrid aspen due to the expression of a single-gene insert resulted from transformation with the *Agrobacterium rhizogenes rolC* gene, which was fused to the CaMV *35S* promoter (Nilsson et al. 1996b) (figure 2F, 2G). The *rolC* gene is responsible for hairy root disease, which is caused by the plant pathogen *Agrobacterium rhizogenes* (Zambryski et al. 1989). Compared with wild-type hybrid aspen plants, *rolC*-expressing plants elongated slower, produced more leaves, generated dwarfed leaves, and displayed an altered phyllotaxis. In addition, the apex branched at irregular intervals to produce multiple leading shoots, which resulted in a bushy phenotype. Following a period of environmentally induced dormancy, outgrowth of axillary buds in the second growing season always produced short shoots in the *rolC* transformants, whereas wild-type plants formed long shoots. Another conspicuous feature of the *rolC*-expressing hybrid aspen plants was the formation of fasciated stems, which occurs when the apical meristem loses its radial symmetry and expands in 1 plane to form a ridge-like meristem (Gorter 1965). The flat stem produced by such meristems occasionally grew in a spiral (figure 2G). Overall, expression of the *35S-rolC* construct in a hybrid aspen resulted in a phenotype with drastically altered appearance.

Endogenous levels of IAA, gibberellins (GAs), and cytokinins were measured in fasciated and nonfasciated shoots of *rolC*-expressing hybrid aspen plants. Like IAA, GAs and cytokinins are plant hormones involved in controlling cell division and elongation, which are important for shoot and leaf expansion (Davies 1995). A marked reduction in the ratio of IAA to the cytokinin zeatin riboside ([9R]Z) was found in the transformants, which resulted from a small decrease in free IAA and a large increase in the cytokinin. Moreover, GA_1 was decreased. Because the biochemical activity of the *rolC* protein is unknown (Costantino et al. 1994), any observed changes in hormone levels as a primary effect of its presence is only speculation. Nevertheless, the large alterations in the auxin/cytokinin ratio and the GA_1 level, together or separately, could explain several observed changes in phenotype.

Improvement of Trees by Gene Technology

Examples presented show that growth and development in woody species can be altered by genetic engineering. This has considerable potential for accelerating tree improvement and offers the possibility of directly altering specific parts of the genome either by manipulating the expression pattern of endogenous genes or by inserting genes from other organisms. Several levels of control can be genetically modified to obtain phenotypic alterations. These include the: 1) perception of external stimuli, as in the *phyA* transformants; 2) internal balance of signal substances, as in the plants expressing the IAA-biosynthesis genes; and 3) expression of specific cellular proteins determining developmental fates, as in the *LFY* transformants. The gene pool available for tree improvement by genetic engineering is not limited to the species of interest because traits outside the genetic range of the species can be incorporated. The potential of this approach to alter plant development is best exemplified by the severely altered phenotype of the *rolC*-expressing hybrid aspen plants.

Potential Applications

LEAFY and Early Flowering

If the resulting flowers are fertile, ectopic expression of the *Arabidopsis* gene *LFY* in a tree species, which reduces the flowering time from 8 to 20 years to a few months, will have a major impact on tree breeding programs. Reducing flowering time would encourage construction of homozygous inbred transgenic lines and characterization of molecular markers over several generations and would accelerate existing tree-improvement programs. Traditional breeding techniques have increased the yield of many agricultural crop species that have a short generation time. One example is the development of cereals from

their original long, slender, low-yielding grass species to today's short, sturdy varieties with a dramatically increased seed production. However, such breeding programs have operated for thousands of plant generations, whereas programs involving tree species are much less developed due to their long time to flowering.

Recently, techniques for the construction of saturated genetic maps were improved tremendously. These techniques also detect small structural differences between DNA of individuals. This technology is used to link so-called "quantitative trait loci" (QTLs) with specific DNA markers (e.g., Bradshaw and Stettler 1995; Grattapaglia et al. 1995; Groover et al. 1994). Once a QTL has been tagged, growing full-sibling progeny to maturity to select a particular trait is unnecessary. Instead, the trait of interest can be sought at the seedling stage based on its association with a molecular marker. Using this approach, the limiting factor for the breeding program will again be time to flowering. Coupling early screening for QTLs with the application of *LFY* to shorten the generation time would accelerate traditional tree-improvement programs. Incorporating an inducible promoter system would be an additional refinement because flowering could be induced when wanted; for example, by applying a harmless chemical (e.g., Wilde et al. 1992).

Plant Hormones and Tree Growth

The morphological and anatomical changes in hybrid aspen plants expressing either the IAA-biosynthesis genes or the *rolC* gene were associated with an altered hormone balance. This is not surprising as hormones are involved in the regulation of many aspects of plant growth and development. Using genetic engineering to modify the endogenous hormone balance has obvious potential for obtaining a desired phenotype. However, the dramatic alteration of growth and development observed in our hybrid aspen transformants emphasizes the need to express factors involved in developmental control in the appropriate cells at the proper time. Depending on the target cell competence, this is particularly true with plant hormones that have distinct functions in different tissues.

Controlling modification with such factors requires promoters capable of targeting gene expression both spatially and temporally. Incorporation of an inducible promoter system would be most beneficial. The molecular tools for manipulating hormone homeostasis in specific tissues of intact plants are available because the levels of IAA, cytokinin, ethylene, and GA have been altered by genetic engineering (e.g., Hedden et al. 1995; Klee and Romano 1994). Moreover, the number of tissue-specific promoters available for experimentation is increasing rapidly. Exploiting these tools is valuable to evaluate the role of hormones in wood formation and for future gene engineering of tree growth.

Modification of Wood Properties

An obvious target for tree breeding is to improve the chemical and physical properties of wood itself (Whetten and Sederoff 1991). To date, efforts toward genetic engineering of wood have focused on the lignification process; van Doorsselaere et al. (1995) showed that lignin composition in *Populus* can be modified. However, our discussion is restricted to wood structure, which is a primary determinant of wood density and the most important factor for yield and quality of fibrous and solid wood products (Zobel and Jett 1995). The role of plant hormones in regulating wood formation was investigated for many years. Auxin, cytokinins, ethylene, and GAs are all involved to varying extents in regulating xylem cell production and structure and in determining the proportion of each xylem cell type formed in time and space (Aloni 1991; Little and Pharis 1995). IAA, whose transport is basipetally polar from crown to root in the cambial region, is key to this regulation because it maintains the structure of the vascular cambium and induces cambial cell mitotic activity and xylem cell development (Little and Pharis 1995). Therefore, it seems likely that IAA is involved in controlling the integration of crown structure and stem form and in serving as a link between external stimuli such as photoperiod, gravity, and wood formation. Underlying mechanisms, by which auxin or any other plant hormone acts in regulating wood formation, remain to be determined; however, wood structure was altered in hybrid aspen plants expressing the IAA-biosynthesis genes. Although the potential for genetically engineering wood structure is evident, other possible tools besides plant hormones are available for modifying wood density. These prospects are briefly discussed.

Wood density is determined by a complex of factors related to the: 1) structure of individual xylem cells (i.e., their secondary wall thickness and radial diameter); 2) proportion and distribution of the various types of xylem cells (i.e., fibers, vessels, and parenchyma in hardwoods, and tracheids and parenchyma in conifers); and 3) proportion and distribution of different types of wood (e.g., reaction-wood, earlywood, and latewood). Xylem cell structure is determined as cambial derivatives differentiate. During differentiation, each derivative passes through several successive developmental phases, including primary wall expansion, secondary wall formation, and programmed death for the supportive fibers and the water-conducting vessels and tracheids (Larson 1994) (figure 4). Xylem cell size and wall thickness reflect the rate and duration of the expansion and secondary wall deposition phases (Dodd and Fox 1990; Wodzicki 1971). These phases probably are independently regulated, because wide- and narrow-lumen tracheids with thick and thin walls have been induced experimentally (Larson 1969). This variability supports the idea that xylem cell structure can be manipulated in specific ways by genetic engineering.

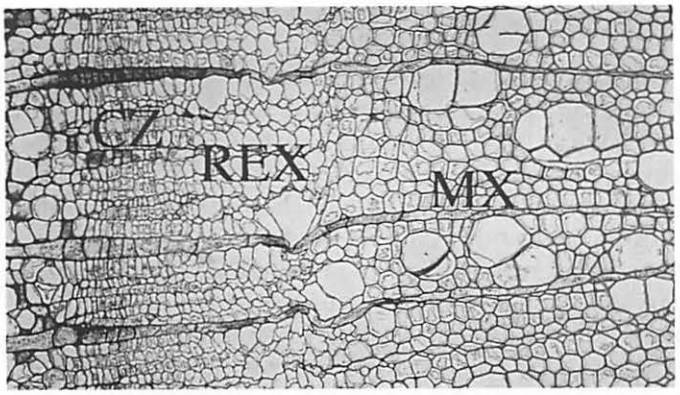


Figure 4. Transverse section of the cambial region in a current-year shoot of wild-type hybrid aspen plants showing xylem cells in different developmental phases. CZ=meristematic cambial cell zone; REX=radially enlarging xylem cells undergoing primary-wall expansion; MX=maturing xylem cells undergoing secondary-wall formation.

Natural variability of traits related to wood density is high. Many wood properties have high heritability (Zobel and Jett 1995), but wood formation is also very plastic, which suggests the importance of physiological factors in controlling wood density (figure 5). The plasticity of wood formation shows that environmental cues, such as photoperiod, temperature, gravity, mineral availability, and water status, affect wood structure and sometimes result in different wood types such as latewood or reactionwood

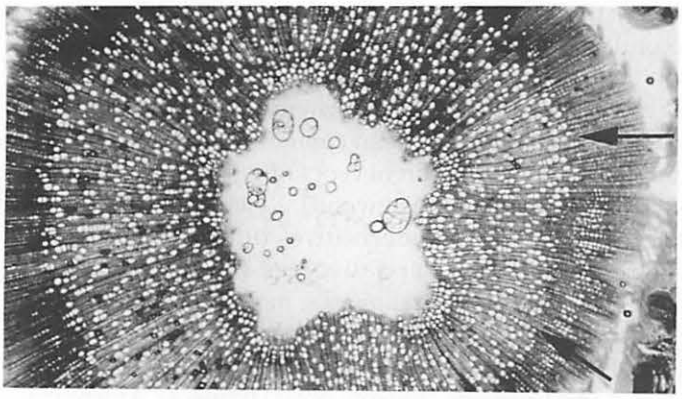


Figure 5. Transverse section of a current-year shoot of wild-type hybrid aspen plants in which the IAA balance was disturbed by applying the auxin-transport inhibitor *N*-1-naphthylphthalamic acid. Note the marked change in the type of wood formed after the treatment began (arrows), which exemplifies the plasticity of wood formation and highlights the major role of IAA in controlling wood properties.

(Creber and Chaloner 1990; Denne and Dodd 1981; Timell 1986). Moreover, wood structure varies spatially in the stem; for example, wood with juvenile characteristics is formed within the crown while more latewood to earlywood is produced at the base of the stem rather than at the top of the tree. Relative amounts of juvenilewood and latewood significantly influence wood density (Zobel and Buijtenen 1989). Variability of naturally formed wood provides additional evidence that wood formation processes are amenable to manipulation by genetic engineering. In fact, all processes that are directly or indirectly under genetic control can in theory be manipulated by this approach. Obvious targets are factors associated with controlling xylem cell production (see Savidge 1985).

Development of New Tools for the Genetic Engineering of Wood

Currently, the main factor limiting genetic engineering for wood improvement is insufficient knowledge about the regulation of wood formation. Better understanding is required of the mechanisms that operate at the molecular and cellular levels, and of the mechanisms that integrate environmental cues responsible for the seasonal and spatial changes in wood production at the whole-plant level. Further, characterization of key internal factors, which serve as links between external stimuli and wood formation and control the cascade of cellular events that determine the properties of individual xylem cells, is essential.

Many aspects of xylem cell formation are best studied using simple experimental systems that are easily cultivated and manipulated such as cell or tissue cultures of herbaceous model species (Fukada et al. 1994). For example, cultured *Zinnia* mesophyll cells were useful for investigating gene expression and molecular function of key factors involved in tracheary element differentiation (e.g., Fukada 1994; Stacey et al. 1995). Several genes induced during the differentiation process were characterized, and their spatial expression pattern was visualized in intact *Zinnia* seedlings by *in situ* localization (Demura and Fukada 1994; Ye and Varner 1994). In another approach to isolate genes involved in xylem cell differentiation, chemical mutagenesis was used at the John Innes Institute (Norwich, England) to characterize several *Arabidopsis* mutants with aberrant xylem development (M. Bevan and K. Metzlaff, personal communication). Molecular cloning of genes corresponding to known mutations in *Arabidopsis* is promoted by dense genetic and physical maps that cover known markers. Because *Arabidopsis* has become the most important model system for studying basic plant biology, it will be the major source of genes coding for conserved factors involved in plant growth and development regulation (Gibson and Sommerville 1993). As previously discussed, the usefulness of this source for the genetic engineering of woody species was recently shown with

the *Arabidopsis LFY* gene that induced early flowering in our hybrid aspen model system. Knowledge about xylem cell formation in herbaceous model systems should also be applicable in woody species.

Although experimental systems that are easily manipulated are attractive, using tree species to identify biochemical events and specific genes related to xylem cell development also has advantages. For example, due to the size of trees, specific cambial region tissues and cell types can be identified and isolated by microdissecting techniques (Hampp et al. 1990). This approach enables monitoring of the cellular status of enzymes, nutrients, and hormones with sensitive microanalytical techniques at varying stages of the differentiation of cambial derivatives (Catesson et al. 1994; Uggla et al. 1996). Similarly, polymerase chain reaction (PCR)-based techniques can be used to isolate genes that are uniquely expressed in time and space within the cambial region (Hertzberg and Olsson 1995). In addition, using tree species allows for the characterization of cambial derivatives differentiating into different wood types. For example, the transition from earlywood to latewood and from normalwood to reactionwood can be induced experimentally by photoperiod and tilting, respectively. Thus, changes in cellular events and gene expression related to these wood types can be characterized. Furthermore, using *Populus* as a model system will enable isolation of genes and characterization of their function in transgenic plants.

A combination of biochemical, molecular, and genetic approaches will be required to obtain the tools for improving wood properties by genetic engineering. Unraveling metabolic pathways, characterizing their enzymes, and cloning the corresponding genes will provide the means for manipulating known end products. Using this approach, lignin biosynthesis, carbon metabolism, and hormone balance was manipulated (Klee and Romano 1994; Stitt 1995; Whetten and Sederoff 1995). However, genes determining developmental fates, such as *LFY*, will not be isolated by this approach. For this purpose, screening for mutants with aberrant development is attractive. Additional advantages of the mutant approach include: 1) the effect of gene inactivation can be determined at both the whole-plant and cellular levels; and 2) very weakly expressed genes can be detected. Isolation of differentially expressed genes during defined phases of xylem cell formation, as with the *Zinnia* system, or during the production of specific wood types may result in unexpected and exciting discoveries that may lead to the characterization of novel cell- or tissue-specific promoters.

Undoubtedly, an increasing number of genes, enzymes, and other factors involved in xylem cell formation will be characterized in herbaceous and woody model systems. In addition, control of the activities of these factors will be better understood (Catesson 1994; Northcote 1995; Savidge 1995). This knowledge will give us tools to unravel the control of wood density and, ultimately, to use genetic engineering for wood improvement. Although the stem's main functions of storage, transport, and support must be maintained, the variability of wood structure found in nature shows that considerable modification of the wood formation process is possible.

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Chapter 12

Transgene Expression in Field-Grown Poplar[1]

Gilles Pilate, David D. Ellis, and Simon Hawkins

Introduction

Genetic engineering is a powerful tool used to modify plants that are of economic interest. This is accomplished by introducing genes that confer desirable traits such as pathogen resistance, herbicide tolerance, and modification of key metabolites. The introduction of foreign genes (transgenes) or the transformation of plants is routine for many annual crop species. Many examples are undergoing field trials and commercialization (Dale 1995). Similar transgene research on forest trees is less advanced, primarily because of difficulties manipulating woody plants, which are generally less responsive in tissue culture than herbaceous material. Also, trees are not well analyzed or understood at the genetic level. Poplar is a model species for pioneering such biotechnological procedures because it is relatively easy to manipulate *in vitro* and has a small genome. Since 1987, when the first transgenic poplar was obtained (Fillatti et al. 1987), several techniques were developed, which are now routinely used, to transform different *Populus* species or hybrids. Availability of such techniques has allowed the introduction of many genes aimed at conferring or modifying particular traits. For example, genetic transformation was used to improve herbicide resistance (Brasileiro et al. 1992; Donahue et al. 1994; Fillatti et al. 1988), insect tolerance (Leplé et al. 1995; McCown et al. 1991), and lignin metabolism (Baucher et al. 1996; Van Doorsselaere et al. 1995). Most of the above studies were conducted on plants grown under controlled greenhouse conditions. The promising results obtained from these studies need careful field evaluation before commercialization.

Trees are grown for multiple years before harvest; they are large, complex plants, which presents sampling problems; and they undergo a phase change (juvenile to mature). All of these factors could significantly influence the expression of foreign genes. Furthermore, care must be taken to prevent foreign genes from spreading into native, nontransformed populations. The situation is further complicated because, depending on the nature of the crop, different growing practices are used for different species. Thus, designing a field trial that represents specialized commercial conditions is difficult. Initial commercial exploitation of transformed trees may be restricted to high-technology, agroforestry plantations with intensive, short-rotation times. Poplar is a model system since clonal material is already grown commercially under such conditions allowing establishment of representative field trials. Additionally, the desirable traits cited above involve modifying plants grown under field conditions.

Field trials must include an evaluation of the level and stability of transgene expression. Limited information is available about transgene stability in long-lived species such as trees. This information is critical because transgene expression in annual plants is less stable than originally believed (Finnegan and McElroy 1994; Meyer et al. 1994). Similar problems may occur in perennial woody species such as trees.

Field Studies of Transgene Expression

Seasonal and Developmental Influences

Our laboratories at INRA, Orléans, France and the University of Wisconsin have established field trials of transgenic trees, including poplar and spruce hybrids, to analyze the stability and variation of the transgene expression. In the first field trial, the poplar hybrid clone 'INRA 717 1B4' *(P. tremula x P. alba)* was transformed by

[1] Klopfenstein, N.B.; Chun, Y. W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

Agrobacterium tumefaciens with the gene *uidA* coding for the protein β-glucuronidase or GUS. After supplying tissue with an appropriate enzymatic substrate, expression of the *GUS* reporter gene is detectable by blue coloration or other quantitative assays. The *uidA* gene under control of the cauliflower mosaic virus *35S* promoter, was stably integrated into the host genome. Twelve plants representing 4 independently transformed lines were regenerated, characterized at the molecular level, and introduced into the field in 1991 (Pilate et al. 1994). Leaf and stem samples from these plants were harvested at regular intervals (1992-1995), and transgene expression levels (GUS activity) were determined by GUS fluorometry and histochemistry (Jefferson 1987). After 4 years in the field, all transformed lines continued to express the *GUS* gene in all living cells and no gene silencing was observed (Pilate et al. in prep.). Significant differences in transgene expression levels were observed among different transformants, but not among different individuals from the same line (Pilate et al. in prep.). GUS activity apparently was not correlated with the copy number of the introduced *GUS* gene. Similar variation was observed among 27 transformants (*35S-GUS*) grown under greenhouse conditions (Pilate et al. in prep.); observing expression of these lines in the field is of further interest.

Field trials were initiated in 1993 with the poplar hybrid 'NC5339' (*P. alba* x *P. grandidentata* cv. 'Crandon') that was transformed by particle bombardment with a similar *GUS* construct that was under the control of an enhanced *35S* promoter. Analyses performed in 1994 and 1995 showed significant differences in expression levels among different transformed lines (Ellis et al. 1996). Although gene silencing was observed *in vitro*, it did not occur after plants were transferred to field conditions. Similarly, silenced plants *in vitro* did not initiate transgene expression after transfer to the field (Ellis et al. 1996). This suggests a degree of stability of transgene expression in the field.

In poplar clones 'NC5339' and 'NM6' (*P. nigra* x *P. maxmiowiczii*), and in several transformed lines of spruce (*Picea glauca*), the highest level of GUS activity and the least variation in *GUS* expression occurred with *in vitro* plants (Ellis et al. 1996). As plants were transferred to greenhouse, cold frame, and field conditions, the levels of *GUS* expression decreased while variation in expression among the different lines increased (Ellis et al. 1996). These differences in gene expression among different environments are possibly due to differences in relative growth rates, plant structure, and leaf anatomy.

Leaves of field-grown poplar are more robust and morphologically different than leaves from *in vitro* cultures. Leaves from greenhouse-grown plants are morphologically intermediate between tissue culture- and field-leaves. These observations help design *in vitro* selection strategies for transformants for subsequent field growth. Despite this variation, transgenic lines showing the highest expression *in vitro* also showed the highest expression level in the field (Ellis et al. 1996). With *in vitro*- or greenhouse-grown plants, minimal variation in GUS activity occurred among different individuals of the same transformant line. However, the variation increased substantially after these transformants were transferred to the field, despite using the leaf plastochron index to reduce variation due to differences in developmental stage (Ellis et al. 1996). One problem is that leaves in the field are highly variable in development, morphology, and physiology, which may reflect the variation in *GUS* expression. Relatively little variation in *GUS* expression of mature leaves from different individuals of the same transformant line indicates that developmental differences may be important in gene expression.

While the above work indicates that the developmental stage of leaves may influence expression levels, studies on poplar clone 'INRA 717 1B4' help explain the effect of the leaf spatial position within the tree on transgene expression. Leaf samples from branches at 4 different tree heights showed no significant differences in *GUS* expression. However, significant differences were observed between leaf samples harvested either at the apex or close to the trunk of the same branch (Pilate et al. in prep.). In that study, the highest expression was observed in leaves closest to the trunk. Significant differences in the transgene expression levels also were observed among different organs harvested from similar tree positions (Pilate et al. in prep.). Seasonal events also influence *GUS* gene expression in field-grown poplar trees. In poplar clone 'INRA 717 1B4,' expression was significantly higher in summer than autumn (Pilate et al. in prep.).

In the leaves of clone 'NC5339,' *GUS* expression was initially very high immediately after bud break, but within 2 weeks expression was 5 to 8 times lower than that originally observed (Ellis et al. 1996). After this drop, *GUS* expression stabilized, although considerable variation still occurred among leaves of similar developmental stages. Overall, younger leaves that were still expanding showed higher and more variable levels of *GUS* expression throughout the year in comparison with older leaves (Ellis et al. 1996). This contrasts with field expression patterns of *GUS* observed with another hybrid poplar 'NM6,' where the older fully expanded leaves exhibited higher *GUS* expression (Francis et al. 1995). In older leaves of 'NC5339,' no significant differences were observed in *GUS* expression between the 2 years and GUS levels were significantly different in younger leaves on only a few dates. Although these studies suggest a general stability of transgene expression in field-grown trees, external factors (environment) and internal factors (organ, developmental stages, metabolism, source-sink relationships, etc.) affect such expression. In addition, these observations indicate the sampling difficulties associated with tree studies and illustrate the need to collect comparable material for subsequent analyses of transgene expression.

Targeted Gene Expression

Until now, most tree species tested in the field were transformed with a reporter gene (usually *uidA* coding for GUS) under control of the cauliflower mosaic virus *35S* promoter or derivatives. The *35S* promoter reportedly induces transcription of the transgene coding sequence in all cells providing constitutive expression. However, recent results show that expression controlled by the *35S* promoter is not always constitutive. At the University of Wisconsin, poplar and spruce lines transformed with the *uidA* gene under control of the *35S* promoter exhibited tissue-specific (vascular) expression (Ellis et al. 1996). Similarly, at INRA, Orléans, of 27 poplar lines transformed with the *35S-GUS* construct, 5 lines showed some type of tissue specificity in plants grown under greenhouse conditions (Pilate et al. in prep.). It should be emphasized that the expression pattern in all transformants appeared stable. Transformants expressing *GUS* in a tissue-specific manner continue to do so, as do transformants expressing *GUS* in a constitutive manner. We have never observed a switch from one pattern of expression to another. Although the use of a *35S* promoter does not guarantee constitutive expression of an introduced gene, careful screening will probably result in the selection of transformed lines exhibiting the desired pattern of gene expression.

While constitutive promoters can be used for controlling the expression of certain genes (e.g., bacterial endotoxins from *Bacillus thuringiensis* for insect resistance), transformation with other genes may involve other strategies. In these situations, effective transgene expression may require careful spacial and temporal regulation. For example, when sterility was induced in tobacco by using an RNase gene fused to a tapetum-specific promoter (Mariani et al. 1990), the use of a constitutive promoter was impossible.

Little is known about the stability of tissue-specific or inducible promoters in trees, especially under field conditions. However, we recently began studies of such expression patterns at INRA, Orléans. Because it constitutes the timber, xylem is an interesting tree target tissue and is an ideal target for introducing genes aimed at modifying wood quality or quantity. A recent study showed that the Eucalyptus *CAD* (cinnamyl alcohol dehydrogenase) promoter *(EuCAD)* directs the expression of a reporter gene in mainly young xylem tissue (Feuillet et al. 1995). As an example of a tissue specific promoter, we used this promoter with the *uidA* gene to study the stability of targeted transgene expression in field-grown poplar trees. A number of transformants were analyzed under *in vitro* and greenhouse conditions. While differences in *GUS* expression levels were observed among different transformed lines, all transformants examined exhibited tissue-specific (vascular) expression. The pattern and stability of such expression will be analyzed under field conditions and will allow an evaluation of seasonal affects on expression controlled by a tissue specific promoter.

Similar field trials are underway at the University of Wisconsin where the *uidA* coding sequence has been functionally fused to the potato wound inducible *PIN2* promoter and introduced into 'NC5339.' Initial results indicate that the *PIN2* promoter is induced by wounding under field conditions and is active in a developmentally regulated manner (Ellis et al. 1996). While the *GUS* system is undoubtedly the most useful reporter gene for characterizing promoter expression patterns, such expression patterns should be independently confirmed. We intend to study the expression controlled by the *EuCAD* promoter under field conditions using another reporter gene (Green Fluorescent Protein-*GFP*; Haseloff and Amos 1995).

Commercial Application and Environmental Impact

Field trials with trees transformed by reporter gene-promoter combinations are useful for analyzing the expression pattern, level, and stability of any particular promoter. The potential of specific promoters to control the expression of genes of economic interest can be thoroughly evaluated by such studies. If a plant is transformed with a coding sequence for a gene of economic interest that is fused to a well-characterized promoter, we expect that the transgene expression should be controlled similarly to the previously studied reporter gene. However, interactions between the introduced gene or gene product (messenger RNA, protein) and the plant could affect the expression of the gene in an unexpected manner. Preliminary field trials using trees transformed with the actual gene of economic interest should be conducted on a scale and under conditions comparable to that of a commercial operation before commercial application. Such trials would help determine whether results from young plants grown under controlled conditions are consistent with those from field-grown trees.

Since the range of possible transformations is almost infinite, the environmental impact of each transformation must be thoroughly evaluated. For example, a transformation conferring insect resistance could strongly impact the ecosystem and result in: 1) an adaptive advantage for the trees should they become weeds; 2) the possible evolution of resistant insect biotypes; and 3) altered competition among different insect species. Conversely, modifications of lignin metabolism may have less environmental impact, although such modifications could impact the transformed tree's disease and / or stress resistance.

To assess field performance and environmental impact of trees transformed for insect resistance, Leplé et al. (1995) separately introduced 2 genes into a poplar clone (*P. tremula* x *P. tremuloides* cl. '353-38') and investigated the affect on the clone's resistance to a poplar leaf beetle, *Chrysomela*

tremulae (Coleoptera: Chrysomelidae). A gene encoding a cysteine protease inhibitor (*OCI*) was used first. Then, a gene encoding an endotoxin from *B. thuringiensis* (*Bt*) (*cryIIIA*) was used. Both of these gene products were shown to be effective against Coleoptera. Following molecular characterization, 3 transformants were selected that overexpressed the *OCI* gene. Greenhouse assessments of this approach showed that target insect mortality was increased by approximately 40 percent (Leplé et al. 1995). Following this initial success, field trials were established under growing conditions comparable to commercial plantations, with 10 individuals from each of 2 transformed lines (Leplé et al. 1995). The performance of these transformed trees will be evaluated over an 8-year period for growth, resistance to the target insect, and effects on target and nontarget insect species. Such a study should provide valuable information on the long-term stability of expression with such constructs, and their environmental impact under field conditions. Over the last 3 growing seasons, field trials at Wisconsin with poplar hybrid 'NC5339' containing a *cryIA*(a) gene, have revealed no loss of *Bt* gene expression. This indicates that expression of an economically interesting coding sequence and reporter genes is apparently stable over multiple growing seasons (Kleiner et al. 1995).

Lignin metabolism is another target for genetic transformation. When making paper pulp, lignin is separated from fibers by chemical processes that are economically and environmentally costly. In a European-funded research project (ECLAIR-OPLIGE (N° AGREE 0021 - C)/FAIR-TIMBER (PL95424)), a poplar *CAD* coding sequence was introduced into poplars in an antisense orientation to try to reduce the endogenous activity of CAD and modify lignin content (refer to chapter by Boerjan et al.). As part of the FAIR-TIMBER program, field trials are underway using transgenic poplar plants that show a reduced activity in CAD or *O*-methyltransferase (OMT), another enzyme involved in lignin biosynthesis (refer to chapter by Boerjan et al.). The effects of these transformations, which were evaluated in plants grown under controlled conditions (Van Doorsselaere et al. 1995), on lignin quality and quantity will be investigated in field-grown plants after 4 and 8 years. Transgene expression in such transformed plants can be followed by monitoring enzymatic activity (CAD, OMT) and, in plants transformed with the antisense *CAD* sequence *(ASCAD)*, by the phenotypic red coloration induced in the xylem. These genetically modified poplars were transformed with the antisense coding sequences *(CAD, OMT)* under the control of the *35S* promoter (Van Doorsselaere et al. 1995). However, future commercialization of transgenic poplars will probably use an antisense sequence under the control of a tissue-specific promoter.

As part of the FAIR TIMBER Program, poplar plants are being transformed with the *ASCAD* coding sequence under the control of the *EuCAD* promoter to investigate the above combination. The stability of transgene expression in these plants will be followed under *in vitro*, greenhouse, and field conditions. Such experiments should provide information about the interactions among a tissue specific promoter, an endogenous plant coding sequence in an antisense orientation, and in the field environment.

Future Prospects

Future research work should focus on the underlying mechanisms of transgene instability (Matzke and Matzke 1995; Meyer et al. 1994) and ways to reduce such variation (Bhattacharyya et al. 1994; Dejong et al. 1994; Mlynarova et al. 1994) under controlled conditions. Research efforts should also focus on understanding the complex environmental effects, including temperature, light, abiotic stress, and biotic stress, on transgene expression in field-grown trees. Another important research area from a commercial viewpoint is the frequency of gene silencing and the reduction in expression levels that can occur after transfer from *in vitro* conditions to the field. Is this a genuine problem or can lines that are prone to expression instability be detected and eliminated before the field stage?

Many commercial transformations may require targeted transgene expression using tissue-specific promoters. Thus, studies that characterize other tissue-specific promoters are an important area of future research. The rapid rate that new plant genes are being characterized will provide new coding sequences for desirable traits that can be combined with tissue-specific/inducible promoters to expand the range of plant improvements. Such advances will require continued trials to investigate these promoter-coding sequence combinations in the field.

Biotechnological research with transgenic trees is less advanced than with many other shorter-lived species. This situation is reflected in the strict regulations governing establishment of field trials and field introduction of genetically modified trees (GMTs). In Europe, the poplar root system, which is superficial, is contained by burying plastic sheeting in the soil surrounding the plantation and maintaining a plant-free zone. All flowers must be manually removed as soon as they appear. Although these labor-intensive precautions may seem detrimental to future commercialization of GMTs, strict regulations are not in force everywhere. In the United States, the regulations concerning the release of GMTs into the field are less strict than in Europe. In addition, Strauss et al.(1995) have presented a solution to the concerns about the spread of foreign genes by using transformation technology to produce sterile trees. Hopefully, risk assessment studies will be refined and enlarged to encompass more tree species and additional knowledge will be gained about the environmental impact of particular introduced coding sequences.

This information should allow streamlining of regulatory procedures to facilitate transgenic tree commercialization (Dale 1995).

Most field trials involving transgenic poplar have used clones that are not of immediate economic interest but are easy to transform. In contrast, poplar clones of commercial interest ('NM6') and potential commercial interest ('NC5339') were used in field trials at Wisconsin, where transgene expression was stable (Ellis et al. 1996). Present efforts to transform poplar clones of economic interest will intensify, and field trials with such clones will likely be initiated shortly after transformation protocols are available. Another step toward the commercialization of transgenic poplar is to develop protocols and technology to introduce more than 1 transgene. The introduction of multiple genes may allow modification or introduction of completely different traits or affect a single trait controlled by several genes. For the most efficient expression of these transgenes, different combinations of coding sequences and promoters will probably be used. Field trials should prove useful and are essential for evaluating the complex interactions between the environment and introduced genes.

Another important question concerns the future exploitation of these introduced genes and the plants containing them. Will such modified trees be propagated solely *in vitro* or be used as parents in crosses with other nontransformed or transformed elite plants? One prerequisite for evaluating the use of transformed plants is knowing what happens to the introduced gene(s) in such crossings; how it segregates and how stably it is expressed in the progeny. Pilate et al. (in prep.) are analyzing the results of a controlled cross involving 1 of the field-grown, transgenic *(35S-GUS)* poplar and will soon address this important point.

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Chapter 13

Transgenes and Genetic Instability[1]

M. Raj Ahuja

Introduction

Genetic improvement of forest trees by traditional breeding and selection methods is a slow process. Because of long life cycles, these approaches may have limited application in forest trees. Even in annual plant species, traditional approaches involving hybridization and backcrosses may take several generations for transfer of desirable genes. Genetic engineering, on the other hand, offers prospects for genetic improvement at an accelerated pace in herbaceous and woody plants.

A major problem regarding gene transfer is how to regulate integration and expression of foreign genes in plant and animal genomes. Although many promoters of viral, bacterial, and plant origins have been tested in herbaceous plants, little is known about their regulatory control in forest trees. Another problem presented by gene transfer is stability of the integrated transgenes in forest tree genomes. Although originally transgenes were considered stably integrated and expressed, recent reports on herbaceous crop plants suggest that transgene expression is not always stable in the transgenic plants. Gene silencing, partial or complete inactivation of recombinant genes or endogenous genes, has been observed in several herbaceous transgenic plants (Finnegan and McElroy 1994; Jorgensen 1992, 1995; Kooter and Mol 1993; Matzke and Matzke 1993, 1995; Meyer 1995; Paszkowski 1994). Furthermore, transgene expression levels vary among independent transformants, which may be determined by copy number and/or integration transgene site (Hobbs et al. 1990, 1993; Jones et al. 1985; Pröls and Meyer 1992). This suggests that it is difficult to predict if a transgene would be stably integrated or expressed in plants, whether herbaceous annuals or woody perennials. Does a chimeric gene integrate either at a specific site or at any available slot in the genomic landscape? Are these locations assigned by specific endogenous genetic sequences, or does the alien gene choose any location in the genome? Although the answers to these questions are currently unknown, research in genetic engineering is progressing so rapidly that these questions may soon be resolved.

Forest trees have long life cycles, with an extended vegetative phase ranging from 1 to several decades. Because trees are firmly anchored in 1 location, they are exposed to changing environments over long periods that may influence their physiology and morphogenetic processes. For long-term survival, trees must adapt to the new challenges posed by the changing environment. Under such conditions, genes conferring low fitness in trees, for example some transgenes, may be silenced or eliminated. Therefore, genetic transformation in woody plants must be investigated to understand genetic instability on a short- and long-term basis. Questions on the genetic instability of transgenes in long-lived forest trees (Ahuja 1988a, 1988b, 1988c) include: Will the foreign genes be stably integrated and expressed immediately in a specific tissue or in all tissues? Will foreign genes be expressed immediately but remain inactive for a long time, then be re-expressed? Will foreign genes undergo rearrangement or be lost during the long vegetative phase of forest trees? Will foreign genes cause genetic changes in the host genome via a position effect involving 1 or multiple copies of a transgene in the host genome?

In this review, I will discuss published experimental data and present theoretical arguments on the stability and instability of transgenes in woody plants using published reports on transgene inactivation in annual herbaceous plants. Recent investigations are also presented on transgene stability and expression in *Populus* and other woody plants. Because the techniques and methods for genetic transformation by *Agrobacterium*-mediated gene transfer or DNA delivery through a microprojectile-bombardment system have been adequately described in ear-

[1] Klopfenstein, N.B.; Chun, Y.W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

lier reviews (Binns and Thomashow 1988; Charest and Michel 1991; Chupeau et al. 1994; Hooykaas and Schilperoort 1992; Jouanin et al. 1993; Kung and Wu 1993; van Wordragen and Don 1992) and in other chapters in this volume, they are not discussed here.

Genetic Transformation

The following conditions must be present before gene transfer is possible in plants: 1) an efficient *in vitro* regeneration system; 2) a vector system for the transport of the chimeric gene(s); and 3) an efficient gene transfer system. Once the transgenic plants are regenerated, there is a risk from genetic instability (internal), and a risk to humans, animals, and the ecosystem (external). I will address the genetic risk, whether caused by gene interaction or external factors.

Regeneration System

Many excellent *in vitro* regeneration systems were developed in multiple plant species, including woody plants. Some of the reasons that *Populus* is a model system in forest biotechnology and genetic engineering include:

- fast growth,
- short-rotation cycles,
- growth on marginal sites,
- vegetative phase normally lasts 7 to 10 years,
- *in vitro* technology offers prospects for early maturation that may reduce the vegetative phase to 3 to 4 years,
- unisexual flowers,
- *in vitro* regeneration systems are developed for many species,
- rejuvenation from mature trees is achievable by tissue culture,
- relatively small genome (1/10th the size of a pine genome),
- molecular genome maps are available, and
- genetic transformation and regeneration of transgenic plants are achievable in certain aspens and poplars.

Excellent regeneration systems are available in aspens (Ahuja 1983, 1986, 1987a, 1993) and poplars (Chun 1993; Ernst 1993), which lend them to genetic transformation and regeneration of transgenic plants (Chupeau et al. 1994; Confalonieri et al. 1994; Devillard 1992; Donahue et al. 1994; Fillatti et al. 1987; Fladung et al. 1996; Howe et al. 1994; Klopfenstein et al. 1991, 1993; Nilsson et al. 1992; Tsai et al. 1994).

Chimeric Gene and Vector System

Several chimeric genes have been transferred to *Populus* by the *Agrobacterium*-mediated gene transfer system, particle acceleration DNA delivery system (McCown et al. 1991), and electroporation of plasmids into poplar protoplasts (Chupeau et al. 1994). In most studies, at least 2 chimeric genes were transferred simultaneously, 1 as a selectable marker and the other as a reporter gene. The selectable marker genes normally used are antibiotic-resistance genes that confer kanamycin or hygromycin resistance to the transformed tissue. Kanamycin resistance is conferred by a bacterial neomycin phosphotransferase (*NPTII*) gene and 2 promoters, *NOS* (nopaline synthase) and *OCS* (octapine synthase), which are used to control its expression. Alternatively, several alien genes have been used as reporter genes. These may include those that are biochemically detectable, and others that have a characteristic phenotypic expression. One biochemically detectable gene, the *GUS* (β-glucuronidase) gene from *E. coli* bacteria, has been extensively used in many plant genetic transformation studies. In most studies, *GUS* expression is driven by a *35S* promoter from the cauliflower mosaic virus (*35S-GUS*). *GUS* expression can be histochemically detected in transgenic plant tissues. Several different chimeric gene constructs were used to confer pest and herbicide tolerance in poplars. A proteinase inhibitor II (*PIN2*) gene from potato conferring pest tolerance under the control of a *35S* or *NOS* promoter (*35S-PIN2* or *NOS-PIN2*) was expressed in a transgenic hybrid poplar clone (*P. alba* x *P. grandidentata* cv. 'Hansen') (Klopfenstein et al. 1991, 1993). Another bacterial mutant gene, *aro*A coding for 5-enolpyruvyl-shikimate 3-phosphate (EPSP), driven by a *35S* promoter, conferred herbicide tolerance in another hybrid poplar clone (*P. alba* x *P. grandidentata* cl. 'NC5339') (Donahue et al. 1994). In an earlier study using a chimeric gene fusion carrying the *aro*A gene with a *MAS* (mannopine synthase) promoter (p*MAS-aro*A) (Fillatti et al. 1987), herbicide tolerance was not well expressed in hybrid poplar leaves (Riemenschneider and Haissig 1991). As with *NPTII* or *GUS* genes, the morphological effects of *PIN2* or *aro*A expression were undetected, except for the action of the introduced gene product.

In a recent investigation, we used the *rolC* gene from *A. rhizogenes*, under the expressive control of *35S* and *rbcS* (from potato) promoters (*35S-rolC* and *rbcS-rolC*), for ge-

netic transformation of European aspen (*P. tremula*) and hybrid aspen (*P. tremula* x *P. tremuloides*) clones (Ahuja and Fladung 1996; Fladung et al. 1996). Expression of the *rolC* gene is detected at the phenotypic level by reduced leaf size and reduced chlorophyll content that produces a pale-green colored leaf in comparison to the dark green leaves of untransformed aspens. Since expression of *rolC* is detected at the morphological level, it offers prospects for continuously monitoring expression during growth and development of transgenic plants. Consequently, *rolC* can be used to address questions regarding genetic stability of transgenes in woody plants at the phenotypic and molecular levels. Transgenic plants carrying other chimeric genes are also suited for similar analysis. In cases such as *aro*A and *PIN2* genes, those lacking phenotypic expression must be analyzed at specific intervals by biochemical methods and then exposed to pests or herbicide for tolerance monitoring.

It could be argued that chimeric genes, driven by promoters from viruses, bacteria, or plants, then introduced in herbaceous or tree species, are subject to rejection by the plant genome as foreign entities. Because of specific integration sites or sequence homology in the genome, integrated transgenes are not a mainstream part of the genetically and evolutionarily adapted plant species in space and time. Such transgenes may be subject to gene interaction, inactivation, and loss depending on the endogenous and exogenous environments. Genetic variation occurs in callus cultures and tissue culture-derived plants. Transgenic plants apparently can inherit genetic instability from the tissue culture process and from foreign gene transfer; both sources are examined.

Tissue Culture Related Genetic Instability

Tissue culture-induced genetic variation has been observed in many plant species (Ahuja 1987b; Bajaj 1990; Kaeppler and Phillips 1993; Larkin and Scowcroft 1981; Phillips et al. 1994; Skirvin 1978). In particular, callus cultures are prone to genetic changes and exhibit different kinds of genetic aberrations. Various terms have been used to describe plant variants regenerated from tissue culture. Plants regenerated from stem callus are called "calliclones" (Skirvin 1978), and those from leaf protoplasts are referred to as "protoclones" (Shepard et al. 1980). Larkin and Scowcroft (1981) proposed the general term "somaclonal variation" for plant variants regenerated by any type of somatic cell culture. In contrast, variation detected in haploid cell cultures are called "gametoclonal variation" (Evans et al. 1984).

The value of somaclonal variation in horticultural and agricultural crops has been adequately described (Bajaj 1990; Evans and Sharp 1983; Evans et al. 1984; Larkin and Scowcroft 1981; Larkin et al. 1984). I will focus on the possible role of the media components, in particular hormones, in the induction of genetic variation during cell culture. I will also argue that using cultured cells for genetic engineering adds a risk of genetic instability in transgenic plants. Somaclonal variation apparently occurs at a higher frequency in cultured cells than in spontaneous mutations in uncultured plant cells. Most commonly observed genetic variations include changes in chromosome number and structure (D'Amato 1978), gene mutations, and epigenetic changes involving DNA methylation (Brown 1989; Kaeppler and Phillips 1993; Müller et al. 1990). In addition, repeat-induced point mutation (RIP) may also contribute to the genetic instability of cultured plants cells (Phillips et al. 1994). Some variation induced in tissue culture is not heritable and may be an epigenetic modification. All types of somaclonal variation are not useful; most genetic variation induced in tissue culture is undesirable.

Factors involved in somaclonal variation in plants include: genotype, donor plant age, explant source (stem segments, meristems, leaf discs, root segments), medium composition, length of time tissues are kept in culture, and culture conditions. Exposing cells in the culture medium to relatively high levels of exogenous phytohormones, such as 2,4-dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA), 6-benzyladenine (BA), or other chemicals, which are several magnitudes higher than the physiological endogenous concentrations, may strongly influence the induction of somaclonal variation. In particular, 2,4-D and other auxins may be involved in increased DNA methylation and may induce RIP in plant tissue cultures (Phillips et al. 1994). Therefore, changes in the genome are expected when cells are removed from their normal surroundings and placed in an artificial tissue culture environment (McClintock 1984). Cultured cells are under constant stress *in vitro*. As a result, the genome adapts to this new environment and becomes more error-prone. This adaptation may depend on the genotype, but many genotypes exhibit genetic instability when placed in the tissue culture environment.

Are Transgenic Plants Genetically Stable?

In a 1987 symposium titled "Genetic Manipulation in Woody Plants" in East Lansing, Michigan (Hanover and Keathley 1988), several reports were presented on the status of biotechnology in woody plants including one on

gene transfer in forest trees (Ahuja 1988a). At that time, genetic engineering in annual plants was newly established but was still in its infancy in forest tree species. The few existing studies mainly involved testing the efficacy of *Agrobacterium* strains for inducing galls on trees (Ahuja 1988a; Chapham and Ekberg 1986; Sederoff et al. 1986). Around that time, the first report was published on *Agrobacterium*-mediated transfer of the *aro*A gene from *Salmonella* bacteria to confer herbicide tolerance in the hybrid poplar clone 'NC5339' (Fillatti et al. 1987). Variation occurred in the type of shoots produced *in vitro* following genetic transformation (Fillatti et al. 1987). Also, *aro*A gene expression was less than expected when transgenic poplars were sprayed with the herbicide glyphosate (Riemenschneider and Haissig 1991; Riemenschneider et al. 1988). These variations may be attributed to genetic or epigenetic changes in the poplar genome, perhaps due to transgene position effect, bacterial hormonal genes, or less than optimal transgene expression.

Based on transgenic poplars and earlier experience with the genetic tumor systems of *Nicotiana* and *Lycopersicon*, which also involved gene transfer from 1 species to another by conventional backcrossings (Ahuja 1962, 1965, 1968; Doering and Ahuja 1967), it was proposed that foreign genes may be unstable or poorly expressed under greenhouse or field conditions. Unexpected changes or expression loss of a foreign gene can occur during the extended vegetative phase in long-lived forest trees. Variability occurring in transgenic plants, possibly associated with an *in vitro* transformation and a gene transfer event, which may be independent of somaclonal variation, was termed "somatoclonal variation" (Ahuja 1988a, 1988b, 1988c). Somatoclonal variation may be epigenetic or heritable and due to any of the following factors (Ahuja 1988c), which occur singly or in combination in the plant genome after genetic transformation:

- loss of expression of a foreign gene,
- rearrangements in the copy number of a foreign gene,
- loss of a foreign gene,
- copy number of a foreign gene inserted in the host genome,
- integration site of a foreign gene in the genome,
- position effect, and
- rearrangements caused by a foreign gene in the host genome.

The first published report on transgene inactivation by another nonlinked transgene was based on a double transformation mediated by *Agrobacterium* in transgenic tobacco (Matzke et al. 1989). Subsequently, many reports on gene silencing have been published involving the inactivation of 1 transgene by another, and widely ranging transgene inactivations involving homologous endogenes or ectogenes (de Lange et al. 1995; Finnegan and McElroy 1994; Flavell 1994; Flavell et al. 1995; Jorgensen 1992, 1995; Kooter and Mol 1993; Matzke and Matzke 1990, 1993, 1995; Meins and Kunz 1995). Levels of transgene expression seem unpredictable in the transgenic plants and appear to vary among independent transformants.

In a recent informal survey of more than 30 biotechnology firms working on the commercialization of transgenic crops, nearly all companies reported observations of transgene inactivation (Finnegan and McElroy 1994). Unfortunately, much of this information may or may not be published.

We used *Populus* as a model system to investigate the stability and expression of transgenes at the morphological, physiological, and molecular levels (Ahuja and Fladung 1996; Faldung et al. 1996). Using an *Agrobacterium* binary vector system, 4 clones of European aspen (*P. tremula*) and hybrid aspen (*P. tremula* x *P. tremuloides*) were genetically transformed with different chimeric genes, including the *rolC* gene from *A. rhizogenes*. A dominant, pleiotropic gene, *rolC* exhibits pronounced phenotypic and physiologic affects on transgenic plants. Since *rolC* expression is detectable at the morphological level, it can be used as a selectable marker for monitoring phenotypic response, molecular expression, and genetic stability in transgenic plants. Two types of promoters, *35S* from the cauliflower mosaic virus and *rbcS* from potato, were used to control *rolC* gene expression. A second gene for kanamycin resistance (*NPTII*) under control of a *NOS* promoter was also included in the gene construct. By using an improved leaf-disc transformation method, putative transformants were regenerated on a kanamycin-containing medium.

During the past 2 years, more than 1,000 transgenic aspens have been regenerated and grown in the greenhouse to investigate their morphologic and genetic stability. Transgenic aspens carrying the *35S-rolC* gene construct had much smaller leaves when compared to untransformed controls. In contrast, *rbcS-rolC* transgenics showed only slightly smaller leaves when compared to the controls. However, transgenic aspens carrying either *35S-rolC* or *rbcS-rolC* transgene exhibited pale-green leaf color in the 1-year-old plants when compared to the dark-green leaf color of the untransformed controls. In the second year of growth, the leaf size remained characteristic of the *35S-rolC* or *rbcS-rolC* transgenic aspens, and the young leaves were pale-green; however, the leaves turned dark green with maturation. Several leaf abnormalities, including 1 leaf showing half pale-green and half dark-green sectors, chimeras, and revertants to normal state, were observed in the transgenic aspens. In addition, a couple of transgenic aspens that tested positive for the *rolC* gene by PCR (poly-

merase chain reaction) were negative for the *rolC* phenotype. Southern blot analysis, using a nonradioactive hybridization method (Fladung and Ahuja 1995), revealed that most of the independent transformants carried a single integrated copy of the *rolC*. However, some transgenic aspens carried 2 or more transgene copies (Fladung et al. 1996).

We will continue molecular analysis of the transgenic aspens showing growth abnormalities including chimeras. In addition, we are investigating the mechanism of transgene inactivation in aspens. To learn how the *35S-rolC* or *rbcS-rolC* transgenic aspens perform under field conditions, it is important to determine if the *rolC*-controlled phenotypic traits are expressed under environmental conditions in the greenhouse and field.

Expression regulation of another chimeric gene, *uidA* (*GUS*) under control of different promoters, was investigated in poplar and spruce (Ellis et al. 1996; Olsson et al. 1995). In the *rolC-uidA* transgenic hybrid aspen (*P. tremula* x *P. tremuloides*), GUS activity was tissue-specific and mainly localized in the vascular tissue before the winter dormancy. However, during the onset of dormancy, *GUS* expression shifted from phloem to include cortex and pith cells. After exposure to temperatures that induced final dormancy changes, *GUS* expression disappeared from all stem tissues. In contrast, *GUS* expression in the stem of *35S-uidA* transgenic hybrid aspen was strong in all tissues, except the vascular cambium and xylem, and did not vary in intensity during growth and dormancy (Olsson et al. 1995).

Variation in *GUS* expression was also monitored in the *35S-uidA* transgenic poplars and spruce (Ellis et al. 1996). In 1 hybrid poplar clone (*P. alba* x *P. grandidentata* cl. 'NC5339'), *GUS* expression was more variable during the growing season in younger leaves than in mature leaves. However, GUS activity was relatively higher in older than younger leaves in another hybrid poplar clone (*P. nigra* x *P. maximowiczii* cl. 'NM6'). In spruce (*Picea glauca*), GUS activity was detected in needles only during their elongation but persisted throughout the growing season in the stems. Based on GUS levels in transgenic poplars and spruce, Ellis et al. (1996) concluded that *GUS* expression was least variable during *in vitro* culture of regenerated transformants and most variable during field growth of transgenic plants. These studies on GUS activity suggest that recombinant gene expression is dependent on promoter type, plant genotype, and environmental conditions.

Inactivation of Transgenes and Endogenous Genes

All alien genes, whether introduced into plants by genetic engineering or introgressive hybridization, are subject to genomic scrutiny. Acceptance or rejection of foreign genes may depend on their interaction with endogenous genes, and on their integration site within the host genome. In a well-adapted organism, new genetic introductions, and most new endogenous mutations, are not well tolerated; transgenes are not exceptions. They are alien, reconstituted, hybrid genes, chimeric for the promoter and reporter genes located between the left and right borders of the T-DNA in a plasmid vector. Transgenes are subject to inactivation by the homologous or nonhomologous endogenes and by environmental factors in the greenhouse, under field conditions, and *in vitro*. Similar parameters may apply to the modification of expression or inactivation of 1 endogene by another nonlinked endogene, a transposon, or alleles at 1 locus.

Gene silencing may be due to *trans*inactivation (unidirectional) or cosuppression (bidirectional) in transgenic plants. Several hypotheses on gene silencing have been recently proposed (Bester et al. 1994; Finnigan and McElroy 1994; Flavell 1994; Jorgensen 1992, 1995; Kooter and Mol 1993; Matzke and Matzke 1993, 1995; Meins and Kunz 1994). Although these modes of gene silencing are not mutually exclusive, no single hypothesis can explain the origins of transgene instability. Alternatively, several different mechanisms for gene silencing might occur depending on: 1) the hybrid chimeric gene construct; 2) the promoter type; 3) the plasmid/DNA delivery system; 4) whether it is a single or double genetic transformation; 5) the extent of homology between the transgene(s) and the endogene(s); 6) the plant species; 7) the genotype; and 8) the hormonal regimes in the *in vitro* regeneration system used. Therefore, transgene inactivation may occur at several different DNA/RNA levels. Possible mechanisms of gene silencing at the transcriptional and post-transcriptional levels are briefly discussed.

Transcriptional Silencing

Transcriptional gene inactivation occurs at DNA/RNA levels presumably by increased methylation, paramutation, or other mechanism(s) (Flavell et al. 1995; Matzke and Matzke 1995; Meins and Kunz 1995). By using a hybrid chimeric gene *35S-uidA/NOS-NPTII* in tobacco, Hobbs et al. (1990) observed intertransformant variation in *uidA* gene expression by monitoring GUS activity in the R_1 and R_2 generations. High and low levels of *GUS* expression were observed in transgenic tobacco leaves. Transformants in the high group had a single copy of the transgene, while those with low GUS levels had multiple T-DNA integrations into the tobacco genome. Plants with multiple transgene copies exhibited increased methylation of the integrated T-DNA (Hobbs et al. 1990). However, transgene copy number and expression were not correlated in other studies (Dean et al. 1988; Hoeven

et al. 1994; Jones et al. 1987; Shirsat et al. 1989). Later experiments by Hobbs et al. (1993) showed that multiple T-DNA insertions *per se* were not associated with low GUS activity. However, the configuration of a particular insert due to T-DNA rearrangement during transformation determined whether the GUS activity was low or high. Further, *trans*interactions between single and multiple inserts caused partial or total suppression of GUS activity. Correlation between T-DNA methylation and inactivation of T-DNA encoded genes was observed in several other studies (Meyer 1995); however, it is unclear whether methylation was the cause or the effect of transgene inactivation.

Another well-studied instance of transgene silencing involves the transfer of the maize *A1* gene, which produces brick-red pigmentation in flowers, into a white-flower mutant of *Petunia hybrida*. Transgenic petunias exhibited a flower color ranging from brick red to variegated to white (Linn et al. 1990; Meyer et al. 1987). Most transgenic plants carrying multiple copies of the *A1* gene were white, while plants carrying a single copy of the transgene had brick-red flowers (designated line RL-01-17). To test transgene stability under different environmental conditions, 30,000 transgenic petunias carrying a single copy of the *A1* gene (line RL-01-17) were planted under field conditions (Meyer et al. 1992). During the growing season, variegation in flower color was observed; the number of plants with white, red, variegated, and weakly colored flowers was variable. Plants with red flowers during the early growing season often produced flowers with weakly colored petals later in the season. Reduction in *A1* gene expression was correlated with increased methylation of the *35S* promoter that controlled transgene expression (Meyer et al. 1992). Hypermethylation was restricted to the transgene and did not spread to adjacent areas of the hypomethylated endogenous DNA in the transgenic petunia (Meyer and Heidmann 1994). Such studies suggest that a transgene is inactivated by the plant genome when it is recognized as a foreign entity.

Environmental factors modify or silence genes introduced into 1 species from another by hybridization. Such introduced genes are not chimeric genes, but are alien genes with some or no homology to the endogenous genes, and they cause genetic instability and developmental changes in host plants. One such gene, frosty spot (*Frs*), was introduced from *Lycopersicon chilense* into the genetic background of *L. esculentum* by repeated backcrossing (Martin 1966). *Frs* is a dominantly inherited gene that causes many phenotypic and developmental abnormalities in the hybrid derivatives such as: 1) dwarfed, weak plants with poorly developed xylem in the stem; 2) tumor-like outgrowths on the abaxiel surface of the leaves; and 3) abortive flower set in the greenhouse (Ahuja and Doering 1967; Doering and Ahuja 1967). The tumor-prone genotype carrying the *Frs* gene behaved quite differently under field conditions; it grew, blossomed, and set fruit like normal tomato plants but failed to develop tumors. On a morphological basis, tumor-prone and normal genotypes were indistinguishable under field conditions. This epigenetic change was reversible when progeny from these plants were grown in the greenhouse (Doering and Ahuja 1967).

Genetic tumors from different interspecific combinations in *Nicotiana* also exhibited a different response to environmental conditions (Ahuja and Hagen 1967). Tumors generally develop on specific genotypes after the flowering stage; however, they can be induced at any stage of plant development by stresses including wounding, hormonal treatment, or irradiation (Ahuja 1965; Smith 1972). Some tumor-prone genotypes are more sensitive to environmental conditions than others. For example, tumor-prone hybrid derivatives carrying a *Tu* gene on a specific chromosome fragment from *N. longiflora* in the genetic background of *N. debneyi-tabacum* (Ahuja 1962, 1968), developed tumors under greenhouse and field conditions (Ahuja and Hagen 1967). In contrast, a nontumor mutant isolated by irradiation of seed from tumor-prone amphidiploid *N. glauca-langsdorffii* (Izard 1957), behaved differently under greenhouse, field, and irradiation environments. Although questions remain regarding the dominant nature of the nontumor mutant (Ahuja 1996; Smith and Stevenson 1961), it or the nontumor F_1 progeny from crosses between nontumor and tumor-prone genotypes developed variable tumors when grown in the greenhouse (Ahuja 1996) or when exposed to irradiation (Durante et al. 1982; Smith and Stevenson 1961). However, because tumors did not develop on these genotypes under field conditions, environmental factors must play an important role in the activation or silencing of another gene class, the tumor genes in *Nicotiana*.

Besides inactivation of the *A1* transgene by methylation of its *35S* promoter in the petunia line 17-R (Meyer and Heidmann 1994), other mechanisms, such as paramutation (Brink 1973), may operate in gene silencing. The transgenic line 17-R carries a single copy of the maize *A1* gene. However, a reduction or inactivation of *A1* gene expression can occasionally be observed in flowers of the same line. A white derivative, line 17-W, was isolated from the homozygous line 17-R in which the brick-red pigmentation was undetectable (Meyer et al. 1993). In line 17-W, the *35S* promoter of the *A1* was found hypermethylated, in contrast to its hypomethylated state in the transgenic petunia line 17-R, which usually remains transcriptionally active (Pröls and Meyer 1992). Following hybridization between the red and white petunia lines, the heterozygous petunias carrying 17-R and 17-W alleles did not exhibit the expected *A1*-mediated, brick-red color. Instead, variable expression of the allelic transgenes

(17-R/17-W) was observed in the flower color. Meyer et al. (1993) proposed that differential methylation of ectopic homologous alleles may cause *trans*inactivation. Presumably the 17-W allele induced a paramutation or a directed epigenetic change of the 17-R allele in heterozygous petunias.

Inactivation of homologous and nonhomologous transgenes can occur in transgenic plants. *Trans*inactivation was observed in transgenic tobacco plants that were sequentially transformed with two selectable markers, kanamycin and hygromycin resistance, encoded by T-DNA I and T-DNA II, respectively (Matzke and Matzke 1990; Matzke et al. 1989). Inactivation of T-DNA I occurred in the presence of T-DNA II, probably by methylation of the promoter in T-DNA I (Matzke and Matzke 1991). Following segregation of the two T-DNAs, transgene (T-DNA I) expression was restored after several generations. This change was accompanied by partial or complete demethylation of the promoters in the transgenic tobacco. Epistatic interaction between the two transgenes occurred again, when both the T-DNAs were brought together in a hybrid following sexual hybridization (Matzke et al. 1994).

Post-Transcriptional Silencing

Transgene interaction usually results in gene silencing at the post-transciptional level. Cosuppression, which is coordinate suppression of the transgene and the homologous endogene, was described previously (Flavell et al. 1995; Jorgensen 1995; Meyer 1995). Usually, cosuppression results from a post-transcriptional process involving RNA turnover. A flower color gene in petunia, chalcone synthase (*CHS*), is necessary for the biosynthesis of the anthocyanin pigment responsible for the red and purple flower color. When a chimeric *CHS* gene was introduced into a purple flowering line of *Petunia hybrida*, the resulting transgenic plants produced either white flowers or variegated flowers with white and pale sectors (Napoli et al. 1990; van der Krol et al. 1990). In the white flowers, both the *CHS* transgene and the endogenous *CHS* genes were seemingly silent and were characterized by a reduction in steady-state mRNA levels (van Blokland et al. 1994). Post-transcriptional silencing was also observed with other chimeric genes. For example, the meiotically reversible silencing of *rolB* in *Arabidoposis thaliana* (Dehio and Schell 1994), β-1,3-glucanase (*GN1*) gene from *Nicotiana plumbaginifolia* in tobacco (de Carvalho et al. 1995), and *NPTII* gene in tobacco (Ingelbrecht et al. 1994). Another variation on the cosuppression of genes invokes an autoregulatory model involving a biochemical switch that determines the degradation of excessive RNA produced by the transgene and its homologous endogene (Meins and Kunz 1994, 1995). Whether the degradation of a specific RNA occurs in the nucleus or cytoplasm remains unresolved in most cases of post-transcriptional gene silencing.

Transgene Stability: Limitations and Prospects

Although transferring recombinant genes into plants is possible, several problems exist with their integration and expression. Investigations on several plant model systems, including *Populus*, indicate that transgenes are less stable in the transgenic plants than originally thought. Transgene inactivation was observed in many plant species. Levels of transgene expression vary among independent transformants, and this variability may be conditioned by the transgene copy number and/or integration sites. This suggests that it is difficult to predict if a transgene will be stably integrated or expressed in plants; whether annual plants or a forest trees. Several hypotheses explain gene silencing in transgenic plants (Flavell 1994; Jorgensen 1995; Matzke and Matzke 1995; Meins and Kunz 1995). Although several of these are mutually exclusive, none can explain all instances of transgene inactivation. Perhaps different mechanisms of transgene inactivation occur that are interdependent on the promoter type, reporter gene, chimeric gene number, or other sequences between the T-DNA borders introduced into the host genome.

An overall picture emerging from transgenic research in plants is that recombinant genes may not be compatible or well integrated with an evolutionary stable plant genome, particularly of a forest tree. However, it is possible that some transgenes will be well expressed, but may still cause instability in transgenic plants. Transgene expression may depend on the type of chimeric genes such as those conferring herbicide tolerance, disease and pest resistance, or causing hormone or phenotypic alterations. Transgene stability may also depend on the T-DNA insert and its sequence homology at the site(s) of integration within the plant genome (Koncz et al. 1994).

Transgene loss or instability acquires a new dimension and perspective for long-lived trees. For example, a transgene conferring herbicide tolerance must be active during the major part of the life cycle of an annual transgenic crop. Alternatively, expression of a herbicide-tolerant transgene may be required only during the first or second year of tree growth, when herbicide is sprayed to kill competing weeds. After a year or two, expression of the herbicide-tolerant gene may be unnecessary, and the gene could become nonfunctional, inactive, heterochromatized, or perhaps discarded from the tree genome during the extended vegetative phase of a tree species. In contrast, disease- or pest-resistant transgenes must remain active for many years to protect against disease or pests during tree growth. Therefore, transgene expression may

depend on its functional use during the life cycle of a woody plant. On the other hand, a transgene may become inactive at any stage of tree development by *trans*inactivation, silencing, or eventually undergoing mutation. It is the fitness and adaptability of a transgene in the tree genome that determines its long-term survival.

Improving transgene stability, particularly under field conditions, is important for commercialization of transgenic crops or forest trees. Many biotechnology firms worldwide are conducting field trials on improved transgenic crops, such as cotton, maize, and potato, to determine their performance before large-scale release. In 1994, the transgenic "Flavr Savr"™ tomato was released into the marketplace in the United States (Ahl Goy and Duesing 1995). Overall, transgene inactivation or gene silencing apparently can occur at any stage of transgenic plant development, clonal propagation, or in the progeny of the transgenic plants. To address this problem, transformation methods should be devised that preferentially insert a single copy of the chimeric gene, perhaps at specific sites in the genome. While such technology is currently unavailable, methods should be developed that select transformants carrying a single integrated copy of a transgene that remains stable over time. Because a transgene promoter may be the target of increased methylation, selecting promoters that are less prone to methylation is worthwhile. Recent studies show that stability of transgene expression may be increased in transgenic plants by including nuclear scaffold attachment regions (SAR) or matrix attachment regions (MAR) to flank the transgene (Allen et al. 1993; Breyne et al. 1992; Mlynarova et al. 1994). Although *Agrobacterium*-mediated gene transfer has served us well (Chilton 1993), insertion of T-DNA, carrying the hybrid chimeric genes and extra genetic sequences between the left and right borders or outside of them, may contribute to genetic or epigenetic instability in transgenic plants. The particle gun DNA delivery method is an alternative, but is not without problems. Therefore, other innovative avenues for gene transfer should also be explored to promote the stability of transferred genes in annual crops and long-lived woody plants.

What factors determine whether 1 or several copies of the chimeric gene will be integrated at 1 or several sites in the genome? Are transgenes regulated in the plant genome by their own promoter, or do plant regulatory genes also control transgene expression? Does increased methylation of the transgene promoter cause transgene inactivation, or is it the result of gene instability? Does *trans*inactivation involve a paramutation or an epimutation? What causes cosuppression of a transgene and the homologous endogenous gene? Is gene silencing due to post-transcriptional events involving reduction in steady-state mRNA levels? These are some questions relevant to transgene instability in annual crops and in woody plants that future research efforts may answer.

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Section III Molecular Biology

Chapter 14

Applications of Molecular Marker Technologies in *Populus* Breeding[1]

Maria Teresa Cervera, Marc Villar, Patricia Faivre-Rampant, Marie-Christine Goué, Marc Van Montagu, and Wout Boerjan

Introduction

In the 17th and 18th centuries, farmers and tree-breeders in Europe selected individual trees for traits such as fast growth and . Traditional breeding work was based on the analysis of phenotypes and has provided a wealth of information on genetic material through multigeneration pedigrees. Recent developments in DNA marker technologies have created the possibility of generating new breeding strategies. In forest trees, these technologies are developed for conifer, *Eucalyptus*, and *Populus* breeding.

Application of molecular markers to forest tree breeding is dependent on the advantages and limitations of tree genetics. The *Populus* genus (poplars, cottonwoods, and aspens) is a good model system for the diverse disciplines of forest tree biology (reviewed by Stettler et al. 1996). *Populus* species have considerable genetic variation; are highly heterozygous; interspecific hybrids can be easily obtained through artificial breeding techniques (Stanton and Villar 1996); and controlled sexual propagation can be conducted in a greenhouse over approximately 3 months. Moreover, the ease of vegetative propagation permits replicated clonal trials to estimate genetic and environmental components of phenotypic variance for traits of interest; the nuclear genome is relatively small (2C = 1.1 pg); and the chromosome number (2n = 38) is the same for all *Populus* species (Bradshaw and Stettler 1993; Wang and Hall 1995).

In the first part of this chapter, we describe marker technologies commonly used in plant breeding, and provide references to their application in forest trees including poplar. The second part focuses on *Populus* spp. and is an overview of current applications of molecular markers in phylogenetic analysis, genetic mapping, and marker-assisted selection.

Molecular Markers Used as Genetic Markers in Breeding

For centuries, morphological traits provided the basis for plant breeding and contributed to the development of theoretical population genetics. The first genome maps were produced by analysis of genetic segregation and linkage between morphological markers. However, because morphological markers can be strongly influenced by the environment and are limited in number, they are less valuable in current breeding programs.

Techniques to analyze proteins (Smithies 1955) have allowed molecular marker identification based on protein polymorphisms (Lewontin and Hubby 1966; May 1992). Through developments in molecular biology, different techniques have emerged to detect molecular markers based on DNA. Using restriction endonucleases (Linn and Arber 1968; Meselson and Yuan 1968) allows evaluation of DNA sequence variation through the analysis of Restriction Fragment Length Polymorphism (RFLP). In 1985, the Polymerase Chain Reaction (PCR) technique was developed based on the amplification of DNA fragments using a thermostable DNA polymerase (Saiki et al. 1985, 1988). Many variants of the PCR strategy have since been established to detect DNA polymorphisms. The objective of the PCR variant strategies was to increase the number of markers analyzed per experiment and the likelihood of identifying markers that display a high Polymorphism Information Content (PIC). Since the plant genotype is analyzed directly by DNA marker technology, environmental or developmental alterations of the phenotype have no effect.

[1] Klopfenstein, N.B.; Chun, Y.W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

Molecular Markers

According to Weising et al. (1995), an ideal marker technology has markers: 1) with a codominant inheritance (homo- and heterozygotic states can be discriminated); 2) that are evenly distributed throughout the genome; and 3) with selectively neutral behavior. Moreover, ideal marker technology: 1) can reveal many polymorphic loci with multiple alleles; 2) is easy, fast, and inexpensive; and 3) is highly reproducible.

Marker technologies that are currently available combine some of these properties. A marker system should be selected on the basis of the genetic analysis to be performed. In the following discussion, we describe the principles, advantages, and limitations of marker technologies commonly used in poplar breeding (table 1).

Isozyme and Allozyme Markers

Allozymes are enzymes encoded by different alleles of the same gene; thus, they can differ in their amino acid sequence, protein structure, and kinetic properties. Allozyme analysis is based on the correlation between differences in mobility in an electric field and differences among alleles encoding these allozymes (Murphy et al. 1990).

Allozyme analysis comprises: 1) preparation of tissue extracts using buffers that allow protein extraction while maintaining enzymatic activity; 2) electrophoretic (starch or polyacrylamide gel) separation of proteins according to net charge and size; and 3) allozyme detection after electrophoresis using specific stains.

Allozyme analysis is easy to perform, inexpensive, and technically accessible. Allozyme expression is typically codominant allowing discrimination between homozygous and heterozygous loci and multiple alleles. However, allozyme analysis has several drawbacks. The limited number of loci that can be analyzed may restrict attempts to locate markers associated with traits of interest, and the level of genetic polymorphism within coding sequences is relatively low. A new allele can be detected as a polymorphism only if nucleotide differences cause amino acid substitutions that affect the electrophoretic mobility. Furthermore, allozymes may be active only at a specific physiological stage, tissue, or cell compartment. Other limi-

Table 1. Comparison of DNA marker systems (adapted from Mazur and Tingey 1995).

	RFLP[1]	RAPD[2]	SSR[3]	AFLP™ [4]
Assay principle	Endonuclease digestion and hybridization	Amplification with random primers	Amplification of SSRs	Selective amplification of DNA fragments
Polymorphism type	Single-base insertions or deletions	Single-base insertions or deletions	Repeat length	Single-base insertions or deletions
Polymorphism level	Medium	Medium	High	Medium
Number of different loci assayed in a single reaction	1-2	5-20	1	40-100
Abundance	High	Very high	Medium	Very high
Dominance	Codominant	Dominant	Codominant	Dominant/codominant
Amount of DNA required	2-10 µg	10-25 ng	25-50 ng	250 ng
DNA sequence required	No	No	Yes	No
Radioactive detection	Yes/no	No	No	Yes/no
Cost	Medium/high	Low	High	Medium

[1] Restriction Fragment Length Polymorphism
[2] Random Amplified Polymorphic DNA
[3] Simple Sequence Repeats
[4] Amplified Fragment Length Polymorphism
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tations, such as post-transcriptional modifications or the presence of isozymes with identical mobility but encoded by different loci, can hamper interpretation of the zymogram (banding pattern) analysis.

Linkage analysis of isozyme loci was performed on many forest trees such as *Populus* spp. (Liu and Furnier 1993a), *Larix laricina* (Cheliak and Pitel 1985), *Eucalyptus regnans* (Moran and Bell 1983), *Pinus* spp. (Conkle 1981), *Pinus radiata* (Moran et al. 1983), *Pinus rigida* (Guries et al. 1978), *Pinus strobus* (Eckert et al. 1981), and *Pinus taeda* (Adams and Joly 1980). Isozyme analysis was used to identify clones of the genus *Populus* (Cheliak and Dancik 1982; Hyun et al. 1987; Liu and Furnier 1993b; Lund et al. 1992; Rajora 1988, 1989a; Rajora and Zsuffa 1989).

Restriction Fragment Length Polymorphism (RFLP)

RFLP analysis, a DNA-based marker technology used in plant breeding and plant genetics (Neale and Williams 1991; Tanksley et al. 1989), is based on polymorphism observed after DNA digestion with 1 or more restriction enzymes. Resulting fragments are electrophoretically separated in agarose or acrylamide gels, according to their molecular weight, transferred to a membrane and visualized after DNA hybridization with probes containing homologous sequences. RFLP technology detects base substitutions within cleavage sites, insertions, deletions, and sequence rearrangements.

RFLPs are codominant markers capable of distinguishing multiple alleles. They have been used to generate genome maps, study the architecture of complex genetic traits and monitor their inheritance, monitor trait introgression through RFLP-backcross breeding, evaluate germplasm diversity, and analyze genome homologies among plant species. Depending on the method used to select the probes, RFLP markers can cover the entire genome. DNA clones used as probes to detect RFLPs from nuclear DNA can be obtained from complementary DNA (cDNA) or genomic libraries.

Although RFLP analysis reveals high levels of polymorphism within a population, it is technically demanding, slow to accomplish, and requires relatively large amounts of DNA. Another limitation is the availability of libraries with useful probes, although libraries are available for several main crops and some forest trees such as *Populus* spp. (Bradshaw and Stettler 1993; Liu and Furnier 1993a), *Eucalyptus nitens* (Byrne et al. 1994), and loblolly pine (Devey et al. 1991). RFLP genome maps were generated with these probes (Bradshaw et al. 1994; Byrne et al. 1995; Devey et al. 1994; Liu and Furnier 1993a). Ahuja et al. (1994) reported that informative cDNA probes for RFLP analysis of loblolly pine were also useful for other conifers. Associations between RFLP markers and traits that display Mendelian inheritance were established for several forest tree species including loblolly pine (Groover et al. 1994) and *Populus* spp. (Bradshaw and Stettler 1995). In addition, RFLP markers were used to study inter- and intraspecific variation in *Populus* (Keim et al. 1989; Liu and Furnier 1993b).

RFLPs can also be obtained from chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA). Two different approaches are useful to study RFLPs in cytoplasmic DNA: 1) isolation of cp or mtDNA, digestion with restriction enzymes, electrophoretic separation, and visualization of RFLPs by ethidium bromide or silver staining; and 2) isolation and digestion of total DNA, electrophoretic separation, and Southern blotting using total cp or mtDNA or specific cp or mtDNA sequences as a probe. Various studies were conducted on *Populus* based on cpDNA RFLPs (Mejnartowicz 1991; Rajora and Dancik 1995a, 1995b) and mtDNA RFLPs (Barrett et al. 1993; Radetzky 1990).

Polymerase Chain Reaction (PCR) Technologies

Many variant methods have been developed since the invention of PCR technology in 1985 (Innis et al. 1990); some allow detection of DNA polymorphisms.

Random Amplified Polymorphic DNA (RAPD) markers are generated by PCR amplification using arbitrary primers. The following research teams established this technique: 1) DuPont, which patented the technique with the name RAPD (Williams et al. 1990); 2) Caetano-Annóles et al. (1991a), who proposed the name DAF for DNA Amplification Fingerprinting; and 3) Welsh and McClelland (1990), who described AP-PCR (Arbitrarily Primed PCR). RAPD markers have been used for DNA fingerprinting, genetic mapping, and localization of genes of interest (Caetano-Annóles et al. 1991b; Neale and Harry 1994; Newbury and Ford-Lloyd 1993; Tingey and DelTufo 1992; Williams et al. 1993).

RAPD protocol is based on use of a single short primer (10 to 12 bases in length with at least 40 percent G+C content) in the PCR reaction. To obtain an amplification product, the distance between both regions complementary to the primer sequence (but in an inverted orientation) should not exceed 1,000 to 2,000 bp. Amplified DNA fragments are electrophoretically separated on an agarose gel and visualized by staining with ethidium bromide. Experiments have shown that the number of amplified DNA fragments observed per primer is independent of the genome complexity. Typically, between 5 and 20 loci can be analyzed per experiment. Thus, when large genomes, such as those of conifers, or small genomes, such as that of *Arabidopsis*, are analyzed, RAPD patterns show similar complexity. These results are due to primer competition and imply that not all amplifications derive from the perfect pairing between the primer and DNA template (Williams et al. 1990).

RAPD technology detects single nucleotide changes, deletions, and insertions within the primer annealing site (Williams et al. 1990). A RAPD marker of interest can be cloned and sequenced for conversion to a Sequence Characterized Amplified Region (SCAR) (Paran and

Michelmore 1993). Each SCAR is a specific PCR-based marker that defines a single locus. Approximately 20 percent of the RAPDs can be converted to codominant SCARs.

RAPD technology does not require probe libraries; instead, direct detection of bands in the gel is possible, which avoids hybridization and autoradiography. Moreover, less (approximately 1,000-fold) tissue is required for the assay, and a universal primer set can be used for genome analysis of any organism. Another important advantage is that RAPD analysis can be automated. Overall, RAPD assays are relatively simple, quick, and inexpensive.

However, this marker technology does present several limitations. RAPD markers are dominant, so they usually cannot distinguish between homozygous and heterozygous loci. This results in a loss of genetic information mainly for highly heterozygous organisms such as forest trees. RAPDs are also difficult to reproduce. This variation can be attributed to mismatched annealing of the random primer to the DNA (Muralidharan and Wakeland 1993; Penner et al. 1993).

Since Carlson et al. (1991) originally demonstrated Mendelian inheritance of RAPD markers based on segregation data in conifers, numerous maps were generated by analyzing haploid DNA from megagametophytes of white spruce (Tulsieram et al. 1992), loblolly pine (Grattapaglia et al. 1991), longleaf pine (Nelson et al. 1994), and maritime pine (Plomion et al. 1995a, 1995b). Grattapaglia and Sederoff (1994) combined the pseudo-testcross strategy and RAPD technology to generate genetic linkage maps of *Eucalyptus grandis* and *E. urophylla*, while Bradshaw et al. (1994) used RAPDs, RFLPs, and STSs to construct a *Populus* genome map. Associations were established between RAPD markers and economically important traits of *Eucalyptus grandis*, *E. urophilla* (Grattapaglia et al. 1995), sugar pine (Devey et al. 1995), loblolly pine (Wilcox et al. 1995), and *Populus* spp. (Villar et al. 1996). Fingerprint analysis based on RAPD markers was also used to reveal inter- or intraspecific variation, and to study taxonomic relationships within the genus *Populus* (Castiglione et al. 1993; Liu and Furnier 1993b).

Simple Sequence Repeats (SSRs), or microsatellites, are tandem repeats of sequence units, which can be as short as 4, 3, 2, or even 1 nucleotide. SSRs are characterized by high levels of genetic polymorphism due to variation in the number of repeats (Hamada et al. 1982; Tautz and Renz 1984).

Database searches of published sequences reveal that SSRs are abundant and widely dispersed in the plant genome with an average frequency of 1 every 50 kb (Morgante and Olivieri 1993). They were detected in 34 plant species. The AT dinucleotide repeat was the most abundant, whereas AC/TG repeats, common in animals, were observed only in 1 plant species. Oligonucleotides containing TG and GATA/GACA repeats were used as probes in RFLP assays and revealed polymorphisms in plants (Lönn et al. 1992; Weising et al. 1989).

SSR-based markers are generated by PCR amplification of the SSR using specific primers (20 to 25 bases) complementary to their flanking regions. The number of repeat units at a locus is highly variable and can be easily detected as polymorphisms when the amplified DNA fragments are electrophoretically separated in polyacrylamide or high-resolution agarose gels. Bands are directly visualized by ethidium bromide staining or by autoradiography when labeled primers are used in the PCR reaction. One locus can be analyzed per specific primer combination.

SSR-based markers have been used for genome mapping, variety identification, and germplasm analysis (Akkaya et al. 1992; Bell and Ecker 1994; Lynn and Heun 1993; Morgante et al. 1994; Thomas and Scott 1993; Wu and Tanksley 1993). SSR-based markers are codominant and detect many different allele sizes per locus. SSRs have the highest PIC of any marker system. However, the effort required to obtain the sequences flanking SSR is the major disadvantage of SSR-based technology. The first step is to generate a genomic library enriched in repeated sequences. Then, clones must be hybridized with oligonucleotides complementary to the target sequence repeat. Positive clones are sequenced to design specific primers flanking the SSR. Due to this limitation, only few data based on characterizing microsatellites in forest trees were reported (Condit and Hubbell 1991; Dow et al. 1995. Steinkellner et al. 1995).

AFLP™[1] (Amplified Fragment Length Polymorphism) marker technology is a powerful DNA fingerprinting technique developed by Keygene N.V. (Zabeau and Vos 1993), which is based on selective PCR amplification of restriction fragments from a complete digestion of genomic DNA. The AFLP technique is as reliable as the RFLP technique but avoids Southern blotting by detecting restriction fragments with specific PCR amplification (Vos et al. 1995).

The assay consists of the following 5 steps (Vos et al. 1995): 1) digestion of total genomic DNA with 2 different restriction enzymes, a rare cutter enzyme (e.g., *Eco*RI) and a frequent cutter enzyme (e.g., *Mse*I); 2) ligation of oligonucleotide adapters (e.g., *Eco*RI-adapter and *Mse*I-adapter) to the restriction fragments to obtain the primary template for PCRs, these oligonucleotide adapters contain the core sequence and an enzyme-specific cohesive sequence; 3) selective preamplification of the primary template using PCR primers that contain 3 characteristic regions in their sequences: a core sequence, an enzyme-specific sequence (both homologous to the sequence of the adapter), and a 3' selective extension (1 nucleotide); 4) selective amplification with labeled PCR primers, similar to those used in the preamplification but with a longer 3' selective extension (2 or more nucleotides); and 5) electrophoretic separation of labeled fragments on polyacrylamide gels followed by autoradiography.

Selective amplification does not require previous information about the sequence because it is conferred by se-

[1] ALFP is a registered trademark in the Benelux.

lective extension of the primers; thus, only those fragments homologous to the nucleotides flanking the enzyme-specific sequence are amplified. The number of amplified DNA fragments can be controlled by the cleavage frequency of the rare cutter enzyme and the number of 3' selective nucleotides. Typically, between 60 and 120 restriction fragments are detected per reaction.

The AFLP technique detects polymorphisms such as the presence or absence of restriction enzyme sites, sequence polymorphisms adjacent to these sites, insertions, deletions, and rearrangements. AFLP technology can be applied to clonal identification, germplasm analysis, construction of high-density linkage maps, and localization of monogenic and polygenic traits when close associations are established between AFLP markers and traits of interest. This technique will contribute significantly to map-based cloning (Cervera et al. 1996a, 1996b; Thomas et al. 1995; van Eck et al. 1995).

The most important advantage of the AFLP technique is the high number of polymorphic markers that can be analyzed per experiment. Fingerprints are produced using a limited set of primers. Another advantage is its high reproducibility. AFLP markers are codominant; using specialized software, it is possible to identify whether a locus is homo- or heterozygous although it is impossible to detect multiallelism. However, AFLP technology is technically demanding, and is more difficult to automate and more expensive than RAPD technology.

In forestry research, close associations between AFLP markers and genes of interest were reported in *Populus* (Cervera et al. 1996a). Clonal identification based on AFLP fingerprints has been performed, and high density AFLP maps are in progress for 3 *Populus* species (Cervera et al. 1996b).

Sequence Tagged Site (STS) markers are those obtained by converting RFLP markers into PCR-based markers (Olson et al. 1989). This conversion requires the terminal sequence of RFLP probes to synthesize specific primers. Codominant STS-based polymorphism can be detected directly as length variation (Amplified Sequence Polymorphism) or may require restriction enzyme digestion of the amplified product to reveal the polymorphism. STS markers are called Expressed Sequence Tags (ESTs) when the sequenced clones are cDNAs (Olson et al. 1989). In forestry research, Bradshaw et al. (1994) generated a genetic linkage map of a hybrid poplar composed of RFLP, RAPD, and STS markers.

Single Strand Conformation Polymorphism (SSCP), a method that enables detection of DNA polymorphisms such as point mutations (Hayashi 1991), is based on differences in electrophoretic migration of single stranded, denatured DNAs in a nondenaturing polyacrylamide gel. SSCPs are codominant markers capable of distinguishing multiple alleles. SSCP was used by Bodénès et al. (1996) to differentiate between *Quercus petraea* and *Q. robur*, 2 white oak species.

Applications of Molecular Markers to Poplar Breeding

Molecular breeding is the application of molecular (protein- or DNA-based) marker technologies to breeding programs. Short-term applications of molecular markers involve identification and discrimination of genotypes, germplasm analysis, and taxonomic studies. Medium- and long-term applications involve: 1) the generation of genetic linkage maps; 2) early selection of individuals with specific characteristics within larger progenies; 3) efficient selection of parents for new breeding programs; 4) efficient trait introgression; 5) genetic mapping of simple or complex traits; 6) the study of the architecture of Quantitative Trait Loci (QTLs); and 7) comparative or syntenic mapping. A more exhaustive list on applications of marker-based technologies in plant breeding has been provided by Beckmann (1991). The use of molecular breeding technologies for forest trees could radically reduce the breeding time, a crucial advantage given the long generation times (Grattapaglia et al. 1994).

Genetic Fingerprints to Identify Genotypes in *Populus*

Marker technologies have been used to: 1) fingerprint genotypes and identify characteristic inter or intraspecific variation (varietal identification can be a tool to legally protect a breeder's rights); 2) perform paternity analysis; 3) identify superior pollen donors; 4) monitor fidelity of controlled crosses by analyzing specific alleles present in the homozygous dominant state in 1 parent and null in the other; and 5) characterize genetic diversity. Molecular information on genetic diversity is useful to screen for redundancy and deficiencies in germplasm collections of forest trees, and to analyze management and use efficiency (Millar 1993).

Isozyme analysis (Cheliak and Dancik 1982; Hyun et al. 1987; Liu and Furnier 1993b; Lund et al. 1992; Rajora 1988, 1989a; Rajora and Zsuffa 1989) and gas chromatography (Greenway et al. 1989) provided the first molecular clonal identification within the genus *Populus*. Cheliak and Dancik (1982), Hyun et al. (1987), and Lund et al. (1992) examined levels and distribution of isozyme variation in several *P. tremuloides* populations. Liu and Furnier (1993b) compared allozyme RFLP and RAPD markers for their ability to reveal inter and intraspecific variation in *P. tremuloides* and *P. grandidentata*. Their results indicated that RAPDs are more powerful for fingerprinting individuals. To estimate taxonomic relationships and discriminate among all tested commercial clones, Castiglione et al. (1993) used RAPD fingerprints to analyze 32 clones be-

longing to 10 *Populus* species. Based on ribosomal DNA (rDNA) RFLP analysis, Faivre-Rampant et al. (1992) obtained characteristic inter and intraspecific variability among 5 different species of *Populus* (*P. deltoides, P. nigra, P. trichocarpa, P. maximowiczii,* and *P. alba*). Interspecific variability was displayed by different major ribosomal unit types, while intraspecific variability was displayed by the length variation of the ribosomal unit types.

Based on RFLP analysis of interspecific poplar crosses, a study on cpDNA transmission revealed a maternal mode of inheritance (Mejnartowicz 1991). Rajora and Dancik (1995a, 1995b) conducted research on inter and intraspecific cpDNA variation in *Populus* (*P. deltoides, P. nigra,* and *P. maximowiczii*) by RFLP analysis using 16 restriction enzymes and 6 heterologous probes. Similar studies were performed by Barrett et al. (1993) using mtDNA RFLPs to examine inter and intraspecific mitochondrial variation among *P. deltoides, P. nigra, P. maximowiczii,* and *P. tremuloides*. Using mtDNA RFLPs, Radetzky (1990) established that mtDNA is inherited maternally in *Populus*. Recently, AFLP fingerprints were used to reveal inter or intraspecific variation in *Populus* (M.T. Cervera unpublished results).

Molecular Systematics in Poplar

Development of biochemical and molecular marker technologies provides new tools for plant systematic studies (Machon et al. 1995; Olmstead and Palmer 1994; Strauss et al. 1992). The genus *Populus* comprises approximately 35 species native to the northern hemisphere (figure 1). The classification in 5 sections, Aigeiros, Tacamahaca, Leucoides, Leuce (currently termed Populus), and Turanga, is based on morphology, geographical localization, and crossability. Although *Populus* is considered a model in forestry, data on systematics are lacking for this genus and published studies involve only a limited number of species. Taxonomic analysis is difficult due to wide intraspecific variability, natural crossability of the members within this genus, and convergent morphology shown by hybrids and species (Eckenwalder 1984a, 1984b; Hu et al. 1985; Keim et al. 1989; Ronald 1982). Because the variation in poplar morphological traits is difficult to interpret, molecular systematics was developed to evaluate genetic diversity in the framework of breeding and gene conservation programs.

The first approach involved 12 enzyme systems used to study *P. deltoides, P. nigra,* and *P. maximowiczii* (Rajora and Zsuffa 1990). The results indicated that *P. nigra* was more closely related to *P. maximowiczii* than to *P. deltoides,* although both *P. nigra* and *P. deltoides* are classified in the section Aigeiros.

Other marker technologies have been used for molecular phylogeny studies, involving nuclear and cytoplasmic markers. Smith and Sytsma (1990) analyzed restriction site

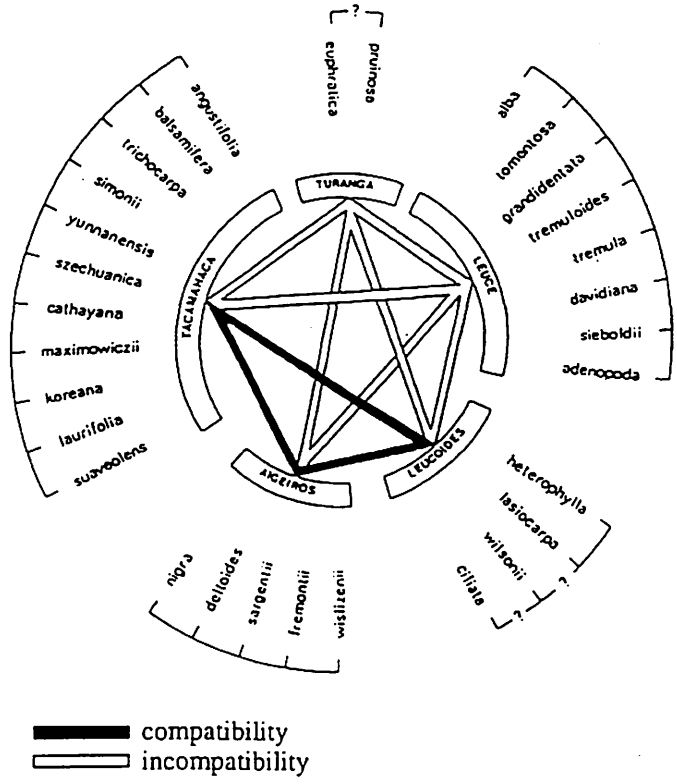


Figure 1. Species of the genus *Populus*, with compatibility and incompatibility relationships shown among the 5 genus sections (Willing and Pryor 1976).

variations in cpDNA and rDNA in 10 poplar species from the sections Aigeiros, Tacamahaca, and Leuce. Based on cpDNA data, a single-parsimonious Wagner tree revealed a close relationship between the European poplars *P. alba* and *P. tremula*. Another cluster was composed of the American species *P. deltoides, P. fremontii* (Aigeiros), and *P. balsamifera* (Tacamahaca). Unexpectedly, the results supported a closer relationship of *P. nigra* to *P. alba* and *P. tremula*, than to the other Aigeiros species. Cladistic analysis performed on rDNA revealed 3 distinct groups. One of these groups included the Leuce species. The second group was composed of Tacamahaca species and Aigeiros species except *P. nigra*. The third group was only represented by *P. nigra*. These results suggest that cpDNA phylogeny is correlated with geographic distribution, while rDNA phylogeny is concordant with current botanical classifications. As suggested by the authors, the analyzed *P. nigra* might have resulted from hybridization/introgression events between *P. alba* and *P. nigra*.

Experiments by Barrett et al. (1993), using mtDNA RFLP, showed a close relationship between *P. deltoides* and *P. maximowiczii*. In addition, they revealed close similarities between *P. nigra* and 1 species of the Leuce section, *P. tremuloides*, confirming the results of Smith and Sytsma (1990).

Using RAPD markers, Castiglione et al. (1993) studied 10 species belonging to the 3 main sections, Aigeiros, Tacamahaca, and Leuce, 19 hybrids *of P.* x *euramericana* (*P. deltoides* x *P. nigra*), and 1 *P. deltoides* x *P. maximowiczii* hybrid. One cluster of *P.* x *euramericana* clones was revealed. All hybrid clones were closer to the maternal species, *P. deltoides*, than to the paternal species. Surprisingly, phenetic analysis revealed no cluster between the species. Nevertheless, the authors pointed out the unusual position of *P. trichocarpa*, which was separated from the other Tacamahaca species. *P. nigra* was also separated from the Aigeiros species. More recently, Rajora and Dancik (1995b) and Castiglione (unpublished data) confirmed dissimilarity between *P. nigra* and *P. deltoides* using cpDNA RFLP.

Investigation of nuclear DNA variation was reported by Faivre-Rampant et al. (1995a). This study involved 18 species and 1 hybrid belonging to the sections Aigeiros, Tacamahaca, Leuce, and Leucoides. STS markers developed by Bradshaw et al. (1994) were used. Sokal and Michener's (1958) genetic distance analysis and cluster analysis based on this distance analysis were performed. The results are presented as a neighbor-joining tree in figure 2. Species of the Leuce section were clearly clustered, except *P. davidiana*. As expected, *P. deltoides*, *P. fremontii*, and *P. wislizenii* formed a group, confirming that the 2 latter species can be considered subspecies of *P. deltoides*. Tacamahaca and Leucoides species, with *P. nigra*, formed a single large cluster. Again, *P. nigra* was isolated from the other Aigeiros species. These results indicate that *P. nigra* is not related to *P. deltoides*, *P. fremontii*, and *P. wislizenii*, and that species of the Tacamahaca and Leucoides sections share a close relationship.

In conclusion, molecular classification obtained by polymorphism analysis of nuclear markers roughly matches the botanical classification. Molecular systematic studies do not classify *P. nigra* in the Aigeiros section. These results coincide with many elements on evolutionary relationships obtained from morphological (Eckenwalder 1977), crossability (Rajora 1989b), pathological (Pinon and Teissier du Cros 1976; Steenackers 1972), and cytological studies (Faivre Rampant et al. 1995b). According to Smith and Sytsma (1990), *P. alba* could be an ancestor of *P. nigra*. Further work is needed to confirm this interesting hypothesis. Recently, Rajora and Dancik (1995b) proposed to assign *P. nigra* in a new section called Nigrae. Additional studies are required on cpDNA, mtDNA, and nuclear DNA with markers equally distributed throughout the genome. Studies are needed on interspecific variation of species that were not previously analyzed, and on intraspecific variation involving individuals from the entire natural range.

Genetic Linkage Maps of *Populus*

Two linkage maps were published and 5 others are currently under construction from different pedigrees and with various objectives.

1) The genetic linkage map generated at the University of Minnesota (USA) is based on an F_1 *P. tremuloides* x *P. tremuloides* family (Liu and Furnier 1993a). This partial map incorporates 54 RFLP and 3 allozyme markers, and comprises 14 linkage groups covering 664 centiMorgan (cM) of the genome. The objective of

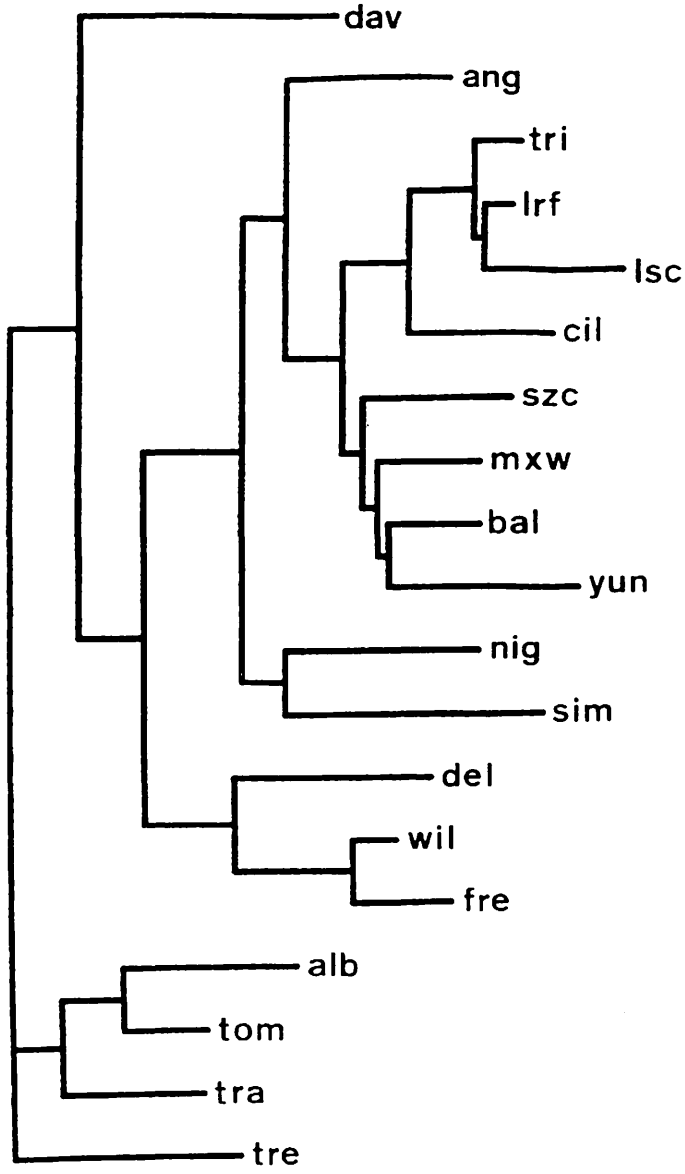


Figure 2. Neighbor-joining tree for some species of the genus *Populus*. The analysis was done with PHYLIP package programs (Felsenstein 1993; http://bimcore.cc.emory.edu/phylip.doc).

alb = *P. alba*
tra = *P. tremula*
tre = *P. tremuloides*
tom = *P. tomentosa*
dav = *P. davidiana*
ang = *P. angustifolia*
tri = *P. trichocarpa*
lrf = *P. laurifolia*
szc = *P. szechuanica*
mxw = *P. maximowiczi*
bal = *P. balsamifera*
yun = *P. yunnanensis*
sim = *P. simonii*
lsc = *P. lasiocarpa*
cil = *P. ciliata*
nig = *P. nigra*
del = *P. deltoides*
wil = *P. wislizenii*
fre = *P. fremontii*

this map was to understand the mode of inheritance and linkage relationships of these genetic markers.

2) The genetic linkage map constructed at the University of Washington/Washington State University (UW/WSU), USA is based on a 3 generation *P. trichocarpa* x *P. deltoides* pedigree. This pedigree consists of an F_2 family of approximately 350 trees. This family was obtained from a controlled cross between 2 full-sib individuals from an interspecific hybridization between a female *P. trichocarpa* and a male *P. deltoides*. The published map is composed of 343 RFLP, STS, and RAPD markers, and the total distance contained within the 19 largest linkage groups is 1,261 cM (Bradshaw et al. 1994). Average spacing between the markers is 6.7 cM (Bradshaw and Stettler 1995). Limitations of this map are its size, which is approximately half the estimated length of the *Populus* genome. The UW/WSU map has been extended to 512 markers (H.D. Bradshaw unpublished data) and is of sufficient scope and density to permit the mapping of QTLs. This map will be used to analyze the genetics of adaptive and commercial traits such as leaf phenology, stem basal area, height, wood quality traits, and disease resistance. Genotype data, available markers, and other data information may be obtained on the World Wide Web at http://www.poplar1.cfr.washington.edu.

3) Linkage maps generated at the INRA (France) are based on an interspecific F_1 *P. deltoides* x *P. trichocarpa* family of 125 individuals (Goué et al. 1996). This map is under construction according to the pseudo-testcross strategy described by Carlson et al. (1991) and Grattapaglia and Sederoff (1994) for conifers and *Eucalyptus*. This mapping strategy results in 2 maps corresponding to each parent. Four types of markers, RFLPs, RAPDs, STS, and allozymes, are currently being mapped. Of 255 Operon primers that were screened, 119 were selected to amplify a total of 274 RAPD markers. These markers were grouped to produce a maternal *P. deltoides* map with a total of 113 markers in 25 linkage groups, and a paternal *P. trichocarpa* map with 121 markers in 28 linkage groups. RFLP markers will be mapped with probes provided by H.D. Bradshaw (UW/WSU). A mixed marker map composed of numerous RAPDs and fewer codominant markers, such as RFLPs and/or allozymes, has been successful (Bradshaw et al. 1994). RFLP probes will also permit the comparison of the 2 maps, searching for syntenic groups. These INRA maps will allow investigation of the genetic architecture of resistance to diseases such as rusts (*Melampsora* spp.), bacterial canker (*Xanthomonas populi*), and leaf spot (*Marssonina brunnea*) (Goué et al. 1996). The family being mapped in this project is connected to other intra and interspecific families by a 9 x 9 factorial mating design, involving 8 *P. trichocarpa* parents and 10 *P. deltoides* parents. This mating design will play a key role in determining QTL stability across genetic backgrounds.

4) The linkage maps constructed at the Flanders Interuniversity Institute for Biotechnology (VIB) (Gent, Belgium) are based on 2 interspecific F_1 families, *P. deltoides* x *P. nigra* and *P. deltoides* x *P. trichocarpa* (Cervera et al. 1996b). The 2 families share a common *P. deltoides* female parent. Three maps are in progress combining the pseudo-testcross strategy with the AFLP technology. The *P. deltoides* x *P. nigra* hybrid family consists of 126 individuals, while the *P. deltoides* x *P. trichocarpa* family consists of 110 individuals. Each map will incorporate about 300 AFLP markers, and other RFLP and STS markers used previously by Bradshaw et al. (1994). RFLP and STS markers will be used to identify syntenic linkage groups. These maps will allow the genetic mapping of resistance to pathogens such as *Melampsora* spp., *Xanthomonas populi*, poplar mosaic virus, and *Marssonina brunnea*, along with other economically important traits such as growth, leaf phenology, wood density, and frost tolerance.

Genetic Analysis of Simply and Complex Inherited Traits

Genetic Analysis of Simply Inherited Traits

Sax (1923) proposed the association between a morphological marker and a quantitative trait as a method for indirect selection. The utility of molecular markers in plant breeding, and more specifically in forestry breeding (because of the long generation times), is based on finding associations between these markers and traits of interest. This linkage allows the screening of a progeny for the presence of a desirable trait by detection of isozymes, RFLP, RAPD, SSR, STS, or AFLP markers. These linkages have been determined for several simply inherited traits (reviewed in Michelmore 1995 for resistance to pests and diseases).

To introduce disease resistance into susceptible varieties, crosses with varieties that carry the resistance gene must be performed. In traditional breeding, progeny screening involves pathogen inoculation. For nursery tests, screening efficiency depends on environmental conditions, pathogen infection pressure, and the presence of other pathogens. For laboratory tests, screening efficiency depends on inoculation efficiency. Furthermore, screening requires large nursery and greenhouse space and several annual replications. In contrast, the use of molecular markers allows rapid screening of progeny to identify resistant clones by analyzing the presence of markers associated with the resistance gene. In ad-

dition, this analysis does not depend on biotic or abiotic factors.

Molecular rust genetics (*Melampsora larici-populina*) resistance was initiated mainly by searching for markers associated with qualitative resistance using Bulked Segregant Analysis (Michelmore et al. 1991). RAPD markers tightly linked to *M. larici-populina* resistance were identified in 3 families of a 2 x 2 factorial mating design (Villar et al. 1994, 1996), and such linkage is currently under investigation in 21 interspecific *P. deltoides* x *P. trichocarpa* families originating from 10 *P. deltoides* and 8 *P. trichocarpa* parents (M. Villar unpublished data). Cervera et al. (1996a) identified 3 AFLP markers tightly linked to *M. larici-populina* resistance in an interspecific *P. deltoides* x *P. nigra* family. Because of their close linkage, these markers are exceptional tools for gene introgression and can be useful in cloning the resistance genes by chromosome landing (Tanksley et al. 1995).

For breeding purposes, qualitative resistance is not as desirable as other resistance forms. In Europe, the breakdown of qualitative resistance has regularly occurred on interspecific F_1 hybrids selected for immunity. When breeding for durable resistance, quantitative resistance must be considered. Durable resistance (horizontal resistance or tolerance), which is assumed to be under polygenic control, is currently under dissection through the search for QTLs on genetic maps (Goué et al. 1996; Lefèvre et al. 1994, 1995). This is one mapping effort objective at the University of Gent (Belgium) and INRA (France). The USA group is focusing on resistance against *M. medusae*, for which markers have been revealed on the UW/WSU linkage map (Newcombe et al. 1996).

Genetic Analysis of Complex Inherited Traits

Most of the heritable characters of economic importance, such as vegetative propagation, growth, or development, are complex inherited traits. These traits are named polygenic or quantitative because they result from a combined action of several genes (Tanksley et al. 1989). Little is known about the number, localization, magnitude of effect, and interaction of genetic loci controlling expression of these traits. Manipulation of polygenic traits, more difficult than that of simple inherited traits, has always been challenging for traditional breeders.

The first premise for identifying markers for polygenic traits is to consider the inheritance of quantitative traits. Quantitative traits depend on genes subject to the same properties and laws of transmission as genes displayed by qualitative (or monogenic) traits (Falconer 1989). The identification of markers associated to quantitative or qualitative traits requires a similar approach when selecting a DNA marker technology. Classical QTL mapping strategy involves analysis of a segregating population derived by crossing 2 progenitors that are genetically distinct for the trait under investigation. Two searches must be conducted; identification of molecular markers associated to the character, and construction of a relatively dense linkage map to analyze the number, chromosomal position, magnitude of effect, and mode of action of genes controlling the expression of the trait. Not all QTLs are detectable through associations with molecular markers; detection of a QTL depends on the magnitude of its effect on the quantitative trait, heritability of the trait, size of the segregating population, and recombination frequency (map distance) between the marker and the QTL.

Different statistical methods can determine the linkages between marker loci and QTLs. One of the most accepted approaches is interval mapping (Lander and Botstein 1989). The single marker approach, based on associations between a molecular marker and a QTL, cannot discriminate between a small-effect QTL that is close to a marker and a large-effect QTL that is further away from the marker. The interval mapping approach solves this problem because it is based on the analysis of 2 linked markers flanking an interval that may contain a QTL (Lander and Botstein 1989). Different software packages have been developed for the analysis of QTLs such as MapMaker-QTL (Lander and Botstein 1989; Lander et al. 1987), QTLStat (Liu and Knapp 1992), QTL cartographer, QGene, and MAPQTL.

QTL analysis is difficult because age, genetic background, and environmental factors can affect phenotypic expression. Moreover, QTL analysis requires scoring the trait and genetic analysis of several hundreds of individuals to obtain a high mapping resolution. The number of progeny that is analyzed can be reduced while keeping a similar statistical power of QTL detection. To accomplish this, a selective genotyping can be carried out by choosing only those individuals showing extreme phenotypic values among the entire sample population (Darvasi and Soller 1992); this might be successful in tagging QTLs of large effect (Wang and Paterson 1994). Or, a genotype replication strategy can be used to reduce the environmental variation. In this approach, clonal replicates are planted and analyzed to increase the precision of the phenotypic measurement, without increasing the number of molecular marker assays. This strategy is especially useful for traits of low heritability (Bradshaw and Foster 1992). When planted in contrasting environments, this strategy allows analysis of QTL by environment interactions.

Results show that large proportions of the total variation for many complex inherited traits may be controlled by a limited number of major genes (3 to 7) (Beavis et al. 1991; Edwards et al. 1987, 1992; Grattapaglia et al. 1995). This is also true for *Populus* hybrids, where QTLs were mapped for stem growth, form, and spring leaf flush (Bradshaw and Stettler 1995). Phenotypic data were collected over a 2-year period from a nursery clonal trial in Puyallup (Washington) containing ramets of the parental trees (*P. trichocarpa* and *P. deltoides*), F_1 progeny, and F_2 progeny. For each trait mea-

sured, 1 to 5 QTLs were responsible for a large proportion of the genetic variance. For example, after 2-years growth, 44.7 percent of the genetic variance in stem volume was controlled by just 2 QTLs (LOD = 3.86). For spring leaf phenology, 5 QTLs were identified that explained 84.7 percent of the genetic variance (multilocus LOD = 16.63). This QTL mapping information provides the genetic basis for understanding the processes governing heterosis in hybrid poplars.

The major positive QTL allele for 2-year height growth was dominant and was derived from the *P. trichocarpa* parent. This result agrees with the observation that hybrid F_1 progeny trees from a *P. trichocarpa* by *P. deltoides* cross are generally as tall as the *P. trichocarpa* parent. Additionally, QTLs controlling stem basal area growth, and those controlling sylleptic branch and leaf area traits were clustered (i.e., sharing a similar chromosomal position) suggesting a pleiotropic effect of QTLs ultimately responsible for stem diameter growth. This result corroborates previous statistical correlations among these traits (Hinckley et al. 1989). The QTL mapping effort at the UW/WSU was strengthened by growing this pedigree and another F_2 family, having the same *P. trichocarpa* maternal grandparent, in 2 other contrasting environments. The Boardman site (Oregon) is drier, more continental, and irrigated, while the Clatskanie site (Oregon) is wetter, coastal, and not irrigated (Stettler et al. 1994). The aim is to analyze QTL stability across genetic backgrounds and environments, and to measure other traits such as wood quality. After 1-years growth, the data indicate that the magnitude of the QTL effect for stem basal area was different for each family and environment (Stettler et al. 1994). However, some QTLs are conserved across different environments because their mapped chromosomal locations coincided. Stettler et al. (1994) identified similar positions for QTLs controlling stem volume, stem height, and height/diameter that were mapped in 2 families with a different male grandparent *P. deltoides*. For some QTLs, different alleles control the trait in different environments, thus a strong genotype by environment (G x E) interaction is evident.

Conclusion

In recent decades, rapid technological evolution in the molecular marker field has allowed development of diverse methodologies for the detection of genetic variability. Choosing the best-suited marker technique should be based on the advantages and limitations of each molecular marker methodology, and other factors such as breeder friendliness, cost, time-efficiency, and automation ease.

The introduction of molecular markers in poplar breeding is providing vast information about genome structure, organization, evolution, and diversity. For breeding programs, the development of high-density linkage maps will increase the efficiency of QTL mapping and consistently facilitate the introgression of more complex traits. In addition, these high-density linkage maps will facilitate positional cloning strategies (Tanksley et al. 1995).

Using molecular markers for gene introgression programs (backcross breeding) is one example of Marker-Assisted Selection (MAS) (Hillel et al. 1990; Hospital et al 1992). Trait introgression is appropriate for monogenic or oligogenic traits (Dudley 1993). In this way, breeding for resistance controlled by a small number of loci could be improved and accelerated through MAS in forest trees (Nance et al. 1992), fruit trees, and other plants (Michelmore 1995).

For the future, a primary challenge is to demonstrate that genetic markers can complement long-term breeding programs focused on improving complex polygenic traits. Determining the full potential of MAS depends on answering: Which QTLs are stable across different developmental stages, genetic backgrounds, and environments?

Recent results on *Populus* (Bradshaw et al. 1995) and *Eucalyptus* (Grattapaglia 1996) are encouraging. In both cases, most of the markers transfer well to other pedigrees, and linkage relationships and map distances are conserved. Furthermore, QTLs with higher LOD scores and larger effects are more stable across environments (Bradshaw et al. 1995; Grattapaglia 1996), and some QTLs are conserved across genetic backgrounds (Bradshaw et al. 1995). Although several aspects need intensive investigation, these results provide interesting prospects for marker-assisted breeding.

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Chapter 15

Differentiation of Poplar Clones Using Random Amplified Polymorphic DNA Fingerprinting[1]

Dolly C. Lin, Martin Hubbes, and Louis Zsuffa

Introduction

For over a decade, interest in poplars for short rotation intensive culture plantations for energy, chemicals, and fiber has been developing (Zsuffa et al. 1984). Due to easy clonal propagation, species and hybrid materials were inadequately defined. Conclusive identification of *Populus* species and hybrids is urgently required to: 1) further genetic improvement and characterization; 2) determine the linkage of important economic traits such as fast growth, superior wood quality, and ; and 3) enable advanced genetic engineering and breeding.

Recent progress in molecular biology has generated new analytical tools that are well suited for taxonomic and genetic investigations. Several approaches have been studied in poplar such as isozyme markers (Rajora 1988, 1989a, 1989b; Rajora and Zsuffa 1989, 1990, 1991; Rajora et al. 1991), restriction fragment length polymorphism (RFLP) markers (Bradshaw et al. 1994; Soltes-Rak 1993), STS (sequence tagged site)/RFLP (Bradshaw et al. 1994), ribosomal DNA (rDNA) polymorphisms (D'Ovidio 1992; D'Ovidio et al. 1990, 1991; Faivre-Rampant et al. 1992a, 1992b), mitochondrial DNA (mtDNA) (Barrett et al. 1993), chloroplast DNA (cpDNA) (Rajora and Dancik 1995a, 1995b, 1995c; Smith and Sytsma 1990), and random amplified polymorphic DNA (RAPD) markers (Castiglione et al. 1993; Lin et al. 1994a, 1994b).

Although isozymes have served as genetic markers for fingerprinting clones, the small number of available alleles and loci made it impractical to identify all the clones under investigation (Bournival and Korban 1987). In recent years, RFLP of rDNA, mtDNA, and cpDNA were used intensively for inter and intraspecies differentiation in *Populus*. However, these processes are labor intensive and require a relatively large amount of tissue. Other limitations of RFLP include low resolution for small size differences between relatively large DNA fragments (Gill et al. 1990) and ambivalent differences in the migration of DNA fragments across an agarose gel caused by band shifting (Landers 1989).

Amplification of DNA sequences using polymerase chain reaction (PCR) (Saiki et al. 1988) is currently used with many organisms for studying populations and systematics, tagging major genes, and constructing genetic maps (Innis et al. 1990). Advantages of PCR-based DNA markers over the RFLPs are their rapidity, simplicity, and requirement for only small amounts of DNA (Castiglione et al. 1993).

RAPD banding patterns were sensitive to slight changes in reaction conditions, which caused reproducibility problems (Ellsworth et al. 1993; Muralidharan and Wakeland 1993; Penner et al. 1993). However, DNA fingerprints can be reproduced with standardized conditions such as consistent use of the same thermal cycler, cycling conditions, and concentrations of the reagents (i.e., template DNA, buffer, dNTPs, and DNA polymerase). DNA fingerprints by RAPD markers were chosen in the present study for species and hybrid differentiation and individual clonal characterization of *Populus*.

Materials and Methods

Plant Material

Fifty-five poplar clones representing 8 species and hybrids (table 1) were analyzed. Clones were selected according to frequency of cultivation in Europe and North America (Castiglione et al. 1993), and for their potential use in plantations and biomass production (Steenackers et al. 1990; Zsuffa 1990). Two dormant cuttings of each clone were collected and rooted in the greenhouse at the Faculty of Forestry, University of Toronto.

[1] Klopfenstein, N.B.; Chun, Y. W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

Table 1. List of poplar species and hybrids for clonal characterization.

Species and hybrids	Number of clones
P. x *balsamifera*	3
P. x *jackii*	3
P. deltoides	5
P. trichocarpa x *P. deltoides*	3
P. deltoides x *P. trichocarpa*	2
P. x *euramericana*	25
P. nigra	6
P. nigra x *P. maximowiczii*	5
P. maximowiczii	3
Total	55

DNA Extraction

Young leaves were collected from rooted cuttings. After washing with distilled water and blotting dry, leaf tissue (0.5 g) was ground to powder in liquid nitrogen, then transferred to 10 ml of preheated 2X CTAB isolation buffer (100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2 percent hexadecyltrimethylammonium bromide 'CTAB,' 0.2 percent 2-mercaptoethanol). After thoroughly stirring, the mixture was incubated at 60 °C for 30 min, extracted twice with chloroform-isoamyl alcohol (24:1), and precipitated with 2/3 volume of cold isopropanol (Doyle and Doyle 1987). DNA concentration was estimated by comparing serial dilutions of DNA with those in agarose gels stained with ethidium bromide.

Primers

Sequences of 17 primers used for the PCR reaction are in table 2.

Amplification of DNA by PCR

The amplification reaction was performed in 15 µl of buffer containing 50 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP and dTTP, 0.5 units of *Taq* DNA polymerase (Boehringer Mannheim), 15 pmol primer and 10 ng of DNA. The amplification reaction was performed in the Perkin-Elmer DNA Thermal Cycler (Model TC-1). After an initial denaturation at 94 °C for 2 min, the reaction program continued for 40 cycles consisting of 1 min denaturing at 94 °C, 1 min annealing at 40 °C, and 2 min extension at 72 °C. This program was followed by an extension phase at 72 °C for 10 min. DNA from all clones was subjected to 3 independent amplifications, and only fragments that were observed in all assays were scored for species and clonal characterization.

Electrophoresis in Agarose Gel

Amplification products (15 µl) were analyzed by electrophoresis in 1.5 percent agarose gel (15 x 15 cm) at 100 V until the tracking dye (tartrazine) migrated toward the end

Table 2. Sequences and sources of primers used in the RAPD analyses.

Name	Sequence	References
Chl-1	5'-GAGGCCTACGCCCCATAGAA-3'	Castiglione et al. 1993
Chl-2	5'-AATGCGTTGAGGCGCAGCAG-3'	Castiglione et al. 1993
Chl-4	5'-TTCCCGTGTCTTCCGGCTTAC-3'	Castiglione et al. 1993
Chl-10	5'-TTCTTCTCCTACCAGTATCG-3'	Castiglione et al. 1993
Deca-2	5'-GCGATCCGGC-3'	Castiglione et al. 1993
Deca-4	5'-CGTTGGCCCG-3'	Castiglione et al. 1993
Deca-5	5'-CCAAGGGGGC-3'	Castiglione et al. 1993
Deca-7	5'-CCGCCCGGAT-3'	Castiglione et al. 1993
Deca-9	5'-TGGCCCCGGT-3'	Castiglione et al. 1993
Deca-10	5'-AGCCGGCCTT-3'	Castiglione et al. 1993
Deca-11	5'-ATCGGCTGGG-3'	Castiglione et al. 1993
Deca-12	5'-CTTGCCCACG-3'	Castiglione et al. 1993
Deca-13	5'-GTGGCAAGCC-3'	Castiglione et al. 1993
2114	5'-GACTGCCTCT-3'	Operon Technologies[1]
2115	5'-GAAACGGGTC-3'	Operon Technologies
2116	5'-GTGACCGAGT-3'	Operon Technologies
2117	5'-CAGAAGCGGA-3'	Operon Technologies

[1] 1000-T Atlantic Ave. Suite 108, Alameda, CA 94501-1147, USA

of the gel. Gels were stained in 0.5 μg/ml ethidium bromide solution and photographed in UV-light.

Results and Discussion

The clone and origin of poplar materials subjected to RAPD analyses are listed in table 3. Of 17 primers tested, only 4 (Deca-10, Deca-2, Chl-1, and Deca-7) (figures 1 to 4) were needed to distinguish all 55 poplar clones into species and hybrids (table 4). The molecular sizes of RAPD markers used for species and hybrid differentiation are in table 4. Individual clones could be characterized by primer Chl-1 (figure 3) and/or Deca-7 (figure 4). DTAC clones from different parental origin (i.e., *P. deltoides* x *P. trichocarpa* and *P. trichocarpa* x *P. deltoides*) could be distinguished by primer Chl-1 (figure 3).

Recently, several specific PCR primers were developed for DNA fingerprinting. These primers were determined from known DNA sequences, which include the M13 universal primer (Chong et al. 1995), simple sequence repeats (SSRs) (Gupta et al. 1994), and microsatellite repeats (Morgante and Olivieri 1993). DNA fingerprinting with these specific prim-

Table 3. Poplar (*Populus* spp.) clones and their origin for RAPD analyses (note figures 1 - 4).

Clone no.	Species and hybrid	Origin
Upper Gel		
TAC8	*P. balsamifera*	Ontario
TAC21	*P. balsamifera*	Ontario
TAC51	*P. balsamifera*	Manitoba
JAC4	*P.* x *jackii*	Ontario
JAC7	*P.* x *jackii*	Ontario
JAC28	*P.* x *jackii*	Manitoba
D35	*P. deltoides* (W-1-2) Larsson	Ontario
D39	*P. deltoides*	Ontario
D196	*P.* x *deltoides* cv. 'Northwest'	Saskatchewan
D391	*P. deltoides* (Mixoploid) (Brockville #C136)	Ontario
DTAC7	*P. trichocarpa* x *P. deltoides* cv. 'Unal'	Geraardsbergen, Belgium
DTAC8	*P. trichocarpa* x *P. deltoides* cv. 'Beaupre'	Geraardsbergen, Belgium
DTAC26	*P. trichocarpa* x *P. deltoides* cv. 'Boelare'	Geraardsbergen, Belgium
DTAC9	*P. deltoides* x *P. trichocarpa* cv. 'Donk'	Wageningen, Holland
DTAC10	*P. deltoides* x *P. trichocarpa* cv. 'Barn'	Wageningen, Holland
DN5	*P.* x *euramericana* cv. 'Gelrica'	W. Germany
DN14	*P. x euramericana* cv. 'Harff'	W. Germany
DN16	*P.* x *euramericana* cv. 'Batarde d'Hauterive'	France
DN17	*P.* x *euramericana* cv. 'Robusta'	France
Lower Gel		
DN93	*P. x euramericana* cv. 'Triplo'; cl. 'I37/61'	Casale Monferrato, Italy
I45/51	*P.* x *euramericana*	Casale Monferrato, Italy
I214	*P.* x *euramericana*	Casale Monferrato, Italy
I455	*P. x euramericana*	Casale Monferrato, Italy
DN21	*P.* x *euramericana* cl. 'I78B' (LW42)	Italy
DN30	*P.* x *euramericana* cv. 'Canada Blanc'	Spain
DN173	*P. x euramericana* (cv. 'Dorskamp') (Koster 925)	Holland
DN177	*P.* x *euramericana* (cv. 'Spijk') (Koster 2195)	Holland
N75	*P. nigra* (#113)	Hungary
N84	*P. nigra* var. *italica* (#555/50)	W. Germany
N91	*P. nigra* cv. 'Purkersdorf' (#44/62(10))	Austria
N100	*P. nigra* cv. 'Kunovice' (312/65(002/66)	Czechoslavakia
NM1	*P. nigra* x *P. maximowiczii*	Japan
NM2	*P. nigra* x *P. maximowiczii* (cl. 'Max-1')	W. Germany
NM8	*P. nigra* x *P. maximowiczii* (#62-7)	Korea
M900	*P. maximowiczii*	
M901	*P. maximowiczii*	
M908	*P. maximowiczii*	

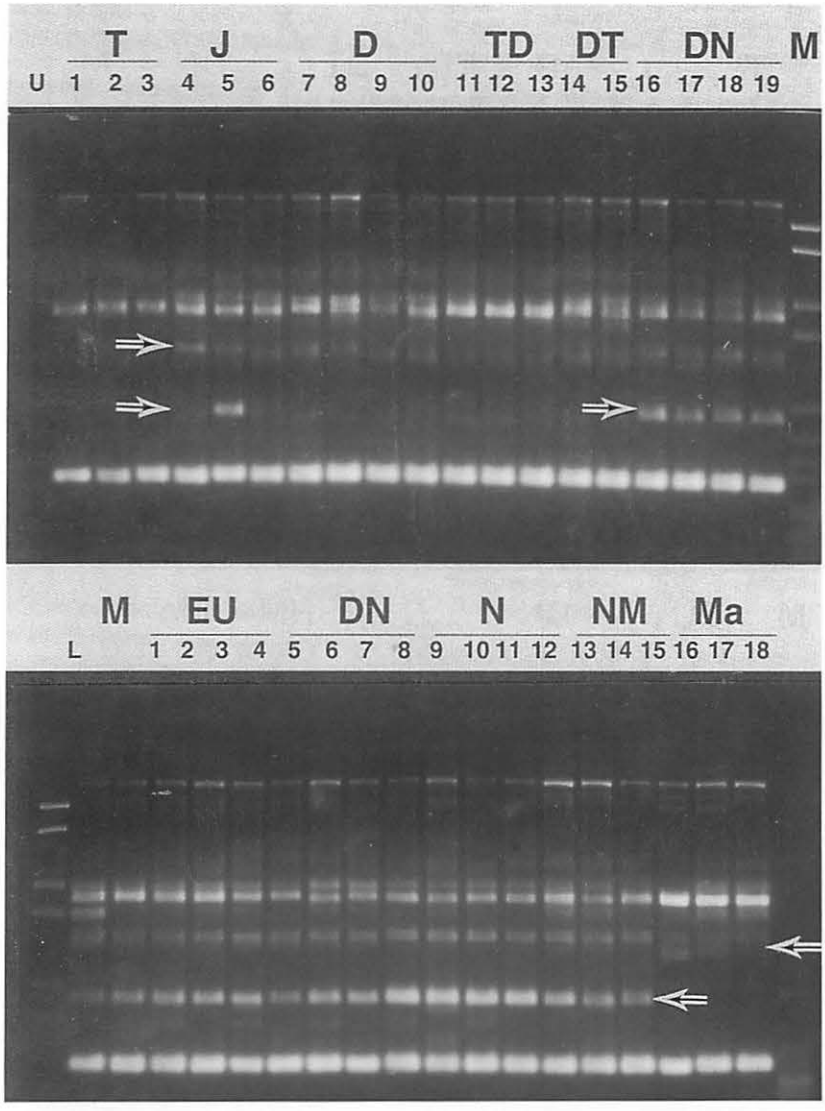


Figure 1. DNA polymorphism of poplar clones amplified with primer Deca-10.

T = *P. balsamifera*
DN = *P. deltoides* x *P. nigra*
J = *P.* x *jackii*
EU = *P.* x *euramericana*
D = *P. deltoides*
N = *P. nigra*
DT = *P. deltoides* x *P. trichocarpa*
NM = *P. nigra* x *P. maximowiczii*
TD = *P. trichocarpa* x *P. deltoides*
Ma = *P. maximowiczii*
M = Molecular Markers VI from Boehringer Mannheim Corporation
→ = species differences

ers was also performed in our study (Lin unpublished data) to generate highly reproducible DNA-banding patterns. However, our results from these experiments (1993 to 1995) indicate that either random or specific primers can be used for DNA fingerprinting without major problems.

The application of RFLP of rDNA, mtDNA, and cpDNA in differentiation of *Populus* is limited to the inter and intraspecies level. Compared to RAPD markers, the recently developed technique of amplified restriction fragment polymorphism (AFLP) (Vos et al. 1995) may provide an advantage in reliability for DNA fingerprinting. In practice, however, DNA fingerprinting by RAPD markers remains an easier and less expensive technique for individual clonal characterization.

Table 4. Identification of poplar species and hybrids by primers and molecular marker.

Primers	Marker size	Species and hybrids[1]
Deca-10	922	J, D, TD, DT, DN, EU, N, NM, M
	807	M
	600	DN, EU, N, NM
	583	J, D, TD, DT
Deca-2	1102	M
	953	T
	884	DN, EU, N, NM
Chl-1	1710	N, NM, M
	1035	TD
	77	DT
Deca-7	1649, 1474	DN, EU
	272	NM, M

[1] T = *P. balsamifera*
DN = *P. deltoides* x *P. nigra*
J = *P.* x *jackii*
EU = *P.* x *euramericana*
D = *P. deltoides*
N = *P. nigra*
DT = *P. deltoides* x *P. trichocarpa*
NM = *P. nigra* x *P. maximowiczii*
TD = *P. trichocarpa* x *P. deltoides*
M = *P. maximowiczii*

Acknowledgments

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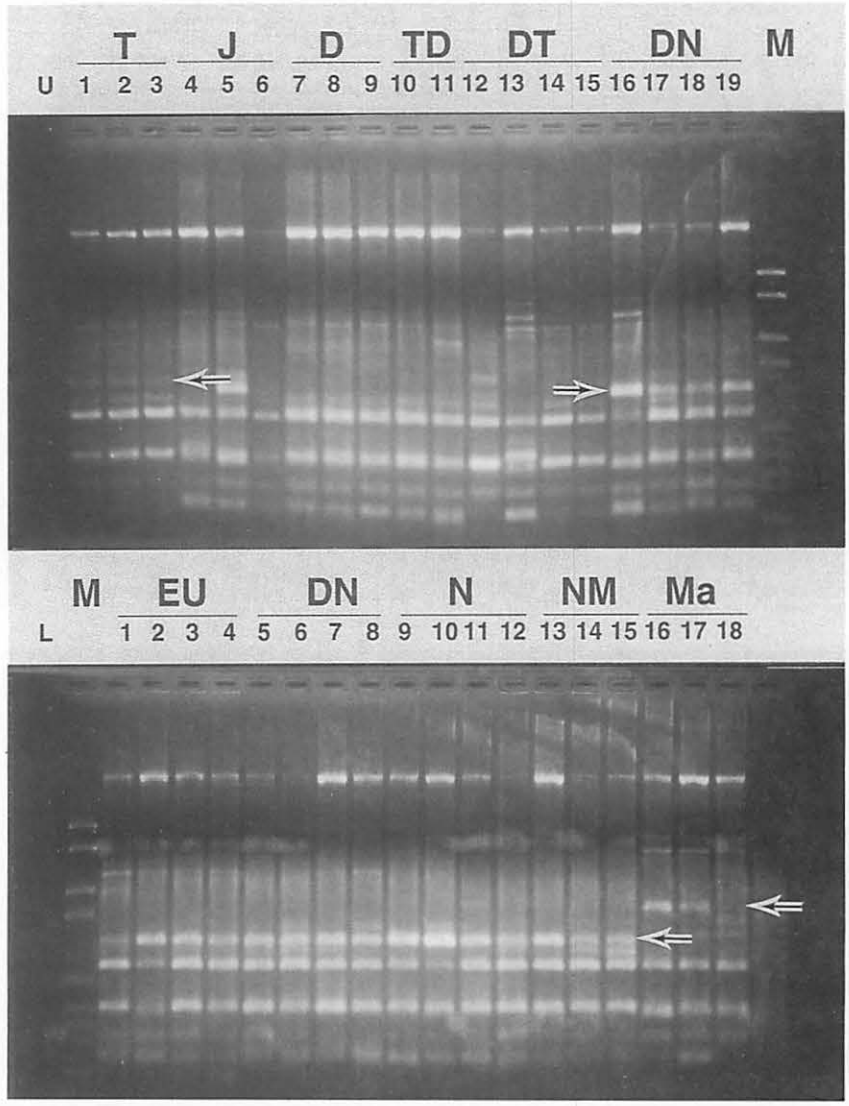


Figure 2. DNA polymorphism of poplar clones amplified with primer Deca-2.

T = *P. balsamifera*
DN = *P. deltoides* x *P. nigra*
J = *P.* x *jackii*
EU = *P.* x *euramericana*
D = *P. deltoides*
N = *P. nigra*
DT = *P. deltoides* x *P. trichocarpa*
NM = *P. nigra* x *P. maximowiczii*
TD = *P. trichocarpa* x *P. deltoides*
Ma = *P. maximowiczii*
M = Molecular Markers VI from Boehringer Mannheim Corporation
→ = species differences

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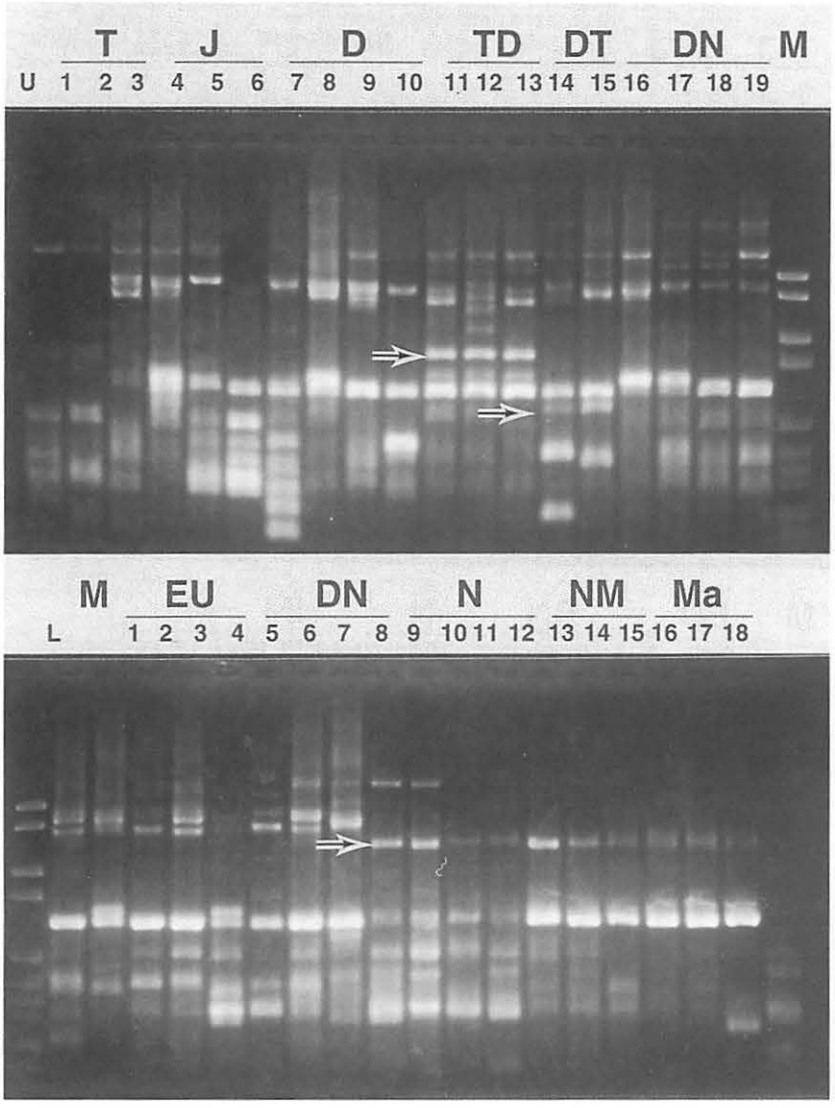


Figure 3. DNA polymorphism of poplar clones amplified with primer Chl-1.

T = *P. balsamifera*
DN = *P. deltoides* x *P. nigra*
J = *P.* x *jackii*
EU = *P.* x *euramericana*
D = *P. deltoides*
N = *P. nigra*
DT = *P. deltoides* x *P. trichocarpa*
NM = *P. nigra* x *P. maximowiczii*
TD = *P. trichocarpa* x *P. deltoides*
Ma = *P. maximowiczii*
M = Molecular Markers VI from Boehringer Mannheim Corporation
→ = species differences

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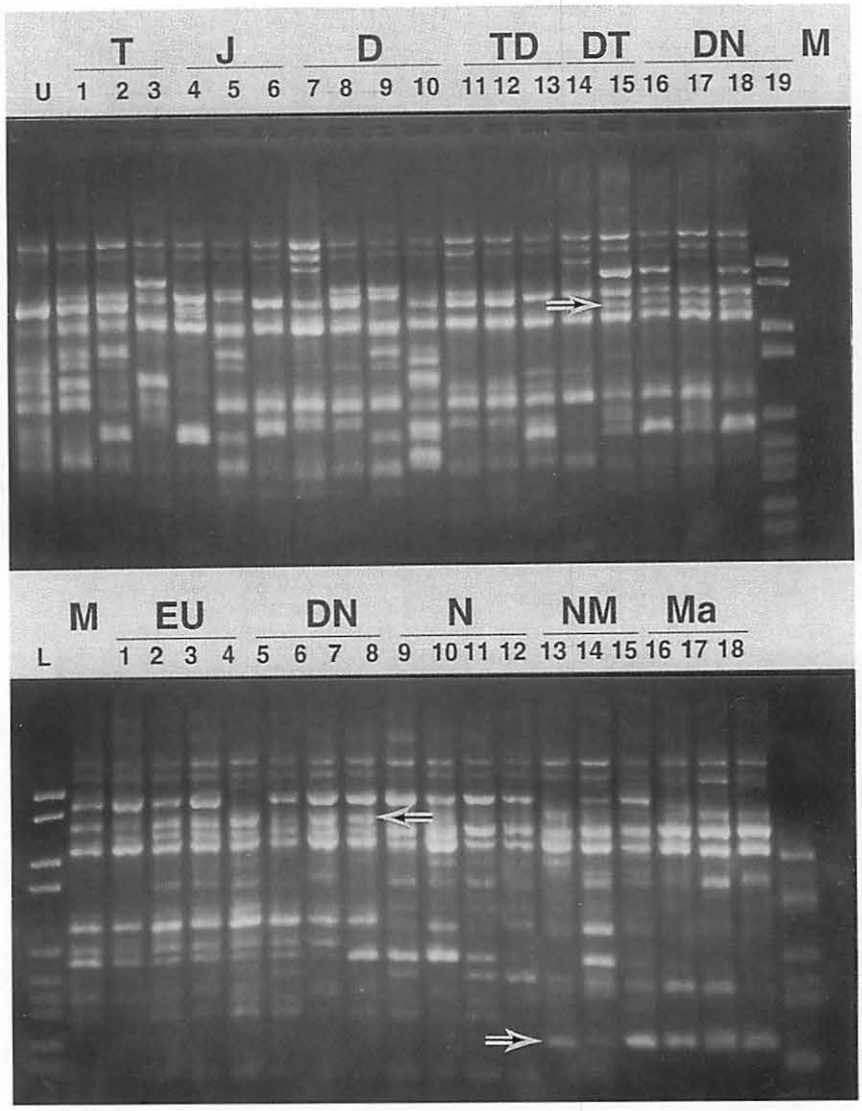


Figure 4. DNA polymorphism of poplar clones amplified with primer Deca-7.

T = *P. balsamifera*
DN = *P. deltoides* x *P. nigra*
J = *P.* x *jackii*
EU = *P.* x *euramericana*
D = *P. deltoides*
N = *P. nigra*
DT = *P. deltoides* x *P. trichocarpa*
NM = *P. nigra* x *P. maximowiczii*
TD = *P. trichocarpa* x *P. deltoides*
Ma = *P. maximowiczii*
M = Molecular Markers VI from Boehringer Mannheim Corporation
→ = species differences

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Chapter 16

Seasonal Vegetative Storage Proteins of Poplar[1]

Gary D. Coleman

Introduction

The availability of nitrogen often limits forest tree growth. Many tree species have evolved physiological strategies to reduce the impact of limited nitrogen availability. One strategy is to resorb nitrogen from leaves during autumn, and store it in perennial tissues during overwintering. During spring, this stored nitrogen is remobilized and used to support spring growth (Ryan and Bormann 1982; Taylor and May 1967). Besides reducing nitrogen loss from leaf fall, nitrogen resorption and storage may also reduce dependence on soil supplies during periods of high demand such as spring growth (Ryan and Bormann 1982). This review focuses on the molecular physiology of seasonal vegetative storage proteins in poplar. Advances in the biochemistry, physiology, and molecular biology of poplar vegetative storage proteins are reviewed. The general topic of woody plant vegetative storage proteins was reviewed in 1994 by Stepien et al. Poplar seed storage proteins are reviewed in this volume by Beardmore et al.

Seasonal Nitrogen Cycling

Nitrogen cycling in forest ecosystems is a complex process that involves nitrogen deposition, leaching, plant and soil uptake and return, and internal plant redistribution (Attiwill and Adams 1993). Internal nitrogen redistribution occurs when: 1) nitrogen is redistributed from source to sink tissue during growth, according to sink demands (Dickson et al. 1985; Vogelman et al. 1985); and 2) nitrogen is resorbed from senescing leaves, overwinter storage in perennial tissues, and spring mobilization to new growth.

Fall nitrogen resorption is a complex physiological process that is not well understood in woody perennials. Generally from 40 to 80 percent of leaf nitrogen is resorbed from fall senescing leaves before abscission. Leaf nitrogen resorption is associated with leaf protein hydrolysis and retranslocation of amino acids (Boerner 1984; Chapin and Kedrowski 1983; Luxmoore et al. 1981; Ostman and Weaver 1982). Protein hydrolysis and amino acid transport can account for 80 to 90 percent of leaf nitrogen removal (Chapin and Kedrowski 1983). Although factors that control fall leaf senescence and abscission are poorly understood in trees, it is know that nitrogen resorption can vary among species (Boerner 1984) and according to leaf position within a tree (Staaf and Stjernquist 1986). Nitrogen recycling is rapidly re-established during forest development (Ryan and Bormann 1982).

Seasonal nitrogen recycling has been extensively studied in tree fruit crops and is reviewed by Titus and Kang (1982). Considerable information is available for apple (*Malus*), which illustrates some general concepts of seasonal nitrogen recycling in deciduous trees. In apple, fall leaf senescence is accompanied by a decline in leaf nitrogen levels. This is associated with the translocation of leaf nitrogen to overwintering perennial storage sites, in particular the shoot bark (O'Kennedy et al. 1975; Oland 1963). During leaf nitrogen translocation, leaf protein levels decline by almost 50 percent while bark protein levels increase by over 200 percent (Kang and Titus 1980). Most of the decline in leaf protein is due to the preferential loss of ribulose 1,5-*bis*phosphate carboxylase/oxygenase (RUBISCO) (Kang and Titus 1980; O'Kennedy and Titus 1979). About 90 percent of the nitrogen in overwintering bark is in protein (Kang and Titus 1980; O'Kennedy and Titus 1979).

Studies indicate that spring growth is correlated with the level of stored nitrogen (Taylor and May 1967). Recent studies using ^{15}N have more precisely determined the importance of stored nitrogen to growth. Sanchez et al. (1991) showed that in pear trees, 45 percent of stored nitrogen was partitioned into new spring growth, and stored nitrogen accounted for 48 percent of the nitrogen in new growth.

[1] Klopfenstein, N.B.; Chun, Y. W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

Similar results have also been reported for walnut trees, where stored nitrogen accounted for 75 percent of the nitrogen in xylem sap during spring flowering and spur leaf development (Deng et al. 1989). Partitioning of stored nitrogen to new growth also seems independent of soil nitrogen supplies (Millard 1994; Millard and Neilsen 1989; Millard and Proe 1991; Millard and Thomson 1989).

Although nitrogen cycling and storage in *Populus* are not as well studied as apple, some information is available. In an aspen-mixed-hardwood forest, it was estimated that about 40 to 60 percent of foliar nitrogen was resorbed during fall leaf senescence (Pastor and Bockheim 1984). Killingbeck et al. (1990) estimated that in aspen over 50 percent of foliar nitrogen was resorbed and stored in perennial tissues during fall, and that the rate of resorption was dependent on leaf senescence since resorption rates were reduced during premature abscission. Total leaf nitrogen content declined nearly 50 percent during fall leaf senescence in *P. deltoides* and coincided with a reduction in salt-extractable proteins and an increase in free amino acid concentration (Côté and Dawson 1986; Côté et al. 1989). Bark nitrogen levels increased by as much as 97 percent during this period and correlated with increased salt-extractable proteins (Côté and Dawson 1986; Côté et al. 1989). Höllwarth (1976) showed that 95 percent of the nitrogen in the overwintering bark of *P. balsamifera* was contained in the protein fraction. Bark protein levels declined during spring growth while levels of soluble nitrogen compounds, including amino acids, increased (Höllwarth 1976). Similar patterns of seasonal nitrogen partitioning have been reported for poplar hybrids (*P.* x *euramericana* cv. 'Eugenei' and *P. tristis* x *P. balsamifera* cv. 'Tristis #1') where it was shown that about 75 percent of tree nitrogen is in the foliage during growth, but 80 percent of the nitrogen is retained in the tree after leaf abscission (Pregitzer et al. 1990).

Seasonal nitrogen cycling occurs in a wide-range of temperate deciduous species and is an important component of tree nitrogen budgets. Seasonal nitrogen storage involves fall resorption of leaf nitrogen before abscission providing a mechanism for conserving plant nitrogen. Although nitrogen can be stored in different chemical compounds, the predominate form is protein.

Poplar Vegetative Storage Proteins

Definition

Vegetative storage proteins (VSPs) are a heterogeneous class of proteins, that when accumulated, temporarily store carbon and nitrogen during periods of excess availability, or by resorption of nutrients that might otherwise be lost during tissue senescence. Stored carbon and nitrogen are available for later use by the plant (see Staswick 1994 for review). O'Kennedy and Titus (1979) proposed that in apple shoot bark a VSP should accumulate to high levels in dormant shoots and decline to low levels or disappear when growth resumes. Seasonal VSPs have been identified in several angiosperm and gymnosperm species (table 1). In poplar, specific VSPs have been identified that, because of their predominance in the bark, are referred to as bark storage proteins (BSP).

Accumulation and Localization

A major 32-kDa (kilodalton) BSP accumulates in bark phloem parenchyma and xylem ray cells during the fall and disappears during spring regrowth (Sauter et al. 1988; Wetzel et al. 1989a). During BSP accumulation, the large central vacuoles of bark phloem parenchyma and xylem ray cells are replaced by small spherical protein storage vacuoles that range from 0.5 to 0.8 μm in diameter (Sauter et al. 1988; Sauter et al. 1989; Sauter and van Cleve 1990; van Cleve et al. 1988; Wetzel et al. 1989b). Sauter and van Cleve (1990) demonstrated that morphological changes in these protein storage vacuoles were correlated with changes in BSP content. Immunochemical localization has demonstrated that these protein storage vacuoles are the sites for 32-kDa BSP accumulation (Sauter and van Cleve 1990; van Cleve et al. 1988).

BSP accumulation is not uniform throughout the tree, is greatest in the bark of 1-year-old shoots and wood, declines basipetally in bark and wood, and then increases in roots (Sauter et al. 1989). Therefore, BSP storage is greatest in tissues adjacent to sites of subsequent spring growth. Storage of nutrients near growing points would reduce the transport distance for these reserves and may improve nutrient reuse efficiency.

Biochemical Characterization

In addition to the 32-kDa BSP (Coleman et al. 1991; van Cleve et al. 1988; Wetzel et al. 1989a), a 36-kDa (Langheinrich and Tischner 1991; Stepien and Martin 1992) and a 38-kDa protein (Stepien and Martin 1992) were reported to accumulate in poplar bark. All of these different molecular weight BSPs are glycosylated (Langheinrich and Tischner 1991; Stepien and Martin 1992). Stepien and Martin (1992) showed that the neutral sugar content of BSP ranged from 10 to 20 percent; galactose-(1-3)-N-acetylgalactosamine and galactose-(1-4)-N-acetylglucosamine were present, but terminally linked mannose and sialic acid were absent. Results from deglycosylation analysis (Langheinrich and Tischner 1991) and peptide mapping (Stepien and Martin 1992) indicate that these different BSPs represent glycolytic isoforms of the "core" 32-kDa protein. Native BSP appears to be a heterodimer composed of 2

Table 1. Occurrence of bark storage proteins in trees.

Species	Tissue	Molecular mass (kDa)	Reference
Angiosperms			
Acer saccharum	bark	16, 24	Wetzel et al. 1989a
Betula papyrifera	bark	24	Wetzel and Greenwood 1991
Populus deltoides	bark	32	Wetzel et al. 1989a
	bark	32	Coleman et al. 1991
Populus x *euramericana*	wood	32	Sauter et al. 1988
	bark	32	Stepien and Martin 1992
Prunus persica	bark	16, 19	Arora et al. 1992
	wood	19	Arora et al. 1992
Robinia pseudoacacis	bark	29, 31	Nsimba-Lubaki and Peumans 1986
Salix x *smithiana*	bark	32	Wetzel et al. 1989b
Sambucus nigra	bark	34.5, 37.5	Greenwood et al. 1986
Sophora japonica	bark	30-35	Hankins et al. 1987
Gymnosperms			
Larix decidua	bark	25, 27, 32	Wetzel and Greenwood 1989
Metasequoia glyptostroboides	wood	32, 34	Harms and Sauter 1991
Pinus strobus	bark	15	Wetzel and Greenwood 1989
Pinus sylvestris	bark	15	Wetzel and Greenwood 1989
Taxodium distichum	wood	35	Harms and Sauter 1991

isoforms with an estimated molecular weight of 58 kDa (Langheinrich and Tischner 1991). Immunological and peptide mapping studies indicate that the 32-kDa protein in bark and wood is likely the same protein (Stepien et al. 1992). Although BSPs are known to be glycosylated, it is unknown if they are modified in any other manner. Unlike soybean VSPs, which also have phosphatase activity (DeWald et al. 1992), it is unknown if poplar BSP has any enzyme activity.

Molecular Cloning

Complementary DNA (cDNA) clones were isolated and sequenced for the 32-kDa BSP (Clausen and Apel 1991; Coleman et al. 1992). Comparison of the predicted amino acid sequence with the NH_2-terminal amino acid sequence from the purified 32-kDa protein suggests that the 32-kDa BSP is synthesized as a preprotein with NH_2-terminal signaling sequences for endoplasmic reticulum transport and vacuolar targeting (Coleman et al. 1992). Analysis of the predicted amino acid sequence indicates that the mature processed protein consists of 288 residues with a calculated molecular weight of 31,502 (Coleman et al. 1992). A potential glycosylation site (ASN-XXX-SER) is present in the mature peptide and is probably involved in the generation of glycolytic isoforms (Coleman et al. 1992). Based upon the predicted amino acid sequence, the protein is rich in the essential amino acids Leu, Lys, and Phe (Clausen and Apel 1991; Coleman et al. 1992).

Poplar BSP is encoded by a small multigene family (Coleman et al. 1992) consisting of approximately 2 to 3 genes (Coleman unpublished results). The BSP multigene family segregates as a single locus and is likely to be clustered (Davis et al. 1993). In addition, the BSP gene family is linked to a related gene family of wound-inducible cDNAs (*WIN4*) from poplar leaves (Davis et al. 1993). A number of *bsp* genomic clones were isolated (Coleman unpublished results) and 1 member, *bspa*, was sequenced (Coleman and Chen 1993).

Bspa is contained within a 3.9-kb *Eco*RV / *Afl*III fragment that includes a 1,646-bp (base pair) coding region, 1,246 bp upstream of the coding region, and 1,022 bp downstream of the coding region. The open reading frame is interrupted by 4 introns of 152, 262, 175, and 144 nucleotides in length. The transcriptional start site is located 71 nucleotides upstream of the translation start site (Coleman and Chen 1993). A number of potential regulatory sequence motifs are located in the upstream region of *bspa* including a putative TATA box that is located 25 nucleotides upstream of the transcriptional start site. Approximately 50 bases upstream of the TATA is a potential basic leucine zipper protein binding site (b-zip) that consists of the G-box core sequence ACGT (Armstrong et al. 1992; Oeda et al. 1991). Further upstream, approximately 300 nucleotides from the G-box core sequence, is a palindromic TGAC motif of 2 identical half-sites for b-zip binding that are identical to the cAMP response element CRE (Katagiri et al. 1989). Between these 2 sites are located several A/T rich

regions that may have a role in *bsp* transcription, since similar sequences had a role in regulating plant gene transcription (Bustos et al. 1989; Datta and Cashmore 1989). The significance of these sequences in regulating *bspa* gene expression is unknown, but current research suggests that transcriptional and post-transcriptional regulation are important in *bspa* gene regulation (Coleman unpublished results).

BSP Gene Expression

Several environmental factors including photoperiod (Coleman et al. 1991; Coleman et al. 1992; Langheinrich and Tischner 1991), nitrogen availability (Bañados 1992; Coleman et al. 1994; van Cleve and Apel 1993), temperature (van Cleve and Apel 1993), and wounding (Davis et al. 1993; Stepien and Sauter 1994) induce *bsp* expression. BSP accumulation is also tissue-specific, occurring primarily in stem bark parenchyma and xylem ray cells. However, regulatory factors governing tissue-specific expression remain undefined (Coleman et al. 1994; Davis et al. 1993).

Overall, the relationship between the above mentioned environmental factors that induce *bsp* expression is unknown. The exception is photoperiod and nitrogen availability, which interact during *bsp* induction and BSP accumulation. Although photoperiod is an important signal for *bsp* expression, nitrogen availability can interact with photoperiod to modulate gene expression and protein accumulation (Coleman et al. 1994). For example, reduced nitrogen availability delays photoperiod induced increases in steady state levels of *bsp* messenger RNA (mRNA). However, this delay is transitory since continued short-day exposure leads to similar levels of *bsp* mRNA in both nitrogen deficient plants and plants with optimal levels of nitrogen (Coleman et al. 1994). Furthermore, although both nitrogen deficient plants and those with optimal levels of nitrogen eventually have similar levels of *bsp* mRNA under short-day conditions, nitrogen deficient plants have reduced levels of BSP (Coleman et al. 1994). In severely defoliated plants, which have reduced levels of foliar nitrogen available for resorption and mobilization, short-day exposure still results in increased *bsp* mRNA levels (Coleman unpublished results). These results suggest that nitrogen availability and photoperiod both influence *bsp* expression, but this may be achieved by different mechanisms. Under short-day conditions, the major influence of nitrogen availability is on protein accumulation, while photoperiod is more closely associated with increases in mRNA levels. This might be explained if short-day photoperiod acts at the level of gene transcription and/or mRNA stability, while nitrogen availability influences some form of translational or post-translational control. Under certain conditions, however, nitrogen availability must also influence other levels of *bsp* gene regulation because both *bsp* mRNA and BSP accumulate to high levels in long-day plants treated with high levels of nitrogen (Coleman et al. 1994). Clearly, *bsp* gene regulation involves complex interactions between photoperiod and nitrogen availability.

VSP gene expression in soybean involves an interaction between nitrogen sinks and sources (Staswick 1994). Carbon source-sinks change during short-day induction of dormancy in poplar (Dickson and Nelson 1982). Under long-day conditions, poplars translocate carbon from mature source-leaves to young sink-leaves. During short-day conditions, carbon translocation changes from young sink-leaves to stem and root tissues (Dickson and Nelson 1982). Besides changes in source-sinks, short-day dormancy induction also leads to reduced carbon translocation from mature source-leaves (Dickson and Nelson 1982). Short-day photoperiod significantly alters source-sink relations in poplar, which probably are important in *bsp* induction.

Environmental regulation likely involves alteration of nitrogen source-sink relations associated with *bsp* expression. One mechanism could involve short-day alteration of nitrogen source-sinks and coincident induction of leaf senescence. Because of altered source-sink relations, available nitrogen from leaf senescence would be mobilized from the leaves to the stems, instead of from leaf to leaf. This increased level of stem nitrogen would then result in *bsp* induction. Although this mechanism provides a system for regulating *bsp* expression, it does not account for *bsp* induction in nitrogen deficient or severely defoliated plants. An alternative mechanism might involve induction of both *bsp* expression and leaf senescence by photoperiod. Short-day induction of *bsp* would provide a sink for nitrogen mobilization from leaves. Sink relations would be altered through reduced leaf sinks, while BSP synthesis and accumulation would increase stem sink strength. This increase in stem sink strength would be accompanied by increased nitrogen availability (nitrogen source) from photoperiod induced leaf senescence. Thus, short-days would induce both *bsp* expression and leaf senescence, thereby leading to source-sink changes. This might be reflected by the differences that nitrogen availability has on short-day accumulation of *bsp* mRNA and BSP (Coleman et al. 1994).

The molecular mechanisms controlling *bsp* expression are not well characterized. Results from nuclear run-off transcription assays suggest that transcriptional and post-transcriptional processes contribute to short-day and nitrogen induced *bsp* expression (Coleman unpublished results). Because BSP is encoded by a small multigene family, it is also possible that different environmental factors (i.e., photoperiod, nitrogen, or wounding) may result in differential gene expression. However, experiments involving S1 nuclease protection assays have failed to detect any evidence for differential gene expression during both short-day photoperiod and nitrogen induced gene expression (Coleman unpublished results).

Clonal Variation in BSP Accumulation

Pauley and Perry (1954) showed that stem growth cessation in *P. deltoides* and *P. trichocarpa* varied by ecotype, and that this variation was inversely correlated with the latitude of origin. Similar to growth cessation, maximum *bsp* mRNA levels of different ecotypes of *P. deltoides* vary inversely with the latitude of origin when grown in natural conditions. The latitude of origin accounted for nearly 70 percent of the variation in the date of *bsp* mRNA accumulation in different ecotypes (Coleman unpublished results)

Besides variation in gene induction, the bark protein content (mg/g fresh weight) of different poplar clones also varies among clones (Coleman et al. 1991; Langheinrich 1993). It seems probable that different poplar clones vary in their ability to resorb and store nitrogen from senescing leaves. The significance of this variation and its influence on resource use are undetermined. If BSP accumulation level is a component of overall nutrient-use efficiency, then its manipulation could be an important component in production.

BSP Degradation and Nitrogen Mobilization

Since BSP stores reduced nitrogen during overwintering for later use, then the plant must have mechanisms for mobilizing the stored BSP nitrogen. For BSP to be used during spring shoot growth the protein must be degraded to either individual amino acids or small polypeptides, so that the nitrogen can be loaded into the xylem and transported to supply new growth. BSP degradation and nitrogen mobilization are controlled by the sink demand from newly elongating buds and shoots (Coleman et al. 1993). Environmental factors, such as day length and temperature, appear to influence BSP degradation indirectly by influencing new shoot growth and nitrogen sink demand (Coleman et al. 1993). Since BSP degradation and nitrogen mobilization are dependent on new shoot growth, newly growing shoots must somehow communicate with BSP storage sites and initiate protein degradation. The nature of this communication is unknown, but it may involve phytohormones, such as gibberellins (GAs) or cytokinins, since these are correlated with bud break (Domanski and Kozlowski 1968; Powell 1987).

Nitrogen mobilization from BSP probably involves protein hydrolysis similar to the hydrolysis that occurs during seed storage protein utilization (Müntz et al. 1985; Shutov and Vaintraub 1987). The nature of the proteases involved in BSP degradation are unknown; however, activity by an endoprotease may be involved since 2 polypeptides of about 12 and 14 kDa appear to be the initial hydrolysis products (Bañados 1992). Furthermore, Sauter and van Cleve (1992) reported that glutamine is the predominant amino acid in the spring xylem sap of poplar, and that it can account for 75 percent of the amino acid sap content. Given this predominance of glutamine, BSP degradation must involve protein degradation to the constituent amino acids that are then used for glutamine synthesis and nitrogen transport.

Final Comments

The ability of poplars to resorb nitrogen from senescing leaves and store it as protein during overwintering makes them an excellent model to investigate the physiology, genetics, and molecular biology of seasonal nitrogen cycling. The ability to manipulate the growth cycle, the ease of vegetative propagation, and the ability to be genetically transformed adds to the value of poplars as a model for woody plant molecular physiology.

Although advances have been made in understanding the physiology and molecular biology of seasonal nitrogen cycling in poplars, significant questions remain. An interdisciplinary research approach that combines physiology, genetics, and molecular biology is required to increase the understanding of seasonal nitrogen cycling. The generation of transgenic poplars for studying the regulation of gene expression and the role of BSP offers exciting opportunities. Such research will further our understanding of seasonal nitrogen cycling in poplars and advance our knowledge of the molecular physiology of woody plants.

Acknowledgments

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Chapter 17

Poplar Seed Storage Proteins[1]

Tannis L. Beardmore, Suzanne Wetzel, and Sharon M. Regan

Introduction

Seed storage proteins have been actively studied since the mid 1700s (Beccari 1745) particularly in agricultural plant species. These studies can be attributed to the historic role of agricultural plants as a primary nutrition source for both humans and livestock. Recently, the study of agricultural plants has shifted toward genetically improving crops to increase stored protein content and enhance the nutritive value of the protein through manipulation of amino acid content (Blechl and Anderson 1996; Ceoloni et al. 1996; Hoffman et al. 1988; Kjemtrup et al. 1994; Lawrence et al. 1994). At a basic scientific level, seed storage protein (SSP) synthesis provides a particularly interesting model for examining gene regulation since storage protein expression is restricted to specific developmental times and cell types. This research area has yielded important information regarding the regulation and control of developmental events in embryogenesis and protein targeting. Seed research has been neglected in woody plants until recently. Although seeds of woody species are not always important for consumption, the study of seed storage proteins is necessary to understand topics such as tree biology, species distribution, phylogenetics, and systematics.

The following review consists of 3 sections. The first section is a general discussion of the current information regarding seed storage proteins. In the second section, woody plant work is discussed with a focus on the *Populus* seed storage proteins. The final section presents future prospects in storage protein research in *Populus*.

[1] Klopfenstein, N.B.; Chun, Y.W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

Seed Storage Proteins

Importance of Storage Proteins

Storage proteins provide a source of reduced nitrogen, carbon, and amino acids to growing tissues. When present in the seed, these proteins are called seed storage proteins (SSPs). When present in vegetative tissues, they are referred to as vegetative storage proteins (VSPs). Seed storage proteins and VSPs are 2 different forms of plant storage protein (Staswick 1988, 1990). For a discussion of the *Populus* VSPs, see the chapter by Gary Coleman (this volume). Seed storage proteins accumulate in the latter stages of seed development and are mobilized during germination and seedling growth (Higgins 1984). Until seeds can conduct sufficient photosynthesis to become fully autotrophic, storage proteins in combination with other stored reserves (i.e., lipids and starch) provide an essential food source as the seedling initiates heterotrophic growth.

Storage proteins were once thought to be metabolically inert and lacking in enzymatic activity (Larkins 1981). However, recently several storage proteins were found to function as storage compounds of nitrogen and carbon and as metabolically active enzymes. For example, phytohemaglutinins, ureases (Casey et al. 1986), ribulose *bis*phosphate carboxylase (Wittenbach et al. 1980), lipoxygenase (Tranbarger et al. 1991), lectins (Rudiger 1984; Spilatro et al. 1996), trypsin inhibitors, and Kunitz inhibitors are often classified as storage proteins. Many of these proteins function in stress responses, specifically in plant defense mechanisms. This could be advantageous for the germinating seed if a requirement develops for the *in vivo* enzymatic function.

Characterization of Seed Storage Proteins

Seed storage proteins are distinguished from other identified proteins based on 2 criteria they: 1) accumulate in the latter stage of seed development and are mobilized during germination and seedling growth; and 2) are present in relatively large quantities in mature seed

(Higgins 1984). These proteins can account for approximately 10 to 40 percent of the dry weight in mature seeds (Larkins 1981). Storage proteins are a complex of individual proteins that can be bound together by a combination of disulfide, hydrogen, ionic, and hydrophobic bonds. Storage proteins are primarily oligomeric, possessing multiple subunits within which are polypeptide chains that are usually disulfide bonded (reviewed in Larkins 1981).

Seed storage proteins are a diverse group of proteins because they are encoded by multigene families and have different post-translational processing (Bevan et al. 1993). This polymorphism can exist both within and among genotypes of the same species (Gonzalez and Henriques-Gil 1994; Hager et al. 1995; Horstmann et al. 1993). Despite this diversity, there are constraints on protein structure, probably related to their function, which result in similarities among the storage proteins. For example, the conservation of specific domains or amino acid motifs may be necessary for their processing, transport, and packaging during development and for their proteolysis during germination. Thus, selection pressure on amino acid sequence is perhaps less important than that on conservation of overall amino acid composition.

Traditionally, SSPs were classified on the basis of their solubility (Osborne 1924). Based on sequential extraction of the proteins, this classification system still provides the primary framework for current SSPs studies. This system is composed of 4 storage protein classifications: 1) albumins (soluble in water and dilute buffers with a neutral pH); 2) globulins (soluble in salt solutions but insoluble in water); 3) glutelins (soluble in dilute acids or alkaline solutions); and 4) prolamins (soluble in aqueous alcohols). Recently storage proteins were classified on the basis of conserved elements in the promoter regions of storage proteins genes (Morton et al. 1995). Interestingly, classification based on conserved elements corresponds closely to classification of storage proteins based on the solubility system of Osborne's (1924). Other parameters often used in classifying SSPs include sedimentation coefficients of the proteins (in Svedburg units, S), amino acid composition, molecular weight, and subunit and polypeptide composition (Larkins 1981). Unfortunately, these diverse classification criteria have increased the complexity of determining phylogenetic relationships.

Seed storage proteins were characterized in many species throughout the angiosperms and to a lesser extent in the gymnosperms. Although generalizations regarding SSPs are difficult, the major seed storage protein groups found in angiosperms are the globulins, prolamins, and albumins, with the globulin SSPs being the most widely distributed storage proteins (Derbyshire et al. 1976; Luthe 1992). The 7S (vicilin type) and 11S (legumin type) proteins are the major types of globulin SSPs based on the sedimentation coefficient of their aggregated forms (Derbyshire et al. 1976). Prolamin storage proteins are primarily restricted to monocotyledonous plants, while albumin storage proteins are found predominantly in dicotyledonous plants (Derbyshire et al. 1976).

Synthesis, Deposition, and Mobilization of Seed Storage Proteins

Synthesis of SSPs primarily occurs during the maturation stage of seed development (Larkins 1981). This stage in seed development is characterized by cell expansion and the deposition and accumulation of stored reserves (i.e., protein, lipid, and carbohydrate). Expression of SSPs is primarily restricted to seed tissue that is terminally differentiated. In angiosperms, sites of SSP synthesis and accumulation are in the embryo, primarily in the storage parenchyma cells of the cotyledons (Panitz et al. 1995; Weber et al. 1978) and in the persistent endosperm (Shewry et al. 1993). These sites of SSP expression and subsequent SSP accumulation provide nutrients for the germinating embryo. Other more transient sites of SSP expression, including the suspensor (Nagal et al. 1991; Newcomb 1973; Panitz et al. 1995) and liquid (nonpersistent) endosperm (Czako et al. 1991; Harris et al. 1989; Quatrano et al. 1986), can also occur during very early stages of seed development to sustain embryogenesis.

Transcription and translation of storage protein messenger RNA (mRNA) occurs during the maturation stage. Storage protein gene expression is regulated mainly at the transcriptional level (reviewed by Bevan et al. 1993; Morton et al. 1995). Several classes of DNA sequence motifs and associated transacting factors are recognized as having a role in SSP gene regulation (reviewed by Morton et al. 1995). Identifying regulatory sequences has been difficult, since these elements appear to reside far upstream from the SSP gene (e.g., greater than 1 kilobase). Also, the regulation of these genes appears to involve the interaction of multiple DNA elements.

Translational regulation of SSP synthesis also can occur. For example, in alfalfa somatic embryogenesis SSP, mRNA is present in the early stages of embryo development, but restricted to the nontranslated ribonucleoprotein particles (RNP) pool (Pramanik et al. 1992). When these embryos reach the early maturation stage, the SSP mRNA are shifted to polysomes. Furthermore, differential transcript stability was suggested as an important regulatory component in SSP mRNA accumulation in soybean embryos (Walling et al. 1986).

Storage proteins are synthesized with a signal polypeptide that is cleaved when the protein is translocated into the lumen of the endoplasmic reticulum (ER) (Muntz 1989). Some storage proteins also contain propeptides that must be cleaved to produce the physiologically active form (Ceriotti et al. 1991). Many storage proteins are synthesized as long polypeptides that are co- or post-translationally cleaved to generate 2 or more subunits (Croy et

al. 1980). Proteases responsible for these cleavages were identified in certain species (reviewed by D'Hondt et al. 1993).

Subsequent processing of these proteins is not well understood. Many processing events occur in the ER; disulfide bond formation, cleavage of the carboxy-terminal peptide, chaperone-mediated folding, isomerization, and N-glycoslyation (reviewed by Shewry et al. 1995). From the ER, most proteins are processed via the Golgi apparatus and are targeted to the vacuole or protein bodies (reviewed by Chrispeels 1991).

Little is known about the regulatory factors involved in the cessation of SSP synthesis. Repression or down regulation of storage protein synthesis occurs toward the end of the maturation stage just before seed desiccation. Chern and coworkers (1996) recently identified a regulatory protein that is apparently involved in repressing SSP transcription in *Phaseolus vulgaris*.

Overall, available information regarding storage protein hydrolysis is limited. It was suggested that multiple mechanisms are responsible for SSP hydrolysis, involving transcriptional and post-transcriptional regulation (Callis 1995). Briefly, the hydrolysis of the storage proteins into their constituent amino acids is induced by proteases (Fincher 1989). The amino acids are reused for protein synthesis or they are deaminated, providing carbon skeletons for respiratory oxidation (Callis 1995). Proteases involved in SSP hydrolysis are apparently synthesized *de novo* during germination and, to a lesser extent, are present in an inactive form in the dry seed, which is activated upon germination (Wilson 1986).

Seed Storage Proteins in *Populus* and Other Woody Plants

Woody Plants

Seed research in woody plants is problematic due to the time required for these plants to obtain reproductive maturity, their long reproduction cycle, and irregular flowering processes. Nonetheless, research on seed storage proteins of woody plants has originated from 2 areas. One area is the development of somatic embryogenesis procedures for tree species of commercial importance (i.e., primarily gymnosperms). In this area of study, SSPs are used as markers to determine the embryogenic potential (Roberts et al. 1989; Sotak et al. 1991) and competency of the embryogenic system to produce a somatic embryo that is biochemically similar to its zygotic counterpart (Flinn et al. 1991b; Hakman 1993; Hakman et al. 1990). The second area of study examines the phylogenetic origin and molecular evolution of woody species (Luthe 1992).

To date, most information regarding characterization of SSPs in woody plant species is related to their accumulation patterns during embryo development (Flinn et al. 1991b; Hakman 1993; Hakman et al. 1990). Such information is determined by electrophoretic and immunological analysis. Alternatively, DNA and amino acid sequence information have been more limited. Current knowledge of SSPs in woody species is in tables 1 and 2. Storage pro-

Table 1. Seed storage proteins in angiosperm woody species.

Species	Family	Native protein (kDa)[1]	Subunits (kDa)	Polypeptides (kDa)	Protein family	Reference
Cercis siliquastrum	Fabaceae	[2]	[2]	168, 144, 104, 100, 72, 68, 54, 52, 36, 34	globulin	Gonzalez and Henriques-Gil 1994
Castanea sativa	Fabaceae	260 to 240	62 to 56, 48 to 41	36 to 31, 23 to 22, 20 to 19, 18	globulin	Collada et al. 1991
Castanea crenata	Fabaceae	260 to 240	34 to 32		globulin	Collada et al. 1991
Quercus ilex	Fabaceae	[2]	62 to 50, 46 to 40, 35 to 32	40 to 30, 24 to 22, 22 to 21	glutelin	Collada et al. 1991
Quercus robur	Fabaceae	[2]	58 to 50, 43, 34 to 31	34 to 31, 23 to 22, 22 to 21, 17	glutelin	Collada et al. 1991
Populus grandidentata	Salicaceae	120 to 100	60, 58	36, 32, 22, 18, 14	albumin	Beardmore et al. 1996

[1] kilodaltons
[2] not reported

Table 2. Seed storage proteins in gymnosperms.

Species	Family	Native protein (kDa)[1]	Subunits (kDa)	Polypeptides (kDa)[2]	SSP[3] family	Reference
Ginkgo biloba	Ginkgoaceae	400	50, 31	28, 20, 13	globulin	Jensen and Berthold 1989
Macrozamia communis	Zamiaceae	260	126	44	globulin	Blagrove et al. 1984
Abies koreana	Pinaceae	[4]	55	43, 28, 22, 16	globulin	Jensen and Lixue 1991
Cedrus atlantica	Pinaceae	[4]	55	32, 21	globulin	Allona et al. 1994b
				45, 33, 22	globulin	Allona et al. 1994b
Larix occidentalis	Pinaceae	[4]	[4]	41, 35, 22	globulin	Misra and Green 1994
Picea abies	Pinaceae	[4]	[4]	42, 33, 28, 22	globulin	Hakman et al. 1990
Piceae glauca	Pinaceae	[4]	44		globulin	Newton et al. 1992
Picea glauca x *P. engelmanii*	Pinaceae	[4]	[4]			
Picea glauca x *P. engelmanii*	Pinaceae	[4]	[4]	35, 33, 24, 22, 17, 16	globulin	Flinn et al. 1991a, 1991b
Pinus monticola	Pinaceae	[4]	55 to 51	35 to 31, 22	[4]	Gifford 1987
				41	albumin	Flinn et al. 1991a, 1991b
Pinus pinaster	Pinaceae	190	47, 27, 22		globulin	Allona et al. 1994a
		175	47, 27, 22		globulin	Allona et al. 1994a
Pseudotsuga menziesii	Pinaceae	63 to 55	35 to 32, 25 to 22		globulin	Leal and Misra 1993a
Biota orientalis	Cupressaceae	[4]	60 to 58	23 to 22	globulin	Allona et al. 1994b
				45, 25 to 24, 21	globulin	Allona et al. 1994b
Calocedrus decurrens	Cupressaceae	[4]	[4]	34 to 30, 25 to 23	globulin	Hager and Dank 1996
Chamaecyparis lawsoniana	Cupressaceae	[4]	60 to 56	34 to 31, 24 to 21	globulin	Allona et al. 1994b

[1] kilodaltons
[2] relative molecular weight
[3] seed storage protein
[4] not reported

teins from such families as Pinaceae, Cupressaceae, Ginkgoaceae, Fabaceae, and Salicaceae have been characterized (tables 1 and 2).

The presence of SSPs homologous to the 11S and 7S globulin proteins were identified in several gymnosperms (Allona et al. 1994a, 1994b; Flinn et al. 1991a; Gifford 1987; Hakman et al. 1990; Leal and Misra 1993a) and a few of the woody angiosperms (Arahira and Fukazawa 1994; Collada et al. 1991). Amino acid sequences of various woody species, such as *Pinus pinaster* (Allona et al. 1994a), *Calocedrus decurrens* (Hager and Dank 1996), *Picea glauca* x *P. engelmanii* (Newton et al. 1992), *Pseudotsuga menziesii* (Leal and Misra 1993a), and *Ginkgo biloba* (Arahira and Fukazawa 1994), show that SSPs are highly conserved plant proteins and that the amino acid identity levels follow taxonomic groupings among closely related species.

In gymnosperms, SSP gene expression can be regulated by the plant growth regulator abscisic acid and the osmotic environment (Dunstan et al. 1988; Misra et al. 1993; Roberts 1991; von Arnold and Hakman 1988). In gymnosperm embryos and megagametophytes, temporal changes in the SSP accumulation are similar to that found in angiosperms, with accumulation occurring in the mid- to late-maturation stage (Flinn et al. 1991a, 1991b; Hakman 1993; Hakman et al. 1990; Misra and Green 1994; Roberts et al. 1989). Protein bodies are most evident in the cotyledons of gymnosperm embryos (Durzan et al. 1971; Hakman 1993; Johnson et al. 1987), suggesting that this is the predominant site of accumulation in the embryo. Leal and Misra (1993b) observed transient accumulation of SSP transcripts very early during development in the embryo and megagametophyte of *Picea glauca*. Possibly, these tran-

sient accumulation sites supply nutrients to the developing embryo.

Populus

Seed storage protein analysis has been conducted for only 1 *Populus* species, *P. grandidentata*. In *Populus*, this area of study is tedious due to difficulties in effectively working with very small seeds and the necessity of working quickly due to the short duration of seed viability (i.e., loss of viability can occur as soon as 5 days after tree collection) (Brinkman 1974; Hellum 1973).

Storage protein composition can be elucidated using two-dimensional, polyacrylamide gel electrophoresis to allow for the selective and sequential disassembly of the storage proteins into their constituent polypeptides. Using this approach, *P. grandidentata* SSP was found to be a 120 to 100 kilodalton (kDa) albumin storage protein (i.e., water soluble) containing 60 and 58 kDa subunits (Beardmore et al. 1996). Refer to table 1 and figures 1A and 1B for a summary of storage protein composition. When these subunits are reduced (i.e., disulfide bonds are broken), they yield polypeptides with molecular masses of 36, 32, 22, 18, and 14 kDa (figure 1B). Stoichiometrically the protein is a dimer, containing 2 hydrogen-bonded subunits of 60 and 58 kDa.

The 36 and 32 kDa polypeptides are glycosylated and occur in approximately 8 to 12 isomeric forms (pI 7.0 to 8.2) (figure 1C). The 18 and 14 kDa polypeptides do not exist as isomers and have a pI of 7.0. The 22 kDa polypeptide is suggested to be either very acidic (< 5.0) or very basic (>8.0), since it does not resolve during conventional isoelectric focusing (Beardmore et al. 1996).

This research has illustrated similarities between the seed and vegetative storage proteins in *Populus* (Beardmore et al. 1996). Protein fingerprint patterns of the 36 kDa polypeptide isolated from seed (sexual) and stem tissue (vegetative) yielded polypeptides of identical molecular masses. However, it is important to note that while the VSP and SSP may be similar with regard to constitutive polypeptides (i.e., both contain 36 and 32 kDa polypeptides), they differ with respect to their native proteins and subunit composition. Specifically, the SSP 36 and 32 kDa polypeptides are disulfide bonded, while the VSP 36 and 32 kDa polypeptides are hydrogen bonded (figures 2A, 2B).

Physiology of *Populus* Seed Storage Protein: Accumulation, Deposition, and Mobilization

Poplar seed consists of an embryo approximately 3 mm in length surrounded by the testa (Schreiner 1974). The embryo is the main area of deposition for storage protein (Beardmore et al. 1996; Breton et al. 1993). Soluble protein content of *P. deltoides* zygotic embryos significantly increased from 25 days after pollination (DAP) to 40 DAP, when the embryo was mature (Breton et al. 1993). Breton and coworkers (1993) suggest that this increase was due in part to storage protein deposition. Polypeptides with a similar molecular mass (approximately 60 to 58, 36, 32, 18, 14 kDa) to those identified in *P. grandidentata* embryos were detectable relatively early (15 DAP) in *P. deltoides* globular embryos (Breton et al. 1993), suggesting that storage protein synthesis may occur early in embryo development.

Storage protein mobilization in *P. grandidentata* occurs while the seeds germinate. The *P. grandidentata* embryos germinated by 72 h after imbibition, coinciding with the disappearance of the storage proteins from SDS-PAGE gels (Beardmore et al. 1996).

Homology of *Populus* Seed Storage Proteins

Putative storage proteins were identified on the basis of their relative abundance in *P. deltoides* and *P. trichocarpa* seeds (Breton et al. 1993). In *P. deltoides* and *P. trichocarpa* seeds, these storage proteins were identified with molecular masses in the range of 36 to 32, 22, and 20 kDa (Breton et al. 1993), similar to the molecular masses of the *P. grandidentata* SSPs.

A survey of the major polypeptides present in selected Salicaceae family members revealed that these seeds contained large quantities of polypeptides with similar or the same molecular masses as the *P. grandidentata* storage proteins (Beardmore et al. 1996). Such similarities suggest that *Populus* species and other Salicaceae family members may have homologous storage proteins. However, more studies are required for proper identification of the SSPs in these various species.

In addition, the SSPs in yellow poplar (*Liriodendron tulipifera* L.) zygotic and somatic embryos (Sotak et al. 1991) also appear to be very similar to those identified in *P. grandidentata*. The putative storage protein in *L. tulipifera* has a molecular mass of approximately 55 kDa (Sotak et al. 1991). Polypeptides with molecular mass in the mid-30 kDa range and in the 22 to 14 kDa range were also present in large quantities and at the same relative abundance as the 55 kDa polypeptide, suggesting that these polypeptides may also be SSPs (Sotak et al. 1991). SSPs in seeds of the Fagaceae family also appear to contain polypeptides with similar molecular masses (table 1).

Homology of the Seed Storage Protein to the Vegetative Storage Protein in *Populus*

The poplar 36 kDa VSP and SSP exhibit extensive homology, based on proteolytic digestion patterns, antibody cross-reactivity, and chemical characteristics (Beardmore

A. i) Protein was separated in the first dimension with nondissociating conditions using polyacrylamide gel electrophoresis (PAGE).

A. ii) Protein was separated in the second dimension with dissociating sodium dodecyl sulphate (SDS) and reducing conditions (dithiothreitol, DTT) (SDS-PAGE + DTT).

B. i) Protein was separated in the first dimension with dissociating conditions (SDS-PAGE).

B. ii) Protein was separated in the second dimension with dissociating and reducing conditions (SDS-PAGE + DTT).

C. Protein was separated in the first dimension by isoelectric focusing (IEF) and in the second dimension by dissociating and reducing conditions (SDS-PAGE + DTT).

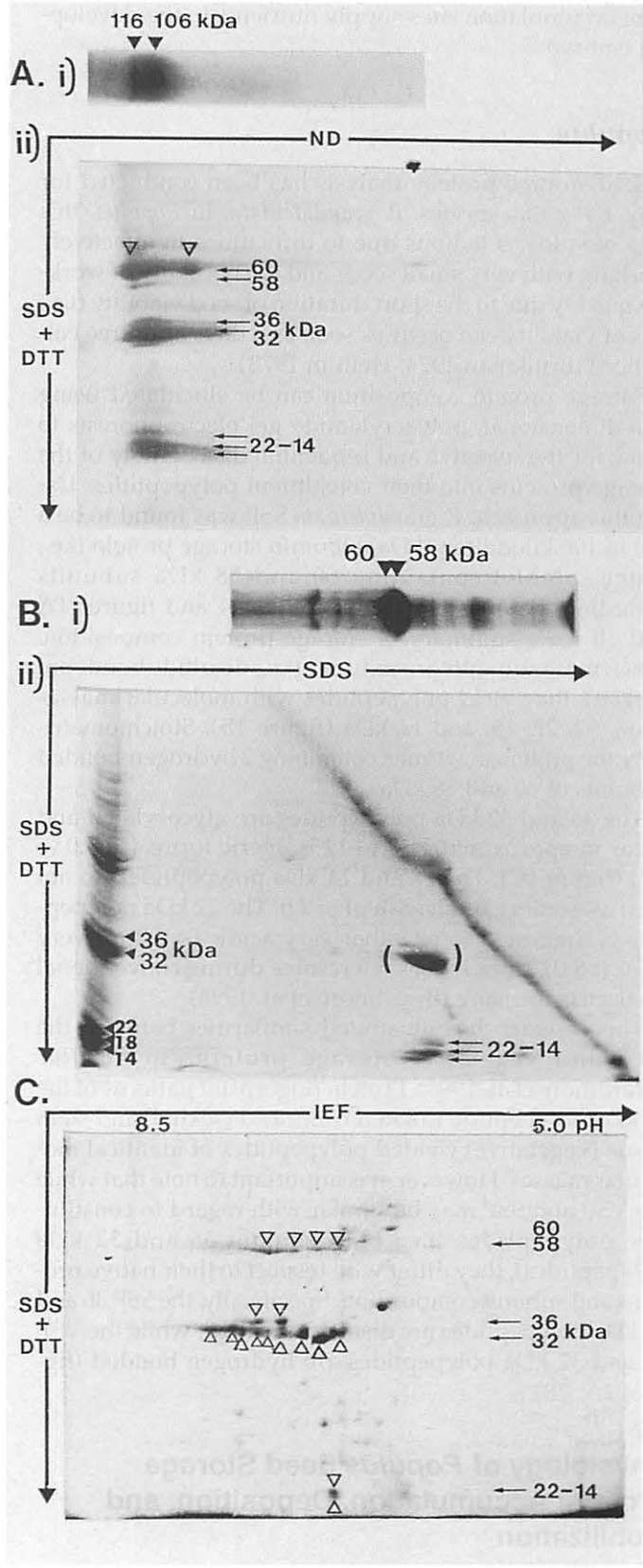


Figure 1. A 2-dimensional, polyacrylamide gel analysis of the seed storage proteins in *Populus grandidentata*. Molecular mass markers in kilodaltons (kDa).

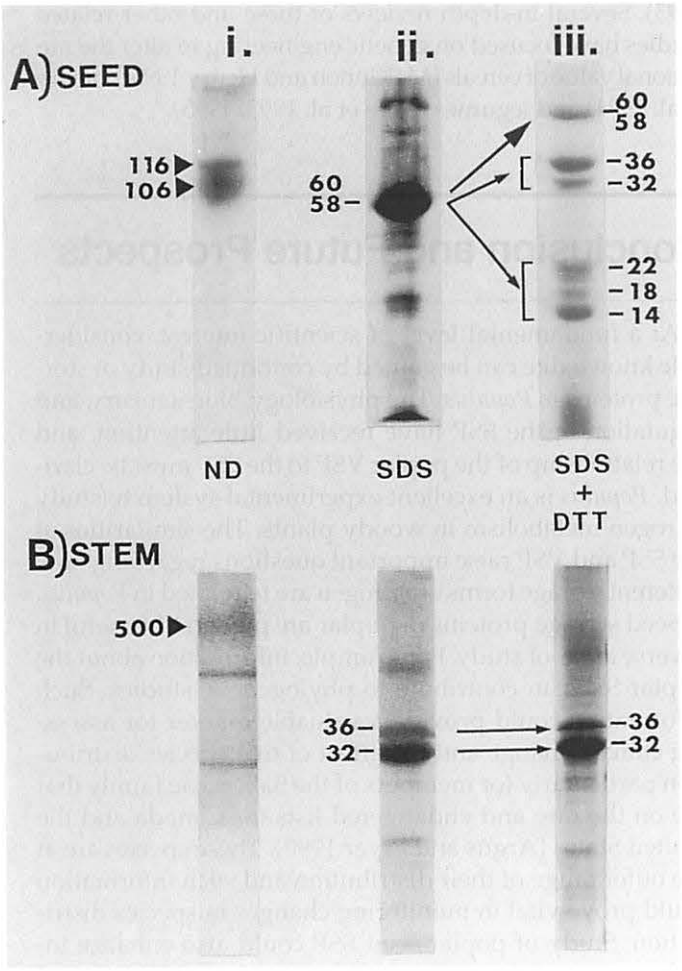


Figure 2. Summary of the seed storage proteins and vegetative storage protein in *Populus*. One-dimensional electrophoresis of: i) nondissociated protein (ND); ii) dissociated protein (SDS-PAGE); and iii) dissociated and reduced protein (SDS-PAGE + DTT). A) Seed storage protein isolated from *P. grandidentata*. B) Vegetative storage protein isolated from *P. alba* stems.

et al. 1996). Such homology indicates that these proteins may be encoded by the same gene. This situation contrasts with the relationship of the VSP and SSP in soybean where the storage proteins are expressed by separate vegetative-specific and seed-specific genes (Staswick 1988). However, differences in post-translational modification of VSP and SSP in poplar (i.e., disulfide bond formation) could result from tissue-specific differential processing of the proteins. Such regulation was suggested for the alternative oxidase in potato, where protein analysis of leaves, roots, and tubers indicated differential processing (Hiser and McIntosh 1995). Isolation of the complementary DNA (cDNA) of the SSP would help clarify the relationship between the VSP and SSP in poplar.

The hypothesis that the same gene codes for VSP and SSP is supported by recent transgenic work with the *Populus* VSP promoter (Gary Coleman preliminary and unpublished results). When the VSP promoter was linked to β-glucuronidase (*GUS*) gene and transferred into tobacco, *GUS* expression occurred in the seed and also the vegetative tissues. Whether this expression can account for all or just a portion of the SSP in the seed is unknown.

Despite the potential relationship between the VSP and SSP genes, at least 3 possibilities for the organization and regulation of the SSP genes exist with poplar. One possibility is that the SSP 36 and 32 kDa polypeptides are encoded by separate genes. These polypeptides are likely related in sequence to each other and to the VSP, since immunogenic analysis could not differentiate these proteins (Beardmore et al. 1996). Because the SSP 36 and 32 kDa polypeptides apparently occur in equal amounts, the respective genes must be expressed at a similar rate. Association of the 2 polypeptides to form the complete native storage protein would, however, depend on intermolecular disulfide bonds. Although identified disulfide bridge formation in plant proteins is usually intramolecular, several examples of intermolecular disulfide bridges are documented. Examples of intermolecular disulfide bridges in storage proteins include castor-seed 2S albumin protein (Shin et al. 1993), wheat high molecular mass glutinin (Shani et al. 1994), soybean seed 34 kDa protein (Komatsu et al. 1992), and lectin from *Eranthis hyemalis* (Kumar et al. 1993). Based on these examples, the SSP 36 and 32 kDa polypeptides could possibly be encoded by separate genes, with their products associated by intermolecular disulfide bonds.

Alternatively, the 36 and 32 kDa SSP could be encoded by 1 large gene, the product of which contains intramolecular disulfide bonds. Similar to many other seed storage proteins, the 2 subunits are subsequently formed as separate 36 and 32 kDa peptides by a specific proteolytic cleavage. The sequence of this larger gene may be related to the VSP gene. Finally, the 36 and 32 kDa proteins could be differentially-processed products of the same gene. This apparently occurs with the VSP; the 36 and 32 kDa proteins are glycoforms, differing only in the extent of glycosylation (Langheinrich and Tishner 1991; Stepien and Martin 1992). A review of this topic is in the chapter by Gary Coleman (this volume).

Currently, evidence supports the hypothesis that 1 gene is differentially processed to produce the 36 and 32 kDa polypeptides that are temporally and spatially regulated to form VSP in vegetative tissues and SSP in seeds. Thus, the poplar storage protein system could be an excellent model to analyze differential regulation at the transcriptional, translational, and post-translational levels. The answer may be resolved by analyzing the gene corresponding to SSP in pop-

lar seeds. Isolation of the putative SSP gene(s) could be achieved with either a lambda gt11 expression library with the SSP antisera or by using the VSP gene to isolate related cDNAs in the seed. Future efforts may also include analysis of the VSP and SSP promoter(s) to determine if tissue-specific elements are responsible for their expression patterns.

Approaches to Understand Seed Storage Protein Gene Expression

Despite many empirical observations about embryogenesis, the molecular and biochemical basis of this developmental pathway is poorly understood. An important limiting factor is the difficulty in obtaining experimental populations of developing seeds that are synchronized in their development and viable year round. Recent innovations in *Populus*, such as the development of somatic embryogenesis protocols (Michler and Bauer 1991) and the successful expression of the Arabidopsis *LEAFY* (*LFY*) gene in poplar transformants (Sundberg et al. this volume; Weigel and Nilsson 1995), provide potentially useful experimental systems that promote laboratory study of poplar SSP gene expression. Somatic embryogenesis protocols are useful because they allow mass quantities of developmentally synchronized embryos to be produced through manipulation of tissue culture conditions. Transgenic poplars expressing the developmental regulator gene *LEAFY*, might provide a convenient source of reproductively mature material for year round controlled pollinations because these plants produce reproductive structures independent of seasonal cues. Until these new approaches prove useful, other model systems will likely serve as surrogate hosts for poplar SSP transgenes. Because tobacco and *Arabidopsis* are easily transformed and have short juvenile phases, they will likely be the species of choice for transgenic work.

Production of an engineered seed storage protein requires accurate processing at all levels of regulation, from tissue-specific expression in the seed, to transport to the appropriate vacuole/protein body. To ensure success of future genetic engineering efforts, significant research has occurred on various aspects of seed storage protein production. These studies have included research on identification of suitable proteins (Mehta et al. 1994), stability of the mRNA (Stayton et al. 1991), stability of the protein (Bagga et al. 1995), post-translational processing of the protein including glycosylation (Sturm et al. 1988), assembly of subunits (Beachy et al. 1985), and other modifications (Ellis et al. 1988). Accurate targeting and assembly of storage proteins were studied by Greenwood and Chrispeels (1985), DeClercq et al. (1990), Kjemtrup et al. (1994), and Zheng et al. (1995). Most transgenic research has involved the production of desirable proteins, but reverse genetics was used in 1 study to alter protein composition of seeds (Kohno-Murse et al. 1995). Several in-depth reviews of these and other related studies have focused on genetic engineering to alter the nutritional value of cereals (McKinnon and Henry 1995; Shewry et al. 1994) and legumes (Tabe et al. 1993, 1995).

Conclusion and Future Prospects

At a fundamental level of scientific interest, considerable knowledge can be gained by continued study of storage proteins in *Populus*. The physiology, biochemistry, and regulation of the SSP have received little attention, and the relationship of the poplar VSP to the SSP must be clarified. *Populus* is an excellent experimental system to study nitrogen metabolism in woody plants. The similarities of the SSP and VSP raise important questions regarding how different storage forms of nitrogen are regulated in *Populus*.

Seed storage proteins in poplar are potentially useful in diverse areas of study. For example, information about the poplar SSP can contribute to phylogenetic studies. Such information could provide a valuable marker for assessing climate change and the effect of tree species distribution particularly for members of the Salicaceae family that are on the rare and endangered lists for Canada and the United States (Argus and Pryer 1990). These species are at the outer range of their distribution and such information could prove vital in monitoring changes in species distribution. Study of poplar seed SSP could also enhance insights into seed development necessary for extending seed viability particularly for many members of the Salicaceae family whose existence may be threatened without effective management and knowledge of their seed.

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Chapter 18

Molecular Genetic Analysis of *Populus* Chloroplast DNA[1]

Eun W. Noh and Jae S. Lee

Introduction

Molecular techniques have been applied to differentiate closely related species and varieties or to study genetic variation of wild populations. Diverse methods have been applied to generate different genetic markers. Therefore, information obtained by the markers varies in amount and type.

Cloned DNA sequences are useful to probe specific regions of genomes for polymorphisms at the DNA sequence level. After DNA digestion with restriction enzymes, these polymorphisms are detected as variation in the length of DNA fragments homologous to a labeled probe. Such variation is termed restriction fragment length polymorphism (RFLP). The RFLP technique was successfully employed in many tree species including poplars. D'Ovidio et al. (1990) distinguished different *Populus* species by analyzing 18S and 25S ribosomal DNA (rDNA) with RFLP. Faivre-Rampant et al. (1992) also reported a simple method of differentiating poplar clones by RFLP, and a donor species in putative hybrids was identified by fragment profiles. Although RFLP generates many polymorphic markers that are useful to differentiate clones and individuals, the technique is time consuming, labor intensive, and expensive.

Another kind of DNA marker called random amplified polymorphic DNA (RAPD) has been developed based on polymerase chain reaction (PCR). In this method, polymorphisms are detected by differential amplification of DNA fragments. This technique provides an advantage over RFLP in its simplicity, rapidity, and requirement for only small amounts of crude DNA. Estimates of genetic similarity based on RAPD profiles in poplar have demonstrated consistency with the present taxonomy of the genus *Populus* (Castiglione et al. 1993; Liu and Furnier 1993). However, a major limitation of this technique is that markers are usually dominant rather than codominant. Reproducibility of RAPD banding patterns also presents a problem because the pattern is affected by different concentrations of reaction components and cycle conditions.

PCR techniques are also used to produce genetic markers based on site-specific amplication. For this technique, known or anticipated sequence information is used to design primers to amplify a specific site. For site-specific PCR, ribosomal RNA (rRNA) genes are widely used because they comprise domains that are conserved across diverse taxa (Sogin 1990). A further derivation of this technique uses restriction endonucleases to cut the amplified fragments (Liston 1992; Nishio et al. 1994). This PCR-RFLP method also locates genomic regions containing variation. When specifically designed primers are used, banding patterns are usually more reproducible than random primers.

Organelle genomes like chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA) offer strong potential to provide markers for tree species. The small size of organelle genomes provides a major advantage. Although the size varies from organism to organism, cpDNA is circular and 135 to 160 kilobases (kb) in size for most land plants (Olmstead and Palmer 1994). Another merit associated with cpDNA is its conserved nature. Evolution of cpDNA has occurred by mutations such as inversions, deletions, insertions, and substitutions. However, comparisons from different taxa have revealed that the cpDNA nucleotide sequences were well conserved during the evolution of land plants. In most angiosperms, including poplars, cpDNA and mtDNA are maternally inherited. Since little variation occurs within cpDNAs and mtDNAs of a species, any polymorphisms in these genomes could be a useful marker.

[1] Klopfenstein, N.B.; Chun, Y.W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

Review of RFLP Analysis of *Populus* Chloroplast DNA

With the availability of many gene probes from well-studied plants, cpDNA from any plant species can be compared by the RFLP technique. Comparisons among species within a genus have shown only a few differences that are often confined to 1 or 2 restriction site polymorphisms. Such comparisons were demonstrated in *Medicago* (Rose and Schlarbaum 1988), *Nicotiana* (Salts et al. 1984), *Solanum* (Kawagoe and Kikuta 1991), and *Picea* (White et al. 1993). Salts et al. (1984) studied cpDNA of 7 American and 4 Australian species of *Nicotiana* by RFLP. With the RFLP profiles, more than 120 polymorphic sites were distinguishable on cpDNA of *N. tabacum*.

With *Populus*, previous results have confirmed maternal inheritance of cpDNA in the genus. However, maternal or paternal inheritance is detectable only when a marker specific to an individual is available. Clones and species were also readily differentiated by the markers developed by RFLP. In addition, *Populus* cpDNAs were characterized by the RFLP technique. Mejnartowicz (1991) analyzed cpDNA of *Populus* species (*P. trichocarpa*), hybrids (*P. maximowiczii* x *P. trichocarpa* and *P. maximowiczii* x *P. nigra*), and F_1 progeny to study inheritance of cpDNA. With each of 5 restriction enzymes (*Pst*I, *Eco*RI, *Hin*dIII, *Bam*HI, and *Xba*I) tested, differences were observed between the 2 parental clones. All test progeny showed the characteristic pattern of the female parent and no paternal or mixed patterns were observed. Rajora and Dancik (1992), studying 2 *Populus* species (*P. deltoides* and *P. nigra*) and their F_1 hybrid progeny, confirmed uniparental maternal inheritance of the cpDNA. Using 13 different enzymes in combination with 4 different probes to detect polymorphisms between the 2 species, they found 36 species-specific restriction fragment polymorphisms. Between *P. deltoides* and *P. maximowiczii*, 16 species-specific restriction fragment polymorphisms were observed. Using RFLP analysis, Lee et al. (1994a) compared cpDNAs of *Populus* spp. (*P. alba*, *P. glandulosa*, *P. alba* x *P. glandulosa*, *P. davidiana*, and *P. nigra*) and reported little intrageneric variation. They used 5 different restriction enzymes (*Pst*I, *Eco*RI, *Bam*HI, *Bgl*II, and *Kpn*I) to find polymorphisms among 5 *Populus* species. With the exception of *P. nigra*, which showed a different fragmentation pattern when cut with the enzyme *Eco*RI, fragmentation patterns appeared similar among the *Populus* species. However, Southern hybridization with the tobacco *rbc*L gene probe produced identical patterns among *Populus* species for all the enzymes tested; suggesting that gene order among *Populus* species is conserved. With all the enzymes tested, the *N. tabacum* reference plant showed different restriction profiles than those of *Populus* species.

PCR-RFLP with *Populus* cpDNA

Recent advances in PCR technology have allowed examination and comparison of specific DNA regions in higher plants. In many DNA regions, variation was frequently observed in noncoding spacer regions rather than in coding regions. Nuclear rDNA spacers exist between genes encoding large and small subunit rRNAs that are ubiquitous among various organisms. This spacer region has been extensively studied in many different organisms by RFLP. Recent RFLP analysis of intergenic spacers (IGS) between the 2 rRNA subunit genes has revealed polymorphisms among various *Populus* species or individuals (Faivre-Rampant et al. 1992). However, little work has focused on the nuclear genome except for the rDNA spacer region perhaps because of the complexity of the nuclear genome and limited sequence information for nuclear DNA.

Unlike nuclear genomic DNA, cpDNA is well studied in higher plants. Sequence information on the chloroplast genome is, therefore, relatively abundant. Complete nucleotide sequences of cpDNAs are published for several plant species (Hiratsuka et al. 1989; Shinozaki et al. 1986). Because many coding regions in cpDNA are highly conserved among higher plants, spacers can be amplified by PCR using primers deduced from coding regions (Liston 1992). This PCR-RFLP method has 2 advantages over conventional RFLP: 1) labeling is unnecessary because a probe is not required and 2) specific DNA regions can be compared in detail using restriction enzymes that recognize tetranucleotides. Employing the PCR-RFLP method, we have analyzed 3 different regions of cpDNA in *Populus* species: 1) 16S-23S rDNA spacer; 2) *rpo*C1C2 region; and 3) *rpl2-psb*A region.

Populus Materials

Our studies of 3 different regions of cpDNA used the following *Populus* materials: 1) 7 clones of *P. davidiana* (6 clones from diverse regions of Korea and 1 clone from China); 2) 1 clone of *P. glandulosa*; 3) 2 clones of *P. nigra*; 4) 2 clones of *P. alba*; 5) 3 samples of *P. maximomiczii*; 6) 3 samples of *P. alba* x *P. glandulosa*; 7) 3 samples of *P. nigra* x *P. maximowiczii*; and 8) 3 samples of *P. koreana* x *P. nigra*. In addition, *Nicotiana tabacum*, *Salix pseudolasiogyne*, and *Quercus accutissima* were used as reference plant material.

Analysis of 16S-23S rDNA Spacer

Ribosomal RNA genes of chloroplasts are arranged as: 16S rDNA-spacer-23S rDNA-spacer-5S rDNA. The *rrn* operon is located within the inverted-repeat region of cp DNA, and 2 transfer RNA (tRNA) genes are contained in the spacer between the 16S rDNA and 23S rDNA. In *Euglena gracilis*, the spacer is 258 base pairs (bp) long, whereas the spacer exceeds 2 kb in maize and tobacco. The

tRNA genes in the 16S-23S rDNA spacer of higher plants contain introns, and the difference in spacer length is due to intron size. In maize, the intron in $tRNA^{Ile}$ is 949 bp and in $tRNA^{Ala}$ is 806 bp (Koch et al. 1981). Corresponding introns in *N. tabacum* are 707 and 710 bp, respectively (Takaiwa and Sugiura 1982).

To amplify the 16S-23S rDNA spacer in *Populus* cpDNA, primer sequences were deduced from cpDNA genes in *N. tabacum* (Shinozaki et al. 1986). The primer sequences are: 5'-GGA AGG TGG GGC TGG ATC CAC C-3' (from the 3' end of 16S rDNA) and 5'-CCT CGT CTC TGG GTG CCT AGG-3' (from the 5' end of 23S rDNA). In all the *Populus* species and *Salix pseudolasiogyne* (reference plant), the spacer spanned 2.3 kb. However, the spacer appeared smaller (2.1 kb) in *N. tabacum* suggesting that *Populus* spp. have a larger intron than tobacco in the 2 tRNA genes within the spacer. Restriction digestions of the spacer with several different enzymes are in table 1. As table 1 shows, enzymes that recognize and cut tetranucleotides typically generate more bands than those that recognize hexanucleotides.

No variation among *Populus* species was observed with the 7 enzymes tested (*Hin*fI, *Hpa*II, *Hae*III, *Taq*I, *Kpn*I, *Sac*I, and *Bam*HI). *N. tabacum* showed different fragmentation patterns for all the enzymes tested except *Sac*I. In addition, no restriction sites were observed in the spacer for the enzymes *Hpa*I, *Pst*I, *Xba*I, *Dra*I, *Hin*dIII, *Eco*RI, and *Sma*I. With *Populus*, no variation is apparent in the spacer between 16S and 23S rDNA (figure 1). Therefore, the sequence of the chloroplast rDNA spacer region seems highly conserved among *Populus* spp.

Table 1. Restriction fragmentation patterns of amplified 16S-23S rDNA spacer in *Populus* spp. and *Nicotiana tabacum*. No variation was observed among *Populus* species.

Enzyme	Species	Fragment size (bp)[1]
*Kpn*I	*Populus* spp.	970; 420; 380; 350; 200
	N. tabacum	1,500; 600[2]
*Sac*I	*Populus* spp.	1,300; 1,000
	N. tabacum	1,200; 960[2]
*Bam*HI	*Populus* spp.	1,000; 410; 360; 340; 190
	N. tabacum	1,575; 575[2]
*Taq*I	*Populus* spp.	1,250; 380; 230; 200; 140
	N. tabacum	1,128; 346; 145; 142[2]
*Hae*III	*Populus* spp.	500; 430; 390; 320; 290; 180
	N. tabacum	764; 553; 302; 295; 246[2]
*Hpa*II	*Populus* spp.	530; 430; 300; 290; 220; 160
	N. tabacum	985; 880; 495; 100[2]
*Hin*fI	*Populus* spp.	530; 400; 220; 160
	S. pseudolasiogyne	530; 400; 220; 160
	N. tabacum	540; 310; 300; 200; 160; 120; 120; 120; 100[2]

[1] Approximate estimation. Bands less than 100 bp are not shown.

[2] The fragment size of *N. tabacum* was calculated from the published map (Shinozaki et al. 1986).

Analysis of *rpo*C1C2 Region

The *rpo*C1 and C2 genes encode RNA polymerase subunits and are located within the large single copy region of the chloroplast genome. The *rpo*C1 gene contains an intron (absent in monocots) and is separated from *rpo*C2 by an intergenic spacer (IGS) (Shinozaki et al. 1986). The intron and the IGS regions are expected to show higher levels of sequence divergence than the coding regions. Primers for *rpo*C1C2 amplification were synthesized according to Liston (1992) and contained the sequences: 5'-AAG CGG AAT TTG TGC TTG T-3' (from the antisense strand of the *rpo*C1 gene) and 5'-TAG ACA TCG GTA CTC CAG TGC-3' (from the sense strand of the *rpo*C2 gene). These primers have successfully amplified 3.7 kb to 4.3 kb products from pine and other dicots (Liston 1992).

Amplification of the *rpo*C1C2 region produced a 4.3 kb band with several *Populus* species (*P. alba*, *P. glandulosa*, *P. alba* x *P. glandulosa*, *P. davidiana*, and *P. nigra*) and *S. pseudolasiogyne*; a smaller band (4.1 kb) was produced with *N. tabacum*. Liston (1992) reported that amplification of the region with the same primers resulted in a 4 kb band from *Astragalus* (Fabaceae). Restriction fragmentation patterns of the amplified *rpo*C gene region in *Populus* spp., *S. pseudolasiogyne*, and *N. tabacum* are in table 2. Restriction digestion of the amplified fragment with the enzymes *Kpn*I,

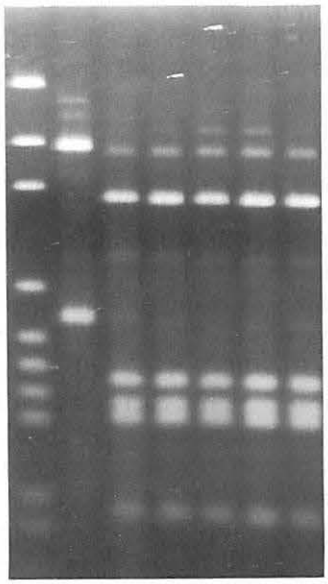


Figure 1. *Bam*HI restriction digestion of the amplified 16S-23S rDNA spacer. Lanes 1 to 7 (from the left) are: pGEM DNA marker, *Nicotiana tabacum*, *Populus nigra*, *P. davidiana*, *P. glandulosa*, *P. alba* x *P. glandulosa*, and *P. alba*.

Table 2. Restriction fragmentation patterns of amplified *rpo*C1C2 region in *Populus* spp., *Salix pseudolasiogyne*, and *Nicotiana tabacum*. No variation was observed among *Populus* species.

Enzyme	Species	Fragment size (bp)[1]
*Kpn*I	*Populus* spp.	2,600; 1,550; 180
	S. pseudolasiogyne	2,600; 1,550; 180
	N. tabacum	2,590; 1,520 [2]
*Sac*I	*Populus* spp.	4,300
	S. pseudolasiogyne	4,100; 200
	N. tabacum	2,700; 1,410[2]
*Bam*HI	*Populus* spp.	2,400; 1,000; 900
	N. tabacum	2,130; 1,980[2]
*Taq*I	*Populus* spp.	520; 450; 390; 340; 240
	S. pseudolasiogyne	520; 500; 450; 430; 290; 240
	N. tabacum	675; 410; 360; 350; 335; 220; 170; 170; 160; 135; 135[2]
*Hae*III	*Populus* spp.	1,450; 1,430; 1,000; 420
	S. pseudolasiogyne	1,450; 1,000; 700; 690; 450
	N. tabacum	1,080; 890; 500; 450; 400; 370; 230; 190[2]
*Hpa*II	*Populus* spp.	2,200; 950; 350; 280; 270; 180
	N. tabacum	920; 760; 660; 540; 340; 270; 250; 200[2]
*Hin*fI	*Populus* spp.	720; 485; 450; 350; 330; 220; 170
	S. pseudolasiogyne	720; 490; 450; 340; 330; 280; 220; 170; 165
	N. tabacum	600; 390; 365; 335; 330; 235; 175; 175; 170;165; 135; 105[2]
*Hin*dIII	*Populus* spp.	4,300
	S. pseudolasiogyne	4,300
	N. tabacum	3,640; 470[2]
*Eco*RI	*Populus* spp.	2,000; 1,550; 750
	S. pseudolasiogyne	2,000; 1,550; 750
	N. tabacum	1,530; 1,380; 1,200[2]

[1] Approximate estimation. Bands less than 100 bp are not shown.

[2] The fragment size of *N. tabacum* was calculated from the published map (Shinozaki et al. 1986).

*Sac*I, *Taq*I, *Hae*III, *Eco*RI, *Bam*HI, *Hpa*II, *Hin*dIII, and *Hin*fI did not discriminate among *Populus* species. However, *Salix* was distinguishable from *Populus* species with the enzymes *Sac*I, *Taq*I, and *Hae*III. In all the *Populus* spp. tested, no *Hin*dIII, *Pst*I, or *Sac*I restriction sites were detected in the region, but *Sac*I and *Hin*dIII restriction sites do occur in the comparable region of *N. tabacum*. In most examples, *N. tabacum* exhibited a totally distinct banding pattern from *Populus* species. An exception was evident when cpDNA was cut with *Kpn*I; 1 *Kpn*I site was apparently conserved among *Populus*, *Salix*, and even *N. tabacum*.

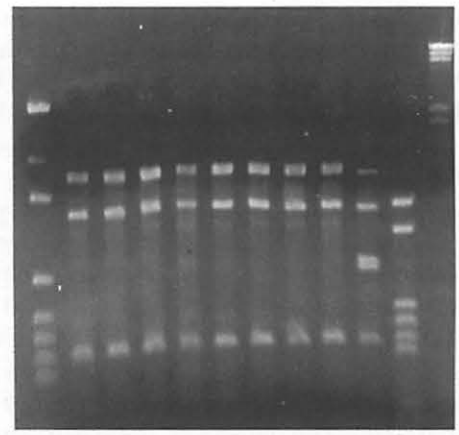


Figure 2. *Hae*III restriction digestion of the amplified *rpo*C1C2 region. Lanes 1 to 12 (from the left) are: pGEM DNA marker, *Populus alba*, *P. alba* x *P. glandulosa*, *P. glandulosa*, *P. davidiana*, *P. maximowiczii*, *P. nigra* x *P. maximowiczii*, *P. nigra*, *P. koreana* x *P. nigra*, *Salix pseudolasiogyne*, *N. tabacum*, and lambda phage (*Hin*dIII cut).

Two *Hae*III sites in the *rpo*C1C2 region are apparently conserved and flank the IGS in tobacco, spinach, rice, and maize (Liston 1992). The distance between the 2 *Hae*III sites was estimated to be 210 bp in *Astragalus*, 193 bp in tobacco, and 234 bp in rice. Because the fragment contains a 35-bp coding region, IGS length in *Astragalus*, tobacco, and rice is 175, 158, and 199 bp, respectively. In *Populus*, the smallest *Hae*III fragment observed was 420 bp. Thus, *Populus* spp. may have a larger IGS since the coding region is highly conserved among organisms. This 220 bp difference could account for the difference in the total length of amplified fragments between *Populus* and tobacco (*Populus* produced a band length of 4.3 kb, whereas tobacco showed 4.1 kb in total length of the amplified fragment). Based on these studies, this region appears very highly conserved among *Populus* species, although some variation occurs between *Populus* and *Salix*.

Analysis of the Region Around the Spacer Between *rpl*2 and *psb*A Genes

In many higher plants, the *psb*A gene is located within the large single copy region close to 1 end of the inverted repeat. The gene encodes a herbicide-binding protein, Qb (Shinozaki et al. 1986). The *rpl*2 gene codes for a ribosomal protein and is located within the inverted repeat region. Although these 2 genes are adjacent to each other,

their transcription directions are opposite in most plants studied. Additionally, the *trn*H gene is located in the spacer between the 2 genes.

Two primers were designed to flank the *psb*A gene, spacer, and *rpl*2 gene: 5'-CCT TGG GGT TAT CCT GCA CT-3' (from the sense strand of *rpl*2 gene) and 5'-ACT GCA ATT TTA GAG AGA CGC G-3' (from the sense strand of *psb*A gene). In *Populus*, observed amplification products of the primers are 2.07 kb. No spacer length variation was evident among *Populus* species and *S. pseudolasiogyne*. With *N. tabacum* and *Quercus acutissima* (reference plant), however, the spacer appeared smaller (approximately 1.73 kb). Restriction fragmentation patterns of the spacer from *Populus* spp., *S. pseudolasiogyne*, *Q. acutissima*, and *N. tabacum* are in table 3. Usually, fragmentation patterns were identical among *Populus* species and a *Salix* species. Restriction digestions with the enzymes *Hae*III, *Hpa*II, and *Taq*I do not discriminate among tested species of *Populus*. However, *Salix* is separable from *Populus* species with the enzyme *Hin*fI (figure 3). The *Hin*fI enzyme also allows differentiation of *P. davidiana* and *P. glandulosa* from other *Populus* species. Also of note is that *P. nigra* exhibited different fragment sizes than other *Populus* species, and no *Bam*HI, *Kpn*I, or *Sac*I restriction sites were detected in the spacer region. Again, *N. tabacum* exhibited totally different fragmentation profiles from *Populus* species with all the enzymes tested. Among the 3 regions analyzed, only this spacer region is polymorphic among *Populus* species.

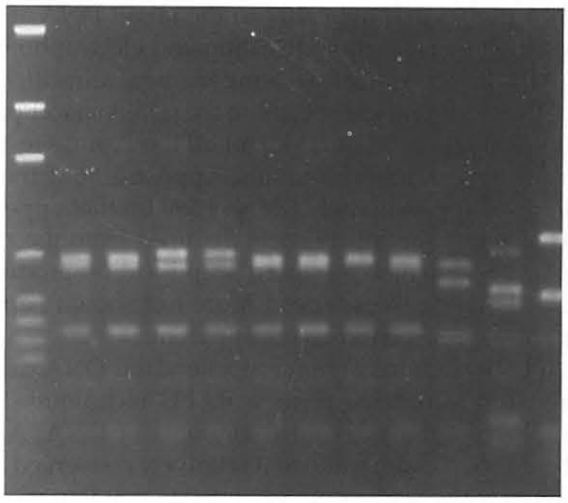


Figure 3. *Hin*fI restriction digestion of the amplified *rpl*2-*psb*A region. Lanes 1 to 12 (from the left) are: pGEM DNA marker, *Populus alba*, *P. alba* x *P. glandulosa*, *P. glandulosa*, *P. davidiana*, *P. maximowiczii*, *P. nigra* x *P. maximowiczii*, *P. nigra*, *P. koreana* x *P. nigra*, *Salix pseudolasiogyne*, *Quercus accutissima*, and *N. tabacum*.

Table 3. Restriction fragmentation patterns of amplified *rpl*2-*psb*A region in *Populus* spp., *Quercus acutissima*, and *Nicotiana tabacum*.

Enzyme	Species	Fragment size (bp)[1]
*Taq*I	*Populus* spp.	1,035; 1,030
	S. pseudolasiogyne	1,035; 1,030
	Q. acutissima	1,040; 700
	N. tabacum	1,040; 415; 187[2]
*Hae*III	*Populus* spp.	960; 765; 300
	S. pseudolasiogyne	960; 765; 300
	Q. acutissima	970; 765
	N. tabacum	768; 668; 138; 100[2]
*Hpa*II	*Populus* spp.	1,650; 270; 130
	S. pseudolasiogyne	1,650; 270; 130
	Q. acutissima	1,560; 180
	N. tabacum	1,500; 238[2]
*Hin*fI	all *Populus* except *P. glandulosa* and *P. davidiana*	675; 670; 440; 167
	P. glandulosa	680; 670; 440; 167
	P. davidiana	680; 670; 440; 167
	S. pseudolasiogyne	650; 550; 400; 167
	Q. acutissima	540; 520; 180; 180; 160
	N. tabacum	747; 527; 167; 120[2]

[1] Approximate estimation. Bands less than 100 bp are not shown.

[2] The fragment size of *N. tabacum* was calculated from the published map (Shinozaki et al. 1986).

The conserved nature of *Populus* cpDNA revealed in our work apparently contrasts with data obtained by other labs. Mejnartowicz (1991) and Rajora and Dancik (1992) reported much variation in cpDNA. This difference in results may be due to general differences in techniques. Whereas their results represented variation in whole cpDNA, our approach was limited to small, localized regions of cpDNA. Therefore, it appears that variability may be found in *Populus* by applying PCR-RFLP to other regions of cpDNA.

PCR Amplification of *Populus* Chloroplast DNA

Many workers have used total DNA to analyze organelle genomes. Major advantages of using total DNA may be the yield and simplicity of extraction procedures. Total DNA also allows flexibility because it can be used for the RFLP analysis of the nuclear, mitochondrial, and chloroplast genome. However, each analysis requires specific

DNA probes that hybridize to the target DNA after fractionation by restriction digestion and electrophoresis.

At a lower annealing temperature, amplification of cpDNA with primers specific to the rDNA spacer produces a thick single band of 2.3 kb for all the *Populus* tested (figure 4). Several other bands also appear, among them are 2 bands of approximately 680 and 690 bp that appear in all *Populus* test species. Primers deduced from the *rpo*C1C2 coding region also produced amplification products that varied among the species (figure not shown), and some species-specific bands were apparent.

If both chloroplast and mitochondrial DNA can be obtained in a purified form, a RAPD technique could be employed even with small amounts of DNA. Although these genomes are small and relatively conserved, current results with *Populus* cpDNA indicate that variation is sufficient for detection by RAPD. For the RAPD method, chloroplasts are isolated from leaf tissue (young or tissue-cultured leaves). After extraction from the chloroplasts, cpDNAs are subjected to PCR amplification using random primers or sequence-specific primers. To generate more bands, a lower annealing temperature is applied. Depending on the primers used, up to 10 bands can be amplified (Lee et al. 1994b). In our studies, arbitrary oligomers (10-mers) generated RAPD patterns when annealing was performed at 35 °C. These RAPD patterns allowed the differentiation of all test species. Overall, RAPD techniques on cpDNA can provide effective markers for tree species when intact chloroplasts are obtainable.

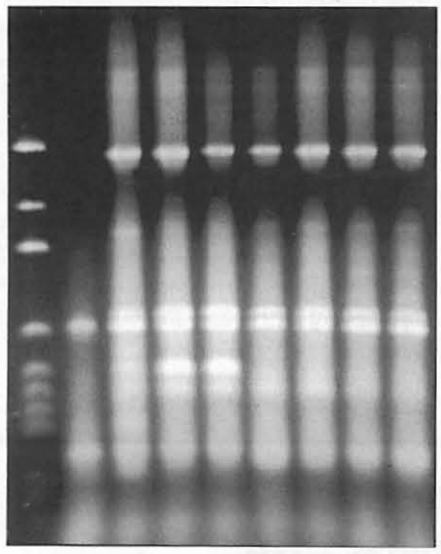


Figure 4. Amplification of *Populus* cpDNA with primers specific to the 16S-23S rDNA region. Annealing was done at 43 °C. Lanes 1 to 9 (from the left) are: pGEM DNA marker, *Populus davidiana* 1, *P. davidiana* 2, *P. glandulosa* 1, *P. glandulosa* 2, *P. alba* x *P. glandulosa* 1, *P. alba* x *P. glandulosa* 2, *P. alba* 1, and *P. alba* 2.

Conclusion

Polymorphisms detected in cpDNA have been used as markers to study genetic variation in wild populations or identify clones of higher plants. Most previous work on cpDNA of tree species including *Populus* has used RFLP. However, the RFLP technique has been used only sparingly for tree species because it is laborious and time consuming. The recently developed technique of PCR-RFLP allows analysis of specific genomic regions. With abundant sequence data available from well-known plants, specific regions of *Populus* cpDNA can be amplified. Using PCR-RFLP, 3 different cpDNA regions were compared among 8 species and hybrids of *Populus*. Among the *Populus* species tested, some polymorphisms were detected in 1 region (*rpl*2-*psb*A), but no variation was detected in the other 2 regions (16S-23S rDNA spacer and *rpo*C1C2 region). Based on these results, *Populus* cpDNA appears highly conserved. However, data from other RFLP work indicate that many polymorphic sites occur in *Populus* cpDNA. If PCR-RFLP is used to examine other cpDNA regions, variable regions may be identified in the future.

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Chapter 19

Molecular Marker Techniques to Study Variability of *Populus* Pathogens[1]

Girma M. Tabor and Harold S. McNabb, Jr.

Introduction

Molecular genetic techniques are commonly used in many laboratories and many have been developed that address diverse problems in plant pathology. In recent years, tremendous advances have occurred concerning the molecular genetics of plant/parasite interactions including the cloning of plant genes for (Jones et al. 1994; Martin et al. 1993; Whitham et al. 1994) and corresponding avirulence genes in pathogens (Huynh et al. 1989; Kearney and Staskawicz 1990; Kobayashi et al. 1989, 1990; Staskawicz et al. 1984). Studies on the molecular genetic basis of in poplar are also underway (Cervera et al. 1996; Goué-Mourier et al. 1996; Newcombe and Bradshaw 1996). However, poplar pathogen populations must be characterized before ecological interactions of pathogenesis and host resistance are fully understood.

The crown gall pathogen (*Agrobacterium tumefaciens*) and its host interactions are genetically and biochemically well described (Beneddra et al. 1996; Binns 1990; Stachel et al. 1985, 1986; Yusibov et al. 1994; Zupan and Zambryski 1995). However, molecular genetic studies of other poplar pathogens and their associated diseases are just developing and are primarily tailored toward basic questions on taxonomy, epidemiology, and population genetics. An understanding of these basic questions is urgently needed so that critical processes involved in host-pathogens interactions will be defined and used to establish coherent selection and breeding programs for *Populus* spp.

Many molecular marker techniques are available to generate diverse genetic information for various purposes. Molecular marker techniques used to study *Populus* pathogens are the same as those used to study the host. Because these techniques are well described elsewhere in this volume (Cervera et al.; Lin et al.; Noh and Lee), a detailed description is not presented here. However, various attributes of these techniques are discussed within the context of this chapter.

Polymerase chain reaction (PCR) methods are the most widely used molecular genetic techniques to study *Populus* pathogens. Although PCR offers a virtually limitless source of genetic information, its use is curtailed because basic information on the taxonomy, etiology, and epidemiology of the pathogens is lacking.

In this chapter, we present some preliminary studies conducted to gain understanding about 2 economically important pathogens of *Populus*, *Septoria* spp. and *Melampsora* spp. Presently, random amplified polymorphic DNA (RAPD) and site-directed PCR are used in *Populus* pathology laboratories to provide basic molecular genetic information of *Populus* pathogens.

Random Amplified Polymorphic DNA (RAPD)

Diseases that are serious problems to many *Populus* clones are leaf and stem diseases caused by *Septoria* spp. In the north central and northeastern United States, *S. musiva* (teleomorph: *Mycosphaerella populorum*) is considered a serious pathogen. Besides leaf spot, *S. musiva* causes lethal stem cankers on *Populus trichocarpa* hybrids. In the Pacific Northwest (PNW) of North America, *S. populicola* (teleomorph: *M. populicola*) causes leaf spot without lethal stem cankers. Of current debate is whether these 2 *Septoria* populations are different species or whether canker development is limited by the environmental conditions in the PNW. Using RAPDs, Ward and Ostry (1996) showed that bulked collections from these 2 pathogen populations exhibit molecular polymorphism, suggesting that these populations may be isolated and distinct. They also found

[1] Klopfenstein, N.B.; Chun, Y. W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

that *Septoria* population from the PNW did not exhibit much polymorphism. In contrast, molecular polymorphism was observed within populations from the North Central United States (figure 1).

RAPD markers can be reliable for typing an individual. In contrast, typing a population using RAPDs is difficult because collected samples must accurately represent all individuals of a population. However, obtaining a representative sample from wild populations is formidable and often impossible. In addition, RAPD markers are not easy to reproduce. The problem of obtaining representative samples of a population, coupled with the reproducibility problems of RAPD markers, can render these markers unreliable for population typing.

RAPD markers linked to plant genes of interest can be successfully used for selection, breeding, and other genetic manipulations (Bradshaw and Stettler 1995; Goué-Mourier et al. 1996; Haley et al. 1993; Michelmore et al. 1991; Newcombe and Bradshaw 1996). Similarly, RAPD markers may be important when working with pathogen isolates that are inbreds or isogenic.

Site-Directed Polymerase Chain Reaction (PCR)

Site-directed PCR requires some prior knowledge of target DNA sequences to be amplified. The fungal ribosomal RNA (rRNA) genes are widely used for site-directed PCR because they comprise domains that are highly conserved among a wide range of taxa. The conserved nature of these domains has allowed the synthesis of general primers that can amplify several regions of the rRNA operon from various fungi. Consequently, this has allowed the synthesis of taxa-specific primers (Gardes and Bruns 1993; Tisserat et al. 1994; White et al. 1990).

A typical eukaryotic nuclear rRNA is polycistronic; coding regions for 18S, 5.8S, and 28S rRNAs are grouped (in the order listed) within a single transcription unit. An entire transcribed unit includes 2 noncoding regions, termed the internal transcribed spacers (ITS), which separate the 3 coding regions from each other. Sequences in the rRNA coding regions are highly conserved among related taxa. Little or no variation is evident among rRNA coding sequences of closely related taxa at the class, family, genus, or species level. This lack of variation in the rRNA coding regions among related taxa limits their use as a diagnostic tool.

Besides the conserved regions, the rRNA operon of several fungi consists of regions that can vary among species in a genus and among subspecies within a species. These variable regions can have practical importance to a *Populus* pathologist. Species, subspecies, and hybrids potentially can be identified based on their differences in the variable regions of the rRNA operon. Depending on the taxa, the ITS regions of fungi may vary in length and DNA sequence (Gardes and Bruns 1993; Tisserat et al. 1994; White et al. 1990) and may be used to identify and classify fungal pathogens of *Populus*.

The other region of the rRNA that may be of practical value to a plant pathologist is the intergenic spacer (IGS) region. In most organisms, rRNA genes occur as multiple copies clustered in long tandem arrays on several different chromosomes. A single transcription unit within a cluster is separated by an IGS that is not transcribed. In some fungi, the IGS regions contain 5S genes, producing 2 IGS regions (Kim et al. 1995). IGS lengths and sequences are known to vary among related taxa, which can be exploited for practical identification purposes.

Coding and noncoding regions of rRNA genes from several fungi can be readily PCR amplified with general primers that target rRNA genes from diverse organisms. PCR products then may be sequenced using automated sequencing machines, and these DNA sequences can be used for diagnostic and phylogenetic studies.

When ITS and IGS lengths are the same from separate pathogen isolates, sequencing may reveal some differences in the DNA. Based on the DNA sequence differences, unique enzyme restriction sites may be identified and used to generate DNA restriction patterns that are unique to these pathogens. In addition, based on DNA sequence differences, specific PCR primers can be designed for selective amplification of DNA fragments from specific pathogen races or species (Gardes and Bruns 1993; Tisserat et al. 1994).

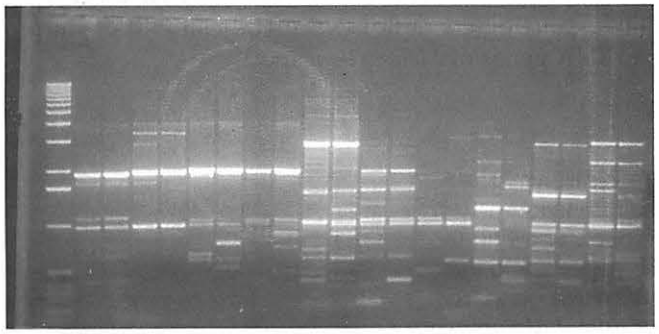


Figure 1. Random amplified polymorphic DNA (RAPD) of *Septoria* spp. isolates from the North Central states, USA. From the left, lane 1=1-kb DNA ladder; lane 2=RAPD control (no template DNA); and lanes 3 through 20=RAPDs various *Septoria* spp. isolates from the North Central states using Operon primer F-10, 5' to 3' - GGAAGCTTGG.

In our laboratory, ITS regions and the 5.8S rRNA genes of the 3 major *Melampsora* species on *Populus* were amplified using general primers. Derived information will be used to identify species and possible interspecific hybrids among *M. medusae*, *M. occidentalis*, and *M. larici-populina*.

Materials and Methods

Template DNA

Template DNA was obtained by either adding a few (10 to 30) urediospores directly to the PCR mixture or by extracting genomic DNA from urediospores. To extract genomic DNA, fresh dry urediospores were mixed with an equal volume of diatomaceous earth product and ground with a plastic pestle operated by an electric drill. The lysis buffer containing 50 mM Tris-HCl (pH 7.2), 50 mM EDTA (pH 8.0), and 3 percent sodium dodecyl sulfate (SDS) was autoclaved for 15 min. The grinding slurry was centrifuged at 800 x g and incubated at 65 °C for 1 h. After incubation, standard phenol/chloroform extraction and ethanol precipitation were conducted to obtain the template DNA.

Primers and PCR Amplification

Primers used in this procedure were ITS4 (TCCTCCGCTTATTGATATGC) and ITS5 (GGAAGTAAAAGTCGTAACAAGG). These primers are universal because they amplify rRNA genes from wide range of fungi (Gardes and Bruns 1993; Tisserat et al. 1994; White et al. 1990).

The amplification reaction was performed in 100 µl volumes containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 µM of each dNTP (dATP, dTTP, dGTP, and dCTP), 0.5 µM of each primer, 2.5 units of *Taq* polymerase, and 1.5 mM $MgCl_2$. Amplification was conducted for 35 cycles of denaturation (1 min) at 93 °C, annealing (35 sec) at 58 °C, and extension (2 min) at 72 °C. Final extension at 72 °C was performed for 10 min. All reagents were obtained from the Perkin-Elmer Corporation (Norwalk, CT, USA).

Restriction Enzyme Digestion

The PCR product was electrophoresed in 1.4 percent agarose gel, stained with ethidium bromide (0.5 µg/l), and visualized under UV light. The PCR product then was digested with several restriction enzymes in attempts to detect any restriction fragment length polymorphism.

Sequencing of PCR Product

The PCR products were purified with spin columns (Amicon, Inc., Beverly, MA, USA). Product concentration was measured using a fluorometer, and the concentration was adjusted for automated sequencing.

Results

Amplified products from intact spores and genomic DNA were approximately 700 base pairs and no difference in product size was observed among tested species of *Melampsora* (figure 2). Restriction digestion products of several enzymes did not produce any detectable polymorphism for use in distinguishing the 3 species. This also was supported by DNA sequence data in that ITS and 5.8S rRNA gene sequences from all 3 species showed a very high degree of similarity. These ITS and 5.8S sequences from *Melampsora* spp. were also highly homologous with those from other rust fungi.

Discussion

Our results indicate that DNA sequences of ITS regions from the 3 *Melampsora* species are identical. Thus far, these sequences have exhibited no distinguishing characteristics for diagnostic purposes. As expected, the 5.8S genes were also identical. Although the ITS and 5.8S sequences cannot be used for distinguishing these 3 species, these DNA sequences are useful for phylogenetic comparisons with other organisms. The ability to amplify rRNA genes without extracting genomic DNA greatly simplifies studies on rRNA genes of *Melampsora* species.

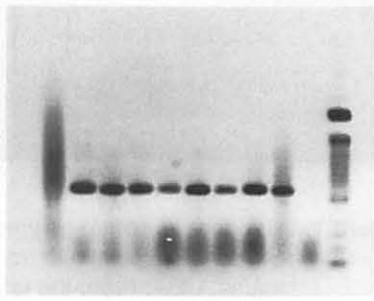


Figure 2. Polymerase chain reaction (PCR) products showing the 2 ITS regions and the 5.8S rRNA gene from 3 species of *Melampsora* with primers ITS4 and ITS5. Intact spores provided as template DNA. From the left, lanes 1 through 3=*M. larici-populina*; lanes 4 through 6=*M. occidentalis*; lanes 7 through 9=*M. medusae*; lane 10=control (no template); and lane 11=100 bp ladder.

Acknowledgments

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Section IV Biotic and Abiotic Resistance

Chapter 20

In Vitro Screening and Selection for Disease Resistance[1]

Michael E. Ostry

Introduction

Poplar improvement efforts are directed at improving tree traits such as rapid early growth, yield, and fiber quality. The goal of improvement programs is to increase resistance to diseases that have the potential to reduce yields or kill trees. Selection, breeding, and testing for poplar disease resistance are hampered by the long generation time of trees, the difficulty in identifying and capturing desired traits using classical breeding techniques, and the limited knowledge of juvenile-mature correlations of desired traits.

Screening for disease resistance in the field is time-consuming, costly, and dependent upon natural fluctuations in inoculum abundance and weather factors that influence pathogen spread, infection, disease development, and disease expression. Trees in field tests are also subject to many pathogens and insect pests that periodically confound test results (Ostry et al. 1989).

Various cultural and chemical control strategies have been suggested for most of the major poplar pathogens. If available, the use of resistant clones is the best long-term management practice. Selection of adapted, highly productive clones with disease resistance is not easily achieved. Host-parasite interactions are complex, dynamic, and affected by many environmental variables and the developmental stage and general health of the host. These factors can significantly affect disease resistance or tolerance.

Application of new techniques in molecular biology and plant biotechnology to poplar improvement may decrease the time necessary to introduce new traits and increase efficiency of new genotype selection and screening (Daub 1986; Helgeson 1983; Miller and Maxwell 1983; Ostry and Michler 1993; Ostry and Skilling 1992). Cell and tissue culture techniques are essential to many of these approaches and most have been successfully applied to poplars (Ostry and Ward 1991). Techniques range from a tissue culture method for eliminating viruses from poplar clones (Berbee et al. 1976) to using aspen root cultures for screening isolates of the fungus *Laccaria bicolor* for their ability to form ectomycorrhizae, which may improve tree survival and growth (Ostry et al. 1994).

The availability of a wide array of *in vitro* techniques for manipulation and regeneration of poplars offers new approaches to study host-parasite interactions. *In vitro* techniques may provide methods to rapidly screen and select poplars for resistance or tolerance to disease with greater efficiency than traditional field tests. Various *in vitro* techniques, including disease resistance screening, can greatly contribute to successful poplar breeding programs (Fröhlich and Weisgerber 1985).

In vitro techniques to screen poplars for disease resistance has many important advantages over screening intact plants in the field, growth room, or greenhouse. In addition to reducing the time and cost for testing, perhaps the greatest advantages of *in vitro* techniques are the precise control of the physical and chemical environmental conditions, the ability to rapidly screen a large number of genotypes in a small space, and the exclusion of other microorganisms. *In vitro* techniques take advantage of simplified experimental host-parasite systems where 1 or a few host cell types can be uniformly challenged by a pathogen or host-specific toxin.

In vitro approaches to disease resistance screening also have some important disadvantages. Resistance is probably determined by multiple factors, and may be governed by a series of biochemical reactions influenced by many host, pathogen, and environmental factors not present during *in vitro* testing. The general health of the host and accompanying stress factors may differ from intact plants in the field. The potential absence of preformed defensive barriers, induced inhibitory compounds, and organized tissues may limit the usefulness of *in vitro* screening techniques, especially if cell and tissue culture systems are

[1] Klopfenstein, N.B.; Chun, Y. W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

used. While these limitations may not exist when plant parts are used, wounds made to the plant part before or during screening may alter the expression of resistance. Major differences may also exist in the physiology and ploidy levels of plant cells or plant parts compared with intact plants. Cells and tissues in culture are actively growing and their reactions to pathogens may differ from mature tissues in intact plants. Also, plant hormones used in tissue culture media may influence host-parasite interactions, altering responses from those normally expressed *ex vitro*.

Tissue cultures are not necessarily free from pathogens; these organisms can occur in cultures and regenerated plants (Cousin et al. 1990). Additionally, *in vitro* selection for a specific trait may inadvertently select undesirable traits. *In vitro* selection techniques may enhance, but not necessarily replace, traditional methods for poplar improvement.

Examples of *in vitro* techniques for screening of poplars are briefly reviewed in this chapter. Some examples include tissue and cell culture techniques where the plant cells or plant cultures are challenged by the pathogen, and others include laboratory challenges of plants or plant parts that were grown in the field or greenhouse. The examples are arranged by the type of stress or pathogen that was studied. Examples of techniques used for screening for resistance to damaging abiotic agents are also provided. In all cases, a brief description of the methodologies and summaries of the results are included.

Abiotic Stress Agents

There are few reports of *in vitro* techniques used to study the effects of abiotic agents on poplars. A tissue culture assay in which the osmotic potential of the media was modified with polyethylene glycol (PEG) was used to determine if poplar callus culture responses to drought stress were similar to assays with whole plants (Tschaplinski et al. 1995). The callus cultures failed to display osmotic adjustment to water stress. The authors concluded that callus cultures could not substitute for assessing water stress at the whole plant level.

Callus cultures of *Populus maximowiczii* were used to compare levels of exogenously supplied lead with culture morphology and anthocyanin accumulation, and to determine the deposition and concentration of lead within the cultured cells (Ksiazek et al. 1984). Cultures were grown under dark or light conditions for 2 weeks, then exposed to aqueous lead solutions at various concentrations for 24 h. Lead reduced both the biosynthesis of anthocyanins and total fresh weight; perhaps because of cell division disturbances. Lead accumulated predominantly in the intercellular spaces and plant cell walls of the cultured tissues.

Callus and cell suspension cultures were employed to study the aluminum tolerance of a hybrid poplar clone (Choi et al. 1987). Fresh weight of callus cultures and dry weight of cell cultures were significantly reduced by increasing aluminum concentrations.

Callus and adventitious shoot cultures were used to screen poplar clones for salt (NaCl) tolerance (Li and Chen 1984). Similarly, shoot tip and bud cultures were used to screen hybrid poplar clones for salt tolerance (Lee et al. 1986). Increasing salt concentrations decreased culture growth. Large differences in growth responses were detected among the clones. Variations in salt tolerance were also detected among individual plants within the clones. The authors suggested that although tolerant clones were identified, they required further testing under field conditions to provide planting recommendations for saline soils.

Screening for Antimicrobial Activity

Callus cultures of aspen *(P. tremuloides)* produced bactericidal substances that stimulated and then inhibited an *Agrobacterium* bacteria species *in vitro* (Mathes et al. 1971). This activity depended upon how long the callus cultures were grown before inoculation with the bacterium (Mathes et al. 1971). Callus was produced from sterilized, stem internodal sections incubated on a basal medium in the dark. The antimicrobial substance was secreted into the medium and was active in the absence of host cells. This type of assay may be useful to screen poplar genotypes for host-specific compounds that may have roles in *ex vitro*. Compounds in the bark of aspen were fungistatic, inhibiting the *Hypoxylon mammatum* pathogen (Hubbes 1966; Kruger and Manion 1994) and other organisms (Mathes 1963). Similar investigations should contribute to the study of host responses and production of preformed and induced defense compounds.

The possible roles of abscisic acid (ABA) in defense reactions were studied using callus cultures of a *P.* x *euramericana* clone (Hrib and Rittich 1992). Callus cultures obtained from stem internodes and leaf blades were cocultured with the fungus *Phaeolus schweinitzii* in Petri dishes, and the fungal growth was monitored. Stem-derived callus had a higher ABA content and a greater inhibition of fungal growth than leaf-derived callus. The authors suggested that ABA intensifies the defense reaction and plays a role in . In contrast, Stopiska (1994) determined that leaves of a poplar clone, found to have the most resistance to *Ceratocystis fimbriata*, had less ABA content than a susceptible clone. It was concluded that the levels and/or ratios of the plant growth regulators that were measured were responsible for the levels of among the poplar clones studied.

Bacterial Canker

The interactions of *Xanthomonas populi* and poplar tissues were studied by inoculating stem explants of one-year-old branches (Lange 1968). Growing the explants under sterile conditions allowed detailed study of the effects of α-naphthaleneacetic acid (NAA), tissue development, and wounding on infection and cell division. Results showed that actively metabolic plant cells and wounding were necessary for canker formation. Bacterial growth stimulated by NAA occurred in the intercellular spaces of callus tissues. The authors suggested that bacterial levels and the developmental state of host tissues were important factors for symptom expression. Callus cultures were used to study the effects of *X. populi* on cell division and growth of callus tissues (Krawiarz and Przybyl 1980). Results confirmed earlier reports that this bacterium induced poplar cell division and stimulated callus tissue formation. In another attempt to develop a technique for disease resistance screening, Kechel and Böden (1985a, 1988) inoculated several clones of *in vitro* grown plants with *X. populi*. Clonal response to inoculation was similar to those of trees inoculated in the field and to young, rooted plants regenerated from tissue culture (Kechel and Böden 1985b).

Melampsora Leaf Rust

Leaf rust, caused by several species of *Melampsora* fungi, is a potentially serious worldwide poplar disease. Investigators have relied on *in vitro* techniques using detached leaves and leaf disks for detailed studies on the interactions of these highly variable rust fungi with many poplar clones and species. Detached leaf cultures offer many important advantages in studying obligate pathogens (Chandrashekar 1982; Shain 1974). Such studies have focused on the: infection process (Shain and Järlfors 1987), pathogen variation (Chandrashekar and Heather 1980; Hsiang and van der Kamp 1985; Hsiang and Chastagner 1993; Pinon et al. 1987; Pinon and Peulon 1989; Shain 1988), effects of ozone on leaf rust interaction (Coleman et al. 1987), and resistance among poplar species and clones (Hamelin et al. 1994; Heather et al. 1980; Lefevre et al. 1994; Prakash and Heather 1986a, 1989; Singh and Heather 1982a).

Races within isolates of *M. medusae* collected from natural stands of *P. deltoides* were identified using a leaf disk assay to reveal differences in latent period, uredial production, and isolate x cultivar interactions (Hamelin et al. 1992; Prakash and Thielges 1987). Inoculation of leaf disks from a set of host differentials also revealed the presence of races of *M. larici-populina*, the Eurasian rust fungus first reported in the United States in 1991 on various species of poplars in California and Washington (Pinon et al. 1994).

Temperature and light were identified as critical factors in disease expression of rust on inoculated leaf disks. This supports the hypothesis that environment plays an important role and emphasizes the importance of field screening (Chandrashekar and Heather 1981; Prakash and Heather 1986b; Prakash and Thielges 1989a; Singh and Heather 1982b, 1982c). Tissue culture and a leaf disk bioassay were employed to demonstrate somaclonal variation in *P. deltoides* for race-specific resistance to leaf rust caused by *M. medusae* (Prakash and Thielges 1989b).

Marssonina Leaf Spot

A laboratory technique was developed that uses excised leaf disks to compare the resistance of poplar clones to *Marssonina* fungi (Spiers 1978). This technique is a modification of earlier methods used to screen poplar clones to *M. brunnea* and leaf rust. A cork borer was used to punch leaf disks from tree leaves and make wells in Petri dishes of 2 percent water agar. Leaf disks were placed in the agar wells, inoculated with conidia of *Marssonina* fungi, and incubated in natural light at room temperature for 8 to 12 days. The level of resistance among test clones was classified using a disease rating scale based on the number of lesions per unit area. Disease resistance varied with clone, and clonal resistance varied with the applied concentration of conidia. This technique is useful to evaluate the relative resistance among clones; however, its use to evaluate field resistance is limited unless the level of natural inoculum is known.

Septoria Leaf Spot

An excised leaf disk bioassay was also used to screen hybrid poplar clones for resistance to *Septoria musiva;* results were compared with the known field reactions of these clones (Ostry et al. 1988). The modified method of Spiers (1978) allowed for separation of clones with similar field resistance to *S. musiva*. The segregation was based on disease progress curves obtained by monitoring progressively enlarging areas of necrosis on inoculated leaf disks that were incubated in the light over 32 days. Results correlated well with those obtained under natural field conditions. Spore concentration was not a factor in the classification of clones and the results were repeatable. However, the authors suggested that this technique should be considered only as a preliminary screen for clones before field tests. This method was also used to identify increased resistance to Septoria leaf spot in poplar plants that were regenerated from tissue cultures of a previously susceptible clone (Ostry and Skilling 1988).

Hypoxylon Canker

Several investigators have utilized tissue cultures to study Hypoxylon canker caused by the fungus *Hypoxylon*

mammatum (Ostry et al. 1990; Race and Manion 1994; Valentine et al. 1988). To screen and propagate aspen resistant to the canker, Wann (1985) challenged aspen hypocotyls and cotyledon explants with a toxin from *H. mammatum*. Surviving explants were elongated, rooted, and transferred to soil. These plantlets were tested for toxin resistance using a leaf puncture bioassay. The result was that toxin resistance expressed *in vitro* was also expressed by intact plants. It was proposed that this resistance trait was not induced by the tissue culture system but was natural in all families tested (Einspahr and Wann 1985). The investigators suggested that this technique has potential value for selecting aspen with canker resistance.

Cambial activity of various poplar clones and species in the presence of *H. mammatum* culture filtrates was investigated (Pinon 1986). Effects on callus proliferation from challenged stem internode sections were compared with the responses of intact trees after field inoculations. Results indicated that the filtrate assay could select among susceptible and nonhost poplar species, but was not sufficiently specific for use in early selection of resistant aspen clones. A toxin-tolerant line of callus was obtained from Leuce (currently termed Populus) poplars and a leaf bioassay indicated that plants regenerated from this line retained the toxin resistance (Antonetti and Pinon 1993).

Culture filtrates of *H. mammatum* and aspen shoot cultures were used to examine the correlation of a toxin bioassay to disease incidence in the field (Belanger et al. 1989a). Although clonal differences in reactions to culture filtrates were detected, the bioassay results did not correlate with disease incidence in the field. This suggests that other variables are involved in this pathosystem. Further investigations using shoot cultures revealed that moisture stress and related changes in amino acids may be important to the susceptibility of aspen clones to infection by *H. mammatum* (Belanger et al. 1989b, 1990).

Using shoot cultures from parent trees and their progeny, Kruger and Manion (1993a) demonstrated that sensitivity to *H. mammatum* culture filtrates was under genetic control. The authors suggested that the demonstrated resistance was horizontal and controlled by a small number of genes. However, the *in vitro* response of selected aspen clones to culture filtrates was not related to their response when inoculated with ascospores (Kruger and Manion 1993b).

Summary

Reports are increasing on the potential of *in vitro* screening for disease resistance in poplars; however, because of technique limitations, more research is needed before they can be practically and reliably applied to poplar improvement efforts. We need a better understanding of the genetics of host-pathogen interactions, the role of environmental variables, and juvenile-mature correlations to host resistance. Many investigators strongly suggest that *in vitro* screening can provide preliminary information on host responses to pathogens, but these techniques should not be considered as suitable substitutes for field tests. Although there have been several reports of strong correlations with *in vitro* screening to field results, field verification is frequently lacking.

The examples reviewed include *in vitro* techniques to screen entire plants or detached plant parts for disease resistance. A more powerful technique would involve selection at the cellular level with characterized, host-specific toxins. Thus far, the limited work with crude or partially purified toxins from poplar pathogens has had only partial success.

Advances in biotechnology, the use of molecular genetics, and the availability of poplar pedigrees will provide future opportunities to study host defense mechanisms. These new tools will provide great assistance to develop and refine disease resistance screening and selection techniques to increase yields and reduce poplar production costs.

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Chapter 21

Transformation of Hybrid Aspen for Resistance to Crown Gall Disease[1]

Hiroyasu Ebinuma, Etsuko Matsunaga, Keiko Yamada, and Mikiko Yamakado

Introduction

'Kitakami Hakuyou' are elite clones of hybrid aspen produced by Nippon Paper Industries (NPI) for commercial use. These elite clones were obtained by crossing 5 selected female trees of *Populus sieboldii* with a male elite tree of Canadian *P. grandidentata*, exhibit better growth features than other species or interspecific hybrids belonging to the section Leuce (currently termed Populus), and express significant heterosis (Takayama 1968). These characteristics make them well suited for afforestation and pulp wood in Japan. However, in some geographic areas, 'Kitakami Hakuyou' clones are extremely sensitive to crown gall disease, caused by *Agrobacterium tumefaciens*. This disease can cause considerable economical loss to nurseries growing rosaceous plants, *Rubus* species, grapevines, or various nut-bearing trees. The phytopathogenic bacterium, *A. tumefaciens*, infects a wide variety of dicotyledonous plants and induces tumors on the infected plants (Moore and Warren 1979). Because this bacterium provides a useful method to introduce desirable genes into plants, we have used biotechnology in an attempt to improve characteristics of 'Kitakami Hakuyou.' Our objective was to use an antisense DNA method to improve crown gall disease resistance in aspen clones (Ebinuma et al. 1991, 1992). In this paper, we report on the construction of plasmids containing antisense DNA, and on a highly efficient transformation procedure for hybrid aspens.

Construction of Vectors Containing Resistance Genes to Crown Gall Disease

A. tumefaciens introduces genes located on the transfer DNA (T-DNA) of the tumor-inducing plasmid (Ti-plasmid; Kim et al. this volume) into the plant genomic DNA, and induces tumors in the infected regions (Bevan and Chilton 1982). In the tumors, plant hormones, auxin and cytokinin, are over produced due to the expression of 3 integrated oncogenes (*iaaH*, *iaaM*, and *ipt*) in the plant genome. We used the Ti plasmid of the pathogenic *A. tumefaciens* strain PO22, which was isolated from a natural crown gall on hybrid aspen, to make antisense DNA constructs (Wabiko et al. 1989). We constructed the binary vector based on the plasmid pBI121, which contained the 1.1-kilobase (kb), *Eco*RI fragment 34 of the *iaaH* gene and the 0.7-kb, *Hin*dIII fragment 41 of the *iaaM* gene in the sense (S) or antisense (A) orientation under the control of the cauliflower mosaic virus *35S* promoter (figure 1). The plasmid was transferred to *A. tumefaciens* strain LBA4404 and used to transform tobacco.

A dramatic inhibition of gall formation was observed after infection by a virulent *A. tumefaciens* strain in 1 of the 5 transgenic tobacco plants expressing a part of *iaaM* gene in the sense orientation (41s4) (table 1, figure 2). Another of the 5 transgenic tobacco plants with the antisense orientation (41a3) showed weak, but distinct, resistance. We investigated the copy number and expression of the integrated genes in transgenic tobacco plants by the Southern and northern hybridization analyses (Sambrook et al. 1989). Southern analysis revealed that the resistant transgenic plant 41s4 contained 4 copies of the sense insert, the nonresistant plant 41s2 had 1 copy, and 41s3 had 6 copies. The amount of transcript in the resistant plant (41s4) was lower than that in the nonresistant plants (41s2 and 41s3); however, no correlation between transcriptional activity and resistance was detected. Although the molecu-

[1] Klopfenstein, N.B.; Chun, Y.W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

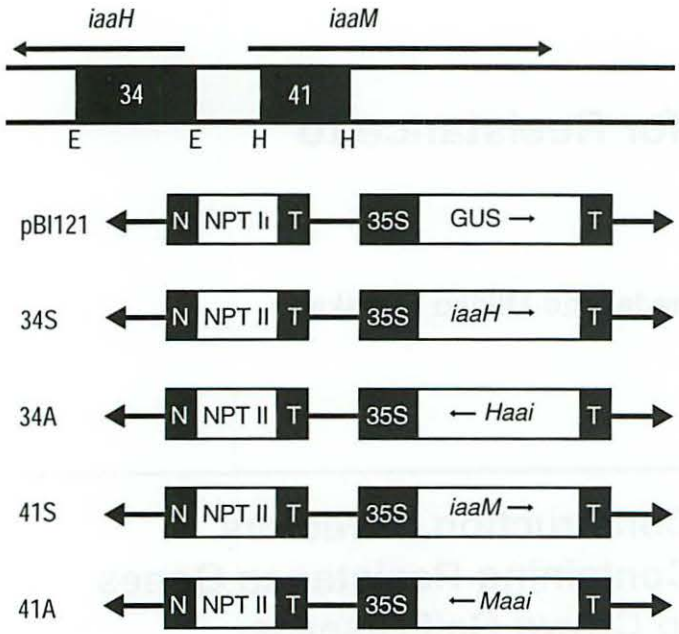


Figure 1. Plasmid construction. *iaaH*=indoleacetamide hydrolase gene; *iaaM*=tryptophan monooxygenase gene; *GUS*=glucuronidase gene; *NPT II*=neomycin phosphotransferase gene; *35S*=*35S* promoter of cauliflower mosaic virus; N=promoter of nopaline synthase gene; T=polyadenylation signal of nopaline synthase gene; E=*Eco*RI restriction site; H=*Hin*dIII restriction sites.

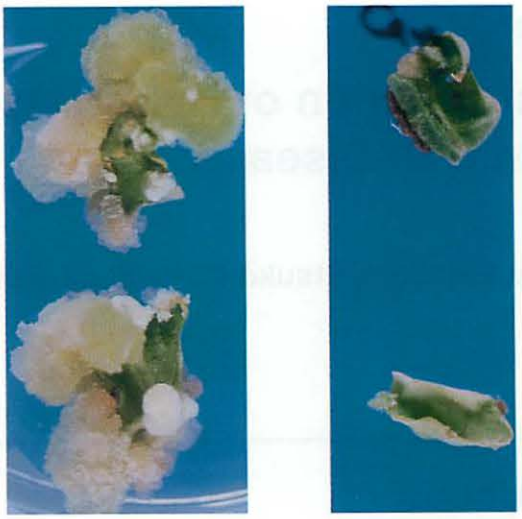


Figure 2. Leaf disc tests of transgenic tobacco. Control=stem segments test; 41s4=stem infection test.

Table 1. Bioassay of tumorigenicity using leaf discs of transgenic tobacco.

Tobacco clones[1]	Number of leaf discs inoculated[2]	Percentage of discs producing tumors[2]
C1	15	93
Lc2	15	73
41a3	15	80
41s4[3]	15	0

[1] C1 = normal tobacco plants

Lc2 = transgenic tobacco plants transformed by pBI121

41a3 = transgenic tobacco plants transformed with antisense DNA fragment 34

41s4 = transgenic tobacco plants transformed with sense DNA fragment 41

[2] Leaf discs were inoculated with pathogenic *Agrobacterium tumefaciens* and placed on agar plates (5 leaf discs/plate, total of 3 plates) and gall development was examined on the 15 leaf discs.

[3] Nine little shooty teratomas developed on leaf discs.

lar mechanism is not fully understood, our findings indicate that crown gall disease in hybrid aspen may be reduced by the 0.7-kb, *Hin*dIII fragment 41 of the *iaaM* gene in the sense orientation under the control of the *35S* promoter.

Highly Efficient Transformation Procedures for Hybrid Aspens

To improve the genetic transformation of hybrid aspens, we developed improved regeneration procedures. Because shoot regeneration frequencies can vary from 0 to 100 percent depending on the variety of *Populus* spp., commercially useful trees in the genus *Populus* frequently exhibit lower regeneration frequencies than model trees used in research. To help overcome this problem, we applied an *Agrobacterium* transformation method, developed for tobacco, to our commercial hybrid aspen clones (Matsunaga et al. 1992). This transformation procedure requires 2 steps, induction and growth of transgenic callus from infected stem segments, and shoot regeneration from the callus. This method has 3 potential problems: 1) the callus growth stage presents a high risk of generating genetic mutants; 2) a relatively long time is required for shoot regenera-

tion; and 3) the efficiency of obtaining transgenic trees with the desired gene is very low. To alleviate these problems, we developed an improved tissue culture method that increases the frequency of adventitious shoot differentiation of our commercial hybrid aspen clones.

By investigating the composition of the nitrogen source in the tissue culture medium, we found that concentrations of both nitrogen sources, ammonium and nitrate, influence the physiological state that induces differentiation of adventitious buds. Molar ratios from 1:2 to 1:5 of ammonium to nitrate were tested in the culture medium. The ratio of 1:3 was particularly effective for highly efficient regeneration of adventitious buds. By using a culture medium with this nitrogen composition, we could directly differentiate adventitious buds from infected internodal segments of hybrid aspen clones, without employing a preliminary step for inducing and growing callus, and subsequently regenerate plantlets. This method should reduce risks of genetic mutations during culturing, and requires a shorter regeneration time because extra steps are not required to induce and grow callus.

The binary vector plasmid, pBI121, which contained the 0.7-kb, *Hin*dIII fragment 41 of *iaaM* gene in the sense (S) orientation under control of the *35S* promoter, was transferred to *A. tumefaciens* strain LBA4404. Under aseptic conditions, stems of flask-grown plants of hybrid aspen clone 'Y63' (*P. sieboldii* x *P. grandidentata*) were cut into internodal segments of 5 mm in length. Stem segments were further cut longitudinally into 2 pieces, then inoculated with the *A. tumefaciens* vector strain. Infected stem segments were transferred to modified Murashige and Skoog (MS) (Murashige and Skoog 1962) agar solid medium (2 percent w/v sucrose, 0.5 mg/l zeatin, 500 mg/l carbenicillin, 100 mg/l kanamycin, and 0.8 percent w/v agar) in which nitrogen was supplied as 10 mM ammonium and 30 mM nitrate. After culturing for 2 months at 25 °C under 3,000 lux, 20 to 40 percent of the inoculated stem segments regenerated adventitious buds. After culturing an additional month under similar conditions, the foliage from each bud had grown to a length of 2 to 3 cm. These stems were aseptically excised and subcultured to a modified 2/3-strength MS medium, in which zeatin was replaced by 0.05 mg/l indole-3-butyric acid (IBA) for rooting. The rooting culture continued and 85 plantlets were obtained after 1 month.

Resistance of transgenic aspens to crown gall disease was evaluated by bioassay tests of tumorigenicity. Stem segments of transgenic and control hybrid aspens were inoculated with a pathogenic *A. tumefaciens* strain PO22 with the same method used for transformation. Inoculated stem segments were placed on MS agar medium to observe gall formation. In resistant clones, we observed the development of teratomatous shoots (figure 3a). For another bioassay, we wounded 5 internodes of greenhouse-grown, transgenic aspens with a needle at 1 point per internode, and inoculated with *A. tumefaciens* strain PO22. In approximately 10 percent of the transgenic plants, characteristic symptoms of crown gall disease were inhibited (figure 3b); however, considerable variability in gall development was apparent. Although several aspects need intensive investigation, these results provide interesting prospects for use of an antisense DNA method to improve crown gall disease resistance in aspen clones.

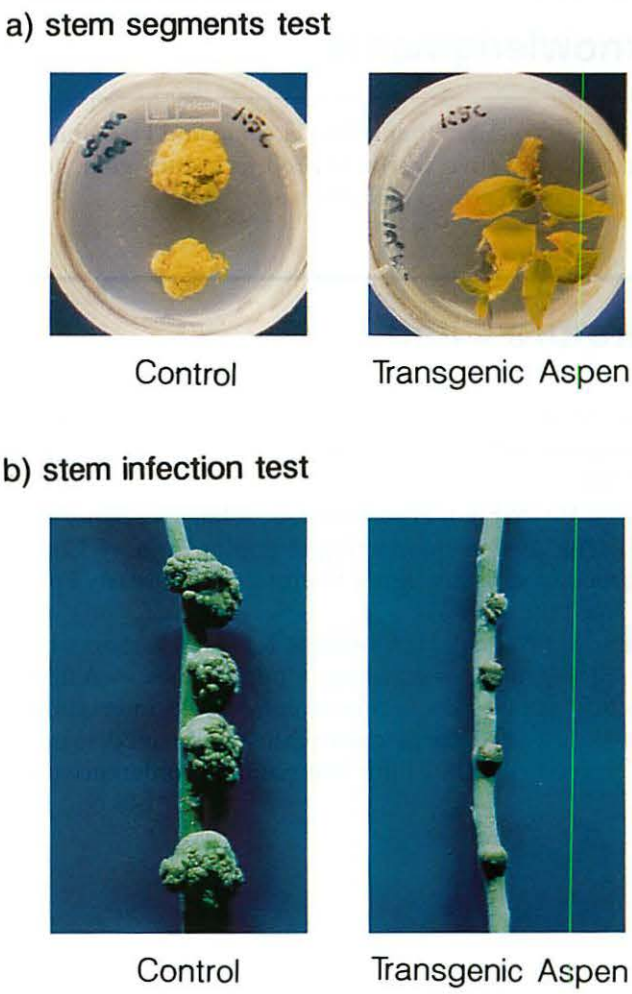


Figure 3. Bioassays of transgenic hybrid aspen. Transgenic aspen with sense DNA fragment 41. a) Internodal stem segments were infected with pathogenic *Agrobacterium tumefaciens* strain PO22 and placed on agar plates. b) Internodes of stems were wounded with a needle and inoculated with *A. tumefaciens* strain PO22.

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The authors want to thank Drs. H. Sano and H. Wabiko for their collaborative works to produce a transgenic tobacco resistant to crown gall disease.

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Chapter 22

Designing Small Antimicrobial Peptides and Their Encoding Genes[1]

William A. Powell and Charles A. Maynard

Introduction

This chapter describes an ongoing process to genetically engineer durable pathogen resistance into poplar and other tree species. The process involved designing, testing, and selecting antimicrobial peptide sequences to use as transgenic plant gene products. The synthetic peptide sequences were then encoded into DNA. Gene promoters, translation initiation and termination sequences, codon preferences, messenger RNA (mRNA) secondary structure, peptide stability, and peptide targeting were considered when designing gene constructs to test in transgenic plants. At this writing, the first of several gene constructs are being used to transform hybrid poplar.

Identifying Opportunities for Genetic Engineering

Modifying the genotype of a plant through conventional breeding is a slow process, especially if several cycles of breeding and selection are necessary to achieve the desired objectives. If the plant is a long-lived woody species that may take years to flower and may not express the character of interest until it reaches maturity, the problems and expenses can be astronomical. Genetic engineering techniques have the potential to eliminate years from conventional development time.

The preceding argument has been made many times, and in broad measure it is true. However, modifying genotypes using genetic engineering may be at least as costly as conventional breeding. Armed with a few breeding lines, a small plot of land, a few hundred pollination bags, and a handful of artist's brushes, the conventional plant breeder can assemble an incredible range of genotypes in a few years. In contrast, the biotechnological alternative of direct gene transfer requires a laboratory equipped for plant tissue culture and molecular biology, and a team of tissue culture and molecular biology specialists. With model plant species such as tobacco and petunia, the process of creating transgenic plants and testing them can be completed in a year or less. However, in many agronomically useful crop species or in tree species, the process may require several years. Given the equipment, supplies, and personnel involved, total costs per gene transferred can be hundreds of thousands of dollars. When contemplating the molecular biology approach to genotype modification, it is important to carefully choose the plant species and variety, the trait of interest, and the genes used to convey that trait.

Identifying Suitable Plant Species and Traits

When engineering genes encoding several antimicrobial peptides for use as pathogen-resistance genes, the model plant species should be one that has high economic value, is susceptible to *Agrobacterium* infection, and is easily regenerated from single cells or small pieces of tissue. The ideal trait is one that cannot be obtained through conventional breeding and is controlled by a single genetic locus. The ideal gene is fully characterized, produces a gene product or products that can be easily assayed, and is expressed only in the desired tissue (e.g., anthers, fruit, roots, cambium, leaves) or under the desired developmental or environmental stimulation (e.g., flowering, wounding, insect attack, heat stress).

There are several other constraints to consider. For example, if the plant species will be grown as a food crop, the gene product should be nontoxic and nonallergenic. Also, if there is any question of human toxicity, the gene product should only be expressed in portions of the plant that are not consumed.

[1] Klopfenstein, N.B.; Chun, Y.W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

Field Testing and Release of Engineered Woody Plants

For more than a decade, the regulatory requirements for field testing and eventual commercial use of engineered organisms have been debated (OAT 1989). The consensus of the scientific community is that transgenic plants should be evaluated by the same sets of procedures and the same criterion as any new variety developed through conventional breeding (Miller et al. 1990; Miller 1994). Although the debate continues, the trend is toward relaxing oversight (Hoyle 1996). For instance, in 1993 the USDA Animal and Plant Health Inspection Service (APHIS) changed from a petition and permit process to a notification and acknowledgment process. Under the old system, all field trials were reviewed in advance and had to comply with National Institute of Health guidelines (NIH 1994 and previous versions) before a permit was granted. Under current guidelines, researchers who want to field test transgenic organisms must still comply with NIH field-testing guidelines, but must only notify the USDA of the field test.

As of September 1995, APHIS has approved or acknowledged 1,818 field trails at 6,762 locations (Schechtman 1995). More than 40 different species have ongoing or completed field trials. The process has become so routine that it can now be conducted through the Internet using electronic mail (Reding 1995).

The final step in successful development of a transgenic plant is approval for commercial use, or receiving what APHIS refers to as nonregulated status. As of January 1996, 14 transgenic crop lines had been granted nonregulated status. Approximately 30 more have gone through the field-testing phase and are awaiting release. Most relevant to the eventual release of transgenic poplar or other tree species is the virus-resistant squash developed by Asgrow Seeds, which is fertile and will be grown in regions known to have native interbreeding wild relatives. APHIS granted the petition because the reviewers concluded that virus-resistance traits have "no potential to increase the 'weediness' of either the crop or its relatives" (Medley et al. 1994).

Although the trend could reverse if unforeseen problems arise with ongoing field tests or releases, we feel that by the time transgenic poplar trees are ready for field testing and release, the guidelines will have relaxed further and compliance will be uncomplicated. However, if genetic isolation is required for nonregulated status or to avoid problems with public acceptance, there are several possible approaches. Sterile genotypes may be chosen for transformation or the ploidy level of the transgenic plants may be altered, rendering them unable to cross with local wild populations. In addition, other researchers are actively working on sterility-inducing genes to incorporate in the transgenic plants along with the primary genes of interest (Strauss et al. 1995).

Among tree species, the genus *Populus* is close to being a model system because it has several species and species hybrids that have been transformed. Most reports deal with hybrids between *P. alba* and *P. grandidentata* (Chun et al. 1988a, 1988b; Chung et al. 1989; Fillatti et al. 1987; Klopfenstein et al. 1991, 1993; McCown et al. 1991; McNabb et al. 1990). Several other species and hybrids have also been transformed, including *P. tremula* (Lee et al. 1989), *P. tomentosa* (Wang et al. 1990), *P. tremula* x *P. tremuloides* hybrids (Nilsson et al. 1992), *P. koreana* (Kim et al. 1989), *P. nigra* (Tian et al. 1993), and *P. alba* x *P. tremula* (Brasileiro et al. 1992; De Block 1990; Devillard 1992; Leple et al. 1992).

One phenotypic modification that has received attention from conventional plant breeders and molecular biologists is increased pathogen resistance (Cornelissen and Melchers 1993). In the United States, it is estimated that $9.1 billion in agricultural crops are lost each year to pathogens (Agrios 1988). Among trees, chestnut blight, Dutch elm wilt, and cottonwood leaf spot, caused by *Cryphonectria parasitica* (formerly *Endothia parasitica*), *Ceratocystis ulmi* (*Ophiostoma ulmi*), and *Septoria musiva* (teleomorph: *Mycosphaerella populorum*), respectively, have caused major damage to important timber and ornamental tree species. All tree species represent a $19 billion annual crop in the United States (Echt 1995). Thus, studying antimicrobial peptide expression as a means of disease resistance is a high priority.

Antimicrobial Peptides

Various groups of membrane-active peptides found throughout the plant and animal kingdoms have useful antimicrobial properties. Since there have been several recent reviews describing the properties and uses of antimicrobial peptides (Cornelissen and Melchers 1993; Destéfano-Beltrán et al. 1993; Raikhel et al. 1993; Rao 1995), we will not attempt to cover the whole topic. Reports of transgenic tobacco expressing antimicrobial peptides, although promising, demonstrate the need for more experimentation. Increased disease resistance was reported in transgenic tobacco expressing α-thionin (Carmona et al. 1993) and a modified cecropin B peptide, Shiva-1 (Jaynes et al. 1993). However, other reports showed no significant increase in disease resistance in transgenic plants expressing other cecropin peptides (Hightower et al. 1994; Montanelli and Nascari 1991). Considering these reports, *in vitro* antimicrobial activity does not necessarily mean *in vivo* pathogen resistance; therefore, genes must be carefully designed to ensure that the encoded peptides are stable, expressed at effective levels, and localized in the proper plant cell parts.

In this chapter, we emphasize the process of selecting promising antimicrobial peptides and designing genes encoding them for use in tree species. We limit our discussion to peptides that are small (<60 amino acids in length), nonenzymatic, linear, without any unusual or modified amino acids, and to those that demonstrate an inhibitory activity to the growth of a plant pathogen. These peptides are interesting because their small size: 1) allows synthesis of analogs with amino acid sequence changes that can be analyzed for antimicrobial activity change; 2) reduces the expense of constructing genes that encode them; and 3) allows possible construction of a single gene that would encode 2 or more peptide products.

The strategy for antimicrobial peptide use is to have transgenic plants express these inhibitory peptides at wound or infection sites and stop the spread of the pathogen. The use of these peptides can be envisioned as infection court-targeted plant pesticides. There are 2 advantages of this disease control strategy over current pesticide use: 1) only a small fraction of the compounds would end up in the food chain or in the environment; and 2) these peptides breakdown rapidly.

Selecting and Designing Antimicrobial Peptides

When selecting antimicrobial peptides for use in pathogen-resistance genes, we looked for those that differed in structure and in mode of action. The first peptide chosen, ESF12 (Powell et al. 1995), is a synthetic analog of PGLa, which is a natural antimicrobial peptide produced in the skin of the African clawed frog (*Xenopus laevis*) (Soravia et al. 1988). ESF12 is an 18-amino acid peptide that at micromolar concentrations inhibits conidial germination of 3 selected plant-pathogenic fungi: *Septoria musiva*, *Cryphonectria parasitica*, and *Fusarium oxysporum*. These organisms cause Septoria leaf spot and Septoria cankers on poplars (*Populus* spp.), chestnut blight on many chestnut species (*Castanea* spp.), and Fusarium wilt of tomato (*Lycopersicon esculentum*), respectively. ESF12 also inhibits the growth of the bacterial plant pathogens *Agrobacterium tumefaciens*, *Erwinia amylovora*, and *Pseudomonas syringae*.

ESF12 is structurally similar to the lytic peptides, magainins and cecropins. These contain an amphipathic α-helix thought to form channels in biological membranes (Christensen et al. 1988; Matsuzaki et al. 1994; Vaz Gomes et al. 1993). Genes expressing analogs of cecropin B enhanced resistance of transgenic tobacco to bacterial wilt caused by *Pseudomonas solanacearum* (Jaynes et al. 1993). We expect that properly constructed genes encoding and expressing ESF12 will also enhance pathogen resistance in poplars and other tree species.

The second antimicrobial peptide chosen, Ac-AMP1.2, is an analog of Ac-AMP1 (Broekaert et al. 1992) with the amino terminal Val replaced with Met to facilitate expression. The Ac-AMP1 peptide was originally identified in the grain amaranth (*Amaranthus* spp.) where it is expressed in the seed coat and apparently helps to protect the seed from microbial degradation. Ac-AMP1 inhibited fungal growth at micromolar levels and inhibited the growth of Gram-positive but not Gram-negative bacteria (Broekaert et al. 1992). Ac-AMP1 is a defensin-like peptide containing 3 disulfide bonds, and is homologous to the cysteine/glycine rich domains of many chitin-binding proteins. Ac-AMP1's ability to bind to chitin suggests that it has a different mode of inhibitors from magainins and cecropins, and is therefore different from the synthetic peptide ESF12.

Testing Antimicrobial Peptides for Phytotoxicity

An antimicrobial peptide would be of little use as a pathogen-resistance mechanism if it was toxic to the plant expressing it. For this reason we also used a germination assay with pollen of *Castanea mollissima*, *Salix lucida*, *Malus domestica*, and *Lycopersicon esculentum*. ESF12 and several other peptides assayed were nontoxic at 50-fold higher concentrations than those inhibiting fungal spore germination (Powell et al. 1995). Ac-AMP1 was not tested *in vitro* due to the cost of its synthesis and, since it is naturally found in plant tissue, it is expected to be nontoxic to plants. This hypothesis will be tested when it is cloned and expressed in transgenic poplar.

Designing Genes for Correct Expression in Plants

All of the peptides we evaluated were synthesized from chemical components, not extracted from biological samples. Synthesizing and testing different amino acid sequences allowed us to experiment widely. We made several amino acid changes from the original model before arriving at ESF12; therefore, there was no gene available that coded for the peptide and a new gene was designed and constructed.

Codon Choices

Since no natural source of ESF12 exists, a gene capable of expressing it in a transgenic plant had to be designed and constructed. Several items were considered to ensure that design flaws would not interfere with optimal expression. Because of redundancy in the genetic code, 1 to 6 codons may specify the same amino acid. For example, 6

different codons specify arginine but only one specifies methionine. Although the genetic code is common among all eukaryotes, there are differences in frequency of use among organisms. Since the final destination of our gene constructs will be 1 or more tree species, we chose from a set of 44 dicot-preferred codons (Campbell and Gowri 1990) to encode the peptides ESF12 and Ac-AMP1.2 into DNA sequences.

Each open reading frame includes an efficient translation initiation site. In most plant mRNA, the 5' proximal AUG is the initiation codon (Joshi 1987), but initiation is also determined by the context of the AUG (Kozak 1986a, 1989). The animal initiation consensus sequence (CCACCAUGG) and the plant initiation consensus sequence (UAACAAUGGC) initiate translation equally well in tobacco mesophyll cells (Guerineau et al. 1992). The animal consensus sequence has the advantage of containing a *Nco*I restriction endonuclease recognition site (CCATGG) in the genes DNA sequence. The location of this restriction site can be used in future modifications, such as the addition of leader sequences that target the gene product to various parts of the cell.

Next, a termination codon was chosen. In dicots, the mRNA termination codon UAA is preferred (46 percent) over UGA (36 percent) or UAG (18 percent) (Angenon et al. 1990). Data suggest that efficient termination in plants might require a tetranucleotide sequence of UAAA, UGAA, or UAGA where A is preferred (41 percent) and C is avoided (6 percent) in the last position (Angenon et al. 1990). Plants contain transfer RNAs (tRNAs) capable of misreading UAG and therefore the UAGA sequence might be used to prevent this stop codon from producing an oversized product.

Lastly, the resulting nucleotide sequences were tested using computer modeling and modified to produced minimal mRNA secondary structure. Folding of mRNA into hairpin loops or other secondary structures can inhibit translation (Kozak 1986b). Minimizing secondary structures also aids the synthesis of the DNA used to produce the gene construct.

Post-Translation Factors

Post-translational processes, regulating peptide stability and targeting for cellular export, must be considered in addition to optimizing factors involved with translation. If the peptides need to be stable within the cell, the amino acid sequence should follow the N-End Rule. Proteins containing Met, Thr, Ser, Gly, or Val at the N-terminus are relatively stable. Proteins containing Lys, Arg, Glu, Asp, Gln, or Asn are rapidly degraded by the ubiquitin system (Varshavsky 1992). Another post-translation process to consider is the targeting of the peptides. If it is desirable to have the peptides exported out of the cell, there are several leader sequences identified. PR-1a, b, and c leaders from tobacco (Pfitzner and Goodman 1987), sCEC leader from the insect *Hyalophora cecropia* (cecropia moth) (Denecke et al. 1990), and the *ap24 leader* from tobacco (Melchers et al. 1993) are examples of leader sequences that direct the export of fused proteins in transgenic plants. We are presently evaluating the ap24 leader for promoting export of our antimicrobial peptides.

Promoter Evaluation

To ensure proper expression of the gene construct, several promoters are being tested. First, the constitutive cauliflower mosaic virus (CaMV) *35S* promoter is being used in the vector pCEA1 (figure 1). The *35S* is a strong constitutive promoter and will be used primarily to determine the effects on the plant tissues to express peptides. If high concentrations of the peptides damage or kill plant cells, we would be unable to regenerate transformants. If the gene is sublethal, but still toxic, we would expect to see severely altered phenotypes among the transgenics. Existing transformants of petunia, willow, and poplar have been obtained. The only consistent effect observed was an increased difficulty in rooting the transgenic plants. This work is still in progress.

Constitutive expression of the peptides will probably be unnecessary for protection against pathogens. We are examining a set of wound-inducible promoters that should confine expression of the antimicrobial peptides to the tissues most susceptible to pathogen attack. The poplar wound-inducible promoters we are evaluating include *WIN*3.12 and *WIN*6.39b (Bradshaw et al. 1989; Davis et al. 1991; Hollick and Gordon 1993).

Creating Multiple-Gene Cassettes for Durable Resistance

Plant breeders, geneticists, and pathologists have been combating pathogens for more than a century and have produced many hundreds of disease-resistant varieties. In many cases, within a few years to a few decades of commercial release, the resistant variety is successfully attacked by a new strain of the pathogen. The length of time a variety retains its resistance is called durability. Many variables affect durability, but one of the most frequently cited causes of low durability is resistance based on a single gene product. This problem may be more pronounced in woody perennials than in annual crops because individual plants are exposed to a pathogen population for years to decades. This long-term exposure enhances the probability of selecting pathogens that can

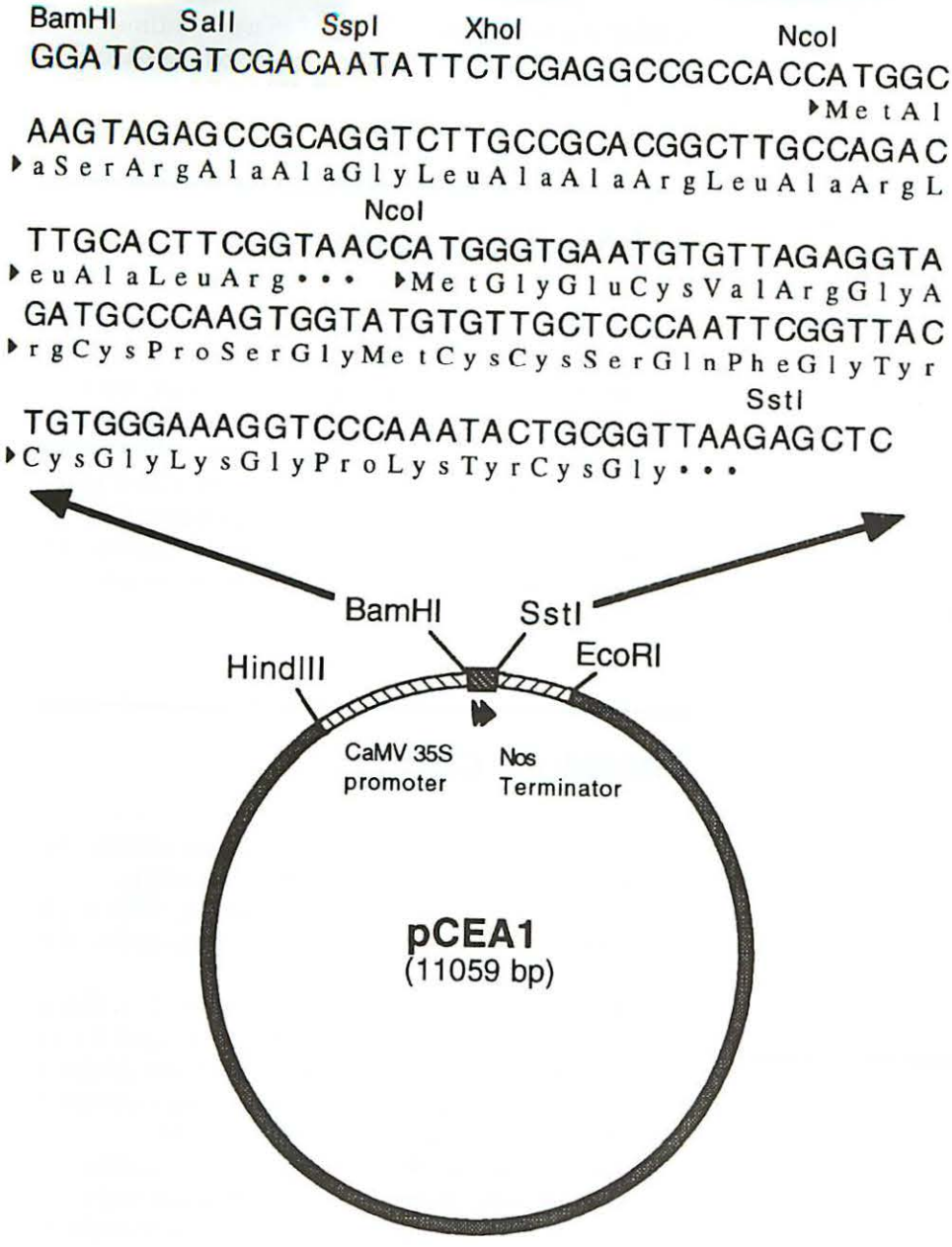


Figure 1. DNA and encoded amino acid sequences of the ESF12/ACAMP1.2 gene construct in the pCEA1 plant binary vector.

overcome the single-gene resistance. Antimicrobial peptides are the simplest of single-gene traits. Therefore, it could be expected that pathogens could evolve quickly to overcome resistance based on a single antimicrobial peptide.

To genetically engineer durable resistance, a multi-layered defense system should be employed using 2 or more gene products that employ different inhibitory mechanisms. A single mutation overcoming the effects of just 1 antimicrobial peptide would have no selective advantage because the plant's other peptide would prevent propagation of the mutated pathogen. For a pathogen to overcome 2 or more different antimicrobial peptides would require multiple mutations to simultaneously overcome the effects of each. It is highly improbable that such multiple mutations would simultaneously occur in a single microorganism. The probability decreases with each additional antagonistic gene product. Therefore, a genetic engineering strategy using several antimicrobial gene products should produce highly durable resistance.

Multiple-Gene Transfer

There are at least 4 options for genetically engineering plants to produce multiple gene products. The first option is to include multiple genes in a single construct with separate promoters to drive the production of each gene. This is a difficult and complex task. The final construct size may be so large that it strains the limit of the *Agrobacterium* vector system. The large size also increases the chance for rearrangement and inactivation of 1 or more of the genes.

A second option would be to transform different plants, genetically cross them, and evaluate the progeny to find 1 or more individuals expressing both genes. For annual plants, this approach holds promise, but for long-lived woody perennials, the time required is prohibitive. In addition, this approach does not genetically link the genes, and allows for offspring to contain only 1 of the genes. This could jeopardize resistance durability by allowing the propagation of pathogens that have mutated and can overcome 1 of the gene products. The presence of these pathogens in the population would effectively reduce the layers of resistance in plants still producing multiple pathogen-resistance gene products.

As a third option, after recovery of a whole plant expressing the first gene, a second round of transformation and selection is initiated to incorporate the second gene of interest. This approach could be used on poplar or other tree species if the genes of interest can be evaluated early and if different selectable markers are available for each round of transformation and selec-

tion. This approach has the disadvantage of not genetically linking the genes.

The fourth option is to express multiple gene products from a single gene construct under the control of a single promoter. This option has the advantage of linking the coding regions, keeping the gene construct small, and minimizing the transformation procedures. We are testing 2 approaches to this option. The first approach takes advantage of a second initiation in translation. If 2 open reading frames are located within a few base pairs of one another and the first open reading frame is small, the second open reading frame can be expressed at a reduced rate (Putterill and Gardner 1989). We have designed a gene construct in which the 2 open reading frames encoding the antimicrobial peptides are next to one another and contain efficient translational initiation sites (figure 1). We are testing this construct for expression of both peptides.

A second approach is to encode both antimicrobial peptides into 1 open reading frame that includes the self-cleaving protease TEV-Nla (Marcos and Beachy 1994; vonBodman et al. 1995). This will produce a polyprotein that will self-cleave in the transgenic plant, releasing the 2 peptides. The disadvantage to this approach is that residual amino acids are left on the cleaved peptides. Therefore, it must be determined if these added amino acids will have any effect on the antimicrobial activity of the peptides. The advantage of this approach is that the peptides are linked and would be released in essentially equal molar quantities. We are presently evaluating this approach for transgenic poplar.

Summary

In this chapter we have described a process of using genetic engineering to develop durable pathogen resistance for poplar and other tree species. Toxicity tests of various amino acid sequences, designed on the basis of structural properties of natural antimicrobial peptides, provided information on which substitutions enhanced or reduced activity against various plant pathogens. Similar tests using pollen showed that the peptides appear to be nontoxic to plant cells even at 50 times the concentration needed to inhibit fungi. Initiation and termination sequences, codon preferences, secondary structure, peptide stability, and multiple product expression were considered in designing a gene to express the antimicrobial peptides *in vivo*.

Present efforts focus on transformation and recovery of nonchimeric whole plants expressing the peptide(s) of interest under the control of either the *35S* constitutive promoter or a wound-inducible promoter. The goal is to develop a gene cassette to express 2 or more antimicrobial peptides that will confer durable pathogen resistance in transgenic plants.

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Chapter 23

Potential of Proteinase Inhibitors for Enhanced Resistance to *Populus* Arthropod and Pathogen Pests[1]

Scott A. Heuchelin, Lise Jouanin, Ned B. Klopfenstein, and Harold S. McNabb, Jr.

Introduction

Proteinase inhibitors have recently been the focus of considerable research and speculation about their potential role as plant defense chemicals in host-pest interactions. These small molecular weight proteins, and the genes that code for them, may soon be a source of resistance to arthropod and pathogen pests for tree improvement programs. Detailed information on proteinase inhibitors and their origins can be obtained from the reviews of Ryan (1981, 1990) and Barrett and Salvesen (1986). In general, proteinase inhibitors interfere with the active sites of proteolytic enzymes by slowing or halting the rate of hydrolysis by a protease on its protein substrate. Proteases cleave proteins into smaller polypeptides and their constituent amino acid residues by hydrolyzing the peptide bonds between amino acids. To help understand the different groups of proteinase inhibitors and the types of proteolytic activity they suppress, we will consider the diverse proteases potentially used by biotic antagonists in the environment.

Classification of Proteinases and Proteinase Inhibitors

Two types of proteases are used in the degradation of a typical protein. Endopeptidases, specific for peptide bonds between distinct amino acid groups (residues), are responsible for the initial stages of protein breakdown and subsequent degradation of polypeptides. Exopeptidases are not specific for the internal bonds that hold together an amino acid chain, but rather for the bonds of the terminal amino or terminal carboxyl residues. Cleavage of terminal residues by exopeptidases generates free amino acids from peptides. For protease nomenclature, the term "proteinase" is used for proteolytic enzymes possessing endopeptidase activity (Barrett 1985).

Proteinases and their inhibitors are classified according to the mechanism by which the peptide bonds are hydrolyzed. Six proteinase families have been determined and are named according to specific amino acid residues that are active in the catalytic mechanisms or compounds crucial to the catalytic activity (Neurath 1984). The 6 families can be placed into the following 4 groups: 1) aspartic, 2) serine, 3) cysteine, and 4) metalloproteinases. For detailed information on proteinases, reviews by Beynon and Bond (1989), Dalling (1986), and North (1982) are informative.

Aspartic proteinases are active in acidic environments and typically have optimal enzyme activity in the range of pH 3.5 to 5.5. Acid aspartic proteinases are produced in most eukaryotic organisms and have been found in many fungi. Inhibitors of aspartic proteinases include the protein pepstatin, epoxy propane, and diazoacetyl-compounds such as the diazoacetyl-norleucine methyl ester-copper ion complex (Beynon and Bond 1989; Dalling 1986; North 1982).

Serine proteinases are active in neutral to alkaline environments and typically have optimal activity in the pH range of 7.5 to 9.0. Alkaline serine proteinases are in almost all prokaryotic and eukaryotic organisms including pathogenic fungi and bacteria and phytophagous insects. Specific inhibitors of serine proteinases include the: 1) proteinase inhibitor (PI) I and PI II proteins of the Solanaceae; 2) soybean trypsin inhibitor and its relatives in the Leguminosae; 3) protein aprotinin; and 4) irreversible chemical inhibitors such as phenylmethylsulfonyl fluoride (PMSF) and diisopropyl-

[1] Klopfenstein, N.B.; Chun, Y.W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

phosphofluoridate (DIFP) (Beynon and Bond 1989; Dalling 1986; North 1982).

Cysteine proteinases are a diverse group and are active in environments with widely ranging pH values. Optimal activity has been observed from pH 4.0 to 8.0, depending on the organism and its environment. Cysteine proteinase activity is typically activated or enhanced in the presence of cysteine, low molecular weight thiol groups, and other reducing agents. This group of proteinases has been found in almost all organisms including phytophagous insects. To date, reports of cysteine proteinases in fungi are few. Specific inhibitors of cysteine proteinases include the OCI (oryzacystatin) protein of rice, iodoacetamide, iodoacetate, N-ethyl maleimide, and heavy metals (Beynon and Bond 1989; Dalling 1986; North 1982).

Metallo-proteinases are typically active in near neutral pH environments; however, some metallo-proteinases have optimal activity in pH conditions as low as pH 5. Most metallo-proteinases contain a crucial zinc atom within their catalytic site. These proteinases are widespread in prokaryotic and eukaryotic organisms including some pathogenic fungi. Inhibitors of metallo-proteinases include phosphoramidon, hydroxamic acid derivatives, mercaptoacetyldipeptides, ethylenediaminetetraacetic acid (EDTA), and other metal-chelating agents (Beynon and Bond 1989; Dalling 1986; North 1982).

Of these inhibitors, the small molecular weight proteins are of particular interest to researchers. Usually these inhibitor proteins are tight-binding reversible inhibitors of their proteinase class that have little reactivity with other types of proteinases. Since they are reversible inhibitors and follow an equilibrium dissociation constant, their activity is dependent on their concentration in the system studied. Because they have exhibited almost no toxicity to nontarget organisms, and their affect on the target organism is rarely acute, they are ideal for biological systems.

Of even greater interest, is the potential to create transgenic trees that can express inhibitor proteins in their leaf, root, and/or cambial tissues. Once the gene for a particular inhibitor protein is cloned, it can be incorporated into a transformation vector and used to create transgenic trees that may possess higher resistance to a particular arthropod or pathogen pest (Klopfenstein and Hart 1997). Research in this direction was recently initiated using *Populus* as a model transformation system for woody plants. Overall, such research has focused on 2 inhibitor proteins; PI II, a serine proteinase inhibitor from potato (Heuchelin et al. 1997; Klopfenstein et al. 1991, 1993, 1997) and OCI (Cornu et al. 1996; Leplé et al. 1995). A summary of the research conducted with these inhibitors, including transformation and expression studies, pest susceptibility assays, and *in vivo* resistance bioassays follows.

Expression of the Serine Proteinase Inhibitor, PI II, in Poplar

The serine proteinase inhibitor, PI II, is a low molecular weight protein (monomer Mr 12,300) (Plunkett et al. 1982) that exists in dimeric form and inhibits the proteolytic activity of trypsin and chymotrypsin (Ryan 1981, 1990; Ryan and An 1988). PI II is the gene product of the proteinase inhibitor II (*PIN2*) gene, which was first cloned from tomato and potato (Graham et al. 1985; Sanchez-Serrano et al. 1986).

In theory, PI II could effectively reduce the vigor of fungi by depriving them of necessary amino acids (Ryan 1990). Lorito et al. (1994) proposed the use of proteinase inhibitors as a novel class of fungicides after observing deleterious effects of serine proteinase inhibitors on fungi. A similar study at Iowa State University (I.S.U.) using PI II, supports the fungistatic effects of serine proteinase inhibitors (Heuchelin and McNabb 1995). Proteinase inhibitors have reduced the activities of fungal proteinases in *Colletotrichum lindemuthianum* (Mosolov et al. 1979) and *Fusarium solani* (Mosolov et al. 1976). Our studies show that *Mycosphaerella populorum* (*Septoria musiva* anamorph) produces proteinases *in vitro* that are inhibited by PI II (Heuchelin and McNabb 1995). Proteinases also have a significant role in the pathogenesis of host tissues. Movahedi and Heale (1990) concluded that proteinases may act synergistically with the cell wall degrading enzymes in pathogenesis. Inhibition of these proteinases may reduce infective capacity, colonization, and sporulation of the fungus. Reductions in any of these areas could slow fungal spread decreasing disease pressure.

Insect growth, development, and reproduction are also potentially influenced by PI II. PI II in the diet of insect species reduced growth in *Heliothis zea* and *Spodoptera exiqua* (Broadway and Duffey 1986), and *Manduca sexta* (Johnson et al. 1989). A potential role of PI II in resistance to arthropod pests is apparent.

Hybrid poplar clones cv. 'Ogy' (*Populus* x *euramericana*) and cv. 'Hansen' (*P. alba* x *P. grandidentata*) were used in studies at I.S.U. and the USDA Forest Service National Agroforestry Center (NAC). In North America, the major defoliating insect pest of 'Ogy' and other *Populus* hybrids of Aigeiros parentage is the cottonwood leaf beetle, *Chrysomela scripta* (Coleoptera: Chrysomelidae). Although *C. scripta* is not a major pest of 'Hansen' and other hybrids of the Leuce (currently termed Populus) section, the imported willow leaf beetle, *Plagiodera versicolora* (Coleoptera: Chrysomelidae), can cause considerable defoliation and is a major pest of white poplar, aspens, and their hybrids (Ostry et al. 1989).

To study *PIN2* gene expression, poplar clones were transformed with an *Agrobacterium* binary vector system. Initially, 'Hansen' was transformed with the binary vector plasmid pRT45 to test the wound-inducible *PIN2* promoter with the chloramphenicol acetyltransferase (*CAT*) reporter gene. Using northern analysis, these studies demonstrated inducible expression of *PIN2-CAT* when leaves were artificially wounded (Klopfenstein et al. 1991).

To test the importance of proteinases in host-parasite interactions, transgenic 'Ogy' and 'Hansen' were produced containing a chimeric fusion of the *PIN2* structural gene with the cauliflower mosaic virus *35S* or bacterial nopaline synthase (*NOS*) promoter (Heuchelin et al. 1997; Klopfenstein et al. 1993, 1997). An *Agrobacterium* binary vector system, containing either the pRT102 or pRT104 transfer DNA (T-DNA) vector and a disarmed tumor-inducing (Ti) plasmid (EHA 101) as the helper plasmid, was used to transform poplars with *PIN2* gene constructs. The T-DNA also included a *NOS* promoter linked to a neomycin phosphotransferase (*NPTII*) gene as a selectable marker (Heuchelin et al. 1997; Klopfenstein et al. 1997). The *PIN2* coding region is regulated by a *NOS* promoter in vector plasmid pRT102 and by the CaMV *35S* promoter in vector plasmid pRT104. Transgenic plantlets were regenerated under kanamycin selection. Putative transgenic lines were analyzed for transgene incorporation by Southern analysis and/or polymerase chain reaction. *PIN2* expression was verified by enzyme-linked immunosorbent assays (ELISA) and/or western blot analysis.

Three lines of transformed 'Ogy' and the untransformed material ('Ogy' control) were used in an *in vitro* feeding assay with *C. scripta*. In addition, 11 lines of transformed 'Hansen' and the untransformed material ('Hansen' control) were used in a separate *in vitro* feeding assay with larvae of *P. versicolora*. *C. scripta* larvae grown on the 3 transgenic 'Ogy' lines consumed less leaf area and had lower larval weights than those reared on control material (Kang et al. 1997). On 1 transgenic 'Hansen' line (Tr665), *P. versicolora* larvae consumed significantly less tissue than larvae on the control material. Overall, some *PIN2*-expressing 'Hansen' lines also showed trends for lower pupal weights and longer development times for *P. versicolora* (Klopfenstein et al. 1997).

Expression of a Cysteine Proteinase Inhibitor in Poplar

Poplars, important trees in western Europe and France, are sensitive to many pathogens and pests including the poplar leaf beetles, *Chrysomela tremulae* and *C. populi*, which are 2 important defoliating insects (Augustin et al. 1993). In nurseries and short-term plantations, these insects can completely defoliate the plants and drastically reduce growth. During wide outbreaks on young plantations, larvae and adults have attacked the bark and caused plant mortality. Chemical treatments must be applied in the nursery to reduce attacks, but these treatments are expensive and potentially hazardous to the environment. Some natural resistance mechanisms are known, but selecting these traits in classical improvement programs is difficult. Genetic engineering is a new strategy being explored at the Institut National de la Recherche Agronomique (INRA), France, to introduce into poplar a gene conferring resistance to these beetles.

Major proteolytic activity in the gut of *C. tremulae* was used to define a strategy for controlling larval growth and development using an inhibitor of digestive proteinases. Proteinases from midguts of third instar larvae were extracted and characterized. Basal proteinase activity showed optima at pH 7 and pH 9. In the presence of cysteine proteinase activators (dithiothreitol or 2-mercaptoethanol), a strong increase (approximately 7 fold) in the proteinase activity at pH 7 was observed. The efficiencies of a serine proteinase inhibitor (soybean Bowman-Birk Inhibitor, BBI) and a OCI to inhibit the proteinase activity were tested. Results indicate that cysteine proteinases sensitive to OCI constitute the major pool of gut proteinases of *C. tremulae* larvae (Leplé et al. 1995). Consequently, *OCI* gene expression in transgenic poplars should produce a deleterious affect on *C. tremulae* survival *in planta*.

The cDNA of *OCI* (Abe et al. 1987) was introduced in a transformation vector under the control of the CaMV *35S* promoter with a double enhancer sequence (Kay et al. 1987). An *NPTII* gene was used for selection of the transformed cells on kanamycin containing medium. Transformation was performed on a Leuce hybrid poplar (*P. tremula* x *P. tremuloides*) clone 'INRA 353-38,' which is very sensitive to *C. tremulae*. Two *Agrobacterium*-mediated transformation procedures, co-inoculation (Brasileiro et al. 1991) and co-cultivation (Leplé et al. 1992), previously established for a *P. tremula* x *P. alba* hybrid, were adapted to the *P. tremula* x *P. tremuloides* hybrid. Twelve independent lines were obtained and characterized at the molecular levels (Southern and northern analyses). These experiments determined which lines were expressing the *OCI* messenger RNA (mRNA) at a high level and which contained a low (1 or 2) T-DNA copy number. Western blot analysis was performed on leaf extracts using an antisera raised against OCI. In the best expressing lines, the OCI protein represented 2 percent of the total soluble proteins. Selected clones were micro-propagated and transferred to the greenhouse for *C. tremulae* feeding tests (Cornu et al. 1996; Leplé et al. 1995). Feeding tests were conducted from egg hatch to the pupation of larvae on young leaves. From the third day of feeding, we observed significant

differences in larval mortality on the selected transformed lines compared to the control plants. These differences increased until the pupation stage (table 1) (Cornu et al. 1996; Leplé et al. 1995).

A deleterious effect was observed after a long delay, which allowed the larvae to cause considerable damage to the leaves. However, development and growth of larvae were delayed and adults emerging at the end of the pupae instar were often abnormal. Therefore, a longer development time will influence the number of generations and promote parasitism in natural conditions, and lower weight of egg-laying parents may adversely affect fecundity resulting in less offspring. Even if the protection conferred by *OCI* in transgenic poplars is not absolute, the addition of these different effects should decrease the size of future *C. tremulae* populations. A field trial of selected *OCI*-expressing lines and control plants was planted in 1995 at INRA, Orlèans, France, which will allow further experimentation under natural conditions.

Conclusions

Proteinase inhibitors show great promise as a tool to help manage arthropod and pathogen pests of *Populus*. However, interpreting ecological interactions of proteinase inhibitors is complex. More studies are needed to evaluate the role of specific proteinase inhibitors in managing specific pests using intact plants under field conditions, where interactions with other pest management practices, other host resistance mechanisms, natural enemies, and less-favorable environments exist. Deployment strategies must be devised to maximize resistance stability and minimize adverse environmental effects. *Populus* is well suited for addressing these issues.

Table 1. Percentage of mortality of *Chrysomela tremulae* feeding on oryzacystatin (*OCI*) transgenic poplar (*Populus tremula* x *P. tremuloides*) foliage.

Feeding day Larval stage	3rd 1.1	7th 1.2	15th 1.3	25th Pupae
Control	0.25	1.75	3.75	4.50
OCI-4	3.50	7.25	21.50	29.75
OCI-33	1.00	11.75	27.00	42.50
OCI-51	0.75	17.00	38.00	43.50

Source: Cornu et al. 1996; Leplé et al. 1995

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Chapter 24

Expression of Transgenic *Bacillus thuringiensis* δ-endotoxin in Poplar[1]

David D. Ellis and Kenneth F. Raffa

Introduction

Bacillus thuringiensis (*Bt*) is one of the most widely used pesticides, with more than 2.3 million kg of spores applied worldwide (Rowe and Margartis 1987). *Bt* represents 74 percent of the total pesticides applied to Canadian forests for spruce budworm and 75 percent of the pesticides sprayed for gypsy moth (*Lymantria dispar*) control in North America. As a pesticide, it is considered environmentally friendly due to: 1) a high specificity of the toxin proteins against target insects; 2) the natural occurrence of the bacterium; 3) a lack of polluting residues; 4) safety to nontarget insects, humans, or other animals; and 5) the short-lived nature of the toxin. The short-lived nature of *Bt* provides a margin of application safety by decreasing residual buildup. However, this feature hinders proper administration and partly contributes to a history of insect control failures.

The high specificity of *Bt* is due to the production of different δ-endotoxins, or crystal proteins, that display activity against certain groups of insects. *Bt* strains, loosely divided into 4 major classes based on toxin structural similarities and activity against certain insect pests, are: 1) *cryI*, that exhibit activity against lepidopterans; 2) *cryII*, with activity against lepidopterans and dipterans; 3) *cryIII*, with activity against coleopterans; and 4) *cryIV*, with activity against dipterans (Hofte and Whiteley 1989). Insecticidal activity of these classes is not absolute. For example, *cryIB* genes show activity against coleopterans and lepidopterans.

Isolation of new *Bt* strains is critical because of large variations in the toxicity of each *Bt* strain to different groups of forest pests (MacIntosh et al. 1990; Ramachandran et al. 1993b; van Frankenhuyzen et al. 1991, 1993). In 1 study (van Frankenhuyzen et al. 1991), 3 subclasses of *cryIA* endotoxin were tested and were equally toxic against the forest tent caterpillar (*Malacosoma disstria*). However, in 3 species of budworm (*Choristoneura fumiferana, C. occidentalis, C. pinus*), *cryIA*(a) and *cryIA*(b) were 5 times more toxic than *cryIA*(c). Further, in gypsy moth, *cryIA*(a) and *cryIA*(b) were 100 times more toxic than *cryIA*(c). These toxicity tests are complicated because toxicity varies with the insect developmental stage. With cottonwood leaf beetle (*Chrysomela scripta*), susceptibility to purified *cryIB* toxin decreases with increasing larval age. Specifically, the first instar larvae are 2 to 4 times more susceptible than third instar larvae (Ramachandran et. al. 1993a). In addition, only 30 percent mortality occurred in adult leaf beetles, even with the highest *Bt* dose.

Bt δ-endotoxins disrupt the midgut after insects ingest *Bt* spores containing a high concentration of the crystal protein. The crystal protein is solubilized in the midgut into a protoxin that is activated by a protease to form a toxin that binds a specific receptor in the midgut (Adang 1991). Toxin binding causes the midgut adenosine triphosphate (ATP) level to decrease within 1 min of ingestion, and 78 percent of potassium ion transport is inhibited within 10 min. The resulting osmoregulatory imbalance causes cells to imbibe water, burst, and form lesions in the midgut (Reichelderfer 1991).

Specificity of the toxin to different insect orders is due to different toxins that bind to distinct receptors in the midgut. Defining the receptor domain of each toxin is an active area of research to increase toxin specificity and engineer toxins for multi-pest activity (Feitelson et al. 1992). Specificity of receptor binding is an important factor because mutations that alter the receptor binding region can render individuals resistant to the toxin. Therefore, engineering toxins with multiple receptor binding sites is one strategy to decrease the evolution of resistance in insect populations. However, the evolution of resistance is not

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eliminated by mixtures of *Bt* toxins (McGaughey and Whalon 1992).

A major factor in the short-lived nature of *Bt* spray effectiveness is that the toxin in *Bt* spores is photo labile. Several strategies have been tested to increase the effective life of the pesticide in the field, such as the expression of the active toxin in other bacteria like *Pseudomonas fluorescens* (Feitelson et al. 1992).

Another option for increasing field effectiveness of *Bt* is by genetically engineering plants to express *Bt* δ-endotoxin. This is effective in several plant species (Adang et al. 1993; Barton et al. 1987; Ellis et al. 1993; Fischoff et al. 1987; Koziel et al. 1993; Perlak et al. 1991, 1993) including poplar (McCown et al. 1991). However, the problem with this approach is insufficient transgene expression and/or instability of the δ-endotoxin RNA or protein in genetically engineered plants (Adang et al. 1993; Perlak et al. 1991; Wong et al. 1992). While the endotoxin comprises up to 50 percent of the total protein in sporulating *Bt* cultures, it is difficult or impossible to detect the δ-endotoxin in genetically engineered plants with an unmodified δ-endotoxin gene. This frequently occurs even in plants where transgene expression produces toxicity in insect feeding trials.

Truncation of the gene, leaving the N-terminal half of the protein unaltered, results in protein expression at barely detectable levels. A comparison of the *Bt* δ-endotoxin sequences with other plant genes reveals significant differences in G/C content, A/T rich regions that resemble plant introns, the presence of potential polyadenylation sequences, -ATTTA- motifs shown in other systems to destabilize plant messenger RNA (mRNA), and the presence of codons rarely used in plants. Resynthesis of the gene to eliminate these sequences increased *Bt* toxin levels in the plants up to 100-fold (Perlak et al. 1991).

Currently, several crop plants genetically engineered with a *Bt* δ-endotoxin gene have received or are close to receiving regulatory clearance for commercial production. Field tests with transgenic poplar containing and expressing the *Bt* δ-endotoxin gene are ongoing in North America and are planned for in Europe.

Bt in Transgenic Poplars

The first report of *Bt* δ-endotoxin gene expression in poplar was the over expression of a partially modified *cryIA*(a) gene controlled by an enhanced cauliflower mosaic virus (CaMV) *35S* promoter in *Populus alba* x *P. grandidentata* cv. 'Crandon' (cl. 'NC5339') (McCown et al. 1991). The *cryIA*(a) gene was truncated so that the gene encoded approximately 55 percent of the amino-terminus of the original protein. This truncation allowed the entire toxin region to remain intact. In addition, the amino-terminal 141 codons were synthetically changed to correspond to preferred plant codons. In this study, 1 of 4 transformed lines expressed the *cryIA*(a) gene at a level high enough for significant effects on the survival and larval weight gain of forest tent caterpillar. However, plants from this transgenic line (NOD 11) did not significantly affect gypsy moth survival, although larval weight gain and leaf area consumed were significantly reduced. Interestingly, the 25 percent level (1 in 4) of transgenic poplar lines that expressed *Bt* δ-endotoxin at lethal levels is similar to the percentage of transformed tobacco plants that displayed insect toxicity after transformation with the same construct (Barton et al. 1987).

Plants from the NOD 11 transformed poplar line were used for several other studies, including greenhouse (Robison et al. 1994) and field (Kleiner et al. 1995) testing for *Bt* expression. In the greenhouse study, the transformed NOD 11 poplars were grown for several asexual generations, throughout which they maintained constant morphology and growth. The *Bt*-expressing transformed line (NOD 11) continued to cause growth reductions in forest tent caterpillar and gypsy moth, and mortality in forest tent caterpillar. Gypsy moths showed a higher ability to recover from *Bt* exposure following feeding on the NOD 11 plants. Forest tent caterpillars consumed a lethal dose after 5 days, yet a 2-day exposure was not lethal. In both cases, development was delayed; however, the gypsy moth could completely recover relative to control insects after 20 days (Robison et al. 1994).

In the field, NOD 11 maintained expression of *cryIA*(a) δ-endotoxin at toxic levels, even after winter dormancy. Insect bioassays with forest tent caterpillar and gypsy moth during June, July, and August of the second field-growing season showed uniform inhibition of feeding and decreased growth. Mortality of the forest tent caterpillars in the June bioassay was higher than that of the gypsy moth in either of the subsequent assays (Kleiner et al. 1995). Observed differences between control and transformed plants in the field were less than that in the previous greenhouse study. These differences are perhaps attributable to reduced feeding on the field-grown, control leaves due to increased toughness.

In France, a *cryIIIA* gene was inserted and tested in a *P. tremula* x *P. tremuloides* hybrid. The toxin encoded by this gene is effective against Coleoptera such as the leaf beetle *Chrysomela tremulae*. Detection of *cryIIIA* gene expression was difficult and reverse transcriptase-polymerase chain reaction (RT-PCR) provided the only reliable detection method other than insect feeding assays. Non-choice feeding assays with leaves from transformed plants showed increased mortality of *C. tremulae* larvae after 18 days. (Cornu et al. 1996). Current work with a synthetic *cryIIIA* gene is expected to yield higher expression (G. Pilate, personal communication).

Several transformed lines of a *Populus nigra* x *P. maximowiczii* hybrid cl. 'NM6' that contain a partially modified *cryIB* gene were regenerated (Francis 1996). This gene is derived from *Bt* strain HD-290-1, which was toxic to cottonwood leaf beetle and forest tent caterpillar when painted on leaves (Ramachandran et al. 1993a). Two transformed lines were planted in the field and monitored for transgene expression throughout 2 growing seasons. Feeding assays with cottonwood leaf beetles revealed increased mortality, although the differences were not significant when compared to the controls. However, a significant decrease in beetle weight gain was observed in 1 transgenic line suggesting decreased larval feeding (Francis 1996).

Managing Biotype Evolution

One concern with the genetic engineering of *Bt* δ-endotoxin in plants is the evolution of resistant insect biotypes. With poplar, and other forest trees, an additional concern is long-term nature of the crop. However, these long-lived perennials also offer alternative options for managing biotype evolution, which may be unavailable or uneconomical with agricultural crops. Studying agricultural crops can be valuable when evaluating these management strategies because insects have historically evolved resistance to agricultural chemical pesticides. This experience has shown that resistant biotypes will evolve, if selection pressures are strong. Further, if selection pressures are strong, every physiological mode of insecticide action can be overcome by insect populations (Raffa 1989). Based on this, it is reasonable to assume that genetically engineered resistance can be overcome by biotype evolution, as has occurred with chemical pesticides under conditions of strong selection pressures.

A simplistic view of biotype evolution is that resistance to an insecticidal agent arises in an individual insect by a single spontaneous genetic mutation that gives that individual a selective advantage when exposed to that insecticide. Such mutations may occur whether the insecticide is present or not, but only confer a selective advantage when the insecticide is present. Due to this selective advantage, this individual's resistant genes are passed on to the next generation at a higher incidence. These resistant genes become dominant in the insect population as long as they continue to confer a selective advantage. This process could render an entire population resistant because only resistant individuals will survive to pass on their genes to the next generation. With genetically engineered poplar, the continual pressure to give resistant genotypes a selective advantage will favor evolution of resistant populations.

Resistance to *Bt* has been documented in more than 500 species of insects (Tabashnik et al. 1994). The most studied case is that of the diamond back moth (*Plutella xylostella*) in Hawaii, where strains with up to a 2,800-fold resistance to *Bt* were isolated. Resistance in the diamond back moth is due to decreased binding of the toxin in the midgut, which is inherited as an autosomal recessive trait (Tabashnik et al. 1994). When selection pressure is lifted, by removal of the *Bt*, the moths become susceptible after several generations. This may be partly due to the resistant alleles conferring decreased fitness in the absence of *Bt*, compared to the wild-type moths.

The diamond back moth is an excellent experimental case to study biotype evolution to *Bt* and factors that influence this resistance, such as the continual and frequent application of *Bt*. Because resistance decreases with removal of *Bt*, management strategies using temporal, spatial, or other crop refugia can be used to decrease the selective pressures for resistant insect populations to evolve. Several other management strategies to decrease the prevalence of resistant biotypes dominating in the population were proposed (Alstad and Andow 1995; Coombs 1993; Raffa 1989) and are discussed below as they pertain to the genetic engineering of poplar for the expression of a *Bt* δ-endotoxin gene.

It is important to review some evidence for the evolution of field resistance to *Bt* (Koziel et al. 1993). Usually, biotype evolution: 1) resulted in only moderate resistance levels; 2) was developed under spraying conditions that provided variable toxicity exposure due to rapid breakdown of the *Bt* δ-endotoxin between applications; 3) involved a single insecticidal agent over several generations; and 4) involved relatively little immigration between resistant and wild populations. This last factor is crucial to the interpretation of lab results because relatively high immigration between populations of most poplar pests would be expected. Thus, many studies may only demonstrate the genetic potential for resistance to arise, not the rate at which such resistance would occur. However, these studies show that insect behavior, insect physiology, delivery mode of the *Bt* toxin, availability of refugia, and many other factors play a major role in determining the actual degree and speed at which resistant biotype populations evolve.

A discussion on risk assessment by Raffa et al. (this volume) addresses some management and deployment strategies for lessening or changing selection pressures on insects to evolve resistance to *Bt*. These and other strategies are detailed below because they pertain specifically to the genetic engineering of poplar for the expression of a δ-endotoxin gene.

Multiple Resistances

As previously discussed, the lethal effect of a *Bt* δ-endotoxin is due to toxin binding of a single receptor in the midgut. Therefore, a single mutation that changes the receptor binding site would alter or diminish binding and

affect toxicity. An alteration in the binding site was a cause of resistance in the diamond back moth. One strategy to overcome the selective advantage of a single mutation is to mix different forms of insecticidal activity in plants. If, for example, plants are genetically engineered with 2 or more *Bt* δ-endotoxin genes that had different and distinct binding sites in the midgut, spontaneous mutations must occur at both sites in the same insect for resistance to occur. This strategy, however, may not be as effective as sequential spraying or exposure to individual *Bt* strains where simultaneous exposure to multiple *Bt* endotoxins is minimal (McGaughey and Whalon 1992).

One consideration with forest trees, such as poplar, is whether multiple resistances in a single tree are as effective as a mosaic of trees with different resistances. Both strategies potentially delay biotype evolution by exposing insect populations to more than one form of toxin. However, if sequential spraying and/or the sequential exposure of different *Bt* toxins are preferred, using genetically engineered trees in a mosaic planting design is advantageous. With this strategy, sequential exposure to different *Bt* δ-endotoxins occurs in a spatial mosaic as insects move from 1 tree to another, whereas a temporal mosaic exists with sequential spraying. The outcome is similar in that insects are exposed to only a single toxin at a time; however, the extent of exposure depends on the degree of insect movement between trees within a forest mosaic.

Management strategies influence whether single tree or stand management for insect resistance is practical. Usually a resistance based on stand management is favorable. However, depending upon the availability and difficulty of transforming different commercially important poplar hybrids, deployment of single transgenic trees with multiple resistances may currently be the only economically feasible strategy.

One pitfall to the genetic engineering of different *Bt* δ-endotoxins is that cross resistance to different δ-endotoxins has been documented in numerous insects (McGaughey and Whalon 1992). For example, a strain of *Heliothis virescens* resistant to *cryIA*(c) was cross resistant to *cryIA*(a), *cryIA*(b), *cryIB*, *cryIC*, and *cryIIA* toxins. This suggests that mixtures, rotations, or mosaics of different *Bt* δ-endotoxin may not be the most effective biotechnological tool to control resistant biotypes. However, other reports (Koziel et al. 1993) note that such resistance is only marginal, and that resistant biotypes are unable to survive under continual *Bt* exposure or when feeding on a transgenic plant expressing *Bt*.

An alternative strategy is to combine *Bt* δ-endotoxins with other heterologous insecticidal genes, such as a proteinase inhibitor gene. With this strategy, cross resistance to different *Bt* δ-endotoxins is not an issue. An example of this strategy would be genetic engineering with a *Bt* δ-endotoxin gene in combination with a proteinase inhibitor gene. By inserting these 2 insecticidal genes, the expression of the proteinase inhibitor would decrease nutrient uptake in the insect, weakening it, while *Bt* would be lethal. This might work with marginally *Bt*-resistant individuals by increasing their susceptibility to the δ-endotoxin. Further discussion on the use of a proteinase inhibitor gene for insect resistance in poplar is presented by Heuchelin et al. (this volume).

A final strategy in the use of multiple resistances is the stacking of *Bt* δ-endotoxin with natural insect resistance. This strategy is mentioned (Raffa et al. this volume; Robison et al. 1994) for management of multiple pest complexes with a mosaic of genetically engineered and natural resistance, using a genetically engineered trait directed against 1 pest and the natural resistance against another. This strategy could alter selection pressures on insects to evolve resistance to an engineered trait by targeting the same pest with both natural and engineered resistance. An example would be to mix a trait for high phenolic glycoside levels, a deterrent to many lepidopterans, with the genetic engineering for *cryIA* δ-endotoxin.

Again, the question of the benefit from single-tree versus stand resistance can be asked. What must be avoided is imposing a selection pressure on the insect population so that the insects must overcome both resistances to achieve reproductive success. Raffa (1989) used a similar scenario when arguing that using genetically engineered *Bt* to target bark beetles might lead to a physiological immunity to both the natural and engineered protection, thereby eliminating natural resistance as a mode of protection.

Altered Expression Patterns of the *Bt* Insecticide

Selection pressures for the evolution of resistant insect populations are greatest when all insects in a population are subjected to very high and constant insecticidal levels. Spraying *Bt* on poplars when insects are present can be accomplished, with only limited success, and selective spraying of only specific tissues on impacted trees is impractical. With genetic engineering, the opportunity exists to express the endotoxin either in an inducible manner or only in the tissues affected or most valuable to the tree. These 2 strategies are referred to as temporal and spatial refugia, respectively (Gould 1988). In both cases, some insects within the population would escape toxin exposure providing individuals on the same tree that are not subjected to selection pressures for the evolution of resistant biotypes. This is a similar strategy to that occurring naturally in a tree for the production of secondary compounds that inhibit insect feeding (Gould 1991; Ramachandran et al. 1994).

The effectiveness of inducing *Bt* δ-endotoxin for insect protection in plants was demonstrated with a chemically induced *PR-1a* promoter controlling a partially modified *cryIA*(b) gene (Williams et al. 1992). In this study, insect tolerance in plants was induced by spraying a chemical inducer that activated the *PR-1a* promoter. While no one is advocating the induction of *Bt* δ-endotoxin expression in plants with the use of a chemical spray, this report shows the potential and efficacy of inducible expression of insecticidal proteins in plants.

Wound inducible promoters were used in poplar to express chloramphenicol acetyltransferase (*CAT*) (Klopfenstein et al. 1991) and β-glucuronidase (*GUS*) (Ellis et al. 1994) using a proteinase inhibitor (*PIN2*) promoter from potato (Thornburg et al. 1987). The degree and method of mechanical wounding profoundly affected the level of *GUS* induction, confirming that this promoter is induced by wounding after a given injury threshold level is reached. This is important because trees can tolerate a mild level of insect attack. By not exposing feeding insects to *Bt* δ-endotoxin during low infestation periods, a subpopulation of insects would be maintained that were never exposed to selection pressures to evolve resistance. These individuals could then serve to dilute resistant alleles in the population.

However, it is unknown whether the *PIN2* promoter can be sufficiently induced in poplar for *Bt* δ-endotoxin expression to impart adequate insect protection. However, our studies found significant increases in *GUS* expression following feeding by forest tent caterpillar on greenhouse-grown transgenic 'Crandon' (data not shown). Field tests with these *PIN2-GUS* transformants have maintained wound-inducible expression of *GUS* even after 3 years in the field; however, wound-inducible expression levels may vary during the year. To date, we know of no studies where this or other inducible promoters were used to control expression of *Bt* δ-endotoxin in poplar.

A good example of the efficacy of spatial refugia with *Bt* was provided with the cottonwood leaf beetle (Ramachandran et al. 1994). Susceptibility of the cottonwood leaf beetle to *cryIB* decreases with age; first instars have a 4-fold lower LC_{50} than third instars (Ramachandran et al. 1993a). Cottonwood leaf beetle eggs are oviposited on the lower mature leaves and the young larvae are relatively immobile. Only as the larvae mature, do they feed on the upper younger leaves. This distinction is important because loss of mature leaves has a significantly lower impact on growth than loss of younger leaves. Furthermore, the stage of the beetle life cycle that is the most susceptible to *Bt* δ-endotoxin, and hence most vulnerable to selection pressures for the evolution of resistant biotypes, feeds almost exclusively on the mature tree leaves. By expressing *Bt* δ-endotoxin only in the younger leaves, the most susceptible stage of the beetle life cycle would not be exposed to the δ-endotoxin and the selection pressure on the insects to evolve resistance would decrease. The lower leaves would be sacrificed, but tree productivity may not be substantially affected.

To test protection afforded by *Bt* expression in only the younger leaves of poplar, a *Bt* spore solution (M-one) was applied to only the young leaves of poplar hybrid cl. 'NM6.' This application compared untreated plants to those with *Bt* treatment of only the mature leaves or the whole plant. The whole-plant treated control was superior because it had the greatest height growth, lowest number of egg masses, and lowest larval survival. However, treatment of only the younger leaves offered significantly greater protection than untreated plants. Further, plants with only the young leaves treated had greater growth than either the untreated controls or plants in which only the lower leaves were treated (Ramachandran et al. 1994). These results suggest that spatial refugia within the same tree can be an effective management strategy for decreasing the selection pressure on insects to evolve resistance to *Bt*, while not substantially sacrificing tree production.

Precise tissue-/development-specific expression of *Bt* δ-endotoxin necessary for spatial refugia in poplar has not been demonstrated. However, several promoters that offer such expression are being tested. An *Arabidopsis* ubiquitin (*UBQ-1*) promoter (Callis et al.1990) is expressed significantly higher in the younger leaves of transgenic tobacco than in the older leaves and is one candidate promoter for testing in the above study with transgenic *Bt* δ-endotoxin. Another promoter that would offer a pattern of spatial refugia is the ribulose-1,5-*bis*phosphate carboxylase (*RUBISCO*) small subunit promoter that could offer differential expression in different aged leaves, and could target *Bt* endotoxin specifically to photosynthetically active tissues (Manzara and Gruissem 1988). In addition, the use of the *Arabidopsis* Rubisco small subunit, *ats1A* promoter, with its transit peptide sequence, increased *cryIA*(c) mRNA 10- to 20-fold compared with a CaMV *35S* promoter. Further, increases in *cryIA*(a) were observed when the untranslated leader and transit peptide sequences were used with CaMV *35S* (Wong et al. 1992).

Results with the untranslated leader and transit peptide sequences are significant because many tissue-/development-specific promoters express genes relatively weakly compared with CaMV *35S*. Therefore, unenhanced promoters may not provide expression at high enough levels for use with *Bt*. Modification of the coding sequence as with the synthetic *cry* genes are crucial; however, the use of chimeric constructs, as demonstrated with CaMV *35S* and the *ats1* sequences, will also be important tools for enhancing expression with weaker promoters.

Lethal vs. Sublethal *Bt* Expression

Some lepidopteran larvae, such as the spruce budworm, avoid *Bt*-treated leaf discs in feeding assays when given a choice of *Bt*-treated versus non-treated discs (Ramachandran et al. 1993b). With genetically engineered spruce, the budworm detected the presence of *Bt* δ-endotoxin and stopped feeding on tissue that expressed a *cryIA*(a) gene at sublethal levels (Ellis et al. 1993). This avoidance of *Bt* by insect pests could be used as a management strategy for lessening selection pressures for resistant biotypes to evolve (Gould 1988). Avoidance decreases feeding, delaying the time to pupation, and rendering the larvae more susceptible to predators, desiccation, and parasites. Sublethal *Bt* expression would lessen selection pressures on the insect populations to evolve resistance; however, it would not prevent resistant biotypes from evolving, and resistance at the population level would inevitably evolve. Therefore, interplanting of the stand with non-*Bt* expressing trees, or the availability of ample other food sources, is also important to maintain a non-*Bt* exposed insect population.

Continued exposure to a sublethal *Bt* level may accelerate the evolution of tolerance in a population so that a higher level of *Bt* is required for mortality. This conclusion is based on experimental and modeling data from *Bt* and chemical insecticides where it is crucial to avoid fixing resistance alleles in homozygotes (Koziel et al. 1993). Because resistant genes are generally recessive, and dominant resistant genes for *Bt* δ-endotoxins have not been identified, a high-dose approach would kill nearly all of the population and eliminate the heterozygotes. Also, this strategy would target the most susceptible or earliest larval stage. Thus, this principle argues against the sublethal *Bt* approach and the use of spatial refugia where the target is not the most susceptible stage of the insect, as mentioned above for the cottonwood leaf beetle.

Bt Interaction With Plant Secondary Compounds

Plant allelochemicals play a large role in the interaction of plants and insects. These effects can be either positive or negative and may be toxic or inhibitory such as with steroidal glyco-alkaloids and protein binding of tannins. Raffa et al. (this volume) discuss the preference of cottonwood leaf beetle versus the forest tent caterpillar to different poplar hybrids based on the foliar content of phenolic glycosides. Several studies have looked at the interaction of *Bt* endotoxin with plant allelochemicals. Similar to the effects of the allelochemicals on insects, both positive and negative effects occur. For example, the phenolics resorcinol and gallic acid increase the activity of *Bt* (Sivamani et al. 1992), while tannins and nicotine decrease the effect of *Bt* (Krischik et al. 1988; Luthy et al. 1985). Effects of different plant extracts were also compared. Compared with alder extract, less mortality occurred with a *Bt*-supplemented diet when Douglas-fir extract was added. This agrees with the reports above as Douglas-fir is higher in tannins and alder is higher in phenolics (Moldenke et al. 1994).

Allelochemical interactions have management implications for poplar plantations genetically engineered with the *Bt* δ-endotoxin because these plant secondary compounds are strongly influenced by the environment. For example, poplars are typically higher in tannins when grown under stress conditions, such as lack of water or nutrients. Under these stressed conditions, a higher tannin content may actually decrease the efficacy of genetically engineered *Bt*, even when expression levels are unchanged. Although the trees are also more susceptible to insect attack under weakened conditions, these allelochemical interactions can be useful tools for managing insect biotype evolution.

Most poplar stands have traditionally relied on relatively few inputs, although management practices are changing. In stands where water and fertilizer are supplied sporadically throughout the year, applications could be timed to increase or decrease growth. Such management practices could change the allelochemical profile of tissues to coincide with and manage yearly insect cycles. The increase and decrease of tannins in slow growing versus rapidly growing tissues, respectively, is a good example of how this might work. Add to this, the expression of a *Bt* δ-endotoxin whose efficacy can be modulated by the management of tannins and other allelochemicals in the leaf. During periods of high insect populations, rapid growth could be encouraged by water and fertilizer applications to decrease tannin levels and increase the efficacy of the *Bt* δ-endotoxin. During periods when insects populations are not threatening, water and fertilizer resources could be withheld. Although this would decrease growth, a multidisciplinary management program for sustained fiber production may interpret this strategy as being in the long-term interest of the stand. Such precise manipulation of plant chemistry currently is impossible, however, with proper monitoring it might be possible in the future.

What is currently possible, is using our knowledge of plant secondary compounds to aid in understanding the spatial refugia set up by the natural plant allelochemicals. For example, if higher *Bt* δ-endotoxin expression in the younger leaves is desired, one option is to use a promoter construct to express *Bt* in only the young leaves as suggested with the *UBQ-1* promoter. Depending on the poplar hybrid used, such a pattern of expression may be inherently established with the natural chemistry of the plant. For example, in poplar hybrid cv. 'Crandon,' the level of phenolic glycosides in young leaves is relatively high compared with older leaves, and the reverse is true with condensed tannins (McCown et al. 1996). These changes

correlate with the mortality observed with gypsy moth in leaf disc assays.

Refugia

The idea of refugia where non-exposed individuals in the population can develop, is perhaps the most important element in the management of biotype evolution on a population level. Because resistant alleles are recessive, constant interbreeding with individuals not carrying the resistant alleles helps ensure that the alleles do not become dominant in the population. For this process to occur, the resistant allele must provide no other selective advantage. Therefore, maintenance of nearby plant populations that allow the development of non-exposed individuals is crucial. These non-*Bt* expressing plants could be interspersed throughout the stand by interplanting genetically engineered trees with non-genetically engineered trees, or by establishing nonproduction stands surrounding production stands.

For control of codling moth on walnuts, a variation of this strategy was proposed where apple trees genetically engineered with *Bt* are interplanted within walnut plantations (Dandekar et al. 1994). Since apple is the preferred host of the codling moth, these will be "bait trees" in the orchard and will control most of the codling moth population. A certain level of codling moth will attack the walnut, but hopefully walnut damage will be small and the *Bt*-expressing apple trees will sufficiently reduce the codling moth population. Any resistant biotypes that survive from the apples will interbreed with the individuals that survived on the walnut. Although this strategy has not been implemented, it could also work in poplar where insect-preferred hybrids are genetically engineered with the *Bt* δ-endotoxin and interspersed among the production clones. However, if target insects avoid *Bt* exposure, like the spruce budworm, this strategy might attract insects that feed on desirable production clones within stands.

As previously mentioned, the use of temporal or spatial refugia is another approach. Theories on application and utility of these refugia are the same as that for non-engineered trees. The important aspect is to provide an area where non-exposed individuals can complete their life cycle to interbreed with individuals that were subjected to the selection pressure of *Bt* toxicity.

Conclusion

Genetic engineering, whether for insect resistance, herbicide resistance, sterility, lignin modification, or increased growth, is a powerful and valuable tool for the forest manager. However, each genetically engineered trait will require modifications to the management of poplar plantations. Use of genetic engineering for insect resistance with *Bt* δ-endotoxin gene(s) in poplar is no exception. Incorporation of genetic engineering with an "Integrated Pest Management" (IPM) strategy for controlling biotype evolution is crucial for the success of this technology. An overriding management objective is to lessen selection pressures so that resistant biotypes do not dominate in the insect population. A constant influx of non-selected individuals is important to dilute resistant alleles and lessen the impact of resistant biotypes.

Several different genetically engineered *Bt* δ-endotoxins were tested in transgenic poplar, and a few were tested in the field. Improvements in the expression of *Bt* δ-endotoxins in crop plants through resynthesis of the coding region, improved expression constructs, combining of multiple toxin domains, and identification of new strains have dramatically improved the insecticidal activity in plants. Soon these advances will be incorporated into trees, including poplar, and commercial plantations will be established, much like the *Bt*-containing maize and potatoes currently available.

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Chapter 25

Genetic Engineering for Air-Pollutant Resistance in Hybrid Aspen[1]

Saori Endo, Etsuko Matsunaga, Keiko Yamada, and Hiroyasu Ebinuma

Introduction

Preservation of the natural environment, a growing worldwide concern, is being approached in various ways. Forests are useful because they continuously eliminate air pollutants while remediating and preserving the natural environment. Forest trees have a high capacity to absorb air pollutants and detoxify toxic substances produced by air pollution, but they do not adequately resist harmful air pollutants and other environmental stresses. An effective way to preserve the natural environment is to improve the resistance of forest trees to air pollutants and other environmental stresses.

Damage to plant cells caused by environmental stresses, including herbicide exposure, high intensity light with low temperature, or air pollutants such as ozone and sulfur dioxide, is related to the over production of active oxygen in the cells. Active oxygen, such as singlet oxygen ($^1O_2$), superoxide (O_2^-), and hydrogen peroxide (H_2O_2), are the by-products of many biological oxidations. For example, the electron transport chain of mitochondria and chloroplasts is a well-documented source of active oxygen. In plant cells, detoxification systems for active oxygen have evolved to facilitate immediate removal of active oxygen. However, under stressful situations, the equilibrium between the oxidative and antioxidative capacity can change within the plant cells, so that over production of active oxygen is induced. As the oxygen detoxification system of higher plants has become better understood, we have attempted to improve the detoxification system by using genetic engineering to increase resistance to environmental stress.

Detoxification System of Active Oxygen

The production and destruction of active oxygen species, such as singlet oxygen ($^1O_2$), superoxide (O_2^-), and hydrogen peroxide (H_2O_2), occur during normal plant cell metabolism and are regulated processes. In plant cells, photosynthesis is the major source of active oxygen species. Chloroplasts generate highly active oxygen species by direct donation of excitation energy, or electrons, to oxygen from the photosynthetic electron transport chain. To counteract the toxicity of active oxygen species, plants possess an efficient antioxidative defense system composed of both nonenzymatic and enzymatic constituents. The typical detoxification system for active oxygen is described in figure 1. Superoxide, which is produced via the electron transport chain, is catalyzed to H_2O_2 by superoxide dismutase (SOD; Enzyme Commission number, EC 1.15.1.1). The H_2O_2 is destroyed in the ascorbate peroxidase (APX; EC 1.11.1.11) reaction, and ascorbate is regenerated either directly by the electron transport chain or by the ascorbate-glutathione cycle.

Increased activities of these antioxidative enzymes in the detoxification system may be related to additional stress-defense capacity. Thus, several research efforts are seeking to improve the natural antioxidative defense capacity by over expression of an antioxidative enzyme in transgenic plants. We are interested in improving the antioxidative defense capacity in woody plants by producing transgenic hybrid aspen with over expression of an antioxidative enzyme, glutathione reductase (GR; EC 1.6.4.2).

[1] Klopfenstein, N.B.; Chun, Y. W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

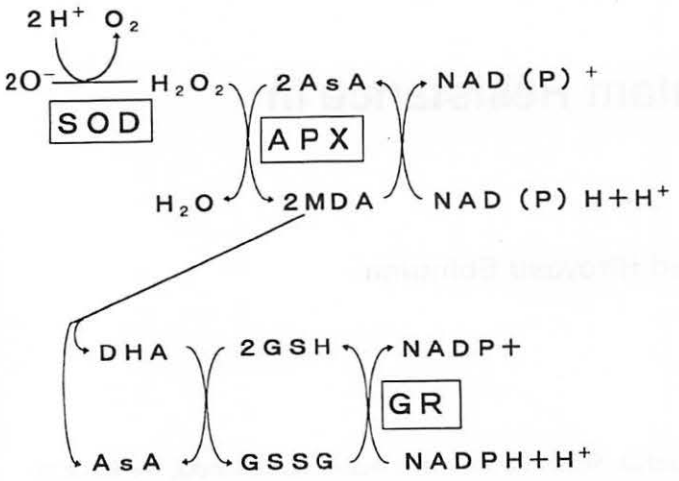


Figure 1. Detoxification system of active oxygen species in higher plants. SOD=superoxide dismutase; APX=ascorbate peroxidase; AsA=ascorbate; MDA=monodehydroascorbate; DHA=dehydroascorbate; GSH=reduced glutathione; GSSG=oxidized glutathione.

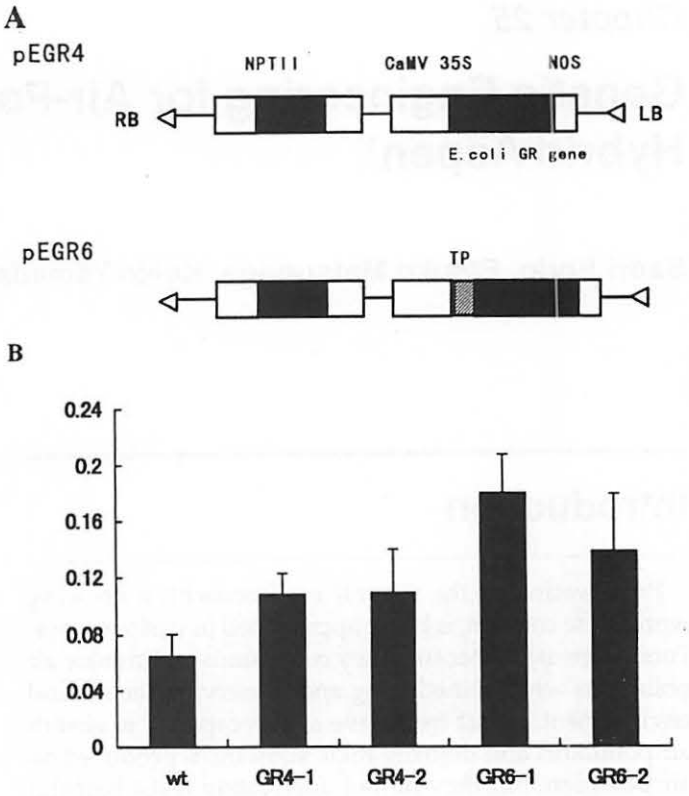


Figure 2A. Construction of the T-DNA region of pEGR4 and pEGR6. RB=right border; LB=left border; NPT II=a chimeric gene for neomycin phosphotransferase; CaMV 35S=35S promoter of cauliflower mosaic virus; NOS=nopaline synthase terminator; TP=chloroplast transit peptide.

Figure 2B. GR activity of transgenic aspen (GR4-1, GR4-2, GR6-1, and GR6-2) and nontransgenic aspen (wt). GR activity of leaf extracts was measured in a reaction mixture, which contained 0.1 M potassium phosphate (pH 7.8), 0.2 mM GSSG, and 0.2 mM NADPH in a final volume, and was monitored by the decrease in absorbance of NADPH at 340 nm.

Glutathione reductase, which exists in chloroplasts and cytoplasm of plant cells, catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) by accompanying oxidation of NADPH (Foyer and Halliwell 1976; Gillhan and Dodge 1986; Rennenberg 1980). Furthermore, GR can be either a limiting factor in the recycling capacity of an antioxidant, such as glutathione, or responsible for the actual amount of antioxidant in the cell. Transgenic tobacco plants containing a transferred *GR* gene have exhibited elevated GR activity and improved resistance to environmental stress associated with active oxygen (Aono et al. 1991, 1993).

Production of Transgenic Plants With the *E. coli GR* Gene

Two derivatives of the binary vector plasmid pBI121, which contained the *E. coli GR* gene under control of the *35S* promoter (pEGR4 and pEGR6), were constructed. In the pEGR6 construct, the DNA fragment coding for a chloroplastic transit-peptide was inserted between the *35S* promoter and the *GR* gene to transport GR protein from the cytosol to the chloroplast (figure 2A). Using our transformation method (Ebinuma et al. this volume), we introduced these genes into a hybrid aspen clone 'Y63' (*Populus sieboldii* x *P. grandidentata*). We subsequently obtained 86 transgenic plants, 28 lines transformed with GR4 and 58 lines transformed with GR6. Expression of the *E. coli GR* gene was confirmed by immunochemical analysis.

The glutathione reductase activity of transgenic plants was assayed in leaf extracts by monitoring absorbance decreases of NADPH at 340 nm. The GR activity of transgenic aspen, GR4 and GR6, ranged from 1- to 3-fold higher than that of nontransgenic plants. Seven lines of GR4 transgenic aspen and 16 lines of GR6 transgenic aspen showed significantly higher GR activity in comparison with nontransgenic aspen. The GR

activity of the transgenic plants, used in tolerance experiments, is shown in figure 2B. Elevated GR activity in the transgenic plants confirms expression of the introduced *E. coli GR* gene. These results indicate that increases in GR activity were caused by *GR* over expression.

Resistance to Environmental Stress Caused by Over Production of Active Oxygen Species

When plants are exposed to stressful conditions, such as a high intensity light, air pollutants, some herbicides, and metals, the generation of active oxygen species is increased. Increase in active oxygen production under stressful conditions results from either the inhibition of the photosynthesis pathway to decrease CO_2 assimilation, or the direct involvement in free radical production. So, plants under stressful conditions have increased susceptibility to photoinhibition with the subsequent development of chlorosis or necrosis.

Transgenic aspens with elevated GR activity (figure 2B) should exhibit higher resistance to active oxygen when compared to nontransgenic aspens. To examine the resistance of transgenic aspens to environmental stresses, we exposed transgenic aspens to paraquat, sulfur dioxide (SO_2), and high intensity light at low temperature.

Paraquat

Several herbicides may cause production of active oxygen species, either by direct involvement in radical production or by inhibition of the biosynthetic pathways. Paraquat (methyl viologen; 1,1-dimethyl-4,4-bipyridium dichloride) is a herbicide that produces active oxygen in plant cells under light. Upon accepting an electron from Photosystem I, paraquat forms a free radical. The paraquat radical is recycled to paraquat by reacting with O_2 to produce active oxygen, which then causes the disruption of cellular membranes and visible leaf damage.

To assess the resistance to paraquat, leaf discs from aspen were incubated with paraquat and illuminated. This treatment caused visible damage to leaf discs of nontransgenic plants, as exemplified by a disruption in the cellular membranes and a color change to dark brown. The extent of cellular damage was quantified by solute leakage, which is a measurement of membrane disruption. Leaf discs of transgenic and nontransgenic aspen were incubated with various concentrations of paraquat (0, 1, 10, and 100 μM) for 1 h at 25 °C in darkness. After paraquat treatment, they were washed and floated on H_2O under light (6,000 lux). Solute leakage was assessed by conductivity of the floating solution. Increased solute leakage of cellular components is indicative of cellular membrane disruption. Leaf discs of nontransgenic aspen showed a dose-dependent increase in membrane damage at 1 μM paraquat, and were nearly completely disrupted at 10 μM. Transgenic aspen leaf discs showed no damage at 1 μM paraquat (figure 3); however, at 10 μM, the extent of damage in transgenic leaf discs was not significantly different from that of nontransgenic aspen. In addition, after the 1 μM paraquat treatment, leaf discs of nontransgenic aspen showed visible damage, whereas those of transgenic aspen did not.

These results indicate that leaf tissues of the GR transgenic aspen are resistant to paraquat when compared to nontransgenic aspen plants. Using paraquat treatment, we tested 2 lines of cytosolic transgenic aspen (GR4-1 and GR4-2) and 2 lines of chloroplastic transgenic aspen (GR6-1 and GR6-2). We obtained similar results in 4 independent experiments. However, we were unable to detect any significant difference in tolerance to paraquat between cytosolic GR transgenic aspen (GR4) and chloroplastic transgenic aspen (GR6).

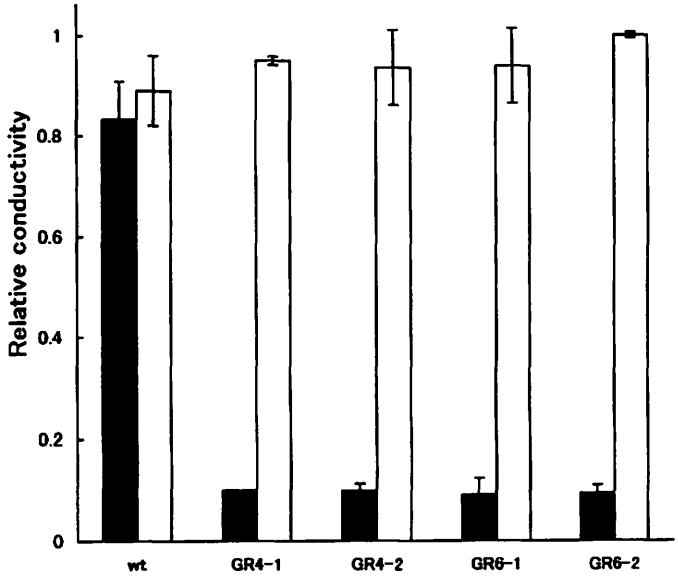


Figure 3. Electrolyte leakage from paraquat-treated leaf discs. Electrolyte leakage was assessed by conductivity of the floating solution. Conductivity was measured after illumination for 20 h, and relative values at 1 μM (filled columns) and 10 μM (unfilled columns) were determined against conductivity at 100 μM paraquat.

Sulfur Dioxide

Air pollutants, such as ozone (O_3) and sulfur dioxide (SO_2), are thought to be major factors influencing modern forest decline. Sulfur dioxide injury, which may occur in many plant species, is potentially severe under light or daytime conditions. Damage by SO_2 to biological systems is probably caused via the generation of radicals, such as O_2^-, $OH\cdot$, and SO_3^-, during SO_2 oxidation to sulfate. In the chloroplasts, SO_2 oxidation can be initiated by superoxide generated from the photosynthetic electron transport chain, this process can induce the production of active oxygen species and sulfur trioxide radicals.

We exposed transgenic and nontransgenic aspen grown in a growth chamber for 4 months to 1 ppm SO_2 at 25 °C under light (550 $\mu E \cdot m^{-2} \cdot s^{-1}$) with 70 percent relative humidity. Three samples of each nontransgenic and transgenic (GR4-1, GR4-2, GR6-1, and GR6-2) aspen line were used for the SO_2 fumigation experiment. After fumigation for 7 h, all nontransgenic plants showed symptoms of foliar damage such as necrosis and dehydration (figure 4A); however, leaves of transgenic plants with elevated GR activity (GR4 and GR6) exhibited lower damage levels (figure 4B). Similar results were obtained from independent replications. Transgenic plants with GR activity levels similar to those of nontransgenic plants also showed severe damage. The SO_2 resistance of transgenic plants is apparently dependent on the increased GR activity from over expression of the *E. coli GR* gene.

We quantified the extent of cellular damage caused by SO_2 fumigation by solute leakage, which is an indicator of membrane disruption. Leaves at the same position were removed, cut into leaf discs, and incubated in H_2O at 30 °C for 1 h. Solute leakage was then measured. After fumigation for 4 h, leaf discs of nontransgenic plants showed increased solute leakage. Nontransgenic leaves fumigated for 7 h showed complete disruption. Leaves of nontransgenic aspen had SO_2-dependent membrane damage, whereas leaves of transgenic plants had less damage. Damage exhibited by cytosolic GR transgenic plants was not significantly different from that of chloroplastic GR transgenic plants. These results indicate that over expression of the *E. coli GR* gene contributes to SO_2 resistance.

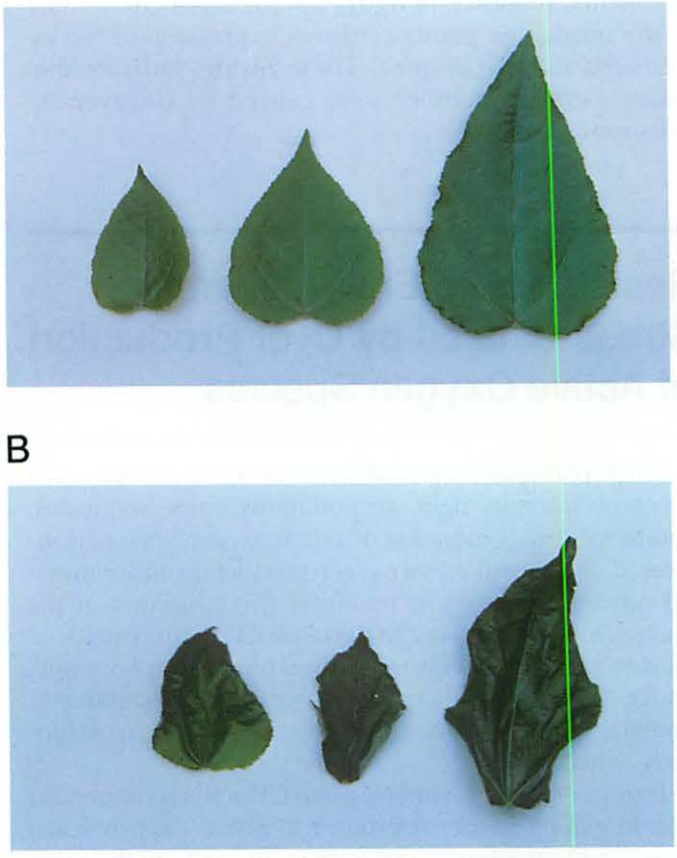


Figure 4. Effect of SO_2 fumigation. Leaves from the same position in transgenic and nontransgenic aspen after exposure to 1.0 ppm SO_2, 25 °C, light (550 $\mu E \cdot m^{-2} \cdot s^{-1}$) and 70% relative humidity for 7 h. A) Leaf of nontransgenic aspen. B) Leaf of transgenic aspen (GR6-1) with elevated GR activity.

High Intensity Light With Low Temperature

When plants are exposed to high intensity light, active oxygen species are over produced. Exposure of plants to high intensity light can exceed the capacity of photosynthesis, leading to over production of active oxygen. Injury resulting from high intensity light is potentially increased with low temperature.

To induce photoinhibition, we exposed leaves of transgenic and nontransgenic aspen to high intensity light (10,000 lux) with low temperature (5 °C). Leaves at the same position in transgenic (GR4 and GR6) and nontransgenic aspen (2 clones of every line) were exposed to the stress conditions that cause photoinhibition. After treatment for 6 h, leaves of the nontransgenic plants showed an increase in solute leakage. In contrast, leaves of transgenic aspen with over-expressed GR showed less (ca. 50 percent) damage. These results are similar to those from transgenic tobacco with over-expressed SOD. We believe that transgenic aspen with over-expressed GR are resistant to photoinhibition.

Conclusion

The results presented here demonstrate that *GR* transgenic aspens exhibit higher tolerance to active oxygen. *GR* transgenic aspens may also exhibit resistance to other environmental stresses, such as ozone and metal, that result in the production of active oxygen species. We believe that "environmental stress-resistant" trees produced by genetic engineering represent a very promising approach to help preserve the natural environment.

Acknowledgments

We thank Dr. N. Kondo, H. Saji, and M. Aono (National Institute for Environmental Studies) for their collaborative studies.

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Section V Biotechnological Applications

Chapter 26

Genetic Modification of Lignin Biosynthesis in Quaking Aspen and Poplar[1]

Wout Boerjan, Marie Baucher, Brigitte Chabbert, Michel Petit-Conil, Jean-Charles Leplé, Gilles Pilate, Daniel Cornu, Bernard Monties, Dirk Inzé, Jan Van Doorsselaere, Lise Jouanin, and Marc Van Montagu with Chung-Jui Tsai, Gopi K. Podila, C.P. Joshi, and Vincent L. Chiang

Introduction

Lignin is a complex aromatic heteropolymer and, second to cellulose, the most abundant organic polymer in the biosphere. In different tree species, lignin content is between 15 and 36 percent of the dry wood weight (Higuchi 1985). During plant development, lignin is deposited in secondary cell walls of mainly xylem cells, providing rigidity and structural support to the cell walls and enabling solute conductance in the vascular system (Higuchi 1985; Monties 1989). Moreover, lignin plays a role in by providing a barrier to prevent the spread of invading pathogens (Walter 1992).

Despite its important biological role, lignin is undesirable in paper manufacturing (Dean and Eriksson 1992; Whetten and Sederoff 1991). For instance, during kraft pulping, the most widely used process for chemical pulping, lignin is separated from the cellulose by cooking the wood chips in NaOH (sodium hydroxide) and Na_2S (sodium sulfide). To remove 1000 kg of lignin, 800 kg of NaOH and 300 kg of Na_2S are required (Chiang et al. 1988). Subsequently, the cellulose fraction containing residual lignin is bleached. Bleaching conditions are determined by how much lignin remains linked to the cellulose after the cooking step. Cooking and bleaching partially depolymerize the cellulose fibers. Wood with a high lignin content requires longer cooking and bleaching times and different bleaching sequences; hence, a lower pulp yield is obtained. Due to the high lignin content in the raw material, chemical pulping results in low biomass use and pulp yield. In addition, residual wastes are hazardous to the environment (Biermann 1993). Reduction of lignin content or changing its composition or reactivity to facilitate lignin extraction would produce economical and environmental benefits through lower pulping costs, higher pulp yields, and less pulp-mill waste.

During mechanical pulping, wood chips are ground and most of the biomass is effectively converted to pulp. Disadvantages of this procedure are yellowing of the paper after sunlight exposure, reduced paper strength, and imperfect paper surface structure (Biermann 1993). Following mechanical pulping, it would be beneficial to reduce the lignin amount and/or change the lignin composition to produce paper with an improved quality.

Lignin is not always undesirable. The lignin content in wood is an important factor determining its intrinsic heat content for use as fuel (Whetten and Sederoff 1991) because the carbon content of lignin is 50 percent higher than that of polysaccharides (Brown 1985). This feature is particularly relevant to poplars and willows because they are cultivated as nonfossil, energy crops (Pearce 1995). Poplars and willows are excellent energy crops because they are fast-growing, easily coppiced, and grow well on diverse soils. Burning 1000 kg of dried coppice can generate as much electricity as 650 kg of coal, but avoids the release of up to 500 kg of carbon into the atmosphere (Pearce 1995). Therefore, increasing the intrinsic heat content of trees by increasing their lignin content might be a useful goal for tree genetic engineering.

Furthermore, the amount and structure of lignin may determine some mechanical properties of wood. Mechanical defects of wood, such as deformation after sawing or drying, were correlated with reaction wood that is fre-

[1] Klopfenstein, N.B.; Chun, Y. W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

quently observed in some poplar clones. These defects are associated with variations in cellulose patterns and lignin deposition in the cell walls (Bailleres et al. 1995).

Lignin is derived from the oxidative polymerization of 3 different hydroxycinnamyl alcohols or monolignols. These monolignols, *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (figure 1), differ from each other only by their degree of methoxylation and generate the *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units of the lignin polymer, respectively. Angiosperm (e.g., poplar) lignin consists of coniferyl and sinapyl alcohol and is classified as G-S lignin. Gymnosperm lignin contains mainly coniferyl alcohol and is classified as G lignin; whereas, lignin of grasses also has significant amounts of H-type lignin (Higuchi 1985).

Lignin biosynthesis was recently reviewed in considerable detail by Boudet et al. (1995) and Whetten and Sederoff (1995). In this chapter, we present a brief description of the lignin biosynthesis pathway and an update on the complementary DNAs (cDNAs) and genes isolated from poplar that code for enzymes involved in lignin biosynthesis. Furthermore, we will focus on several examples in which lignin structure modification was obtained by genetic engineering.

Lignin Biosynthesis

Biosynthesis of the monolignols in angiosperms (figure 1) starts with the deamination of phenylalanine, derived from the shikimate pathway, to cinnamic acid, and the hydroxylation of cinnamic acid to *p*-coumaric acid. These enzymatic conversions are catalyzed by phenylalanine ammonia-lyase (PAL) and cinnamate-4-hydroxylase (C4H), respectively. As listed in table 1, cDNAs for *PAL* were isolated from *P. x interamericana* (*Populus trichocarpa* x *P. deltoides*) (Subramaniam et al. 1993) and *P. kitakamiensis* (*P. sieboldii* x *P. grandidentata*) (Osakabe et al. 1995a). Genomic sequences for *PAL* (Osakabe et al. 1995a, 1995b) and *C4H* (Kawai et al. 1994) were isolated from *P. kitakamiensis*. The acid *p*-coumaric is hydroxylated at its C-3 position by 4-coumarate-3-hydroxylase (C3H) to produce caffeic acid. No cDNA or genomic sequences encoding C3H have been isolated from poplar or from any other plant species. This compound is methylated at the C-3 hydroxyl group to form ferulic acid by bispecific caffeic acid/5-hydroxyferulic acid-*O*-methyltransferase (COMT). Ferulic acid is then hydroxylated at its C-5 position by ferulate-5-hydroxylase (F5H) to form 5-hydroxyferulic acid, then the C-5-hydroxyl group is methylated by the same bispecific COMT to create sinapic acid. cDNAs for *COMT* were cloned from *P. tremuloides* (Bugos et al. 1991) and *P. trichocarpa* x *P. deltoides*

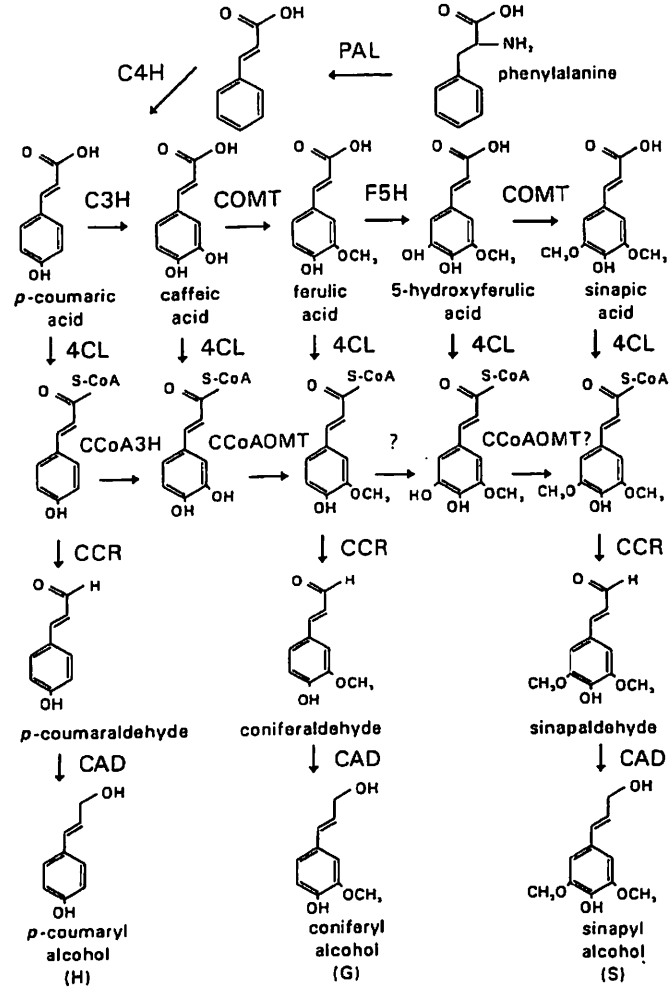


Figure 1. Lignin biosynthesis pathway. PAL=phenylalanine ammonia-lyase; C4H=cinnamate-4-hydroxylase; C3H=4-coumarate-3-hydroxylase; COMT=bi-specific caffeic acid/5-hydroxyferulic acid-*O*-methyltransferase; F5H=ferulate-5-hydroxylase; CCoA3H=coumaroyl-CoA-3-hydroxylase; CCoAOMT=caffeoyl-CoA-*O*-methyltransferase; 4CL=4-hydroxycinnamate-CoA ligase; CCR=cinnamoyl-CoA reductase; CAD=cinnamyl alcohol dehydrogenase.

(Dumas et al. 1992). Genes encoding COMT were isolated from *P. tremuloides* (Tsai et al. 1995) and *P. kitakamiensis* (Hayakawa et al. 1996). In addition, a cDNA for *F5H* was recently isolated from *Arabidopsis thaliana* (C. Chapple, personal communication).

Subsequently, *p*-coumaric acid, ferulic acid, and sinapic acid are converted to the corresponding coenzyme A (CoA) esters by 4-hydroxycinnamate-CoA ligase (4CL). cDNAs encoding 4CL were isolated from *P. trichocarpa* x *P. deltoides*

Table 1. cDNAs and genes from *Populus* spp. involved in lignin biosynthesis. Numbers refer to the different cDNAs or genes that have been cloned.

Gene	cDNA/gene number	Species	Reference
PAL	cDNA 2	*P. trichocarpa* x *P. deltoides*	Subramaniam et al. (1993)
	cDNA 1[1]	*P. kitakamiensis* (*P. sieboldii* x *P. grandidentata*)	Osakabe et al. (1995a)
	gene 2	*P. trichocarpa* x *P. deltoides*	Subramaniam et al. (1993)
	gene 5[2]	*P. kitakamiensis*	Kawai et al. (1994) Osakabe et al. (1995a, 1995b)
C4H	gene 1[3]	*P. kitakamiensis*	Kawai et al. (1994)
COMT	cDNA 1	*P. tremuloides*	Bugos et al. (1991)
	cDNA 1	*P. trichocarpa* x *P. deltoides*	Dumas et al. (1992)
	gene 1	*P. tremuloides*	Tsai et al. (1995)
	gene 2	*P. kitakamiensis*	Hayakawa et al. (1996)
4CL	cDNA 2	*P. trichocarpa* x *P. deltoides*	Allina and Douglas (1994)
CCR	cDNA 1	*P. trichocarpa*	Leplé et al. (1995)
	gene 1	*P. trichocarpa*	J. Van Doorsselaere (unpublished)
CAD	cDNA 1	*P. trichocarpa* x *P. deltoides*	Van Doorsselaere et al. (1995a)
	gene 1	*P. trichocarpa*	J. Van Doorsselaere (unpublished)
Peroxidase	cDNA 3	*P. kitakamiensis*	Kawai et al. (1994) Osakabe et al. (1994)
	cDNA 4	*P. trichocarpa*	Christensen et al. (1995)
	gene 4	*P. kitakamiensis*	Kawai et al. (1993) Kawai et al. (1994) Osakabe et al. (1995c)
	gene 4	*P. trichocarpa*	J. Christensen (unpublished)
CCoAOMT	cDNA 1	*P. tremuloides*	Meng and Campbell (1995)
	cDNA 4	*P. trichocarpa*	Meyermans et al. (1995)
	gene 2	*P. trichocarpa*	Chen et al. (1995)

[1] 1 partial *PAL* cDNA was isolated.
[2] 2 independent genes or gene fragments were isolated.
[3] 4 independent clones were isolated, 1 was sequenced.

by Allina and Douglas (1994). These CoA esters are converted through 2 successive reductions to the corresponding alcohols by cinnamoyl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD). Recently, at the University of Gent (Gent, Belgium), a cDNA and a genomic clone encoding CCR were isolated from *P. trichocarpa* (Leplé et al. 1995; J. Van Doorsselaere, unpublished results). Also, cloning of a cDNA for *CAD* from *P. trichocarpa* x *P. deltoides* was reported recently (Van Doorsselaere et al. 1995a), and a corresponding *CAD* genomic clone was isolated from *P. trichocarpa* (J. Van Doorsselaere, unpublished results). Resulting monolignols are subsequently dehydrogenated with the formation of mesomeric free phenoxy radicals that polymerize and produce the complex lignin heteropolymer. Several cDNAs and a gene encoding peroxidases with potential roles in lignification were isolated from *P. kitakamiensis* (Kawai et al. 1993, 1994; Osakabe et al. 1994, 1995c) and *P. trichocarpa* (Christensen et al. 1995). Potential roles of peroxidases, laccases, and coniferyl alcohol oxidases in lignification were reviewed and discussed by Boudet et al. (1995).

Recently, more attention has focused on alternative pathways involved in the biosynthesis of the monolignols. One such pathway involves the hydroxylation of *p*-coumaroyl-CoA to form caffeoyl-CoA by *p*-coumaroyl-CoA-3-hydroxylase (CCoA3H), and the subsequent methylation of caffeoyl-CoA to produce feruloyl-CoA by caffeoyl-CoA-*O*-methyltransferase (CCoAOMT). It was strongly suggested that in *Zinnia*, CCoAOMT is as important in lignification as is COMT (Ye and Varner 1995; Ye et al. 1994). Recently, *CCoAOMT* cDNAs were isolated from *P. tremuloides* (Meng and Campbell 1995) and *P. trichocarpa* (Meyermans et al. 1995), and genomic clones were isolated from *P. trichocarpa* (Chen et al. 1995).

Matsui et al. (1994) and Daubresse et al. (1995) focused attention on a previously unknown pathway in lignin bio-

synthesis. These groups performed feeding experiments on several angiosperm and gymnosperm plants with radioactively labeled *p*-glucocoumaryl alcohol, coniferin, and syringin, which are the β-glucoside storage forms of *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, respectively. They found evidence for an interconversion of these compounds at the monolignol, glucoside, or lignin level. Moreover, this pathway exists in gymnosperms and angiosperms, in which the conversion from H to G and H to G to S was observed, respectively (figure 1). Since the level at which the hydroxylation and methylation reactions occur is not fully established, these reactions are not shown in figure 1.

Strategies to Modify Lignin Biosynthesis

Two broad categories of linkages between building units in the lignin polymer are the ether linkages and the carbon-carbon linkages (figure 2). Ether linkages, such as the β-O-4 linkage, are easily broken during the delignification process. In contrast, C-C linkages, such as the 5-5 linkage, are very resistant to chemical attack (Boudet et al. 1995; Chiang and Funaoka 1990a, 1990b; Chiang et al. 1988). Gymnosperm lignin is composed mainly of G units that are methoxylated only once; therefore, the C-5 aromatic position is available for a C-C linkage during the polymerization process (e.g., with a C-5 or C-β from another coniferyl alcohol). Due to S units that have no free C-5 positions, angiosperm lignin has less 5-5 and β-5 linkages than gymnosperm lignin and is, therefore, less condensed than gymnosperm lignin. This could be one reason why it is easier to delignify angiosperm wood (hardwood) by kraft pulping than gymnosperm wood (softwood). However, the methoxy groups that are more abundant in syringyl lignin are converted to air-polluting mercaptanes by the reaction with sulfides during chemical pulping (Biermann 1993). A genetically engineered change in the S:G unit ratio of the lignin polymer could potentially modify extraction properties of lignin for chemical pulping.

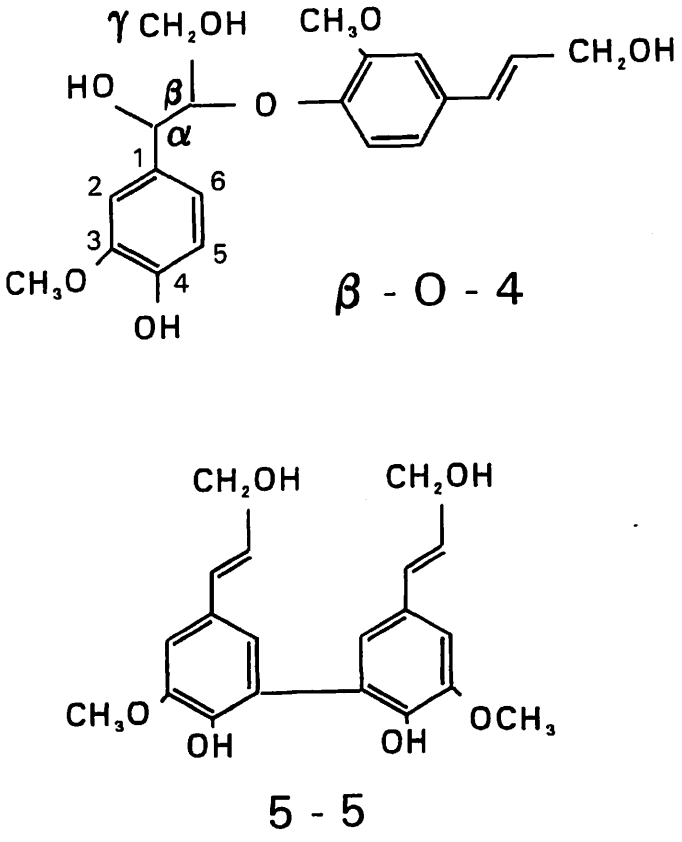


Figure 2. Schematic representation of the β-O-4 ether linkage and 5-5 carbon-carbon linkage. The structures are represented as dimers. For an overview of the different linkages refer to Adler (1977).

Two genetic approaches have been proposed to modify the degree of methoxylation in lignin. In gymnosperm wood, a genetically induced methoxylation at the C-5 position could render the lignin less condensed. In angiosperm wood, substitution of -OCH3 groups by -OH groups could result in the incorporation of 5-hydroxyguaiacyl (5-OH-G) units, thereby increasing lignin solubilization in the pulping liquor (Boudet et al. 1995). Because COMT catalyzes methylation reactions in lignin biosynthesis, manipulation of the COMT activity by genetic engineering would likely result in a modified degree of methylation. Increased COMT activity might result in an elevated S content. Manipulation of COMT has also been proposed to reduce lignin content since brown-midrib mutations in maize (*bm*) and sorghum (*bmr*) were associated with a reduction of COMT activity and of COMT and CAD activity, respectively (Grand et al. 1985a; Pillonel et al. 1991). In both cases, the mutation conferred lower lignin content. Moreover, the maize *bm3* mutant was shown to have a defective *COMT* gene (Vignols et al. 1995). Both mutants have an altered lignin composition with the incorporation of the 5-OH-G unit into the lignin polymer (Chabbert et al. 1993; Lapierre et al. 1988). These mutants are characterized by a reddish-brown coloration of the midrib.

Down-regulation of the *COMT* gene expression was obtained in transgenic tobacco (Atanassova et al. 1995; Dwivedi et al. 1994; Ni et al. 1994) and transgenic poplar (Van Doorsselaere et al. 1995b); the most marked results were a drastic reduction of the S:G ratio and an incorporation of the 5-OH-G residue in the lignin polymer. For more details, see below.

CAD was also targeted to manipulate the lignification process because a reduced CAD activity might limit monolignols available for polymerization. Evidence for this

hypothesis was derived from chemical-inhibitor studies and mutant analysis. First, inhibition of CAD activity in poplar stems by 2 CAD activity inhibitors resulted in a 45 percent reduced incorporation of ^{14}C-labeled cinnamic acid into lignin (Grand et al. 1985b). Second, the sorghum *bmr6* mutant with reduced CAD and COMT activities, exhibits lower lignin content with an increased incorporation of cinnamaldehydes (Pillonel et al. 1991). In addition, the maize *bm1* mutant has a lower lignin content and strongly reduced CAD activity (Holt et al. 1995).

Modifying *CAD* gene expression using an antisense strategy was described for tobacco (Halpin et al. 1994; Hibino et al. 1995; Higuchi et al. 1994). Lignin content and composition were not significantly modified, but a different lignin containing cinnamaldehydes was obtained. Moreover, this change in lignin structure impacted lignin extractability.

Other enzymes from the lignin biosynthesis pathway were up- or down-regulated. Transgenic tobacco plants with decreased PAL activity (20 percent residual activity) had a reduced lignin content and severely affected development (Elkind et al. 1990). An anionic peroxidase, reportedly involved in lignification, was overproduced and down-regulated in tobacco (Lagrimini 1991). However, no significant changes in lignin content or composition were observed in plants with a down-regulated peroxidase level (Chabbert et al. 1992). In addition, plants with an overproduced peroxidase synthesized more lignin.

In this chapter, genetic engineering of quaking aspen with antisense *COMT*, and poplar with antisense *COMT* and antisense *CAD* constructs are discussed along with the effects of down-regulation of these enzymes on the lignin content, composition, and structure.

Manipulation of Lignin Biosynthesis in Transgenic Aspen and Poplar via an Antisense *COMT* Strategy

Manipulation of *COMT* Gene Expression in Transgenic Aspen

In a study at Michigan Technological University (Houghton, MI, USA) transgenic tobacco (*Nicotiana tabacum*) plants expressing an antisense aspen *COMT* gene (Ptomt1) driven by the cauliflower mosaic virus (CaMV) *35S* promoter, showed a pronounced inhibition of COMT activity in the developing xylem tissue. These plants exhibited a marked reduction (up to 20 percent) in S lignin units with no significant reduction in V lignin building units (Dwivedi et al. 1994). The S (amount of syringaldehyde):V (amount of vanillin and vanillic acid) ratio is a measure for the monomeric composition of lignin as determined by nitrobenzene oxidation (Adler 1977; Monties 1989). Down-regulation of *COMT* gene expression in these transgenic plants also reduced lignin content from 5 to 12 percent when compared to the wild type. The antisense approach was subsequently applied to quaking aspen (*P. tremuloides*) to investigate the effects of antisense down-regulation of the *COMT* gene on lignin biosynthesis in a woody angiosperm species. For this purpose, efficient *Agrobacterium tumefaciens*-mediated gene transformation and a whole-plant regeneration system was developed using greenhouse plants as explant material (Tsai et al. 1994).

A 5' 470-bp (base pair) fragment from the aspen *COMT* cDNA (Bugos et al. 1991) was cloned in antisense orientation behind the CaMV *35S* promoter (figure 3), and this gene construct was introduced into aspen according to Tsai et al. (1994). More than 100 transgenic aspen plants were obtained, most of which were phenotypically indistinguishable from the wild-type aspen. These plants were analyzed by polymerase chain reaction (PCR) to investigate whether the T-DNA was present. For all transformants, positive amplification of the neomycin phosphotransferase II (*NPTII*) gene was observed. From these plants, 31 were randomly selected for further analysis. Using the *NPTII* gene, *35S* promoter, or *COMT* gene as a probe, DNA gel blot analysis demonstrated T-DNA integration in most of the plants, and further showed that the 31 plants were derived from 17 independently transformed lines.

Expression of the antisense *COMT* gene in the transgenic plants was demonstrated by RNA gel blot analysis. Individual plants derived from 13 independent transformed lines were selected for the analysis. A sense *COMT* RNA probe was synthesized to detect only antisense transcript derived from the introduced antisense *COMT* gene. Seven lines (numbers 7, 15, 17, 18 19, 23, and 30) showed a hybridization signal of 0.7 kb, the size expected for the antisense *COMT* mRNA. No steady-state antisense *COMT* mRNA was detected for the other transgenic lines, perhaps indicating an effective interaction with the native sense transcript. Based on the observations of several other studies, such plants might display the strongest antisense effect (Atanassova et al. 1995; Van Doorsselaere et al. 1995b).

COMT enzyme assays were conducted on xylem tissue from individual plants derived from the 13 independent transformants. Both caffeic acid and 5-hydroxyferulic acid were used as substrates. In most of the transgenic plants assayed, COMT activity toward caffeic acid and 5-hydroxyferulic acid was reduced by 5 to 50 percent and 10 to 60 percent, respectively.

However, lignin content of these transgenic plants, as determined by the Klason method (Effland 1977) and the

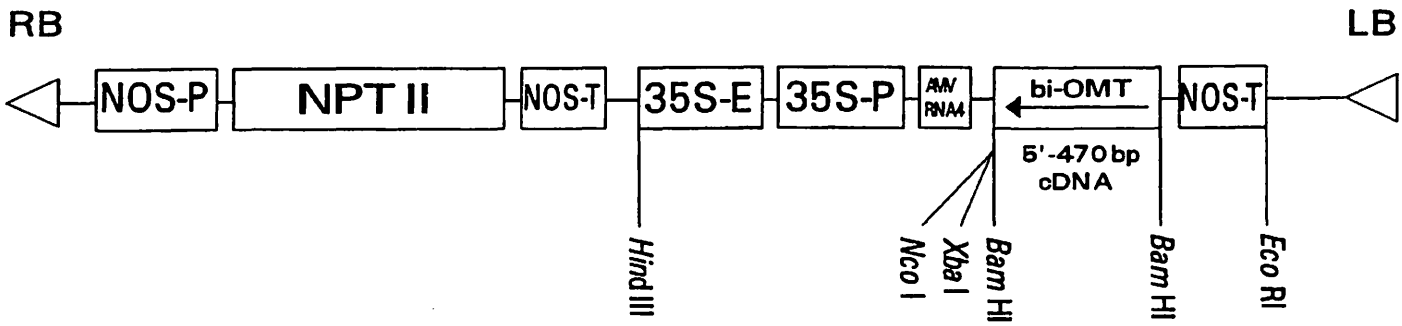


Figure 3. Diagram of the aspen antisense *COMT* gene construct. RB and LB=right and left border of the T-DNA, respectively. *NOS*-P and *NOS*-T=promoter and terminator of the nopaline synthase gene, respectively; *NPTII*=coding sequence of the neomycin phosphotransferase II gene; *35S*-E and *35S*-P=enhancer and promoter of the cauliflower *35S* gene, respectively; AMV-RNA4=alfalfa mosaic virus RNA 4; *bi-OMT*=bi-specific caffeic acid/5-hydroxyferulic acid-*O*-methyltransferase.

acetyl bromide method (Iiyama and Wallis, 1988), did not differ markedly from wild-type lignin content. It is possible that expression of the antisense *COMT* gene may affect the lignin structure with respect to methylation. The antisense *COMT* plants are currently being analyzed for lignin composition.

Manipulation of *COMT* Gene Expression in Transgenic Poplar (*P. tremula* x *P. alba*)

A poplar *COMT* cDNA plasmid (pPCL4) (Dumas et al. 1992) was used to create several constructs under the control of the CaMV *35S* promoter, which drives constitutive expression in poplar (Leplé et al. 1992). Different constructs with 5' and 3' *COMT* fragments in sense and antisense orientation were made. Results of poplar transformation with the pASB plasmid, containing a 0.9-kb 3' fragment of the *COMT* cDNA cloned in antisense orientation, are previously reported (Van Doorsselaere et al. 1995b).

The plasmid pASB and the control plasmid pBI121 (*35S-GUS* construct; Jefferson et al. 1987) were introduced into hybrid poplar (*P. tremula* x *P. alba*) by *Agrobacterium tumefaciens*-mediated transformation (Leplé et al. 1992). Fifteen independent transformants were generated for each construct. All antisense and pBI121 transformants were analyzed for stable integration of the T-DNA by DNA gel blot analysis. The T-DNA copy numbers for the transformants were between 1 and 4. No morphological abnormalities were observed in the different 3-month-old, greenhouse-grown, transgenic lines. However, 2 ASB transformants (ASB2B and ASB10B) appeared to have a different wood color when the phloem was peeled off. The xylem of these trees was pale rose, whereas the xylem of wild-type poplars was white/yellow. As previously mentioned, a red-brown coloration of the xylem occurred in brown-midrib mutants from maize and sorghum with an altered lignin content.

RNA gel blot analysis of xylem tissue was performed to investigate whether expression of the antisense *COMT* transcript reduced the sense steady-state *COMT* mRNA level. In 4 ASB transgenic poplars (ASB4A, ASB6A, ASB2B, and ASB10B), steady-state *COMT* transcript was significantly reduced with the lowest levels occurring in ASB2B and ASB10B. No antisense transcript was detected in these 4 ASB lines.

All ASB transformants and controls were analyzed for their COMT activity toward caffeic acid in xylem tissue. Plants ASB4A and ASB6A had a 50 percent reduction in COMT activity compared with the control (figure 4). In xylem of ASB2B and ASB10B plants, COMT activity was reduced by 95 percent (5 percent residual activity). On protein gel blots of ASB2B and ASB10B, where xylem proteins bound tobacco anti-COMT-II antibodies (Hermann et al. 1987), COMT protein level was very low when compared to the control, thereby confirming the enzyme assay data.

Xylem tissue was harvested from controls and ASB plants and subjected to thioacidolysis followed by gas chromatography (Lapierre et al. 1986) to investigate the effect of a decreased COMT activity on the monomeric composition of lignin. This technique allows analysis of the noncondensed lignin fraction (i.e., determination of those units linked by arylglycerol aryl ether linkages) (figure 2). Due to decreased S units and increased G units in xylem from plants ASB2B and ASB10B, the S:G ratio was significantly reduced (3- to 4-fold) when compared to the control plants (table 2). In plants ASB4A and ASB6A, the S:G ratio was also reduced but only by 1.3- to 2-fold (data not shown). Transgenic poplars with no reduction in COMT activity exhibited an S:G ratio comparable to that of con-

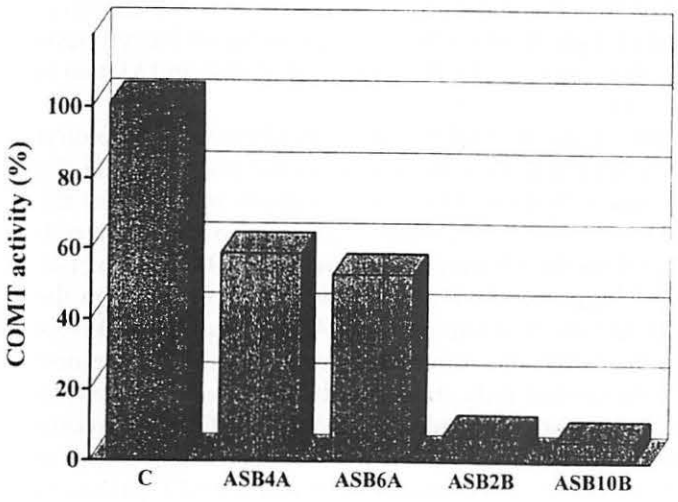


Figure 4. COMT activities, in percent compared to the control value, towards caffeic acid in stem xylem from transformants ASB4A, ASB6A, ASB2B, ASB10B, and control (C).

trol plants (data not shown). Considerable variation existed in the amount and composition of the lignin among different sampling dates, perhaps indicating differential expression of the lignin biosynthesis genes throughout development (Van Doorsselaere et al. 1995b). In plants ASB2B and ASB10B, the partially methoxylated 5-OH-G unit was detected among the thioacidolysis products. No significant differences between control plants and the different transgenic lines were detected when lignin amount was determined by the Klason method and the acetyl bromide method (table 2).

Discussion

Two independent studies to modify lignin content and composition in aspen and poplar were conducted to investigate the *in vivo* role of bispecific caffeic acid/5-hydroxyferulic acid *O*-methyltransferase (COMT) in lignin biosynthesis. The cDNA sequences of *COMT* genes isolated from *P. tremuloides* and *P. trichocarpa* x *P. deltoides* show 99 percent identity at the amino acid level (Dumas et al. 1992). Transgenic aspen and poplar plants were obtained that constitutively express *COMT* gene fragments in an antisense orientation. This provides a unique opportunity to compare and examine effects of similar gene constructs in 2 distinct species of *Populus*.

At Michigan Technological University, more than 100 transgenic aspen trees were produced that constitutively expressed the 5' 470-bp of the aspen *COMT* gene in antisense orientation. A subset of these transgenic plants was analyzed for the presence and expression of the *COMT* gene fragment in antisense orientation. Several lines had a reduced COMT activity (up to 50 percent) using both caffeic acid and 5-hydroxyferulic acid as substrate. This reduced COMT activity did not affect the morphology, growth, or overall lignin content of these plants. Currently, these transgenic aspen trees are being analyzed for their lignin composition.

At the University of Gent (Gent, Belgium), in collaboration with French laboratories of the Institut National de la Recherche Agronomique (INRA) and as part of a European project (OPLIGE), transgenic poplar trees expressing a 3' 900-bp part of the *COMT* gene in antisense orientation were obtained. Four transgenic lines showed a reduced transcript level of endogenous *COMT* and reduced COMT activity. The most distinct results were that an inhibition of COMT activity did not reduce lignin quan-

Table 2. Klason lignin content and lignin composition of xylem from control and antisense *COMT* plants. Data are expressed as mole g^{-1} of cell wall residue (CWR), which is that left after successive extractions of the xylem with toluene ethanol, ethanol, and water. Lignin composition was analyzed by thioacidolysis. The data are the mean of two independent measurements of 1 or 2 plants. Standard deviations for KL, G, S, and 5-OH-G were 3% and 6%, at the most, respectively.

	KL (Klason lignin)	G (guaiacyl unit)	S (syringyl unit)	5-OH-G (5-hydroxyguaiacyl unit)	S:G
June 1994					
PBI121	19.0	133	189	[1]	1.42
ASB2B	16.8	150	34	16	0.23
ASB10B	17.1	160	51	13	0.31
October 1994					
PBI121	19.6	132	207	[1]	1.57
ASB2B	19.3	197	50	16	0.25
ASB10B	19.2	179	84	6	0.47

[1] = not detected
PBI121 = control
ASB2B and ASB10B = plants with 95% reduced COMT activity

tity. However, the overall S:G composition was dramatically reduced, and incorporation of the 5-OH-G unit was reduced in the lignin of ASB2B and ASB10B. In wild-type plants, 5-hydroxyferulic acid is converted into sinapic acid, and the corresponding 5-OH-G monomeric unit cannot be detected via thioacidolysis. Our data show that this unusual monomeric unit can be incorporated into the arylglycerol aryl ether lignin structures of the transformed poplars. In addition, 5-hydroxyferulic acid is not incorporated as such but rather as 5-hydroxyconiferyl alcohol. These results suggest that 5-hydroxyferulic acid is converted into the corresponding alcohol in the same manner as *p*-coumaric acid, ferulic acid, and sinapic acid. Remarkably, the G content in plants ASB2B and ASB10B was higher than the controls.

A similar relative variation in content of noncondensed S and G monomeric units was previously observed with brown-midrib *bm3* mutants of maize (Barrière et al. 1994). Indeed, the maize *bm3* mutant has reduced COMT activity and synthesized modified lignin with a 4- to 5-fold reduced S:G ratio (due to an increase in G and a decrease in S units) when compared to the wild-type (Lapierre et al. 1988). Moreover, an accumulation of the 5-OH-G unit is observed in this mutant.

Recently, Dwivedi et al. (1994), Ni et al. (1994), and Atanassova et al. (1995) reported on down-regulation of COMT from tobacco by expressing aspen, alfalfa, and tobacco *COMT* gene fragments, respectively. Dwivedi et al. (1994) observed a 45 percent reduction in COMT activity that resulted in a slightly lower content of S units. The S:V ratio was reduced by 20 percent. In contrast, Ni et al. (1994) showed that the expression of an alfalfa *COMT* antisense RNA in tobacco reduced lignin content without modifying lignin composition (S:V). No significant amounts of the 5-OH-G unit were found by these authors. This can be explained either by the absence of such monomers in lignin, or more likely, by the oxidative degradation of the corresponding product during nitrobenzene oxidation (Rolando et al. 1992). Partially methoxylated monomeric units containing ortho-diphenolic functionality, such as the 5-OH-G monomers, are lost after nitrobenzene oxidation under standard conditions (Lapierre et al. 1988). Atanassova et al. (1995) showed that antisense *COMT* tobacco plants with a strongly reduced COMT activity, are significantly affected in the proportion of syringyl units in their lignin and accumulate 5-OH-G units, whereas the total lignin content was not modified. These results support those obtained in poplar.

Clearly, reduced S content in the transgenic poplars was due to the down-regulation of COMT. However, lignin quantity was not modified and G unit content was increased, suggesting that G units in the noncondensed lignin fraction are synthesized by an alternative route (e.g., via CCoAOMT). We hypothesize that the CCoAOMT enzyme plays an important role for lignification in poplar, as in *Zinnia* (Ye and Varner 1995; Ye et al. 1994). Involvement of more than 1 OMT activity in lignin biosynthesis was also suggested by Dwivedi et al. (1994) and Matsui et al. (1994).

Down-regulated COMT poplars showed a discolored xylem that may be comparable to the phenotype of the *bm3* maize mutant. The *bmr6* sorghum mutant and the CAD down-regulated tobacco plants also showed a reddish xylem discoloration (Halpin et al. 1994; Hibino et al. 1995; Higuchi et al. 1994; Pillonel et al. 1991). When the CAD activity in xylem tissue of ASB2B and ASB10B trees was measured, no differences were observed compared with the control indicating that the red coloration was not correlated with a reduced CAD activity. Further investigations are needed to determine whether the red coloration in down-regulated CAD and COMT plants is attributable to similar biochemical alterations.

Kraft cooking (pulping) experiments on wood of 1-year-old ASB2B and ASB10B trees were recently performed and showed, consistent with the lower level of S units, a higher kappa number after the cooking step. This observation indicates that the lignin is more difficult to remove from poplars with down-regulated COMT activity, and that the expected positive effect of the 5-OH-G unit on lignin solubilization does not counterbalance the negative effect of the lower S:G ratio on lignin extraction.

Manipulation of Lignin Biosynthesis in Transgenic Poplar (*P. tremula* X *P. alba*) via an Antisense *CAD* Strategy

Manipulation of *CAD* Gene Expression in Transgenic Poplar (*P. tremula* x *P. alba*)

As previously mentioned, cinnamyl alcohol dehydrogenase (CAD) is considered an important target enzyme for reducing lignin content by genetic engineering because it catalyzes the conversion of cinnamaldehydes to cinnamyl alcohols, the last step in monolignol biosynthesis. Therefore, a study was initiated at the University of Gent, in collaboration with the INRA, to modify *CAD* gene expression in poplar and investigate its affect on lignin biosynthesis.

A cDNA for *CAD* from *P. trichocarpa* x *P. deltoides* was isolated by heterologous screening with a tobacco *CAD* cDNA (Van Doorsselaere et al. 1995a). The full-length cDNA was expressed under the control of the CaMV *35S* promoter in sense and antisense orientation. These constructs were introduced into *P. tremula* x *P. alba* using an

Agrobacterium-mediated transformation technique (Leplé et al. 1992). For both constructs, 15 independent transformants were isolated for which T-DNA copy numbers were between 1 and 5.

To investigate transgene effectiveness in overproducing or down-regulating CAD, CAD activity measurements and RNA gel blot analyses were performed. For the poplars transformed with the antisense construct, the CAD activity in xylem was reduced by a maximum of 70 percent (30 percent residual activity). This response was observed for lines ASCAD14, ASCAD21, and ASCAD52. In these lines, a very low steady-state sense *CAD* transcript level was observed by RNA gel blot analysis. For the poplars transformed with the sense construct, the maximum overproduction was about 30 percent more than the control. Two lines, CAD1 and CAD4, showed a 70 percent reduction in CAD activity. This reduced activity was due to a co-suppression phenomenon because only very low levels of steady-state sense *CAD* mRNA were detected by RNA gel blot analysis. All these transformed plants displayed normal growth and development.

Interestingly, transformants with a minimum 50 percent reduction in CAD activity displayed a red coloration of the xylem tissue. In 2-year-old transgenic poplars, red coloration was observed in the outer, young xylem tissue not in the internal, older xylem. A reddish-brown coloration was observed for brown-midrib mutants with a reduced lignin content, and for genetically engineered tobacco plants with reduced CAD activity (Halpin et al. 1994; Hibino et al. 1995). When the red poplars and controls were stained with phloroglucinol (indicative for aldehyde end groups), a different red-brown coloration was seen in the down-regulated CAD plants when compared to the controls, which stained pink. This result suggests an altered level of aromatic aldehyde groups in the lignin of CAD down-regulated plants.

No lignin reduction (Klason method) was observed in the xylem from poplars with reduced CAD activity (table 3). Despite the obvious phenotypic difference, no modification in the lignin composition (S:G ratio) was detected. As for COMT, a considerable and significant variation in the lignin content and composition was observed among different sampling dates. We speculate that this variation is due to differences in the activities of lignin biosynthesis enzymes over the different growing seasons and years. To determine the origin of the red color, dioxane-soluble lignin fractions were isolated from red and white poplar xylem, then analyzed by spectrophotometry (250-500 nm). Dioxane was used to extract lignin without ionizing phenolic groups. The absorption spectrum of the dioxane lignin from red poplars showed a higher absorbance in the range from 300 to 340 nm when compared to the spectrum of dioxane lignin from white poplar. This absorbance was decreased by sodium borohydride ($NaBH_4$) reduction. Such UV spectral behavior is indicative of increased amounts of conjugated aromatic aldehydes in the lignin polymer (Tollier et al. 1995).

Clear differences between red and white plants were obtained after alkali treatment of the xylem cell wall residue (CWR). CWR is the residue corresponding to insoluble

Table 3. Klason lignin content and lignin composition of xylem from control and antisense *CAD* poplars. Data are expressed as mole g^{-1} of cell wall residue (CWR), which is that left after successive extractions of the xylem with toluene ethanol, ethanol, and water. Lignin composition was analyzed by thioacidolysis. The data are the mean of two independent measurements of 1 to 2 plants. Standard deviations for KL, G, S, and S+G were 3% and 6%, at the most, respectively.

	KL (Klason lignin)	G (guaiacyl unit)	S (syringyl unit)	S:G	S+G
June 1993					
PBI121	18.7	133	270	2.03	403
ASCAD3	18.7	113	259	2.29	372
ASCAD14	16.6	133	286	2.15	419
ASCAD21	16.4	136	277	2.05	413
ASCAD52	17.5	107	217	2.03	324
June 1994					
PBI121	18.4	153	261	1.65	413
ASCAD3	19.4	133	200	1.50	333
ASCAD14	19.5	136	233	1.70	369
ASCAD21	18.5	131	173	1.32	304
ASCAD52	20.3	126	196	1.55	323

PBI121 = control
ASCAD3 = wild-type CAD activity
ASCAD14, ASCAD21, ASCAD52 = 70% reduction of CAD activity

cell wall structure remaining after successive xylem extractions with toluene ethanol, ethanol, and water. An alkali treatment of the CWR ionizes free phenolic functions and partially hydrolyzes polysaccharides resulting in partial solubilization of a lignin fraction from the cell wall. This alkali treatment allows the recovery of a saponified residue and an alkali fraction. Saponified residue of the red poplars contained significantly less Klason lignin when compared to the white poplars. Alternatively, substantial quantities of lignin were isolated by acid precipitation of the alkali fraction from the red poplars, which was not so for the white poplars. Spectral characterization of the nonprecipitated alkali fraction revealed higher levels of phenolic compounds for the red poplars when compared to the white poplars. High Pressure Liquid Chromatography (HPLC) analysis of these compounds showed increased amounts of benzaldehydes, mainly syringaldehyde (up to 10-fold) and vanillin (2- to 4-fold). These data show that lignin amount and composition were not significantly modified; however, reactivity of the lignin polymer toward alkali was significantly altered resulting in a higher extractability of lignin.

Discussion

In our European Union-funded project, transgenic poplars (*P. tremula* x *P. alba*) were produced with reduced CAD enzymatic activity. Both cosuppressed and antisense down-regulated CAD plants were obtained. Enzymatic inhibition was correlated with a red coloration of the wood. Although the lignin levels of these transgenic lines were similar to the control, lignin characteristics and structure were modified. Evidence for increased amounts of aromatic aldehydes in the lignin from red poplars was found, and more lignin was extracted upon alkali treatment. No modification of the S:G ratio was observed.

Comparable results were obtained by Halpin et al. (1994), who analyzed antisense *CAD* tobacco plants. CAD activity in these transgenic tobacco plants was reduced to 7 percent of the control value. *CAD* down-regulated red tobacco plants had no lower lignin content; however, evidence for an alteration in lignin composition was demonstrated in that more phenolic material was extracted by NaOH and thioglycolic acid. Pyrolysis mass spectrometry demonstrated increased amounts of coniferaldehyde but especially syringaldehyde.

From the red poplars and the antisense *CAD* tobacco plants, more alkali lignin was released in comparison to the wild type. This result is interesting because alkali treatment together with sulfonation of the wood chips provide the basis for chemical pulping. As previously mentioned by Higuchi et al. (1994), β-O-4 linkages could be broken more easily via the kraft process when the terminal alcohol groups were replaced by aldehyde groups.

In the transgenic poplars, as in the *CAD* transgenic tobacco plants, lignin content was unchanged (Halpin et al. 1994; Higuchi et al. 1994). It is possible that the CAD activity must be further reduced to lower the lignin content. Indeed, Grand et al. (1985b) showed that lignin content in poplar can be reduced with specific inhibitors of the CAD enzyme, and the *bmr6* mutants also have a lower lignin content (Pillonel et al. 1991). It is possible that the 30 percent residual CAD activity in the down-regulated poplars is caused by a different enzyme also having CAD activity.

The chemical cause of the red coloration of the stem has not been clarified. Higuchi et al. (1994) and Tollier et al. (1995) have shown that the polymerization of coniferylaldehyde *in vitro* produces a wine-red synthetic dehydrogenation polymer, whereas the polymerization of cinnamyl alcohols produces a pale-brown polymer. Furthermore, using thioacidolysis, these authors provided evidence for the incorporation of coniferaldehyde units in the synthetic polymer. However, no evidence for the occurrence of these units was found when the same analytical procedures were applied to the reddish CWR from CAD down-regulated poplars. This suggests either the occurrence of coniferaldehyde derivatives not depolymerized by thioacidolysis or, more probably, their rearrangement into benzaldehyde derivatives that exist at increased levels in these plants (Tollier et al. 1995). Red coloration might be caused by an extended conjugated system generated by incorporating aldehydes into the polymer. In the study by Halpin et al. (1994), the red stem coloration was visible during plant growth but disappeared at the flowering stage. In poplar, the red color also disappeared with wood maturation and was only associated with young xylem. A disappearance of the red color during maturation was also observed in the brown midrib mutants. Because the red color is probably due to the presence of conjugated aldehydes in the lignin, this conjugated system may become reduced by an unknown mechanism during aging resulting in the disappearance of the red color.

Kraft pulping experiments are being performed on the transgenic poplars with the lowest CAD activity. Preliminary experiments on young trees indicate that the red poplars are slightly easier to delignify, providing a pulp whose residual lignin content can be significantly lower according to kappa number determinations. Field trials are being established to investigate whether these promising results are reproducible over successive growth years.

Perspectives

Different strategies to modify lignin content in plants have been proposed by several authors (Boudet et al. 1995; Dean and Eriksson 1992; Whetten and Sederoff 1991). Besides *CAD* and *COMT*, several other genes involved in lignin biosynthesis are interesting to manipulate. Such genes

include those encoding laccase and peroxidases involved in the polymerization of the monolignols, *CCR* possibly encoding a flux-regulating enzyme, and *CCoAOMT* encoding the enzyme that is possibly involved in the biosynthesis of the G units. Furthermore, since many cDNAs encoding lignification enzymes have been cloned (e.g., *COMT, CCoAOMT, CAD, CCR*), the altered expression of more than 1 gene (e.g., *COMT* in combination with *CCoAOMT*) might be a realistic goal. Eliminating several of these genes simultaneously may achieve a modification of the lignin composition and a reduction in lignin content. It may be possible to identify new proteins involved in lignin biosynthesis by comparative two- dimensional, protein-gel analysis between wild-type and transformed poplars with altered lignin biosynthesis (e.g., antisense *CAD* plants). Similarly, comparative HPLC analysis of wild-type and transformed plants may uncover new routes in lignin biosynthesis. Another strategy to modify lignin structure might be altering expression of transcription factors that interact with 1 or several genes involved in lignin biosynthesis. Finally, these gene constructs could be transferred to economically important poplar hybrids (e.g., *P. trichocarpa* x *P. deltoides*), or other tree species such as eucalyptus, which are very important for the pulp and paper industry.

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Chapter 27

Potential Use of *Populus* for Phytoremediation of Environmental Pollution in Riparian Zones[1]

Mary Ellen Dix, Ned B. Klopfenstein, Jian-Wei Zhang, Sarah W. Workman, and Mee-Sook Kim

Introduction

Environmental pollution is a serious threat to human life and to our ecosystems. Riparian zones, the narrow band of land between terrestrial and aquatic systems, are especially vulnerable to environmental pollution because many pollutants are transported through these systems via surface or subsurface runoff. Pollutants include fertilizers (e.g., nitrates), pesticides, agrichemical by-products, heavy metals, trichloroethylene, halogenated phenolics, and other waste products (Schoeneberger 1994). Because agricultural and industrial pollutants are widespread, there is increasing interest on organisms that accumulate, detoxify, or degrade these substances. While it is known that plants and microorganisms modify their environments, their potential use as mitigative tools to clean pollutants has only recently gained acceptance (Brown 1995). Woody perennial plants are ideal for remedial purposes because they can be planted over large areas at low cost and can concentrate or degrade environmental pollutants over several years (Moffat 1995), while also providing other economic or ecological services. As metabolic pathways for pollutant detoxification, uptake, and/or degradation are described, woody plants can be selected or engineered to remediate specific environmental pollutants.

Why *Populus?*

Populus is well suited for use in phytoremediation (the use of specially selected and engineered plants for environmental remediation) plantings. *Populus* is easy to establish and grows quickly. Its high transpiration rate and wide-spreading root system make it ideal to intercept, absorb, degrade, and/or detoxify contaminants, while reducing soil erosion. Historically, this widely distributed genus has naturally grown in riparian areas, thus many genotypes are adapted for growth on potential remediation sites. *Populus* plantings are amenable to coppicing and short-rotation harvest, thereby helping to maintain sustained root vigor. Further, if a biofuels or fiber market is available, harvests can generate additional income that helps offset establishment costs (Strauss and Grado this volume). Although *Populus* is not part of the human food chain, many vertebrates and invertebrates use the trees for food, shelter, and reproductive sites. Such increases in biodiversity can contribute to sustained productivity of adjacent aquatic habitat and crop land (Dix et al. in press).

Populus is well studied, with established silvicultural, vegetative propagation, breeding, and harvesting protocols (Stettler et al. 1996). In addition, *Populus* is amenable to tissue culture manipulation, genetic engineering, and genetic mapping (various chapters this volume). Thus, *Populus* is an ideal candidate for genetic engineering and selection for absorption, detoxification, and/or degradation of environmental pollutants such as heavy metals, nitrates, pesticide residues, and other waste products.

Pollutant-Neutralizing Trees

Plants have many mechanisms for neutralizing toxic pollutants including immobilization, absorption, and ac-

[1] Klopfenstein, N.B.; Chun, Y. W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

cumulation or sequestration of contaminants (except cytoplasmic toxins). Plants also support symbiotic, root-associated microorganisms that can contribute greatly to contaminant neutralization (Stomp et al. 1994). One example of phytoremediation is using metal accumulating plants to remove heavy metals from the soil. All plants can accumulate essential heavy metals from the environment and some accumulate nonessential metals such as cadmium (Cd), lead (Pb), and cobalt (Co) (Baker and Brooks 1989; Ernst et al. 1992; Ostry this volume; Salt et al. 1995). Tolerance to aluminum has been reported for several poplar and other plant species (Baker and Brooks 1989; Chung and Chun 1990; Ernst et al 1992). In addition, studies on landfills and municipal sludge recycling systems demonstrate the ability of *Populus* and other plants to take up and tolerate heavy metals and other potential pollutants (Salt et al. 1995; Schultz et al. 1995; Shrive et al. 1994). *Populus* designed for use in riparian remediation plantings also must be able to take up, translocate, and / or resist toxic pollutants. An ideal tree may have densely packed roots for inactivating toxins or removing them from the soil for subsequent translocation to the leaves and storage in roots or stems (Schoeneberger 1994; Stomp et al. 1994).

Nitrates

One of the largest fertilizer pollutants from both agricultural and urban sources is nitrates (NO_3). Large amounts of nitrates have already entered ground and surface waters, adversely affecting the health of aquatic organisms, humans, and other components of the ecosystem (Duda 1982; Komor and Magner 1996; Lowrance 1992). Nitrates are also indirect by-products of livestock operations (Welsch 1991). Large root systems of certain *Populus* genotypes have an affinity for nitrates. For example, in simulated groundwater depletion studies *Populus* x *canadensis* roots substantially reduced the concentration of nitrates continuously over a 2-month period (O'Neill and Gordon 1994). Furthermore, wide-spread root systems of *Populus* are more effective than confined root systems in uptake of these nutrients (Licht 1992). Clonal and species differences in root growth and root physiology are common in *Populus* (Pregitzer et al. 1990). Nitrate uptake, although it varies with environmental conditions, is apparently heritable in selected *Populus* spp. (Nguyen et al. 1990; Pregitzer et al. 1990; Pregitzer and Friend 1996). Thus, planting *Populus* clones selected for their large root masses on sites with potential for nitrate runoff could increase absorption of nitrates. Nitrate uptake in these poplars could perhaps be further enhanced by genetic engineering or selective breeding of poplar for larger root mass or increased protein storage (Coleman this volume). For example, root system growth was stimulated in *P. alba* x *P. glandulosa* (Chung et al. 1989), *P. davidiana* Dode (Lee et al. 1989), *P. deltoides* x *P. nigra* (Charest et al. 1992) and *P. nigra* x *P. maximowczii* (Charest et al. 1992) by transformation with *Agrobacterium rhizogenes*.

Chemical Tolerance and Detoxification

Herbicides are commonly sprayed on crops to eliminate competing weeds. However, herbicides and their by-products that reach streams by direct runoff, leaching, erosion, and other processes can be toxic to aquatic plants and animals. For planting between the crops and streams, herbicide-tolerant poplars could be produced or selected to remove, detoxify, degrade, or tolerate selected pollutants including herbicides. *In vitro* selection, genetic engineering, genetic screening (e.g., marker- assisted selection), and other molecular techniques have potential for producing or selecting genotypes with improved remediation efficiency.

Chemical tolerance

In vitro techniques were developed for detecting somaclonal variation in the tolerance of *Populus* to herbicides (Michler and Haissig 1988). Using such *in vitro* techniques, 4 hybrid *Populus* lines were selected for increased tolerance to glyphosate and sulfometuron methyl (Michler and Haissig 1988). In addition, several *Populus* variants selected for tolerance to sulfometuron methyl were found to have increased acetolactate synthase (ALS) activity (Michler 1993; Riemenschneider and Haissig 1991; Riemenschneider et al. 1988).

Genetic engineering techniques are available for transferring tolerance mechanisms into *Populus*. Mutant acetolactate synthase genes, *crs*1-1 and *als*, that confer resistance to sulfonylurea or the herbicide chlorsulfuron were individually used to transform *P. tremula* x *P. alba* (Brasileiro et al. 1992; Chupeau et al. 1994). Transgenic *Populus* cells containing the mutant *als* gene grew on a selective media containing 200 nM chlorsulfuron (Chupeau et al. 1994), and transgenic plants expressing the mutant *crs*1-1 gene were completely resistant to high doses of chlorsulfuron (Brasileiro et al. 1992). *P. alba* x *P. grandidentata* was genetically transformed with the mutant *aro*A gene for 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase that confers tolerance to the herbicide glyphosate (N-(phosphonomethyl)-glycine) (Donahue et al. 1994; Fillatti et al. 1987; Karnosky et al. this volume). Transgenic *Populus* plants expressing the mutant *aro*A gene demonstrated more herbicide tolerance than the control *Populus* (Donahue et al. 1994).

In work to increase tolerance to air pollution, hybrid aspen (*P. sieboldii x P. grandidentata*) was transformed with an *E. coli* glutathione reductase (*GR*) gene (Endo et al. this volume). *GR*-expressing, transgenic aspen displayed re-

sistance to oxidative stress caused by the herbicide paraquat (methyl viologen: 1,1-dimethyl-4,4-bipyridium dichloride) or sulfur dioxide (SO_2). This approach could potentially confer tolerance to oxidative stresses caused by other environmental pollutants. Pollutant-tolerant *Populus* could be further selected or engineered with remediatory functions, or used to support growth of remediatory microorganisms.

Chemical detoxification

Other remediatory approaches are aimed toward direct degradation or detoxification of toxic pollutants. *P. alba* x *P. tremula*, *P. tremula* x *P. alba*, and *P. trichocarpa* x *P. deltoides* hybrids were transformed with a *bar* gene that codes for the enzyme phosphinotricin acetyl transferase (PAT) (Chupeau et al. 1994; De Block 1990; Devillard 1992). PAT inactivates the commercial herbicide phosphinotricin (glufosinate, Basta) by acetylation (De Block 1990).

Ongoing work at the University of Washington and Washington State University demonstrated that *Populus* hybrids (*P. trichocarpa* x *P. deltoides*) can oxidize trichloroethylene (TCE) to produce carbon dioxide and other metabolites. Further experiments are underway to determine the capacity of *Populus* to remove and degrade TCE from groundwater (Strand et al. 1995). Pioneering work was initiated to enhance environmental detoxification by genetically engineering trees with genes that encode remediatory functions (Stomp et al. 1994). Two genes from *Alcaligenes eutrophus*, *tfd*B and *tfd*C, were isolated and cloned in an attempt to detoxify halogenated phenolics. One gene, *tfdB*, encodes a chlorophenol hydroxylase, and the other gene, *tfd*C, encodes a chlorocatecol 1,2-dioxygenase. Chlorophenols are sequentially hydroxylated by these 2 enzymes to form chlorocatechol. Subsequently, the ring is cleaved to create chloro-*cis*-*cis*-muconate. Initial tests are underway using 2,4-dichlorophenol, a breakdown product of 2,4-dichlorophenoxyacetic acid (2,4-D), and trichloroethylene. Gene constructs have been made with *tfd*B and *tfd*C under the control of a cauliflower mosaic virus (CaMV) *35S* constitutive promoter for transformation of *Populus*, black locust (*Robinia pseudoacacia*), and sweet gum (*Liquidambar styraciflua*). Subsequent studies are assessing active enzyme levels, uptake, and fate of TCE in these trees (Stomp et al. 1994). Such studies in direct detoxification further demonstrate the potential of *Populus* for phytoremediation.

Soil Conditions and Microorganisms

Success of poplar plantings in remediating a riparian site is dependant on soil conditions and microorganisms. Soil chemistry plays a pivotal role in this process with soil pH and chelating agents affecting uptake of metals. For example, many metals in soils are bound to oxides. Plants can dissolve these oxides and enhance their solubility by releasing reductants from the roots. However, soil pH can influence metal bioavailablity and uptake. Plants growing in soils with low pH typically display higher metal toxicity because of decreased metal adsorption to soil particles. This, in turn, can increase concentrations of metals in the soil solution and subsequent leaching (Salt et al. 1995).

Trees support a diverse population of soil microorganisms, including bacteria, ecto- and endomycorrhizal fungi, actinomycetes, and blue green algae. In turn, many of these microorganisms help tree establishment and growth by greatly increasing the uploading capacity of roots. These soil microorganisms are instrumental in the processes of remediation, stabilization, and filtration of water and soil. Like trees, soil microorganisms participate directly or indirectly in these processes. Direct remediation occurs when organisms take up, store, detoxify, or degrade toxic compounds and their derivatives. Indirect remediation occurs through beneficial effects on associated organisms directly involved in remediation. Thus, overall effectiveness of remediation processes is based on interactions among the plant species, the type(s) of pollutants, and the soil microflora (Stomp et al. 1994).

Mycorrhizae, symbiotic associations between soil fungi and roots, can greatly increase the root surface area and provide a low-resistance pathway for water transport (Koide 1990). These symbioses can influence the plant's ability to take-up metals, and possibly influence plant tolerance to heavy metals. However, site conditions can influence the development of mycorrhizal associations. In natural conditions and with advanced stand age, *Populus* roots generally form ectomycorrhizae. When these roots are flooded, in the early stages of stand establishment, or in very fertile soils, they may form vesicular arbuscular mycorrhizae or no mycorrhizal associations (Heilman et. al. 1996).

Genetic selection and manipulation of rhizosphere microorganisms can potentially improve biological remediation of soil and water. Several ectomycorrhizal fungi immobilized the herbicide chlorpropham, while other ectomycorrhizal fungi degraded chlopropham to 3-chloroaniline (Rouillon et al. 1989). *Paxillus involutus*, an ectomycorrhizal colonizer of conifer and hardwood species, was transformed by particle bombardment with the hygromycin phosphotransferase gene (*HPT*) as a selectable marker and the β-glucuronidase (*GUS*) gene as a reporter gene. The transgenes were actively expressed after stable integration into the fungal genome, and the ability to form ectomycorrhizal roots was unaffected (Bills et al. 1995). Thus, the potential to genetically engineer mycorrhizal fungi with remediatory functions is demonstrated. Populations of rhizosphere microflora could be increased indirectly by genetically increasing *Populus* root mass

through transformations such as with *Agrobacterium rhizogenes*. Increased root mass could support larger populations of rhizosphere microorganisms that could also be genetically engineered with improved remediatory functions (Stomp et. al. 1994). However, ethical concerns must be thoroughly addressed before such strategies can be implemented in the field (Yang et al. this volume).

Strategies and Considerations for Plantings

For efficient and sustained remediation, *Populus* planted at remediation sites must tolerate prevailing site conditions such as excess nitrates and herbicides, as well as damage by insect pests and diseases. Accumulation of pollutants is toxic to many plants; thus, these plants must tolerate existing pollution levels and higher concentrations than normally exist within the plant. However, many native poplars traditionally recommended for riparian zones are relatively slow growers and may be intolerant of pollutants. Genetic engineering and selection can potentially improve the tolerance of *Populus* trees to various pollutants. Such pollution tolerance could amplify potential biomass benefits of *Populus* plantings.

Pest outbreaks are common in riparian *Populus* plantings (Ostry et al. 1988), and can threaten remediation activity of the planting. Genetic engineering, genetic selection, and *in vitro* selection can facilitate the development of *Populus* clones with enhanced pest resistance (Cervera et al. this volume; Ebinuma et al. this volume; Ellis and Raffa this volume; Heuchelin et al. this volume; Ostry this volume; Powell and Maynard this volume). Eventually, techniques developed by this research will be used to develop a variety of *Populus* cultivars and clones with improved resistance/tolerance to insects and disease. In addition, planting establishment and maintenance guidelines should include strategies for integration with other pest management techniques (e.g., enhancing natural controls). Such approaches could minimize the need for additional pesticide application to *Populus* plantings.

Because *Populus* biomass plantings usually require intensive management, they should be established at least 1 planting zone away from the stream. These plantings could serve as an effective intermediate buffer zone for absorption and degradation of environmental pollutants. The plant zones adjacent to the stream could be designed to delay or absorb excess chemicals and soil from *Populus* plantings. As mentioned earlier, soil microorganisms found in poplar plantings have a primary role in site remediation. These microorganisms also must tolerate the pollutants and other conditions at the site.

Finally, potential applications of this technology beyond riparian forest buffer are numerous. Similarly designed *Populus* plantings could be used to remediate industrial waste sites, agricultural waste water, sewage, and mine land. For example, in the Pacific Northwest, bioengineers are designing systems that use *Populus* biomass plantings for recovering nitrates and other fertilizers from irrigated waste water, or removing urea and heavy metals from dairy waste, human sewage, and landfill leachate. Most of these practices are exploiting the nitrogen affinity and high water consumption of hybrid *Populus* (Gary Kuhn, USDA Natural Resource Conservation Service, personal communication).

Conclusion

Planting poplar near riparian zones and toxic waste sites has generated considerable interest as an economical method to remediate toxic sites while providing income and environmental benefits. Planting *Populus* in riparian zones may provide unique opportunities for remediation of multiple toxins. *Populus* has high potential for environmental remediation because its biology is well studied, and its management, production, genetic engineering, genetic selection, and *in vitro* manipulation techniques are well developed and readily available. Demonstration plantings have been established in several communities to limit movement of potential ground-water contaminants. Such plantings are used to remediate leachate from contaminated landfill and waste water systems while producing biomass and providing wildlife habitat. However, phytoremediation of pollution in urban and rural landscapes is a long process and is primarily effective only on pollutants near the surface. It is a relatively environmentally safe process that can be used for large areas. Removal of pollutants by this method does not necessarily require much energy (Stomp et al. 1995). Additional research, development and field trials are needed before the specific biochemical processes involved in pollutant uptake, transport, and accumulation are fully understood. Environmental impacts of using *Populus* remediation plantings must also be thoroughly evaluated before such plantings can be fully utilized.

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Chapter 28

Poplar Genetically Engineered for Reproductive Sterility and Accelerated Flowering[1]

Richard Meilan and Steven H. Strauss

Introduction

High-yielding clones of hybrid poplars (*Populus*) were recently developed in the northwestern United States. Although many genes are currently available (e.g., those affecting insect and herbicide resistance) for enhancing the usefulness of these clones (Strauss 1995), a key obstacle to commercialization of genetically engineered trees is the environmental safety of transgenes. Federal regulators will likely require development of a strategy to mitigate environmental impacts from release of transgenes into wild populations. Engineered sterility would help satisfy this requirement (reviewed in Strauss et al. 1995).

In addition to gene containment, sterility offers other benefits. Reproductive growth is an energy-requiring process. By interrupting floral development, energy may be diverted into biomass production. Sterility also reduces genetic pollution. Selected hybrid cottonwood clones are planted on a very large scale in the Pacific Northwest of the United States. Their pollen clouds may potentially affect the genetic structure of surrounding wild stands. Planting sterile versions of these clones would alleviate this concern. Finally, engineered sterility would reduce the level of airborne allergens and undesired litter from reproductive tissues.

Because trees have long juvenile periods, accelerating flowering is essential to verify the effectiveness of introduced sterility-causing transgenes within a reasonable time period. To our knowledge, there are no published methods for inducing precocious flowering and seed production in *Populus*. We include discussion of several techniques used to promote flowering in other woody angiosperms.

Floral Genes

Flower development occurs in 3 phases: 1) conversion of a vegetative meristem to an inflorescence meristem; 2) conversion of an inflorescence meristem to a floral meristem; and 3) emergence of floral organs from the floral meristem (reviewed in Weigel 1995; Yanofsky 1995). Many genes involved in the initiation of flowering were identified through mutational analysis. Flowering-time genes from *Arabidopsis* have been divided into early- and late-flowering categories. Two late-flowering genes that were recently cloned are *CONSTANS* (*CO*) (Putterill et al. 1995) and *LUMIDEPENDENS* (Lee et al. 1994). A model showing control of flowering by time-of-flowering genes was recently proposed by Martinez-Zapater et al. (1994).

Floral homeotic genes, which encode transcription factors and control many aspects of floral development, were isolated from *Arabidopsis*, *Antirrhinum*, and other plant species. These genes are usually divided into 3 functional classes: 1) floral meristem identity genes; 2) floral organ identity genes; and 3) spatial regulators of organ identity genes.

LEAFY (*LFY*) is an example of a meristem identity gene from *Arabidopsis*. It is involved in controlling the transition from an inflorescence to a floral meristem. Its homolog in *Antirrhinum* is referred to as *FLORICAULA* (*FLO*). Strong mutations at this locus cause inflorescence initiation within bract axils resulting in a branchy phenotype with substantial or complete disruption of flower production (Coen et al. 1990; Huala and Sussex 1992; Weigel et al. 1992).

A second floral meristem identity gene, *APETALA1* (*AP1*), encodes a putative transcription factor containing the *MADS* box, which is a highly homologous domain

[1] Klopfenstein, N.B.; Chun, Y.W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

shared by the yeast transcription factor *MCM1*, the *AGAMOUS* gene from *Arabidopsis*, the *DEFICIENS* gene from *Antirrhinum*, and the human serum response factor *SRF*. *APETALA1* is involved in controlling the switch from inflorescence to floral meristems, and is also required for sepal and petal development (Mandel et al. 1992).

Floral organ identity genes are expressed in the floral meristem just before the emergence of the primordia whose development they regulate. These genes are divided into 3 functional groups that are designated A, B, and C. Each of the 4 floral organs emerges from concentric whorls of cells in the floral meristem (figure 1a). The different classes of floral organ identity genes are expressed in 2 adjacent whorls (figure 1b). The location of their expression dictates the type of floral organ produced. For example, when an A-function gene is expressed in whorl 1, sepals form; the combined expression of A- and B-function genes in whorl 2 produces petals; when B- and C-function genes are expressed in whorl 3, stamens are produced; and C-function gene expression in whorl 4 results in pistil development. This general model appears valid for a variety of plant species; however, there are many exceptions and elaborations on this basic scheme including action of partially redundant genes (e.g., *AP1* and *CAL* in zone A) (Yanofsky 1995).

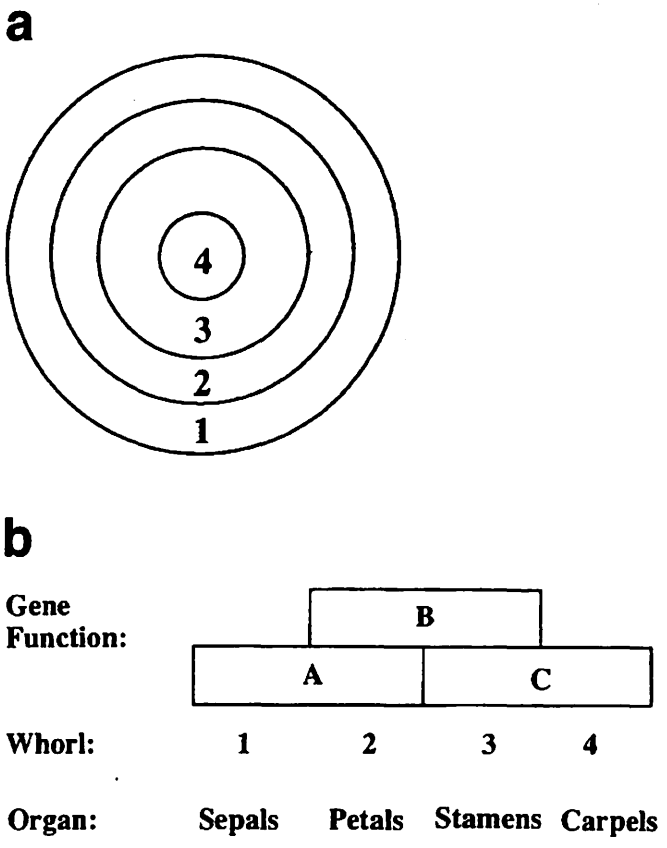


Figure 1. a) Arrangement of concentric whorls of cells in the floral meristem. b) The A-B-C model showing the overlapping zones of expression of the floral organ identity genes (adapted from Coen and Meyerowitz 1991).

The *AGAMOUS* (*AG*) gene is an example of a C-function floral organ identity gene. Mutations in *AG* cause the conversion of stamens to petals and carpels into new flowers (Bowman et al. 1989). *AG* and *AP1* are also examples of spatial regulators. *AP1* is involved in activating *AG* expression and is subsequently down-regulated by *AG*, which results in a loss of *AP1* expression in whorls 3 and 4.

Because mutations in some floral homeotic genes impair fertility at early stages of floral differentiation in male and female flowers, they are attractive targets for engineering sterility. Recently, 3 floral homeotic genes were cloned from *Populus trichocarpa* and identified as putative homologs of *FLO/LFY* (*PTFL*), *DEFICIENS* (*PTD*), and *AG* (*PTAG*) (Sheppard et al.1996). These genes are being used in our ongoing efforts to engineer sterile poplars.

Several floral-specific genes were isolated from highly differentiated floral tissues such as anthers and pistils. Most encode structural or catalytic proteins essential for pollen or carpel function. Examples include stylar transmitting tissue-specific genes (Wang et al. 1993), anther tapetum-specific genes (Goldberg 1988), and genes expressed in both pistils and anthers (Gasser et al. 1989). Although these promoters can be used to engineer sterility for transgene containment, they may not enhance vegetative growth because they are expressed late in flower development.

Engineering Sterility

One primary strategy for engineering sterility involves the expression of cytotoxin genes in a floral tissue-specific fashion resulting in organ-specific ablation or disruption of tissues. Another primary strategy relies on suppression of floral genes that are essential to produce fertile gametes.

Tissue Ablation

Promoters

Many tissue-specific promoters currently in use were isolated from tobacco. The most commonly employed male reproductive tissue-specific promoter is *TA29* (Goldberg 1988). This promoter, which directs expression exclusively in the tapetum of the anther, was used to engineer sterility in tobacco and oilseed rape (Mariani et al. 1990). Without the tapetum, microspore development is arrested. In a similar approach, female sterility was achieved using the

stigma-specific *STIG1* promoter, also from tobacco (Goldman et al. 1994).

Other useful promoters were characterized from genes involved in gametophytic self-incompatibility. These include promoters from *SLG*, an S-locus glycoprotein gene (Thorsness et al. 1991), and *SLR*, an S-locus-related gene (Hackett et al. 1992). Both promoters are expressed principally in stigmatic and stylar tissues but are also expressed in pollen to variable degrees. These provide organ-specific ablation when fused to cytotoxin genes.

Some occurrences of instability in transgene expression are reported. For example, Denis et al. (1993) demonstrated that when male-sterile rapeseed (*Brassica napus*) containing a tapetal-specific promoter was fused to a ribonuclease gene, it frequently reverted to a fertile state. This instability was associated with temperatures greater than 25 °C. If sterility is an important aspect of gene containment for regulatory purposes, it is critical to demonstrate that sterility is maintained under a range of conditions and developmental stages. This could be achieved by carefully selecting transformed lines for stable expression, using redundant constructs (discussed below), or using transgenes flanked by expression stabilizing matrix-attachment regions (reviewed in Spiker and Thompson 1996).

Many floral homeotic genes are floral-specific, required for floral meristem/organ development, and expressed either as the floral meristems emerge or before floral organ primordia develop. Because of their early and frequent floral-specific expression, floral homeotic gene promoters are probably best suited for engineering sterility while also enhancing vegetative growth. The *APETALA3* promoter fused to *DTA* (see below) gave organ-specific ablation of petals and stamens in *Arabidopsis* (Day et al. 1995).

Cytotoxins

Two widely used cytotoxin genes are Barnase, an extracellular ribonuclease isolated from *Bacillus amyloliquefaciens* (Hartley 1988) and the diphtheria toxin subunit A (*DTA*) gene from *Corynebacterium diphtheriae* (causative agent of diphtheria) (Palmiter et al. 1987). The latter encodes an ADP ribosyl transferase, which interrupts protein synthesis by covalently modifying eukaryotic elongation factor 2 (EF-2). With the B subunit of the toxin deleted, it is unable to enter cells autonomously. Although it is a very effective cytotoxin used successfully to engineer reproductive sterility in plants (Day et al. 1995; Koltunow et al. 1990; Mariani et al. 1990; Thorsness et al. 1991), its commercial application may be controversial because of its origin. However, the use of Barnase is technically complicated because, unlike *DTA*, its encoded protein is toxic to eukaryotic cells. Therefore, it is necessary to include a gene encoding an inhibitor in gene constructs that contain Barnase. Barstar encodes a protein that specifically forms a complex with the Barnase gene product to inactivate it to protect the transformation vectors (e.g., *Agrobacterium tumefaciens*) from its ribonuclease activity (Hartley 1988).

Many other cytotoxin genes are available that can be used to avoid some of these obstacles. For example, the *toxA* gene from *Pseudomonas aeruginosa* produces an exotoxin that, at the molecular level, has the same mode of action as DTA (Lory et al. 1988). Another class of cytotoxin, ribosome inactivating proteins (RIPs), interferes with protein synthesis by altering the large subunit of ribosomal RNA (rRNA). Castor bean seeds (*Ricinus communis*) are a rich source of ricin, which is a representative member of a large class of plant-derived RIPs. This protein catalyzes the cleavage of an adenine base from an exposed loop near the 3'-end of 28S eukaryotic rRNA (position 4,324 in rat). This loop is known as the sarcin loop because α-sarcin, a cytotoxin from *Aspergillus*, cleaves the phosphodiester linkage between a guanosine and adenosine directly adjacent to the site where ricin acts (Stripe et al. 1992).

Suppression

Known as co-suppression, transgenes can inhibit their own expression and that of homologous native genes (Flavell 1994). Recently, it was shown that untranslatable versions of transgenes are more efficient at suppression (Smith et al. 1994). Gene silencing can also be achieved by constitutively expressing the transgene in antisense orientation (reviewed by Mol et al. 1994). A disadvantage of the suppression strategy is that the native gene or a highly homologous equivalent is usually needed for efficient suppression, whereas the promoters employed in the ablation approach typically function across diverse classes of plants.

To help ensure that sterility remains stable throughout the life of the plant, building in some redundancy would be prudent (Strauss et al. 1995). This approach could involve targeting multiple genes for suppression. For example, a transcriptional fusion of a floral meristem identity gene, such as *PTFL*, with a floral organ identity gene, such as *PTAG*, could provide strong suppression. Because *PTAG* is a C-function floral homeotic gene, it controls both pistil and stamen development. If *PTFL* function is some how restored in the transgenic plants or allows occasional production of fertile organs, as in strong *LEAFY* mutants of *Arabidopsis*, both male and female flowers will remain sterile by virtue of *PTAG* suppression.

Reversible Sterility

Restoring fertility may be desirable so that valuable transgenic lines could be used in further breeding. For completely sterile trees, it will be necessary to use an inducible promoter (Mett et al. 1993; Weinmann et al. 1994) that stimulates production of a cytotoxin inhibitor, such as Barstar (Mariani et al. 1992), a site-directed recombinase

to remove or deactivate sterility transgenes (Kilby et al. 1993), or antisense versions of cytotoxin genes.

Early Flowering

The long juvenile period of trees severely impedes study of their reproductive biology. Confirmation that engineered trees are sterile requires an examination of their flowers. Surprisingly, little effort has been directed toward developing methods to induce early flowering in *Populus*. However, several techniques were used to accelerate flowering in other woody angiosperms, which hopefully can be adapted for poplars. In addition, ectopic expression of floral regulatory genes was reported to accelerate flowering in several species including poplar.

Chemical Approaches

The most successful inductive treatments for woody angiosperms have been the triazole growth retardants, which includes compounds such as paclobutrazol and uniconazole. These chemicals specifically inhibit the enzymatic conversion of *ent*-kaurene to *ent*-kaurenoic acid in the gibberellic acid biosynthetic pathway (Rademacher 1989). Besides producing short-statured plants, triazole also accelerates flowering. Paclobutrazol, the most widely used triazole, stimulated flowering in apple (*Malus* spp.) (Tukey 1983; Volz and Knight 1986), pear (*Pyrus nivalis*) (Williams and Edgerton 1983), cherry (*Prunus cerasus*) (Edgerton 1986), clove (*Syzygium aromaticum*) (Martin and Dabek 1988), and eucalypts (*Eucalyptus nitens* and *E. globulus*) (Griffin et al. 1993; Moncur and Hasan 1994; Moncur et al. 1994).

Other growth inhibitors, such as daminozide (Alar®, B-9®, SADH) and chlormequat (CCC, Cycocel®), can stimulate flowering. Daminozide has been used for this purpose in apple (Batjer et al. 1963; Luckwill and Silva 1979). There is also an unpublished report of daminozide causing precocious flowering in aspen (Li 1995, personal communication, North Carolina State University, Raleigh, NC 27695 USA). Chlormequat has led to enhanced yields in grape (*Vitis vinifra*) (Bravdo et al. 1992).

Physical Methods

Phloem Restriction

Girdling (removing a swath of bark around the entire stem) stimulated flowering in certain tree species (Eris and Barut 1993; Wesoly 1985), but this treatment ultimately results in tree death. Incomplete, overlapping girdles provide a less severe alternative that was effective for stimulating flowering in white spruce (*Picea glauca*) (Pharis et al. 1985) and loblolly pine (*Pinus taeda*) (Wheeler and Bramlett 1991). A related technique, scoring (cutting completely through the bark around the trunk with a sharp knife without removing any bark), has been used to stimulate flowering, especially in apple (Veinbrants 1972). Wire girdles were also used with some success to stimulate flowering in aspen. This method involves encircling selected branches or the main stem with tightly twisted, steel wire. The wire is adjusted as the plant grows to avoid killing parts distal to the wire. This technique is best applied in the spring or early summer (Li 1995, personal communication, North Carolina State University, Raleigh, NC 27695 USA).

Root Growth Control

Confining root growth can lead to precocious flowering in peach trees (*Prunus persica*) (Richards and Rowe 1977). Copper treatment of pots or of fabric used to envelop roots prevents root circling and penetration. This form of restriction leads to development of more root tips, which frequently seems beneficial, especially in conjunction with nitrogen fertilization (Proebsting 1995, personal communication, Horticulture Dept., Oregon State University, Corvallis, OR 97331 USA). A similar effect may be achieved by root pruning or wrenching, which can also lead to a more densely branched root system. Similarly, Li (1995, personal communication, North Carolina State, Raleigh, NC 27695 USA) has stimulated aspen flowering by growing seedlings in small pots and cutting the roots of potted plants with a large knife in 2 or 3 directions tangential to the stem.

Shoot Training

Fruit tree growers have induced early flowering by training the shoots to grow horizontally. With this technique, it is important to keep the whole shoot horizontal including the leader. If the leader is removed, damaged, or allowed to resume upright growth, the shoot reverts to a juvenile state (Pharis 1995, personal communication, Biology Dept., University of Calgary, Calgary, Alberta, Canada T2N 1N4). After testing a variety of shoot training techniques, Denby et al. (1988) showed that 2 techniques, angle training and spindle training, increase yield and induce precocious flowering in pear (cv. 'Anjou').

Cultural Conditions

Photoperiod

Eucalyptus occidentalis Endl. flowers precociously at less than 1 year of age when grown under a photoperiod of at least 16 h (Bolotin 1975). In addition, photoperiod may modify responses to other environmental factors. For ex-

ample, the stimulation of flower bud formation in *Camellia japonica* by exposure to high temperatures is enhanced by long photoperiods (Bonner 1947). Birch (*Betula verrucosa*) (Longman and Wareing 1959), Japanese larch (*Larix leptolepis*), and blackcurrant (*Ribes nigrum*) (Robinson and Wareing 1969) were all stimulated to flower by long photoperiods.

Nutritional Status

Improved nutritional status is often considered a flower-inducing treatment. With tea crabapple (*Malus hupehensis*) seedlings, Zimmerman (1971) reduced the time to first flowering from 3 years to 9½ months by growth under continuously "favorable conditions" in a greenhouse. This method included weekly treatments with 20-20-20 (N-P-K) water soluble fertilizer. Aldwinckle (1975) obtained nearly identical results with apple watered weekly with 15-6.4-12.4 fertilizer supplemented with trace elements, in addition to the incorporation of a slow release 17-10.8-7.3 fertilizer (Osmocote®) in the upper layer of the soil every 2 to 3 months. Also, the form of supplied nitrogen is apparently important. Grasmanis and Edwards (1974) showed that when ammonium was supplied to liquid-cultured apple, a significantly greater flowering response occurred than when trees received nitrate as their sole nitrogen source.

Moisture Stress

Considerable evidence shows that water stress can enhance flower initiation in conifers, and that hot, dry summers generally induce abundant seed crops in conifers and broadleaf species (Philipson 1990). Water stress was recently used to stimulate aspen flowering in a greenhouse setting. Potted plants were periodically allowed to dry down to predawn moisture-stress readings between -6.9 and -9.7 bars (-100 to -140 psi) before rewatering. This treatment is apparently effective only when applied during shoot elongation (Li 1995, personal communication, North Carolina State University, Raleigh, NC 27695 USA)

Temperature

Low temperatures were used to induce precocious flowering in several tree species (reviewed by Jackson and Sweet 1972) including peach (*Prunus japonica*) (Lammerts 1943), olive (*Olea europaea*) (Hackett and Hartmann 1963), sweet orange (*Citrus sinensis*) (Moss 1976), and lychee (*Litchi chinensis*) (Menzel 1983). More recently, Moncur (1992) showed that transferring *Eucalyptus lansdowneana* seedlings from a heated greenhouse (24/19 °C, day/night temperatures) to a cold regime (15/10 °C) for 5 to 10 weeks before returning them to warm conditions (24/19 °C) was sufficient to induce floral buds.

Molecular Techniques

Recently, over expression of regulatory genes early in reproductive development induced early flowering. When *AP1* or *LFY* (reviewed above) is expressed constitutively under control of the *35S* promoter from cauliflower mosaic virus (CaMV), transgenic *Arabidopsis* plants flower *in vitro* within weeks instead of nearly 2 months (Mandel and Yanofsky 1995; Weigel and Nilsson 1995). In addition, the *Arabidopsis*-derived *LFY* gene expressed under the control of *35S* in hybrid aspen (*Populus tremula* x *P. alba*) also simulated flowered *in vitro* within several weeks (Weigel and Nilsson 1995).

A *MADS* box containing a gene of unknown function, *OsMADS1*, was recently isolated from rice (*Oryza sativa*). When expressed under control of the *35S* promoter in tobacco, transgenic plants flowered 8 days earlier than their wild-type counterparts (Chung et al. 1994). *CO*, a flowering-time gene (discussed above) from *Arabidopsis*, is a zinc finger-containing transcription factor. Transgenic *Arabidopsis* plants containing extra copies of *CO* under the control of its native promoter flowered significantly earlier than wild-type plants (Putterill et al. 1995). Finally, a member of the *SQUAMOSA* binding protein-like gene family (*SPL-3*) was recently shown to bind to the *AP1* promoter. Constitutive expression of *SPL-3* in *Arabidopsis* resulted in early flowering (Saedler et al. 1996).

These early flowering genes have 2 important applications. First, they can be used to retransform existing transgenic plants that contain constructs expected to cause sterility. If the constructs function properly, induced flowers should be sterile. Second, early flowering genes can be used to generate tester lines that are maintained *in vitro*. These lines can then be transformed with newly developed sterility-causing constructs to rapidly indicate their use in trees.

Conclusion

Research on floral development over the last decade has provided a wealth of genes and knowledge on which to base strategies for modifying plant reproductive development. With the diversity of approaches available, a means can be established to genetically engineer reproductive sterility. The primary issues are determining the effectiveness of constructs for inducing stable, bisexual sterility in a variety of genotypes and environments, and identifying a reliable method to accelerate flowering for expedient research. Within the next 5 to 10 years, constructs should become available that can induce highly stable, male and female sterility in poplars and other tree species. Introduction of such constructs into commercially valuable geno-

types will simplify regulatory approval of transgenic varieties and reduce environmental impacts of large-scale tree farming using engineered clones.

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Chapter 29

Integration of Molecular and Classical Genetics: A Synergistic Approach to Tree Improvement[1]

Ronald J. Dinus and Gerald A. Tuskan

Introduction

Commercial tree improvement (i.e., application of classical genetics to tree breeding) involves selection, mating, testing, and propagation of genotypes having desirable growth, yield, wood quality, fiber quality, and biotic and abiotic stress tolerance. Despite significant improvement of many species, progress is constrained by long-generation intervals, poor juvenile-mature trait relationships, and difficulty in identifying and accumulating valuable genes.

Advances are occurring in molecular genetics that can promote tree improvement programs. In this chapter, molecular genetics includes vegetative propagation, DNA-based marker technologies, and genetic transformation. Tissue culture is included because of its effectiveness in genetic transformation and expediting delivery of improved materials, whether manipulated by other molecular techniques or not. These molecular techniques offer much promise to offset or reduce constraints that limit classical tree breeding.

This chapter addresses integration of molecular and classical genetics, with emphasis on the potential impacts of molecular techniques on breeding progress. The discussion builds on writings of other authors (e.g., Cheliak and Rogers 1990; Chun et al. 1988; Ostry and Michler 1993; Riemenschneider et al. 1988; Tuskan 1992) by explaining individual molecular techniques, giving examples of recent accomplishments, highlighting advantages of poplars as model species, and describing critical research needs.

The first section describes molecular techniques and their parallels to classical breeding practices. Several potentially useful techniques (e.g., chromosome transplantation) are not discussed because the research is in the most basic phases. The second section reviews classical breeding strategies for intra and interspecific breeding. The third section describes means for integrating molecular techniques and classical breeding practices, how they complement each other, and how molecular techniques affect genetic gain and breeding efficiency. The last section summarizes and discusses future implications.

Background

Molecular techniques present opportunities but also create challenges for classical tree breeding. The many opportunities and challenges are best addressed by first working with model species; the genus *Populus* is uniquely suited. Using poplars as a model is also appropriate because of their increasing use as a commercial source of fiber and energy.

As is common for most tree species, poplars generally are outcrossing and heterozygous. Most members of the genus are dioecious, which further enhances outcrossing and diversity (Stanton and Villar 1996). Poplars are prolific pollen and seed producers, and wind dispersal ensures wide germplasm distribution. Many species inhabit diverse environments across broad geographic ranges. Variation has been further enhanced by interspecific hybridization.

Breeding methods are available for species and hybrids of *Populus*. Pollen can be collected, stored, and handled with relative ease. Cross pollination can be conducted on branches brought into greenhouses or laboratories (Stanton and Villar 1996). In contrast with many other tree species, *Populus* seed ripens within a few months of pollination. Difficult crosses (e.g., among species in the same or different sections of *Populus*) can be assisted via mentor pollen and/or embryo rescue.

[1] Klopfenstein, N.B.; Chun, Y.W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

Elite genotypes from individual *Populus* species or hybrid populations can be propagated vegetatively by a variety of means, and commercial planting of some selections is done routinely with dormant, nonrooted cuttings (Land and Cunningham 1994). Ease of vegetative propagation has fostered establishment of replicated clonal plantations for precise evaluation of genetic value. Research in such plantations has yielded a wealth of knowledge about genetics, anatomy, physiology, biochemistry, growth, yield, and ecology of *Populus*. Most *Populus* species and hybrids are diploid, have 38 chromosomes, and a relatively small genome (C = 0.55 pico grams and approximately 550,000 base pairs) (Bradshaw and Stettler 1993). For all of these reasons, poplars are well-suited for investigation and manipulation via molecular techniques.

Molecular Techniques

Several examples of molecular techniques that are promising for classical tree breeding are discussed in this section. Vegetative propagation by *in vitro* and more conventional means can shorten the time required and increase genetic gains. DNA markers and genetic fingerprinting can be used to discern and verify identity of breeding materials. Markers can also be used to accelerate breeding via reliable early selection. Genetic transformation offers opportunities for creating and exploiting variants that might otherwise be expensive and time consuming or unavailable.

Vegetative Propagation

Embryo Rescue

Interspecific hybridization is an important tactic in poplar breeding strategies. Many crosses are productive, but some critical combinations have reduced seed yields. *In vitro* culture methods to rescue embryos and increase seedling recovery have been developed (Raquin et al. 1993; Stanton and Villar 1996).

Cell and Protoplast Fusion

This *in vitro* technique can provide a means to enhance genetic variability by creating somatic hybrids among sexually incompatible species and by combining nuclei and cytoplasmic organelles (Kikkert this volume). Various symmetrical and asymmetrical combinations can be produced that may have value to breeders. Efficient methods to regenerate plants from protoplasts of diverse taxa can also facilitate genetic transformation (Charest et al. this volume).

Somaclonal Variation and Selection

Somaclonal variation often occurs spontaneously in tissue culture systems, sometimes yielding plants with considerable variation, phenotypically and/or genotypically, from the donor plant (Fry et al. this volume). If useful genetic change occurs, variants can be added to breeding populations. Stable, lasting variation, even if not heritable, can be exploited via vegetative propagation.

Exposing cell or tissue cultures to selective agents can be used to screen prospective parents for traits such as resistance to biotic and abiotic stress. Coupling somaclonal variation, as previously described, with selective screening is promising since valuable variants may arise under pressure of the selective agent (Ostry this volume).

Dihaploids

Heterozygosity, which is typical of forest trees, has both advantages and disadvantages for breeding. Although breeders have access to many genes, they are hindered in concentrating valuable genes into manageable numbers of genotypes. Creation of dihaploid (homozygous) plants may help overcome such obstacles. Anthers or ovules from elite individuals can be used to establish haploid tissue cultures. Plants regenerated from such cultures, after natural or artificial chromosome doubling, could be used as pure lines. Uddin et al. (1988) reviewed research on anther culture and demonstrated regeneration from *P. deltoides* anther cultures. Further advances were reported by Mofidabadi et al. (1995).

Mass Propagation

Many poplar species can be propagated by rooted or nonrooted cuttings. Although widely used, this approach has the following limitations: 1) relatively small numbers of cuttings can be produced from individual donors during a growing season; 2) rooting ability varies among donors and frequently declines with donor age; and 3) genetic transformation is not feasible. Research has improved cutting yields, but further improvement in rooting efficiencies is needed (Ostry and Michler 1993). Examples of applied programs are provided by Lambeth et al. (1994).

As noted in the chapters of this volume on *in vitro* culture, because poplars are relatively easy to manipulate in culture, significant advances have been recently achieved. *In vitro* propagation can be accomplished by any of 3 approaches: 1) regeneration from existing meristems; 2) organogenesis from adventitious meristems; or 3) somatic embryogenesis.

Regeneration from existing meristems is a common method and is noted for its simplicity and reduced risk of somaclonal variation. Multiplication rates are limited, however, and the approach is not especially amenable to genetic transformation.

Propagation from adventitious meristems (i.e., organogenesis) can be achieved with a large array of explants (Ernst 1993). This approach can produce high multiplication rates and is useful for genetic transformation. Despite significant advances, however, procedures must be tailored to individual genotypes and risk of unwanted somaclonal variation is relatively high.

Somatic embryogenesis offers higher multiplication rates than other *in vitro* methods, generally has less unwanted somaclonal variation, and is ideal for genetic transformation. High frequency embryogenesis has been obtained with various tissues from several poplar species and hybrids (Michler 1995). The approach has also been used for *in vitro* selection for resistance to *Septoria musiva* (Ostry et al. 1994) and tolerance to herbicides (Michler and Haissig 1994).

DNA-Based Marker Techniques

A variety of recombinant DNA techniques can be used to identify DNA markers (i.e., selectively neutral, polymorphic DNA sequences). Such markers can be applied in various phases of classical breeding to fingerprint natural populations, candidates for breeding, and propagules for commercial planting. Additional applications include creation of genetic maps, identification of markers for valuable traits, and isolation of desirable genes.

DNA Fingerprinting

DNA fingerprinting, in the broadest sense, includes any procedure that characterizes genetic variation at the DNA level. In its narrowest form, fingerprinting is a statistical process for identifying individual genotypes (Weising et al. 1995). Several techniques are available for detecting differences in DNA sequences among related and nonrelated individuals. Analysis of restriction fragment length polymorphisms (RFLP) was one of the first such techniques. In this method, a probe or short segment of DNA isolated from a related (homologous) or nonrelated (heterologous) genotype is hybridized to genomic DNA following digestion with restriction endonuclease enzymes (Southern 1975). Overall differences in fragment lengths and/or in homologies at hybridization sites are used to create a unique DNA fingerprint (Cervera et al. this volume).

Recently, polymerase chain reaction (PCR) fingerprinting techniques were developed. These include randomly amplified polymorphic DNA (RAPD) markers (Welsh and McClelland 1990; Williams et al. 1990), minisatellite markers (Jeffreys et al. 1985), simple sequence repeat (SSR) markers (Tautz and Renz 1984), and amplified fragment length polymorphism (AFLP) markers (Zabeau 1993). These approaches use DNA primers in conjunction with DNA polymerase enzymes to create numerous fragments of DNA having lengths dependent upon sequences in the genomic template. Since amplified DNA fragments from populations within species and individuals within populations vary in size, such differences can be used to develop a characteristic fingerprint (Cervera et al. this volume; Lin et al. this volume).

Choices of fingerprinting technique and genotypes to fingerprint vary with breeding objectives. Polymerase chain reaction techniques are faster and less expensive to apply than other approaches, but many PCR markers are codominant and therefore less informative. Even so, PCR-based RAPD markers were used to characterize population differences in several poplar species; for example, aspen (*P. tremuloides*) (Tuskan et al. 1996) and black cottonwood (*P. trichocarpa*) (Reed 1995). Similarly, RAPD and SSR markers were used to fingerprint commercial poplar clones developed in breeding programs around the world (Lin et al. this volume).

Linkage Analysis and Genetic Maps

Linkage analysis is a method for determining the linear arrangement of genes or genetic markers on a single segregational unit (i.e., a chromosome). Recombination frequency is used to establish the extent of crossing over between genes at 2 separate loci. Genes that assort independently are not linked, and the genetic distance is deemed the maximum measure of 100 centiMorgans (cM). The closer that 2 loci are located to each other, the smaller the recombination value. Genetic maps are created by testing the relative colinearity of multiple loci in sequential linkage analyses. Physical and morphological traits, isozymes, RFLP markers, and PCR-based markers can be positioned on genetic maps. Several computer software programs are available to construct genetic maps such as LINKAGE-1 (Suiter et al. 1983), GMENDEL (Liu and Knapp 1990), and MAPMAKER (Lander et al. 1987).

Genetic maps can be used to determine taxonomic relationships among potential breeding candidates, indirectly select candidates based on presence or absence of linked markers, and isolate genes from rare phenotypes (Weising et al. 1995). Creating or acquiring usable populations is the first step in constructing genetic maps. Backcross or F_2 populations typically are used to maximize recombination frequencies. In addition, parents with extreme phenotypes (e.g., those susceptible or resistant to pest attack) can be used to ensure abundant variation. Ideally, 400 to 500 offspring should be available from each generation to ensure precise estimates of crossing over in linkage analyses. Poplars are well-suited as models for genetic mapping because they have a relatively small genome, can be manipulated to produce large numbers of offspring per parent, have relatively short generation intervals, and can be evaluated at early ages.

The relationship between a genetic and physical map depends on the amount of DNA in the haploid genome,

number of chromosomes, and recombination frequency of the particular species. As noted previously, poplars match such criteria well and have exhibited a physical and genetic ratio of approximately 200 kilobase pairs per cM (Bradshaw et al. 1994). Given these advantages, a moderately saturated genetic map was created for poplars, and 500 genetic markers from RFLP and PCR techniques were linked to 30 phenotypic traits (Bradshaw and Stettler 1995). Bradshaw (1996) has proposed reconciling the genetic map with the physical one.

Genetic Markers and Marker-Aided Selection

Research on genetic markers in poplars has advanced rapidly in recent years. Markers were associated with traits governed by 1 or a few genes, for example, disease resistance (Newcombe and Bradshaw 1996; Newcombe et al. 1996), and also with quantitative trait loci (QTLs) that govern characteristics such as height and diameter growth (Bradshaw 1996; Bradshaw and Stettler 1995). Since most commercially important traits in forest trees are quantitative, linkage relationships between genetic markers and QTLs allows indirect selection at early ages. For reliable and economical use, however, such relationships must be verified across families in breeding programs, within varying genetic backgrounds, and across environments (Strauss et al. 1992). Given the time and expense required to identify useful markers, traits for application of marker-aided selection should possess high value, have low heritabilities, and/or be difficult to measure. Some examples are disease resistance, particularly when pathogenic variation exists (Newcombe 1996), complex wood properties (Williams and Neale 1992), and resistance to abiotic stresses that are difficult to replicate (Tauer et al. 1992). Marker-aided selection schemes are being tested with hybrid poplars (Bradshaw 1996).

Genetic Maps and Gene Isolation

Genetic maps can also be used to isolate desirable genes. In this approach, map-based gene cloning flanking genetic markers around a gene of interest are used to "walk" toward the expressed DNA sequence (Tanksley et al. 1995). For greatest effectiveness, markers should be within 1 to 2 cM, or approximately 200 to 400 kilobases, of the gene in question. Thus, high density genetic maps are a prerequisite. Ultimately, genes isolated by this means can be verified and/or used via genetic transformation. Although valuable, map-based gene cloning will probably only be used in the near future for exceptionally rare or valuable traits. Nevertheless, poplars and related genera should be useful for developing and evaluating the approach. For example, the technique is being used to isolate a gene associated with femaleness in *Salix viminalis* (Alstrom-Rapaport et al. 1996). Widespread application will require development of genetic maps, reliable gene transfer techniques, and established associations of specific genes with desirable traits.

Genetic Transformation

Genetic transformation, or nonsexual addition of specific genes, permits acquisition of traits otherwise unavailable. This technique can also be used to incorporate additional copies of indigenous genes that are closely linked to undesirable genes circumventing the need for generations of backcrossing. Bypassing the sexual process offsets time and cost difficulties posed by long generation intervals.

As discussed (Charest et al. this volume; Han et al. 1996; Kim et al. this volume), a variety of gene transformation procedures have been tested and used with poplars. The first successful achievement of such transformation involved a gene conferring herbicide tolerance on a hybrid poplar (Fillatti et al. 1987). Poplars are noted as models for transformation because of the ease, relative to most other tree species, with which they can be manipulated and regenerated in cell and tissue culture (Chandler 1995). Poplar transformation is becoming more routine and is being expanded to include a widening array of genotypes and genes. For example, recovery of transgenic plants of *P. deltoides* was recently reported (Dinus et al. 1995).

Transformation can also be adapted to reduce expression of existing genes that encode undesirable traits. This antisense technology involves inserting a reverse copy of the gene in question. Messenger RNA (mRNA) transcribed from the reverse copy is thought to bind with that from the normal gene inactivating the mRNA and preventing synthesis of a gene product. Early research on antisense technology with forest trees was performed with poplars; for example, control of lignin biosynthesis and deposition (Boerjan et al. this volume; Chiang et al. 1994). Successful deployment of antisense technology will require a better understanding of the extent, stability, and underlying mechanisms of suppressed expression.

Introduced genes are not always expressed, may not be expressed in spatially and temporally desirable manners, and may not persist across the long life cycle of forest trees (Ahuja this volume). Ensuring proper expression is a major technical challenge. In addition, gene insertion may provoke undesirable genetic and/or epigenetic changes; for example, the co-suppression phenomenon. Currently, long-term testing is required to ensure the presence of desirable changes or absence of undesirable changes. Better methods are needed to target gene delivery and verify transformation, gene expression, and genetic fidelity. As noted earlier, cell and tissue culture methods are also needed that are less genotype specific. Research with poplars is helping to fill these knowledge gaps, as discussed in Stettler et al. (1996a) and this volume.

Classical Genetics and Breeding

Early poplar breeders achieved substantial improvement by exploiting variation existing in native populations. Promising individuals were selected from natural stands (i.e., base populations), vegetatively propagated, and evaluated in clonal trials (figure 1). In turn, the best individuals were vegetatively propagated for commercial planting.

Short-Term Strategies

A first step beyond the opportunistic approach involves wide crosses among desirable individuals from the same and/or different species, evaluating offspring in clonal trials, then multiplying the best offspring for commercial planting (figure 1). Matings, intra or interspecific, are repeated and/or adjusted to maintain or increase in accordance with performance of cloned offspring. New parents and crosses are added as needed. Heterosis and/or acquisition of specific traits have been a justification for using interspecific matings. Clonal deployment remains the norm since it captures both additive and nonadditive genetic variation, enhances gain by shortening the time between selection and commercial planting (Matheson and Lindgren 1985), and typically is less expensive than producing and planting seedlings (Foster 1986; Lambeth et al. 1994).

Our informal survey of poplar breeders found that the short-term strategy of wide crossing within and among species, selection, and clonal deployment (figure 1) will be widely used until more sophisticated strategies are deemed necessary and economical. This strategy is lucrative, and the advantages are adequate, especially in view of the associated low cost.

Long-Term Strategies

Most breeders acknowledge that short-term strategies will not pay returns forever and that greater gains are possible from simple recurrent selection within or among species or from reciprocal recurrent selection using 2 or more species. Long-term strategies using such approaches have been advanced by Lambeth et al. (1994), Bisoffi and Gullberg (1996), Stanton (1995), and Li (1995, pers. comm.) and emphasis gradually is shifting toward their implementation. Theoretical arguments favoring such strategies have been presented by Foster (1986, 1993a) and White (1995).

Simple recurrent selection involves selection, mating, and testing of a limited number of parents within a single breeding population (figure 2). Breeding cycles are completed by selecting parents for subsequent generations based on progeny tests of each preceding generation. Simple recurrent selection is easier and less expensive to implement than reciprocal recurrent selection and is well-suited for capturing additive genetic variation. Reciprocal recurrent selection involves selection, mating, and testing a limited number of parents from 2 or more parallel populations; often different species within the genus (figure 3). Parents are selected within populations to produce subsequent generations, while propagation populations are created by matings among populations in each cycle of improvement. Reciprocal recurrent selection is well-suited for creating interspecific hybrids, capturing both additive and nonadditive variation, and reducing inbred materials in commercial populations.

Long-term strategies (figures 2 and 3) call for amassing large, but not overly burdensome, base populations selected from natural stands, earlier breeding work, and/or collections from other breeders. Parents, regardless of generation, may be divided into sublines. Productivity is typically emphasized across generations, but individual sublines can be manipulated to maximize improvement in specific traits, develop complementary phenotypes, and maintain overall genetic diversity (Stanton 1995). Individual organizations undoubtedly will have differing needs and constraints, and are therefore likely to pursue divergent objectives and strategies (Bisoffi and Gullberg 1996). Traits most likely emphasized within sublines include wood properties, disease resistance, bole form, canopy architecture, and reproductive potential. Sexual reproductive capabilities can be stressed in programs relying on reciprocal recurrent selection as recovery of hybrid seedlings is sometimes limited (Stanton 1995; Stanton and Villar 1996; Stettler et al. 1996b). Ease of vegetative propagation can also be emphasized.

Matings within sublines can be arranged as disconnected factorials, with provisions for assortative mating (Foster 1986; Stanton 1995). Parents within factorials and sublines can be grouped according to breeding values estimated from clonal trials of offspring, the best being mated with best in descending order until all selected parents are used. Factorial mating schemes are preferred since they yield reasonable numbers of families from many parents and require relatively few crosses per parent. Inbreeding can be tolerated within sublines but propagation populations, with some exceptions, are derived from crossing among sublines (Bisoffi and Gullberg 1996).

Early and step-wise testing and selection can also be used to reduce numbers of clones tested in field trials. Thus, a several step screening process can be implemented, with each trial eliminating some families from further testing. Overall costs would be reduced since many families would be eliminated in the earliest and least expensive tests (van Buijtenen and Lowe 1989). Poplar breeders are likely to use an initial screening for survival and growth in seedling nurseries. Subsequent evaluation for rooting ability,

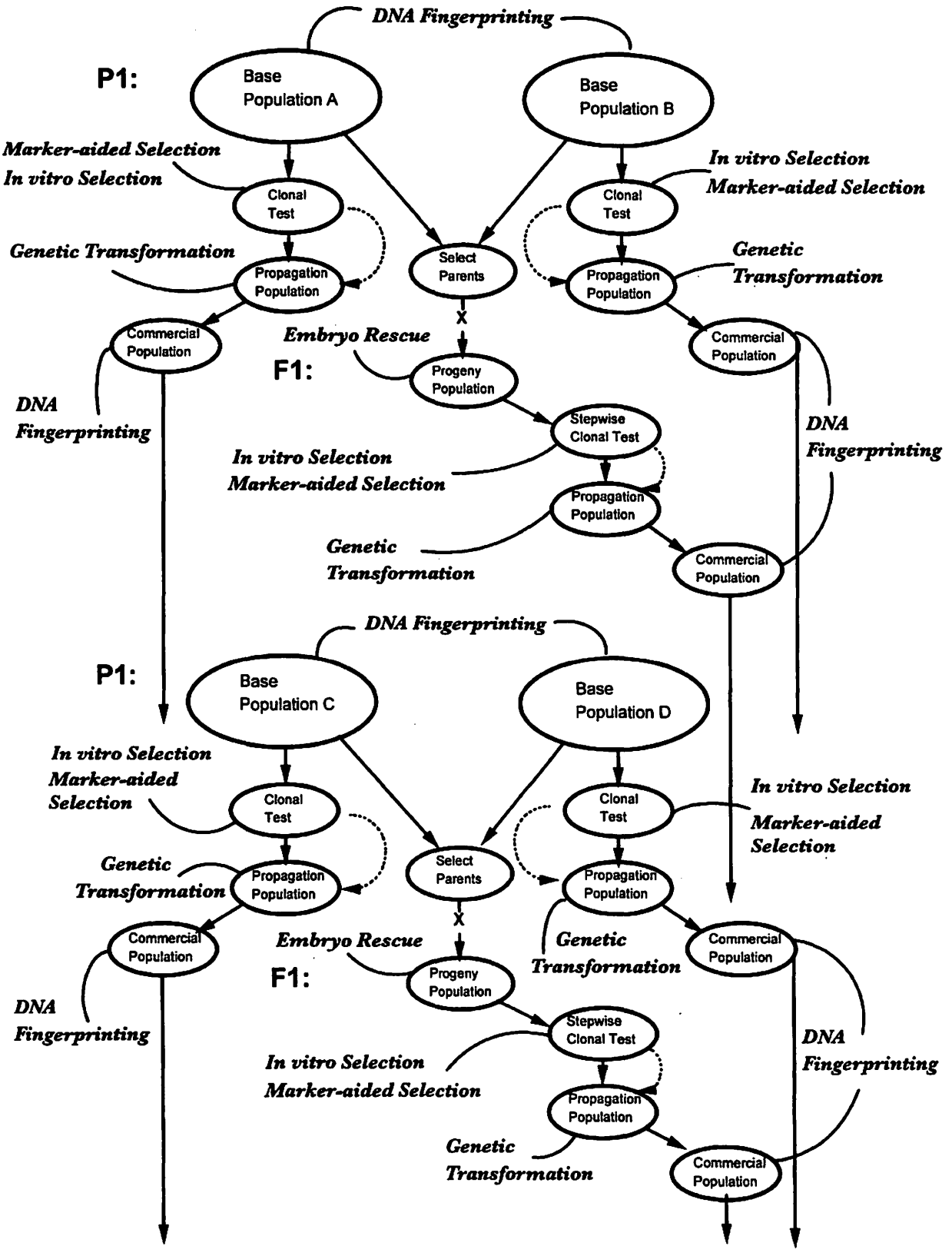


Figure 1. Short-term, *de novo* selection and wide crossing among diverse base populations and/or species. Solid lines=plant material movement; dashed lines=information flow; curved lines=application of molecular genetics techniques.

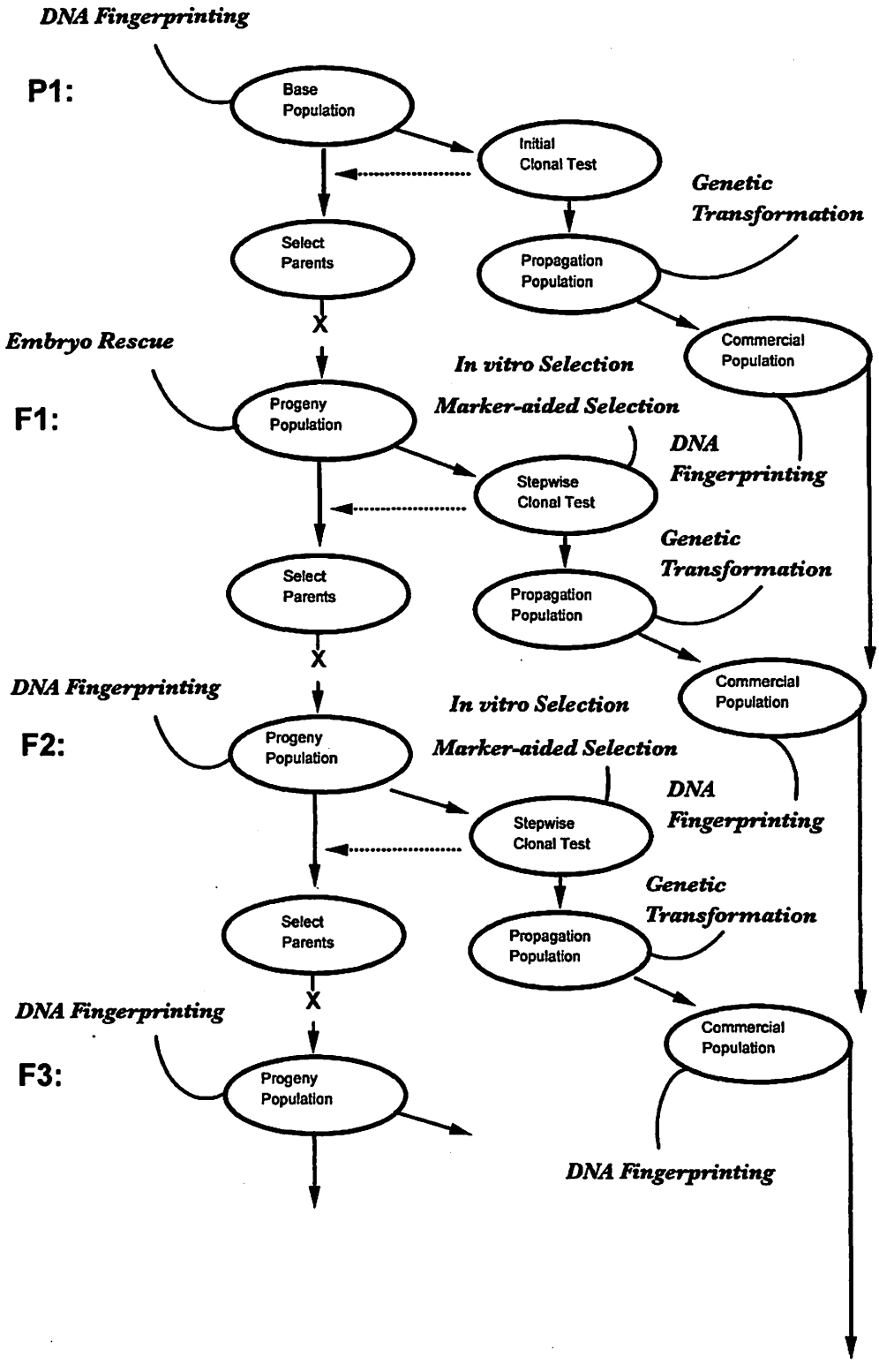


Figure 2. Long-term simple recurrent selection, where initial base populations may consist of one or more species. After the first selection cycle, breeding or parental populations generally are closed, with further selections derived from advanced generation progeny sets. Solid lines=plant material movement; dashed lines=information flow; curved lines=application of molecular genetics techniques.

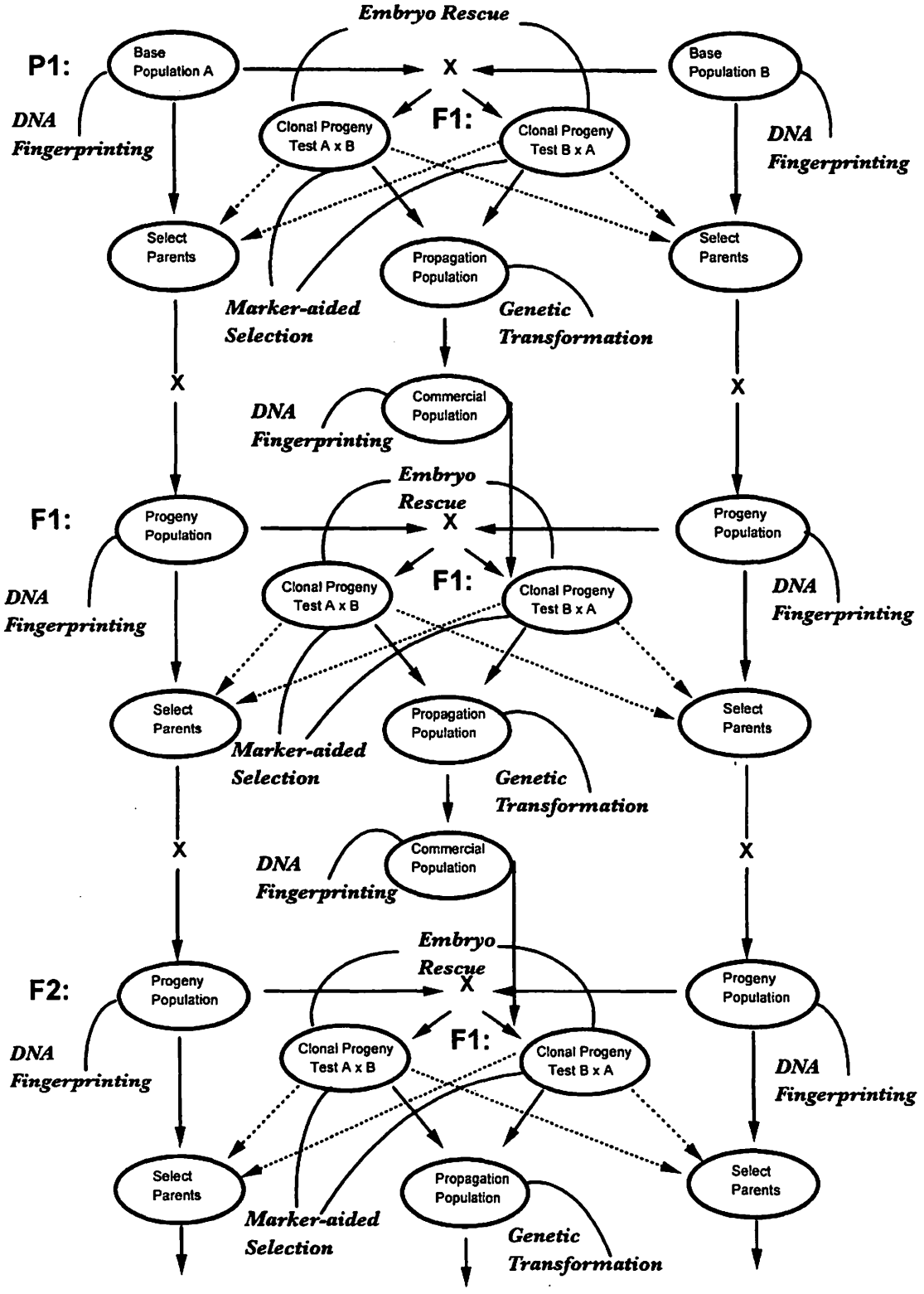


Figure 3. Long-term reciprocal recurrent selection, where inital base populations may consist of one or more species. In each generation, the alternate populations serve as tester parents for creation of new F_1 hybrids. Each commercial population contains newly created F_1 hybrid progeny. Solid lines=plant material movement; dashed lines=information flow; curved lines=application of molecular genetics techniques.

growth, and other traits (e.g., pest resistance) could occur during greenhouse or field multiplication. Later testing for growth, pest resistance, and propagule yields could occur in cutting nurseries. Only the few clones judged as superior in such tests would be evaluated in more costly growth and yield trials.

Parental or breeding populations and continued cycling are intended to exploit additive genetic variation, although correlations between average seedling and clonal performance may be imperfect. Added precision offered by clonal trials, however, can partially offset discrepancies. Quantifying the extent of such correlations remains a vital research need. Individual parents for subsequent generations may be identified via combined family and within family selection. This approach should improve average breeding population quality (i.e., exploit additive genetic variation), while capturing new combinations associated with nonadditive variation.

Propagation populations can be drawn from this same composite of families, seedlings, and clones. Selection of the most outstanding individuals, despite family background, exploits both additive and nonadditive variation. Thus, individuals moved to propagation and commercial populations are selected for their total genetic worth and are not necessarily the same as those selected for breeding. For commercial use, large numbers of related individuals are unlikely if parental or breeding populations are of sufficient size. Should significant relatedness occur, diversity can be maintained by deploying related materials in different planting areas or years.

Strategies for deploying clones in commercial plantings are beyond the scope of this chapter, but are nevertheless a critical issue. Debates continue over the adequate clone number for protection against biotic and abiotic stresses, along with advantages and disadvantages of pure versus mixed plantings. Poplars, whether manipulated by classical breeding and/or molecular techniques, seem ideal for performing this much needed research. Some recent findings can be found in papers by Foster (1993a, 1993b), Knowe et al. (1994), Foster and Knowe (1995), Bishir and Roberds (1995), and DeBell and Harrington (1997).

Integration of Molecular and Classical Genetics

Molecular techniques, ranging from vegetative propagation through genetic markers, genetic mapping, and gene transfer, have parallels and counterparts in classical breeding. Integration is best discussed by emphasizing parallels, then describing how techniques can be applied in each classical breeding phase, what benefits can be expected, and how the approaches are likely to benefit each other.

Acquiring and Infusing Genetic Variation

Acquiring a broad genetic base at the outset of breeding is necessary to minimize reductions in genetic variability over subsequent generations. Additional variability can be infused later by introducing new materials from natural populations, but this will dilute gains captured in preceding generations. Thus, later introductions should be helpful primarily to breeders using short-term strategies such as those in figure 1.

Molecular techniques (e.g., genetic transformation) can produce or ensure new variation but are best employed in short-term strategies. Long-term strategies (figures 2 and 3) could benefit most from application to already improved materials in the mating phase. Potential applications are discussed below.

Breeders spend considerable time and resources defining the genetic architecture of candidate species. While molecular techniques (e.g., DNA fingerprinting) will not replace provenance and progeny tests, they can yield useful information faster, more powerfully, and more precisely than the isozyme methods once used to study genetic architecture (Burdon 1994). Thus, DNA fingerprinting can be used to maximize variation at the initiation of breeding (figures 1 to 3). This technique can also confirm that prospective parents are of the preferred species, provenance, hybrid background (Lin et al. this volume), or gender. With further research, such techniques might be used to determine capacity for sexual and vegetative propagation and for the genetic basis of heterosis. Also, the parents likely to yield offspring with high levels of heterosis.

Gauging impacts on efficiency is difficult, but most molecular applications should raise the quality of initial breeding materials. Also, avoiding errors (e.g., misidentification) will save considerable time and effort.

Selection

Molecular approaches that parallel selection in classical breeding programs, whether following short- or long-term strategies, include direct measures such as inducing and selecting somaclonal variation and indirect ones based on genetic maps and markers (figures 1 to 3).

Selection can be performed in cell and tissue cultures, if the desired trait is expressed *in vitro* and outcomes are directly correlated with field performance. Cultures representing thousands of genotypes can be exposed to a selective agent or environment in modest laboratory facilities. Candidates identified as possessing the trait can be moved quickly into breeding populations. Given reliable culture systems, useful variants could also be trans-

ferred directly to propagation populations. Recent advances in somaclonal variation and selection of *Populus* are reviewed by Fry et al. (this volume) and Ostry (this volume).

Indirect approaches, such as DNA fingerprinting, might be used during the selection phase for some of the same purposes described above (i.e., confirming identity, sex, and reproductive potential). Genetic maps and markers can promote selection of superior genotypes. As previously noted, a genetic map of hybrid poplars is being constructed to locate genes for various traits. Genetic markers, including QTLs, are being developed in the process (Bradshaw 1996; Bradshaw et al. 1994). Advantages of genetic markers include production in large numbers, Mendelian inheritance, and limited influence of environmental conditions or age. Numerous offspring could be evaluated early in life, with the best parents used for breeding and the best individuals transferred, quickly and directly, to propagation populations (Cervera et al. this volume).

Impacts of these several techniques on selection efficiency should be significant. Large-scale, *in vitro* selection can magnify heritabilities and selection differentials and yield time and cost savings. Instead of selecting the best 10 of 100 entries in a field trial, *in vitro* selection could yield similar numbers (from 10 or 100 times more candidates) at earlier ages and in less time. Genetic markers could have similar, possibly greater, impacts. Increased heritabilities and selection differentials from either of these approaches could magnify genetic gain per generation. Gains per unit time would be increased by evaluating more individuals earlier than possible with classical testing procedures. Finally, savings in time and expense should result from culling undesirable or misidentified candidates before they are used for mating.

Marker-aided selection clearly holds much promise, but widespread application awaits verification trials. One to several generations of full-sib offspring are required to develop markers, and extensive collaboration among molecular and classical geneticists is necessary to create useful populations. Since development is costly, sound information on economic advantages compared with classical selection is also needed. Commercial application may occur only if costs and risks are shared via regional, national, and/or international consortia, or if costs of accepting an undesirable phenotype exceed those of marker development and application. Poplar species, with their relatively short times to reproductive maturity and harvest, seem ideal for demonstrating the merits of such techniques.

Mating

Classical breeders recombine and/or add useful genes via intra and interspecific matings. Several molecular techniques parallel and complement these activities, while offering significant advantages in the mating phase. Techniques for gene addition and recombination, however, may provide the greatest advantage. With these new tools, breeders can actually enhance variability rather than merely work to reduce its decline over generations.

Somaclonal variation represents a potentially useful technique for increasing variation. Whether by actual mutation, gene and chromosome rearrangements, or activation of repressed genes, variation has been identified under a variety of cell and tissue culture conditions. After appropriate testing, useful variants can be incorporated into breeding and/or propagation populations.

Although the somaclonal variation approach has potential for enhancing variability and saving time, it also has several drawbacks. Behavior in culture may or may not correlate with that in commercial plantings. Somaclonal variation is viewed as essentially random, thus it may not occur reliably for traits of interest. Useful genes may be linked to undesirable traits and epigenetic effects may accrue. Eliminating such unwanted effects could require generations of selection and breeding. In addition, considerable time and expense may be required to confirm utility, stability, and heritability (Stack 1987). Application to poplars, however, seems more feasible than with other forest tree species. Many poplar variants can be produced in culture and evaluated within 3 to 5 years, which is half the harvest rotation age for many poplars and within which reproductive maturity can sometimes occur.

Somaclonal variation seems a useful addition to classical breeding, and more research on its causes and control seems warranted. For the near term, however, application seems best during selection of propagation populations. Exposing cells and/or tissues from desirable individuals to directed and stringent *in vitro* selection to induce variation is potentially more efficient than large and lengthy field trials.

Several other cell and tissue culture applications that parallel the mating function also have promising potential. Reliable procedures for embryo rescue can enhance seedling recovery from interspecific crosses increasing genetic variability and selection differentials (figures 1 to 3).

In vitro production of homozygous or dihaploid plants would promote selection for native and inserted recessive genes and yield pure lines or true-breeding hybrids more quickly than repeated backcrossing. Research on this promising technique seems worth pursuing.

Protoplast or cell fusion might be used to combine genes and/or cytoplasms from unrelated or sexually incompatible species in special purpose hybrids. Asymmetrical derivatives regenerated from culture may serve as bridges for wide crosses among distantly related species. The dioecious nature of poplars and limitations on crossing between members of different subgenera encourage the use of such techniques. While not presently routine, protoplast and cell fusion are promising for future application. Given their ease of manipulation in culture, poplars can

serve as models for developing methods and demonstrating use.

When genetic variation is minimal or nonexistent, useful traits sometimes cannot be acquired, let alone improved. In such situations, genetic transformation (figures 1 to 3) may enable breeders to enhance genetic variation via gene transfer from other genotypes, tree species, or unrelated organisms. Bypassing the sexual process allows change to occur in a directed manner and in less time than required for reproductive maturity.

Exploiting gene transfer technology during the mating phase requires collaboration between classical and molecular geneticists to ensure that transferred genes are expressed in the acceptable tissue or organ at appropriate times during the life cycle. In addition, collaboration is needed to monitor spread of the gene throughout recipient populations and stability across generations. Transformation using antisense technology may require even longer and more expensive testing.

As noted by Meilan and Strauss (this volume), the most significant drawback of gene transfer is the potential for genetic contamination of native germplasm via pollen or seed cast by transgenic plants. Indeed, current and foreseeable regulations governing release of transgenic plants into the environment may prohibit sexual reproduction of transgenic trees. Thus, sterility should be among the first traits modified via gene transfer.

Application also requires availability of genotypes with proven silvicultural value. Availability of improved genetic backgrounds depends on a thriving classical breeding program. Additionally, the ultimate use of transformants requires a population with sufficient genetic diversity to minimize vulnerability to the many risks in commercial plantations. Transferring genes into all breeding materials could be prohibitively expensive. Until more is known about gene expression, stability, and safety, transformation of individuals only for propagation populations may be the best way to use this particular technology. This approach could also yield significant side benefits; eliminating reproductive structures could divert more photosynthate toward vegetative growth (Meilan and Strauss this volume).

Genetic marker and DNA fingerprinting techniques could also be helpful in the mating phase (figures 1 to 3). As in other phases, these techniques could confirm that prospective parents are of the desired origin, identity, sex, and reproductive capacity. In long-term programs, important applications include ensuring balanced representation of males and females in sublines, monitoring inbreeding within sublines, tracking overall diversity across generations, and verifying hybrids. When genetic transformation is involved, such techniques could be used to verify transformation, confirm persistence of inserted genes across generations, and monitor fidelity for other traits.

Genetic markers might also further interspecific hybridization and backcrossing. While desirable genes can often be obtained via hybridization, backcrossing usually is necessary to concentrate them while diluting or eliminating linked genes that are unwanted. Backcrossing forest trees, however, is an extremely long and costly process. Genetic markers could ease the task by identifying individuals possessing the desired trait and perhaps eliminating less desirable features at early ages (Bernateky and Mulcahy 1992). Matings could then be restricted to individuals known to possess the trait. This application combined with measures to hasten reproductive maturity could increase the benefit of backcrossing in tree breeding.

Despite some drawbacks, molecular techniques clearly have value in the mating phase. First, they have potential for increasing variability and ensuring continued diversity across generations. Second, novel genes not otherwise available in forest trees can be acquired independently of the sexual process preventing undesirable genes from accompanying desirable ones during mating and/or backcrossing. In addition, genetic markers can be used to reduce errors and improve efficiency.

Impacts of introducing previously unavailable genes are difficult to quantify in terms of genetic gain. Outcomes, undoubtedly, will vary greatly with affected traits. Qualitative assays, however, can be made by comparing projected before and after performances. As one example, genetic gain would approach or exceed 100 percent if individuals altered by somaclonal variation or gene transfer survived pest attack while unaltered individuals succumbed. Alternatively, transfer of genes affecting lignin quality and/or quantity (Boerjan et al. this volume; Chiang et al. 1994) may produce relatively small changes. Nevertheless, overall impact could be considerable because measurable change would be obtained within a single generation. Obtaining similar changes via classical breeding would require many generations since genetic variation in lignin content is small and heritabilities are modest at best.

Propagation and Production

Efficient systems for vegetative propagation are essential for using molecular genetics and classical breeding (figures 1 to 3). A variety of propagation methods, ranging from nonrooted cuttings through micropropagation or somatic embryogenesis, can work with poplars. Each approach, however, has specific applications for which it is best suited. For example, nonrooted cuttings are useful for establishing commercial plantings, but are not suited for genetic transformation. In contrast, somatic embryogenesis is useful for transformation, somaclonal variation and selection, and even cell and protoplast fusion. The high cost of organogenesis and embryogenesis, however, may limit their use for mass propagation. Currently, their use

is associated with genetic transformation and regeneration of a few transgenic plants for subsequent multiplication by other methods. As a practical example, regeneration from existing and adventitious meristems is often used to multiply improved materials for testing and further expansion in rooted cutting nurseries. Somatic embryogenesis techniques might also be used to salvage immature embryos that are not amenable to routine embryo rescue. If workable, this application would ensure that more offspring and parents are represented in clonal tests.

Genetic markers and related techniques also have important roles in propagation and production (figures 1 to 3). For selection and mating, genetic markers can be used to identify parents and offspring with high propensities for vegetative propagation. This would encourage moving improved materials into propagation and commercial populations and gradually improve capabilities in breeding populations. In addition, DNA fingerprinting can be used to confirm and maintain identities in propagation and commercial populations, monitor persistence of added genes, and prevent theft of valuable germplasm. Indeed, such techniques may stimulate breeders and growers to create and market "brand name" clones.

Besides the indirect benefits, vegetative propagation can produce at least 2 direct impacts on breeding efficiency and genetic gain. First, vegetative propagation can raise selection efficiency; more individuals from more families can be tested more precisely in less time. Second, all genetic variation, additive and nonadditive, is captured raising heritabilities. With greater heritabilities and selection differentials, gains and returns per generation can exceed those derived from programs relying solely on the sexual process. Breeders using either short- or long-term strategies (figures 1 to 3) can rely on vegetative propagation to generate materials for propagation and commercial populations.

As noted, poplars are easy to propagate, and propagation technology has improved in recent years. Nonetheless, problems persist, primarily for *in vitro* methods, as exemplified by unwanted physiological or genetic variation. As a result, derived plants may require lengthy and expensive field testing to ensure accurate reflection of donor qualities. Additionally, many procedures remain genotype-specific, thereby limiting selection efficiency and raising costs. When only a fraction of elite individuals can be propagated for commercial use, breeders must select, breed, and test more individuals or relax selection differentials. Improved techniques that are less genotype-specific, yet maintain genotypic and phenotypic fidelity, remain an important research goal.

Because capability for vegetative propagation is apparently heritable to some extent, selection and breeding to enhance such capability could yield significant dividends to both classical and molecular geneticists. Breeders would benefit greatly from improved efficiency, and contributions from their molecular counterparts would also be accelerated. Also, genes controlling ease of vegetative propagation may be transferable from other organisms into recalcitrant individuals. Collaboration could lead to synergistic outcomes.

Vegetative propagation is also limited by certain factors that restrict breeding efficiency. Selection in classical breeding programs typically requires lengthy and costly field trials. By the time genotypes are certified as superior, rooting frequencies can decline to inadequate levels. Though not as critical for poplars as for many other trees, such effects can be restrictive. Further research on rejuvenation would benefit poplar breeding efforts.

Discussion and Conclusion

Notable advances have occurred in development of molecular techniques. Application of molecular techniques may offset or reduce many biological and economic constraints on classical breeding. Nonetheless, molecular techniques present challenges and opportunities. Integration with classical breeding will require decisions concerning research priorities, applicability of particular techniques, and resource allocation. Implementing these decisions requires understanding the techniques and conditions under which they can be integrated advantageously and safely.

DNA fingerprinting will help characterize populations, estimate genetic variability within and among populations, and monitor diversity and structure of populations generated, maintained, and manipulated in the breeding process. Discerning and maintaining identity during selection, breeding, and propagation will improve overall reliability. Identification could open markets for and prevent theft of proprietary genotypes. Developing and using such techniques, however, is expensive. Generating populations with appropriate pedigrees requires suitable genetic resources and collaboration with classic breeders.

Genetic markers should lend new efficiency to selection. Large-scale, early selection would help offset constraints imposed by long generation intervals and harvest rotations providing greater genetic gain per unit time. Efficiency of interspecific hybridization could also be improved, since markers can be used to guide matings among parent species. Building requisite genetic maps and identifying useful markers, however, requires that breeders have resources to produce appropriately pedigreed populations. Validating marker value entails precise and lengthy field trials. In view of the associated high costs, research must expand use to more diverse, less specialized pedigrees, and find markers strongly correlated with traits of interest. Viewed from another perspective, costs could be prohibitive unless improved materials are used in large numbers over multiple planting years (Teasdale

1995). Regulations governing permissible minimum numbers of genotypes for commercial planting could worsen the cost-benefit outlook. Poplars were among the first species for which genetic maps and markers were developed; their continued use as models will aid refinement and extension to other tree species.

Genetic transformation offers potential improvement, both quantitatively and qualitatively, beyond classical breeding. Sexual sterility is a trait likely to be added via genetic transformation, since it could become a condition for commercialization of transgenic trees. Regulatory considerations aside, possession of this trait could divert energy from reproductive activities to biomass accumulation and further manipulation of improved materials on a scale larger than possible with classical breeding methods.

Ongoing research on transformation, especially with poplars, will probably yield useful genotypes. Meaningful exploitation, however, remains limited by a delay in identifying valuable genes. Most past efforts relied on genes used in agricultural crops. Valuable lessons were learned, but genes unique to trees may have greater value. This research is critical, especially with regard to organization, structure, and control of genetic systems. Existing knowledge of poplar biology emphasizes the value of poplars for such research.

Determining commercial potential of transgenic trees, sterile or nonsterile, will require extensive, long-term field trials to ensure that desired outcomes are realized and that undesirable effects are minimal or absent (Stack 1987). Transformation with antisense genes will require further research and possibly even more stringent testing. Whatever the approach, materials used for transformation must first be selected and bred for traditional silvicultural values. Having suitably improved and diverse materials available for transformation requires continued maintenance and manipulation of genetic variability.

New and improved systems for vegetative propagation could greatly assist classical breeding. Mass propagation hastens delivery of genetic gains and encourages application of techniques such as genetic transformation. *In vitro* embryo rescue and somaclonal variation and selection have additional potential for supplying new germplasm. Expanding these outcomes, however, depends on developing systems with minimal side effects and maximum applicability to the large array of materials routinely used in breeding programs. Exploiting improved methods, nonetheless, requires existence of genetic variability, and the ability to manipulate it.

Effective integration of molecular techniques requires the existence and maintenance of active classical breeding programs. Molecular techniques are unlikely to replace classical breeding, but instead will enhance the capabilities of breeders to solve practical problems. The opposite side of this issue must not be overlooked, however, since intensive collaboration among disciplines is essential. Integration demands recognition of common problems, raising relevant questions, and understanding the biological, genetic, and economic consequences. Provisions should be established to maintain or expand breeding efforts while also funding efforts to develop and implement useful molecular techniques. Developing individual techniques is costly, but overall costs will be magnified because use of one technique may demand application of others. For example, genetic transformation depends on vegetative propagation. Organizations seeking to exploit molecular techniques should be prepared to provide increased funding for both classical and molecular genetic programs, at least until molecular techniques produce cost savings and increased returns from practical applications in classical breeding.

Support of both classical and molecular efforts is wise. Gains from classical breeding have been substantial and further progress is certain. Reducing breeding efforts could offset the realization of future genetic gains over the near term, diminish potential for future gains, and preclude exploitation of molecular techniques. The more sophisticated the breeding strategy, the more benefit gained from technical integration. Alternatively, failure to fund development and application of molecular techniques could be equally ill fated. Integration holds such high promise that cooperative efforts could capture considerable and lasting advantage. Especially critical is the increasing likelihood that competitors will protect methods and products by securing intellectual property rights (Chandler 1995).

Balanced investments and research are required, with adequate support and funds for ongoing breeding efforts and the development of promising molecular techniques. Clear priorities, consistent goals, and stable support are essential to avoid one area of research outpacing another, with applications hindered because needed techniques are not ready at the appropriate time. With proper foresight and balanced support, useful molecular techniques can be devised and drawn into classical breeding, just as other disciplines (e.g., quantitative genetics) were incorporated earlier.

Likelihood of enhanced returns from technical integration seems more than sufficient to attract support within individual organizations. Prospects for lower overall costs may foster formation and maintenance of centers of excellence via research and development consortia (Griffin 1995). Prudent organization and astute management of cooperative endeavors could hasten research, development, and application. Open, flexible management is essential so that the pursuit of intellectual property rights by individual organizations does not interfere with technical integration or existing cooperative tree improvement programs. Hopefully, involved organizations will realize that overly aggressive pursuit of intellectual property rights can jeopardize collaborative efforts, and that costs of developing molecular techniques, properly integrating them, and covering risks of failure are beyond the capabilities of most individual organizations.

Wise investments coupled with collaboration within and among organizations are necessary to foster productive integration of genetic technologies. Classical and molecular genetics have many parallels and melding the best of each into a new synergistic system depends on mutual support. Only by increased commitment to overall tree improvement can both enterprises contribute to better management of and greater returns from forest tree resources. Individuals working with model species, such as poplars, can contribute much toward these important ends.

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Chapter 30

Application of Tissue Culture Systems for Commercial Plant Production[1]

Kathryn A. Louis and LoriAnn E. Eils

Introduction

Tissue culture propagation or micropropagation in the commercial production of *Populus* species for large scale clonal plantings has limited use due primarily to the high cost per plantlet, especially when compared to species that are readily cloned using cutting propagation. However, two more practical applications of tissue culture propagation of *Populus* species are: 1) propagation of those species that do not readily propagate via other less costly methods (e.g., aspen and hybrid aspen); and 2) multiplying stock plants of newly developed or released genotypes with limited availability. This chapter discusses methods used to clone *Populus* species including: 1) traditional shoot-tip/axillary bud culture; 2) recutting or hedging mini-plants; 3) root suckering *in vitro* and *in situ*; and 4) leaf micro-cross section (MCS) technology.

Traditional Shoot-Tip/Axillary Bud Culture

An excellent bibliography about tissue culture and cell culture of *Populus* species was compiled by the USDA Forest Service (Ostry and Ward 1991). Readers are encouraged to review papers listed in that document. A brief review of the method used at Minn vitro, Inc. is described below and illustrated in figure 1.

Stock plants, which provide explants, are obtained as small plants or dormant branches. Plants are grown indoors under a cool-white, fluorescent (CWF) photoperiod of 16 h until new shoot growth occurs. Dormant branches are stripped of existing leaves, and proximal branch ends are recut and placed in water under a 16 h photoperiod. Branch ends are recut every 2 to 3 days at the time of water replacement. After 2 to 4 weeks, new forced growth occurs. In Minnesota, dormant branches can be successfully forced beginning in March through normal bud break (early May) and again in August through early November. Explant material is not usually field collected because of difficulties eliminating fungal spores and other contaminants on field-grown material.

To initiate *Populus* species cultures, shoot-tip stem cuttings (approximately 2.5 to 7.5 cm long) are collected from actively growing plants or forced branches. Existing leaves are removed and stems with terminal and axillary buds are surface disinfested using a standard commercial bleach

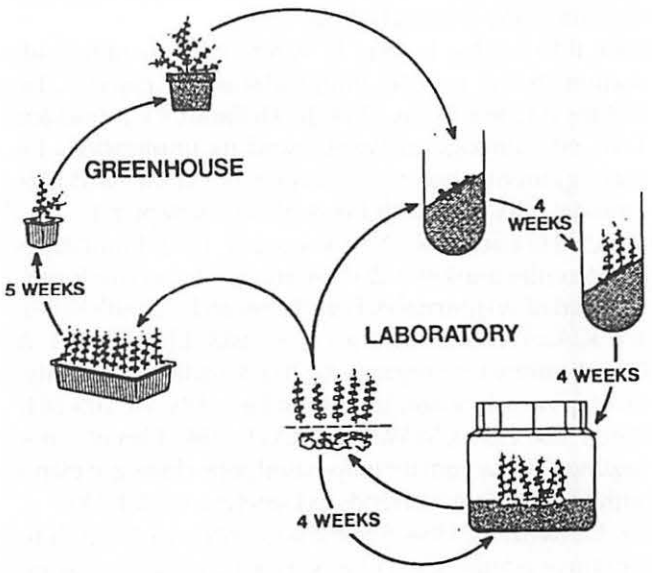


Figure 1. Representation of a traditional shoot-tip/axillary bud culture method used at Minn vitro, Inc.

[1] Klopfenstein, N.B.; Chun, Y. W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

solution (10 percent for 10 min), followed by 3 sterile water rinses. Explants are aseptically cultured on Murashige and Skoog (MS) (1962) basal medium supplemented with 3 percent sucrose, 0.1 to 0.5 mg/l benzyladenine (BA), and 5 g/l agar with a preautoclave pH of 5.75. The culture cycle is 4 weeks with a CWF photoperiod of 16 h. High BA concentrations are used for multiple shoot production during the proliferation cycles, followed by reduced BA concentrations for shoot development before harvest. Although most literature reports the use of Woody Plant Medium (WPM) (Lloyd and McCown 1980) as the basal medium, we have found that MS medium produces superior microshoots (data not shown). During the process of harvesting microshoots from the culture vessel, small microshoots (less than 2.5 cm) and proliferating clusters are transferred to fresh medium for later harvest.

For *ex vitro* rooting, microshoots that are 2.5 cm or longer are harvested and placed in a standard 288 seed germination plug covered tray (trimmed to fit into a standard 1020 flat). The plug tray and flat are covered with a clear dome and placed under a CWF 16 h photoperiod. The plug tray is filled with a rooting medium composed of peat:perlite:vermiculite (1:1:1). All preformed *in vitro* roots are removed before *ex vitro* rooting. (In our experience, *in vitro* formed roots of *Populus* species did not survive after transplanting into peat-based rooting medium. New adventitious roots developed at a slower rate than from microshoots without preformed roots.) Visible roots usually form within 10 days. After 3 weeks of rooting, we recommend that weekly fertilization begin at 200 ppm nitrogen, using a 20:20:20 (N:P:K) formulation. At approximately 4 weeks, acclimation is accomplished by propping open the clear dome slightly for the first day, then increasing the opening each day, until day 7 when the dome is completely removed. Plantlets are then transplanted to larger containers and placed in the greenhouse or nursery bed with 70 percent shade for the first few days. The amount of shade is gradually decreased over a 7-day period, then fertilizer applications can be doubled.

This traditional shoot-tip/axillary bud culture procedure is based on a plant biology that allows existing shoot tips and axillary buds to develop and continue initiation of new shoot tips and axillary buds. These shoot tips and axillary buds then elongate to produce microshoots. This cycle can be repeated indefinitely, provided that transfers occur in a timely manner. Reducing the detrimental effects of systemic bacteria that can become evident over time can be accomplished by a more rapid transfer cycle (every 2 to 3 weeks), using only shoot tips (1 to 2 cm), and/or the addition of antibiotics to the medium. (Based on research by Young et al. (1984) and our in-house research, we found that a combination of 25 mg/l cefotaxime, 25 mg/l tetracycline, and 6 mg/l rifampicin will successfully suppress bacterial growth in most *Populus* species cultures.) Other problems are low rooting rates and poor acclimation for some genotypes. Our overall success rate has been that approximately one-third of the genotypes are readily cloned, one-third of the genotypes are cloned with difficulty, and one-third of the genotypes are not amenable to this method.

Recutting or Hedging of Plantlets

Although this method is not an *in vitro* method, it is worth mentioning because it is often very successful when

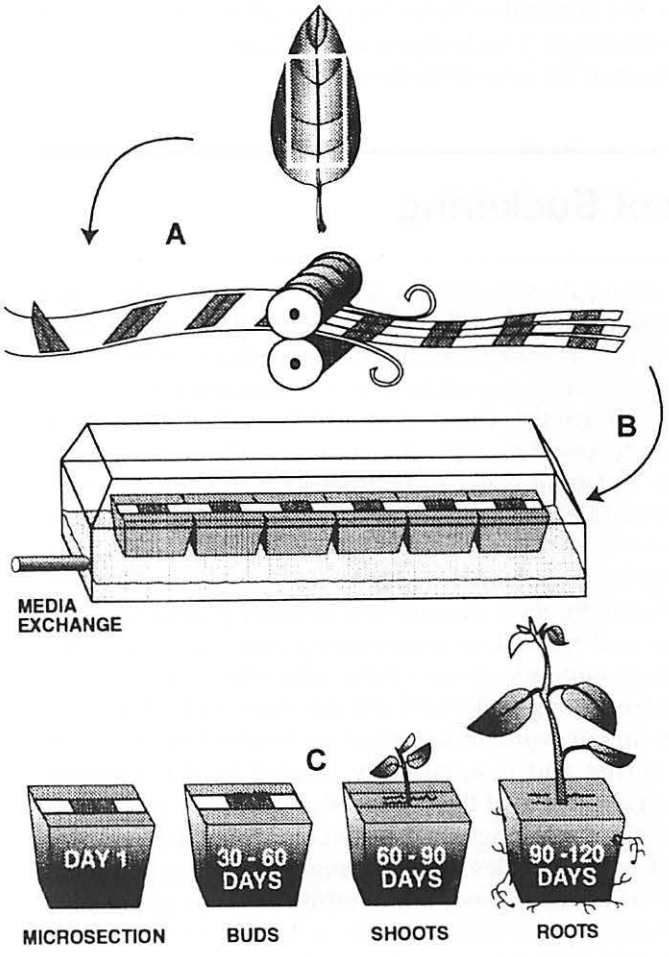


Figure 2. Schematic representation of Micro-Cross Section (MCS) Technology (courtesy of Minn vitro, Inc.). A) Leaf tissue is placed on a tape carrier and sliced into strips. B) The leaf tape strip is placed on a support substrate. The culture medium is liquid (no agar) and the culture vessel has flow-through medium exchange as required. No physical transfer of the explant is required. C) Leaf explant initiates and develops adventitious buds that form shoots, followed by rooting. For details of this method, see Louis and Eils (1994).

tissue cultured plantlets are used. Plantlets that are produced as previously described can be grown in a greenhouse or growth chamber until their main shoots are 8 to 10 cm in height. Cuttings are then collected from these mini-stock plants and rooted as previously described. The remaining portions of the mini-stock plants continue to grow via axillary shoots, which can also be used as mini-cuttings once the axillary shoots are 6 to 8 cm in length.

Mini-cuttings produced in this manner usually root at a higher percentage than those from tissue culture (data not shown). A likely reason for this rooting increase is that mini-cuttings have a more developed cuticle. Thus, although mini-cuttings are less fragile, they apparently maintain the juvenile characteristics of tissue-cultured microcuttings. This hedging method can be successfully performed for several flushes.

Root Suckering

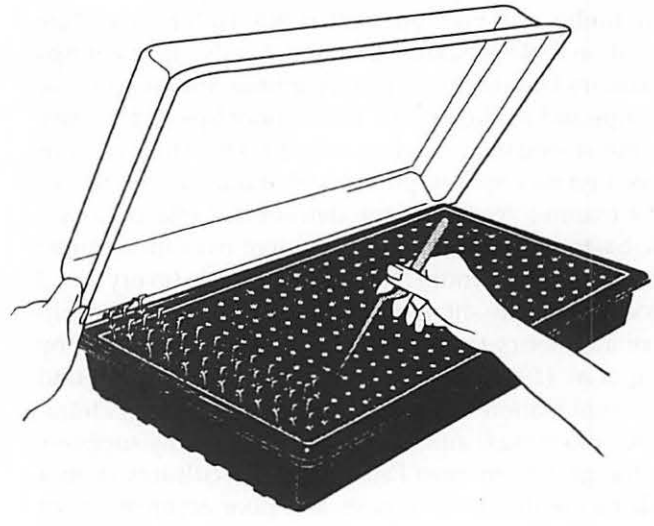
Cloning *Populus* species using root suckers has been successful for species that naturally propagate in this manner (e.g., *P. tremuloides*). The field / greenhouse method begins with digging up roots (1 to 3 cm in diameter and 15 to 20 cm in length). These roots are cleaned with soap and water, followed by a bleach treatment (full-strength for 10 min), and a tap-water rinse. Root segments are then buried in sand and maintained under moist conditions. Within 2 to 4 weeks, shoots develop from preformed and adventitious buds within the roots. When the shoots are 4 to 6 cm in length, they are removed from the parent root segment and treated as mini-cuttings (as previously described). Papers by Schier (1974, 1976, 1981) provide more detail regarding preformed and adventitious root-derived buds. In our work, this method seems most successful in the spring and is apparently limited by the inherent suckering ability of the parent plant. A major problem is correctly identifying which roots belong to what tree. This problem can be alleviated by cloning the identified plant with the usual methods (e.g., cuttings, tissue culture, etc.) then growing the cloned plants in large containers until roots of sufficient size are obtained.

An *in vitro* version of this method was developed at the University of Minnesota (Hanson et al. 1992; Louis et al. 1992a, 1992b) in which *in vitro* growing shoots were treated to induce roots (i.e., BA was removed from the medium). These *in vitro* roots were then cut into 0.5 cm or longer segments and returned to culture. Adventitious shoots were initiated and developed from the root explants cultured on MS medium supplemented with 0.01 to 1.0 mg/l thidiazuron (TDZ). Our attempts to produce large qualities of roots by growing roots without stem tissue were unsuccessful. Although *in situ* root suckering has been used to clone some aspen genotypes, to our knowledge, the use of *in vitro* root suckering as a commercial method to clone plants has not yet occurred.

Micro-cross Section Technology

Micro-cross section (MCS) Technology is a method used to clone plants based on adventitious bud initiation and development from leaf tissue. This method was initially developed by researchers at the University of Minnesota (Lee-Stadelmann et al. 1989). Very small (400 µm to 3 mm) leaf segments are placed onto MS medium supplemented with 3 percent sucrose, 0.8 mg/l BA, and 0.01 mg/l α-naphthaleneacetic acid (NAA). After 4 weeks, adventitious buds begin to form. For microshoot development, the explant is transferred to medium supplemented with a reduced BA concentration (0.1 mg/l) and without NAA. As microshoots develop, they are harvested and rooted as previously described. Because very small pieces of leaf tissue can be used, a large number of explants can be generated for adventitious shoot production. Thus, a proliferation stage is hardly needed and a large number of plants can be produced in a short time.

The uniform size and shape of the explants makes this process amenable to robotics. Continued refinement of this technique along with development of automated handling equipment would probably close the cost gap between tissue culture propagation and seedling propagation. Additionally, the semi-solid medium can be replaced with a liquid medium if a support substrate is used. Five "off-the-shelf" substrates can be used to replace agar: 1) cotton balls; 2) cotton cosmetic rounds; 3) Grodan® rock wool; 4) Isolite® soil amendment; and 5) Sorbarod® cigarette filters. This has led to the envisioned MCS Technology illustrated in figure 2. The results of our continued effort to



develop MCS Technology are summarized in a TAPPI Biological Symposium paper (Louis and Eils 1994). Forty-five different *Populus* genotypes were tested, of which *P. tremuloides* x *P. tremula* (hybrid aspen) genotypes were the most responsive. These results are most promising because hybrid aspen plants do not readily propagate using other asexual methods.

The first commercial trial of MCS Technology was a 10-times scale-up of our research size (i.e., 10 times our standard 100 micro-cross sections), which was replicated for a total of 2,000 micro-cross sections. This scale-up resulted in reduced microshoot numbers per leaf section and extensive fungal growth during acclimation (data not shown). This reduced production was likely caused by using leaves of a less than optimal developmental stage. Fungal growth during acclimation was apparently caused by the residual medium in the liquid support substrates. Even though the substrates were flushed with water before acclimation, adequate sucrose/nutrients apparently remained to support fungal growth.

Discussion

Populus species tissue culture propagation can be classified as either preformed (terminal or axillary) or adventitious meristem culture methods. Preformed meristem culture is the traditional method in which shoot tips with existing meristems are grown in an environment that enhances the production of more meristems at a much faster rate than occurs on a whole-plant basis.

Adventitious bud initiation and subsequent development of microshoots captures the plant's totipotent biology. For example, using *in vitro* root suckering and MCS Technology, a cell, or a small group of cells, divides and differentiates into a new meristem that subsequently develops into a microshoot. Adventitious bud initiation has been used only on a research basis at our laboratory. MCS Technology has shown the greatest potential for producing hybrid aspen at a lower cost than traditional shoot-tip culture.

The most commercially successful method is the traditional shoot-tip/axillary bud method. We have used this method to mass produce 45,000 hybrid aspen for a pilot field planting and 10,000 aspen for horticultural use. We have also used traditional shoot-tip culture to clone unique or rare plants to increase stock plant numbers for cutting propagation.

Generally, regardless of the tissue culture propagation method used, differences occur among *Populus* species and genotypes within a species. Our results have followed the one-third:one-third:one-third "rule" of success; approximately one-third worked very well, one-third worked moderately well, and one-third did not work at all.

Acknowledgments

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Chapter 31

Economics of Producing *Populus* Biomass for Energy and Fiber Systems[1]

Charles H. Strauss and Stephen C. Grado

Introduction

The economics of producing *Populus* biomass is a fairly uncomplicated concept. Some set of inputs, usually land, labor, and capital, is required in the biomass output production. Time is an integral part of this process, and the use of these inputs over time must be considered an investment proposition. Variability in the types and amounts of inputs, length of time, and quantity of output complicates the analysis. Much of the information presented by this book should be factored into the economic analysis of *Populus*.

This chapter provides a general model of *Populus* production and its use in ethanol manufacture. Major components of the model were developed from research sponsored by the U.S. Department of Energy's Short Rotation Woody Crop Program (SRWCP). The model considers the financial and energy costs of producing *Populus* biomass at the plantation sites, and the added costs of harvesting, transporting, and storing the biomass. The resulting raw material costs apply to the conversion of *Populus* biomass for either energy or fiber products.

An expanded model on the conversion of *Populus* biomass to ethanol is in the final section of this chapter and includes all of the production costs, from the plantation site to the final product. Overall, these models are more important in identifying key components of the production process than they are in specifying particular costs. They underscore the critical dimensions of the process and identify where further research and development is needed.

[1] Klopfenstein, N.B.; Chun, Y.W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

Basic Design for *Populus* Plantations

Populus models by Strauss and Wright (1990) and Strauss and Grado (1992) were developed to consider a plantation system established on high quality agricultural sites at a density of 2,100 trees/ha (approximately 2.2 m^2 spacing). Rotation length was from 5 to 8 years, with 2 or 3 rotations anticipated from any given planting. The optimum rotation was selected on a least-cost basis using a discounted cash-flow analysis (Strauss et al. 1990).

Similar to agricultural row crops, plantations were established with a fall and spring planting site preparation and a spring planting of the poplar cuttings. At the onset of the fall season, the site received a total-kill herbicide and mowing operation to remove old field vegetation, followed by plowing (table 1). Lime was added at this time, depending on soil acidity. In the spring, the soil was harrowed, with a pre-emergent herbicide to counter residual weeds. To supply an adequate nutrient base for tree growth, phosphorus and potassium were added before planting. However, nitrogen was not applied until the third and fifth growing seasons to avoid augmenting weed growth in the first 2 growing seasons. Machine planting of the poplar cuttings was assumed within the cost structure of the model.

Additional herbicides were scheduled at the beginning of the first 2 growing seasons to counter potential weed growth. Protection from insect and canker damage was through a biennial insecticide/fungicide spray program. This was a preventive cost against infestations, such as cottonwood leaf beetle (*Chrysomela scripta*) and Septoria canker.

Annual charges were assessed for land rent, property taxes, and managerial supervision of the production effort. These requirements represented specific inputs to the production function.

Harvesting and transportation strategies were developed from previous studies (Stokes et al. 1986; Strauss et

Table 1. Financial and energy costs for the establishment and maintenance of SRWC *Populus* plantations. Costs in U.S. dollars for 1995.

	$/ha	MJ/ha[1]
Establishment		
Fall/spring herbicides (3.3 kg/ha)	210	1,760
Mowing/brushing	24	748
Plowing/harrowing	56	2,547
Liming (1.0 Mg/ha)	49	289
Fertilization (60 kg/ha each P&K)	51	1,729
Planting (2,100 cutting/ha)	152	2,156
Summer herbicides, year 1 and 2[2] (2.2 kg/ha)	140	1,277
Total establishment costs	682	10,506
Maintenance		
Insecticide/fungicide (1.6 kg/ha/appl.)	36	878
Fertilization (120 kg of N/ha/appl.)	42	7,593
Land rent	102	49,820
Land taxes	16	7,620
Managerial	42	261
Approximate average annual maintenance costs	199	61,936

[1] Mega joule per hectare.
[2] Year 2 costs discounted at 5%.

al. 1988; Stuart et al. 1985). Harvesting equipment was designed for the small-diameter, closely-spaced SRWC plantations. After harvest, the biomass was either chipped on-site or at the processing site. Transportation used was either tractor-trailers for delivery of chipped material or flatbed units for delivery of bundled tree stems.

Financial Cost Structure

The cost for each operation was developed from the SRWCP data base (table 1). Charges for contracting for the establishment and operation of commercial-sized plantations compared favorably to previous models of SRWC systems (Lothner et al. 1985; Perlack et al. 1986; Strauss et al. 1988). Cost differences within particular operations were attributed to the type of equipment and amount of material used. All establishment and maintenance costs reflected an agricultural site with good aspect and soil quality.

Establishment totaled $682/ha, including the discounted charge for second-year herbicides (table 1). Herbicide application represented 51 percent of establishment costs; the materials constituted over 90 percent of this expense. Land preparation (mowing/brushing, plowing/harrowing) and planting were 34 percent of the establishment costs; 54 percent of this expense was tied to the poplar cuttings. First-year liming and fertilization was the final 15 percent.

Maintenance included the application of nitrogen during the third and fifth growing season and the use of insecticides/fungicides on an alternate year basis. In addition, an annual managerial cost was assessed for administrative and operational needs (table 1). Land rent represented the opportunity costs, or annual net return, of a good corn production site (7.8 Mg of grain/ha/yr). The capitalized net value of the site at a 5 percent real rate of return was $2,040/ha. This was consistent with the land values used in previous SRWC studies and was 20 percent above the U.S. average for farm real estate (USDA ERS 1988). Annual property taxes were 0.75 percent of this land value.

Energy Cost Structure

The energy accounting system for the proposed SRWC plantations was patterned after other agricultural cropping systems (Pimentel 1980; Roller et al. 1980) and related studies of commercial-sized SRWC plantations (Strauss et al. 1989; Zavitikovski 1979). Equipment costs included the energy: 1) embodied in the equipment's basic materials; 2) employed in equipment fabrication; and 3) embodied in repair parts. Net energy consumption over an equipment unit's life span was 82 percent of its total energy (Pimentel 1980). The division of a unit's net energy consumption by its life span, in hours, provided an hourly equipment cost.

Machinery costs for any given task were the product of operating times and hourly equipment costs. Energy costs for fuel, related material inputs, and labor were added to their respective equipment tasks. Material input costs included the energy embodied in materials such as fertilizer, and the added energy required in its manufacture. Labor's energy cost was based on a net analysis of the energy required by humans as agricultural laborers (Fluck 1981) and was 75 MJ/h (75 mega joule per hour). Although this charge was higher than previous estimates, the energy cost of labor in most operations was small.

The energy cost of land was also organized as an opportunity cost, based upon the net energy secured from corn production (49,820 MJ/ha/yr). This energy value compared favorably to previous estimates of corn production in several U.S. regions (Pimentel 1980). The energy charge for property taxes was based upon the energy to financial cost ratio of land rent and the cost of taxes.

The energy cost of establishing SRWC plantations was 10,506 MJ/ha (table 1). Site preparation and planting was 52 percent of this expense, herbicides were 29 percent, and fertilization/liming was the final 19 percent. Site preparation and planting had proportionally larger energy costs than financial costs due to the higher energy charges for machinery and poplar cuttings. Annual maintenance was substantial because of the energy budgeted for land rent and taxes. The energy cost of poplar cuttings also reflected the land's energy potential, with 40 percent of the cutting cost tied to land use.

Plantation Yields and Unit Costs

A proposed yield was developed from the SRWCP data sets (Strauss and Wright 1990). The 2,100 trees/ha plantation was projected to have a maximum mean annual increment of 16 metric tons, oven dry, per hectare per year (Mg(OD)/ha/yr) by the sixth year.

Unit costs were estimated on a financial and energy basis using an investment analysis approach developed for SRWC plantations (Strauss et al. 1990). Under this approach, unit costs are estimated as a function of the discounted financial costs (or energy costs) of establishing and maintaining the plantation and the discounted plantation yields.

$$\text{Unit cost } (\$/\text{Mg(OD)}) = \frac{\underset{\text{discounted}}{\text{Establishment } (\$/\text{ha})} + \underset{\text{discounted}}{\text{Maintenance } (\$/\text{ha})}}{\underset{\text{discounted}}{\text{Yield } (\text{Mg(OD)}/\text{ha})}}$$

To avoid the time bias associated with longer rotations, the individual rotation lengths were analyzed as a perpetual series. A 5 percent real rate of return was used throughout the analysis, with the optimum rotation length selected on a least-cost basis.

Plantation Costs

Analyses of a two-rotation system placed the least-cost solutions at the sixth year, with unit costs of $19.06/Mg(OD) and 4,381 MJ/Mg(OD) (tables 2 and 3). If the plantations could sustain 3 rotations per planting, the least-cost solutions were still at the sixth year, with costs of $17.71/Mg(OD) and 4,360 MJ/Mg(OD). These reductions were attributed to the extended prorate of the establishment costs over an additional rotation. However, from an energy cost standpoint, the reduction was small due to the large, fixed expense of land in the production model.

Stratification of the financial costs by major inputs (equipment, fuel, materials, labor, and land) showed that the plantation operations were land intensive, with 44 percent of the plantation costs tied to land, 31 percent to material inputs, 18 percent to labor, and 8 percent to equipment and fuel (table 2). Basically, the opportunity cost of good quality land dominated the cash-flow aspects of the biomass production system.

On an energy cost basis, the plantations were again land intensive, with 93 percent of the costs originating from land (table 3). As previously identified, this cost represented the net energy gain available to land from corn production. Although this amount of energy was not actually used by the system, it did represent a minimum energy payment to land from the SRWC system. As for actual energy used by the system, the materials used in establishment, protection from insects, etc., and fertilization were the major expenses, representing 74 percent of these energy costs. Fuel, equipment, and labor were the remaining energy inputs (table 3).

Total Delivered Cost

Unit costs for the harvesting/transportation function were developed from auxiliary studies of these operations and were added to the plantation costs on a current, nondiscounted basis (Stokes et al. 1986; Strauss et al. 1988; Stuart et al. 1985). These amounted to $24/Mg(OD) and 977 MJ/Mg(OD), based upon a SRWC-designed harvesting system and a 40-km one-way truck haul. A 15 percent loss of material was assessed against the harvesting/transportation function (tables 2 and 3).

The total delivered cost on a financial basis, including harvest/transportation and a 15 percent material loss, was $46.42/Mg(OD) (table 2). Nearly 60 percent of the total cost originated from harvesting/transportation and material loss. In reviewing the entire system, equipment was the primary input, representing 31 percent of all costs (table 2).

Table 2. Financial costs by input type and operation for SRWC *Populus* biomass. Costs in U.S. dollars for 1995.

Operation	Unit cost ($/Mg(OD))[1]	Equipment	Fuel	Percentage of cost by input Materials	Labor	Land
Establishment	5.47	13.3	6.8	72.1	7.6	0.2
Insect./fung.	1.30	13.3	3.3	81.4	1.9	0.0
Fertilization,	1.02	11.4	2.9	81.0	4.7	0.0
land rent and taxes	8.30	0.0	0.0	0.0	0.0	100.0
Managerial	2.97	0.0	0.0	0.0	100.0	0.0
Plantation operations[2]	19.06	5.3	2.3	30.6	18.1	43.7
Harvesting and transportation[3]	24.00	55.0	22.1	0.0	22.9	0.0
Material loss (15%)	3.36	5.3	2.3	30.6	18.1	43.7
Total delivered cost	46.42	31.0	12.5	14.8	20.6	21.1

[1] Dollars per metric ton, oven dried.
[2] Operation costs compounded to the end of the rotation at an interest rate of .05.
[3] Strauss et al. (1988), revised for inflation to 1995.

Table 3. Energy costs by input type and operation for SRWC *Populus* biomass.

Operation	Unit cost ($/Mg(OD))[1]	Equipment	Fuel	Percentage of cost by input Materials	Labor	Land
Establishment	84.1	9.4	48.2	34.1	2.3	6.0
Insect./fung.	31.9	8.7	19.0	72.0	0.3	0.0
Fertilization	183.3	1.2	2.4	96.3	0.1	0.0
Land rent and taxes	4,069.9	0.0	0.0	0.0	0.0	100.0
Managerial	11.4	0.0	0.0	0.0	100.0	0.0
Plantation operations[2]	4,380.6	0.3	1.2	5.2	0.3	93.0
Harvesting and transportation[3]	976.9	15.1	82.3	0.0	2.6	0.0
Material loss (15%)	773.0	0.3	1.2	5.2	0.3	93.0
Total delivered cost	6,130.5	2.7	14.1	4.4	0.7	78.2

[1] Mega joules per metric ton, oven dried.
[2] Operation costs compounded to the end of the rotation at an interest rate of .05.
[3] Strauss et al. (1988), with revised labor energy costs.

The total energy cost was 6,130 MJ/Mg(OD) (table 3). As a major input, land was 78 percent of the energy cost. Exclusive of land, 65 percent of the energy was for fuel, 20 percent for materials, 12 percent for equipment, and 3 percent for labor.

Sensitivity Analysis

A review of the financial impact of various inputs on the total delivered cost of SRWC biomass showed equipment having the greatest impact, with a 10 percent change in its cost causing a 3.2 percent change in the delivered cost of biomass (figure 1). Again, over 90 percent of the equipment costs originated from the harvest/transportation. As expected, inputs representing a smaller portion of total cost had a lesser affect on the delivered cost of biomass.

Plantation yields had the greatest affect on unit costs. A 10 percent change in output would shift delivered costs by approximately 5 percent. This key relationship underscored the importance of maintaining or exceeding the targeted production level of 16 Mg(OD)/ha/yr.

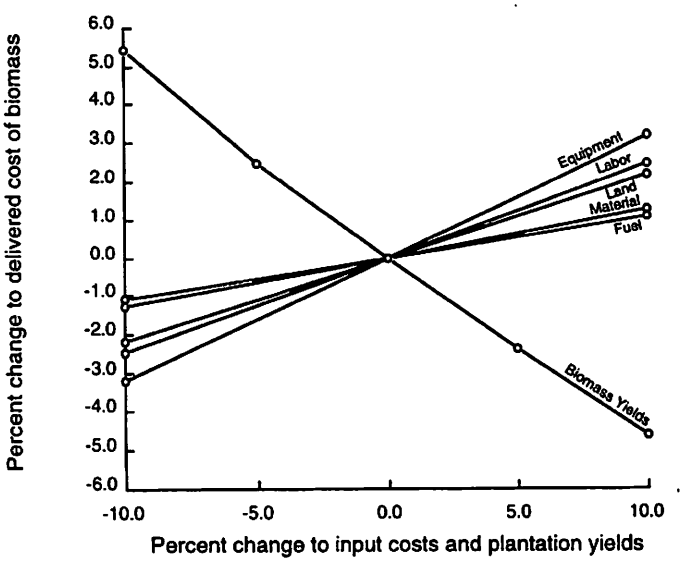


Figure 1. Sensitivity of total delivered cost of *Populus* biomass to changes of input costs and plantation yields.

Ethanol Manufacturing Inventory Control Model

SRWC research has focused on genetic, silvicultural, and economic evaluations of plantation strategies. Research on the conversion of woody biomass into energy products represents another major endeavor. Inherent to the linkage between raw materials and final products is the need to manage the inventories of inputs and outputs.

Forests are unique because trees represent the capital input and product output. As a product, they represent a financial commitment to inventory. Moreover, trees are perishable and may be limited by the duration of the harvest period. The storage of harvested biomass involves additional capital and operating costs, along with fluctuation in the inventory over time. Overall, matching the cyclical and finite nature of plantations with the steady state demands of a processing plant is a complex inventory problem.

General Structure of an Inventory Control Model

An inventory control model was developed by Grado and Strauss (1993) to determine the least costly approach for supplying biomass to a processing plant. A dynamic programming format was used to evaluate the plantation, harvest, and manufacturing components of an ethanol supply system.

The plantation component was based upon *Populus* grown under 4 to 8 year rotations using a strategy similar to that proposed by Strauss and Grado (1992). Harvesting covered a 6-month period that followed the growing season, and employed technologies similar to Stokes et al. (1986) and Stuart et al. (1985). The ethanol manufacturing process was based upon enzymatic hydrolysis of woody biomass as presented by Wright (1989) and Bergeron et al. (1989). The proposed facility could process 10,000 Mg(OD) of biomass per month to meet a maximum output of just over 3 million liters per month. Each component of the model generated particular inventories; various inventories were held during any given time period.

The model identified all costs associated with the plantation, harvest, and manufacturing components. Each component used an accounting format similar to that identified by Strauss and Grado (1992). Establishment and maintenance costs for the plantation were added as capitalized expenses to the harvest and transport costs, which were then added to the manufacturing costs. All costs are reported in U.S. dollars for 1995 on a per metric ton, oven dry (Mg(OD)), or per liter (L) basis.

Model solutions were for an annual operating cycle and provided: 1) the minimum cost combination for plantation, harvest, and manufacturing strategies; 2) the harvesting schedule within a year; 3) a recommended inventory policy for standing trees, harvested raw materials, and final product; and 4) net comparisons of existing supplies to operating demands for each component.

Model Results and Cost Comparisons

The use of inventory control policies as solutions to the model provided more finished product at a lower unit cost under any given rotation. Usually, the optimum harvesting policy provided substantial cost savings for any given rotation. Although longer rotations could provide more raw material and related finished product, there was an increase in the inventories of raw material and product and in raw material deterioration (table 4). Inventory control reduced these expenses for any given rotation. For example, the least costly solution for a 6-year rotation was $0.410/L (table 4), which was 38 percent lower than the highest cost solution for the same rotation.

Among all rotation strategies, the least costly solution was 6 years (table 4). This was consistent with the solution found in the previous model. However, note that this cost was not significantly lower than for the 7- or 8-year rotations. The highest cost items within the system were the manufacturing process, harvest/transportation, lost sales (penalty costs assessed when the supply of final product could not meet market demand), and plantation maintenance. The lowest cost items within the system were plantation establishment, raw material and final product storage, and storage deterioration.

The model solution provided storage policies for standing trees, harvested materials, and finished products, along with a plantation harvest schedule. Average storage time for harvested biomass over an operating year was 2.5 months, with nearly 16,500 Mg(OD)/mo held in storage.

Ethanol inventories were influenced by monthly manufacturing, final product demand, and the inventory policy. Storage of the final product was less costly than for harvested biomass (table 4). For the 6-year rotation, the average storage time for ethanol was only 1 month, with 1.9 million liters held per month. The upper storage level for ethanol was 3.0 million liters, which also represented a minimum long-term storage plant capacity.

The objective of the inventory policies was to coordinate plantation inventories, wood chip storage, and ethanol storage. When viewed in the context of the dynamic market demand for the finished product, these policies were successful in establishing efficient harvesting schedules and in lowering the total costs of the production system.

Conclusions

What have we learned from over 15 years of SRWC research? First, if we only consider the production costs at the plantation site, land is the dominant factor. This is particularly true in cases where plantations are placed on productive soils. Land rent and taxes can represent from 40 to 50 percent of the preharvest biomass cost. The cost of using good quality land is an alternative net return available from other agricultural pursuits. For SRWC systems to compete for land, comparable or higher net returns must

Table 4. Financial costs for an ethanol manufacturing system under alternate *Populus* rotation lengths. Cost in U.S. dollars for 1995.

	Costs ($/liter) Rotation length (years)				
	4	5	6	7	8
Plantation					
Establishment	0.015	0.013	0.010	0.011	0.010
Maintenance	0.068	0.058	0.048	0.046	0.046
Harvest					
Harvest/transport	0.079	0.075	0.074	0.074	0.074
Storage					
Wood chips	0.009	0.013	0.017	0.020	0.020
Penalty					
Deterioration loss	0.002	0.004	0.008	0.009	0.009
Manufacturing					
Processing					
Ethanol	0.242	0.242	0.242	0.242	0.242
Storage					
Ethanol	0.007	0.007	0.010	0.010	0.010
Penalty					
Market loss	0.140	0.060	0.001	0.000	0.000
Total	0.562	0.472	0.410	0.412	0.411

be provided by woody biomass. Lower priced, marginal lands would reduce these costs, but would probably result in lower yields.

Second, the best means for lowering the unit cost of woody biomass at the plantation is to increase plantation yields. Although 16 Mg(OD)/ha/yr was suggested as an average annual yield, sustaining this net average may be difficult. If this annual yield was reduced by 10 percent, preharvest costs would increase by over 12 percent, thereby increasing delivered costs by over 5 percent. A comparison of small-plot research yields to commercial field yields by Hansen (1988) suggested that the current field potential for SRWC plantations may be more in the range of 10 to 12 Mg(OD)/ha/yr. Further increases were considered possible, but these future gains will depend on cultural and breeding research. Higher yields would also require a more precise matching of clonal hybrids to growing sites and the successful implementation of cultural strategies on these sites.

Biotechnology will largely determine the future competitive position of *Populus*. Research efforts should be directed to 2 key areas; growth and yield, and maintenance costs. One method for decreasing costs is developing genetically superior stock with higher yields and lower maintenance requirements. Higher yields are achievable through increased survival rates of planting stock and the overall plantation, improved coppicing between rotations, and increased growth rates. Associated with these enhancements, would be the adaptation of *Populus* to lower quality sites, thereby reducing land costs. Further cost reductions are possible by developing varieties that require less maintenance in terms of site preparation, soil amendments, herbicides, and pesticide sprays. Potentially, these improvements might involve certain compromises such as faster-growing varieties that require higher maintenance costs. Under final analysis, the most cost-effective opportunities for biotechnology will be those that address yield and the major cost components of the production equation.

Separate from the biological forces of growing *Populus* are the technical factors required in the harvest, transport, and storage of this raw material. These items more than doubled the cost of the delivered and stored raw material. Woody biomass is not a convenient material to handle or move and, as such, further innovations are needed. Associated, many harvesting and transportation strategies are adaptations of technologies used in domestic forests. Although further cost reductions are possible, they may require the development of commercial SRWC markets; this precondition could stalemate the development process.

The manufacture of woody biomass into a liquid fuel introduced another set of production costs. As identified in the expanded model, over 50 percent of the final output cost was tied to the conversion process, with the raw material at the plantation site representing less than 15 percent of the total production costs. In the context of today's energy markets, the use of woody biomass for either liquid fuels or direct-burn electric generation cannot compete with abundant and relatively cheap fossil fuels. However, over the past 15 years, *Populus* has been a viable fiber stock for the paper and paperboard industry in selected regions of the U.S. In the Pacific Northwest, *Populus* plantations were established in a relatively short time and have provided a dependable fiber supply to certain niche markets.

One potential shortcoming in this review of *Populus* has been a disregard for the inherent qualities of the raw material. Basically, this woody biomass was viewed as a base of simple sugars or fiber stock for 2 general types of final products. However, inherent to *Populus* is considerable genetic variation for fiber length, chemical composition, and other qualities affecting overall product. Optimization of these qualities for specific applications deserves further attention and should serve as a focus for biotechnology research.

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Chapter 32

Environmental Risk Assessment and Deployment Strategies for Genetically Engineered Insect-Resistant *Populus*[1]

Kenneth F. Raffa, Karl W. Kleiner, David D. Ellis, and Brent H. McCown

Introduction

Most studies on genetically engineered plants have concentrated on efficacy; few have focused on environmental safety (Seidler and Levin 1994). This emphasis reflects the rapid increase in our technological capabilities over the last 15 years and reflects an uncertainty over how best to scientifically generate relative rankings of the likelihood and severity of possible adverse effects. Environmental risk assessment is not an exact science and can only provide direct comparisons between treatments and checks under the most controlled, and therefore least realistic, conditions. Moreover, risk assessment inevitably raises questions that are at least partially subjective, value-laden, and contextual.

This chapter attempts to identify the more significant issues of environmental risk, weigh their relative impacts, and suggest possible strategies for reducing adverse effects. We limit our discussion to potential environmental effects. Impacts on social and economic systems, while important, are beyond our expertise.

Approaches to Environmental Risk Assessment

An appropriate definition of environmental risk is debatable (Morgan 1993; Wilson and Crouch 1987). In this chapter, environmental risk means: "the likelihood that release of a novel material will cause adverse effects such as mortality or reduction in populations of nontarget organisms due to acute, chronic, or reproductive effects, or disruption of community or ecosystem function" (Urban and Cook 1986). Predicting consequences becomes increasingly complex as the scale expands from individual- to community-level interactions. For example, negative effects on individuals do not necessarily translate into reduced populations. Natural and managed systems provide many instances of compensatory feedback where removing substantial numbers of individuals does not affect population density. Conversely, the prospect of indirect effects from community-level interactions, even when no individual effects appear important, is a serious concern. Basic ecological studies provide a wealth of examples. Indirect interactions, across multiple trophic levels, incorporating biotic and abiotic environmental factors, and mediated by a broad range of symbionts, competitors, and alternate hosts, can exert enormous influences and generate unpredicted outcomes (Angle 1994; Bergelson 1994; Ehler 1990; Holt 1977; Price et al. 1980; Simberloff 1985). Similarly, the history of applied ecologies, such as agriculture and forestry, shows that indirect interactions often yield the least predictable yet most damaging consequences.

The issue of ecological complexity is a paradox to risk assessment. Controlled evaluations of acute effects on isolated individuals generate the least variable and seemingly most "reliable" results. Studies that attempt to unravel more diffuse and incipient effects are ultimately more important, yet unfortunately they are less likely to provide definitive answers (Angle 1994). Ironically, current approaches to training, funding, and productivity are strongly biased toward the former approach.

Comprehensive risk assessment must also weigh anticipated benefits against risk. Genetic engineering of *Populus* offers several potential benefits, such as reduced pesticidal inputs, improved carbon sequestration, alleviation of pressures to exploit unmanaged systems, and improved sources of alternatives to fossil fuels (Kleiner et al. 1995;

[1] Klopfenstein, N.B.; Chun, Y.W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

McCown et al. 1991; Raffa 1989; Raffa et al. 1993; Robison et al. 1994). These are potentially enormous benefits; however, they are beyond the scope of this paper.

Potential risks can be categorized based on their spatial and temporal scales. For example, an effect could be limited to the treated site, or it could impact neighboring ecosystems. Effects can be short-term, such as the release of a toxic gene product into the environment, or self-replicating, such as the escape of viable germplasm. Such distinctions can be somewhat blurred and need to be assessed as part of the complete risk evaluation process. Still, there is general agreement that the most serious concerns arise when genetically engineered organisms could cause self-perpetuating injury to commercial or natural ecosystems beyond the immediate area of release.

Criteria for Risk Assessment

Different individuals, agencies, and organizations have advocated different criteria, burdens of proof, and levels of evidence governing the planned release of genetically engineered organisms. Probably the most helpful guidance is provided by a National Academy of Sciences committee headed by Arthur Kelman (NAS 1987). In the opinion of NAS, what matters is the product not the process. According to this perspective, introducing genetically engineered organisms "poses no risks different from the introduction of unmodified organisms and organisms modified by other methods." Therefore, "assessment of risk should be based on the organism, not the method of engineering." Subsequent authors have delineated some important differences between genetic engineering and plant breeding, and hence the need for limits in applying this equivalency (Dale and Irwin 1995; Giampietro 1994; Regal 1994). However, this starting point has proven useful and has withstood the test of time. Similar conclusions are stated by Tiedje et al. (1989) in an Ecological Society of America report: "transgenic organisms should be evaluated and regulated according to their biological properties (phenotypes), rather than according to the genetic techniques used to produce them."

Emphasizing phenotypes over the methods by which they arise has proven useful because it rebuts scientifically unfounded criticisms and focuses on interactions between gene products and their environment. Rather than dismissing environmental concerns, this approach highlights the importance and complexity of predicting responses to gene products at the community level, and the need for ecological expertise in devising scientifically based policies. If the product not the process is critical, then expertise in the methods of genetic engineering is not directly relevant to predicting how novel organisms will interact with ecosystems. Molecular expertise is invaluable, however, in protecting against unintended changes in the genome, incorporating methods of sterility, and controlling and evaluating patterns of expression. The criteria for estimating and the approaches to alleviating environmental concerns require interdisciplinary efforts (Raffa 1989).

Raising every imaginable hazard that could arise from genetically engineered organisms is neither difficult nor helpful. This approach could hinder the enormous value of biotechnology and dilute needed emphasis on legitimate concerns. At the other extreme, the view is sometimes expressed (or implied) that all concerns arise merely from a lack of scientific understanding or breadth. This view seriously underestimates the complexity of scaling from molecular- through ecosystem- level processes. Failure to consider such complexity invariably detracts from the long-term sustainability of new technologies; a costly lesson already appreciated by agrichemical companies. The issue needs to be one of reasonable probability (de Zoeten 1991; Frederick and Egan 1994; Hubbes 1993; NAS 1989; Raffa 1989; Strauss et al. 1991; Tiedje et al. 1989). For example, Tolin and Vidaver (1989) propose that "restrictions should be based on demonstrated, not conjectural risks." However, we would substitute "realistic" for "demonstrated" to promote a more proactive approach to risk management. In our view, the likelihood of risk may be realistic if 2 conditions are met: 1) a clear mechanism, based on known biological processes and verified assumptions, can be delineated; and 2) there is relevant precedent.

Few specific risks meet the above criteria. Those that do can be classified into 3 general categories: 1) escaped plants or genes, 2) evolution and consequences of resistant pest biotypes, and 3) alteration of multi-trophic processes. We first describe how *Populus* systems relate to these questions and then address each risk. Biotype evolution will be developed as a more detailed case study, as this is our primary area of interest. We conclude with an overall synthesis of environmental risk assessment in *Populus*.

For each concern, the potential risk can be addressed by asking 3 questions: 1) "Is there assurance that the proposed event (i.e., gene escape, biotype evolution, altered multi-trophic process) will not occur?"; 2) "Is there assurance that the effects will be harmless if this event does occur?"; and 3) "Are there ways for reducing the likelihood and impact of harmful effects?" These questions place the burden of proof on the novel gene product to be consistent with how other novel products such as biological control agents, introduced plant materials, and pesticides are evaluated (Caltagirone and Huffaker 1980; Charudattan and Browning 1992; FIFRA 1978; Fuester 1993; Harris 1985; Hinkle 1993; Hutton 1992; Upholt 1985; USDA FS 1991; White et al. 1992). During actual experimentation, however, the null hypothesis is one of no effect. Also note that questions 1) and 2) are evaluated in the absence of any ameliorating steps, but the availability

and implementation of such tactics (question 3) could greatly reduce resulting concerns.

Populus Growing Systems

The safety of releasing a genetically engineered organism is determined by the gene product and the environment into which it will be introduced (Abbott 1994; Falk and Bruening 1994; Jepson et al. 1994; Pimentel et al. 1989). In general, risks are lower for intensively cultured short rotation tree crops than for large forested expanses of long-lived species (Raffa 1989). *Populus* occupies a relatively broad range of growing conditions along this continuum. For example, trembling aspen, *Populus tremuloides*, is one of the most widely distributed naturally occurring species in North America. It is a valued forest tree with a number of uses such as soil quality improvement, watershed maintenance, CO_2 sequestration, and wildlife habitat. When used for timber, *Populus* is harvested from self-regenerating forests and grown in commercial stands. *Populus* is also a major component of rapid rotation systems such as biofuel plantations. These intensively managed systems, more closely resembling agricultural than forest production, have short growing intervals, are based on carefully derived clonal material, and are subjected to intensive cultural and chemical inputs.

Populus has also become the focus of intense basic research by molecular biologists, plant physiologists, and ecologists. *Populus* is the preeminent tree model for tissue culture, molecular mapping, and transgenic technology. Concurrently, *Populus* has become a key model for basic ecophysiological and plant-herbivore interaction studies. Thus, *Populus* provides an ideal system for evaluating the role of plant community structure in the efficacy and environmental safety of various deployment strategies and for integrative studies from the molecular- through ecosystem- level scales.

Movement of Transgenes Into Native Populations

The most direct form of proposed environmental harm is that genes encoding novel traits might become established in feral populations and subsequently exert a weedy effect. A variety of mechanisms for gene escape have been proposed such as hybridization into gene pools of wild relatives, crop abandonment, movement of cuttings by animals or water, etc. Escape of transgenes into the environment could occur on several levels, and the risk associated with each level of escape varies. A commonly proposed level of escaped transgenes is via pollen or seed. Another level is the physical movement of plant parts into the surrounding environment. Because virtually all the poplars used in short rotation plantations are clones multiplied by vegetative cuttings, small branches can be moved off site by animals or overland water flow. The root sprouting habit of several poplars poses a similar concern.

Precedents by which to evaluate possible consequences of escaped material are offered by the literature on accidental or injudicious introduction of exotic species. Following the NAS (1987) rationale that introducing genetically engineered organisms should not be treated differently from other unmodified organisms, the literature on exotic introductions provides useful precedents to evaluate the likelihoods and consequences of escapees (Williamson 1994). A large and well-documented data base details numerous instances in which accidentally or deliberately introduced microorganisms, arthropods, nematodes, plants, and vertebrates became established in non-native ecosystems, and subsequently exerted severe economic and environmental consequences (e.g., Liebhold et al. 1995; Lodge 1993; Mooney and Drake 1986; US Congress OTA 1993).

A number of molecular biologists, agronomists, ecologists, and plant protection specialists have cautioned that some escape is likely. For example, Strauss et al. (1995) stated unambiguously, "Gene flow within and among tree populations is usually extensive, which makes the probability of transgene escape from plantations high." Timmons et al. (1995, 1996) expressed a similar conclusion for *Brassica*. Likewise, the ecologists Kareiva et al. (1994) concluded that "the escape of transgenic pollen is inevitable." Williamson's (1994) analysis of historical records of deliberately introduced organisms, concludes that nearly all escape, and of these 10 percent become established.

Our ability to address whether there is sufficient assurance that escaped genes would be harmless is considered case by case (Dale and Irwin 1995). Various authors, including molecular biologists (Strauss et al. 1995), ecologists (Kareiva et al. 1994; Regal 1994; Seidler and Levin 1994; Tiedje et al. 1989), and crop protection specialists (Dale 1994; Williamson 1994), have identified possible adverse effects of escaped transgenes. Some examples include creation of new (or enhanced) pests, harm to non-target species, and disruptions to biotic communities, natural food webs, and ecosystem processes.

In each of these cases, there are well established mechanisms by which such adverse consequences might arise, and substantial literature providing precedents from analogous introductions. Examples of possible mechanisms include: 1) enhanced competitiveness of a genetically engineered organism (due to pest resistance or physiologi-

cal environmental tolerance of stress) that displaces existing or subsequent beneficial organisms (Ellis et al. 1984; Moamad et al. 1984); 2) reductions in seed dispersal, pollination, or biodiversity by insecticidal transgene products (Simmonds 1976; McGranahan et al. 1988); or 3) acquisition of traits that enhance competitive status by existing weed species (Windle and Franz 1979). Again, the historical record with traditional introductions is of some value. Williamson (1994) reports that 10 percent of the 10 percent of escaped species that establish become problematic. It must be emphasized, however, that such figures do not reflect refined deployment strategies (using specific information about target species, transgenes, rotation cropping system, and location) that could accompany planned releases of transgenic poplars. And, although the historical record of planned releases of genetically engineered organisms is still relatively small, to date there have been no known adverse effects.

Despite the potential for adverse effects, a number of attributes of the transgene, the parent organisms, phenotypic expression, and target pest-environment system could reduce risk (Tiedje et al. 1989). For example, risk analysis must consider whether pollen from transgenic hybrids is compatible with surrounding populations and also whether the timing of pollen release occurs when stigmatic surfaces in surrounding populations are receptive. Any impact of escaped genes will likely vary with the novel gene product as well. For example, risks associated with the escape of a *Bacillus thuringiensis* (*Bt*) endotoxin gene may be different than those for a gene modifying lignin. Differences in plant species and growing systems are also pertinent. In agronomic food crops such as soybean, maize, potato, and tomato, measures such as sterility have not been a requirement for registration. Conversely, a case-by-case analysis of each transgene-species-planting site, combinations may be needed.

The third question, whether the risk or impact of escape can be ameliorated, is currently the subject of intense effort. Risk from escape by vegetative material could be reduced by management practices that minimize root sprouting outside the plantation and the distance plant material is moved. This can be achieved by planting buffer strips that are routinely cultivated and/or planted with an annual crop so that escapes can be readily identified and treated with herbicide. One management strategy for contending with pollen or seed dispersal might be to identify late flowering clones for a breeding population, such that harvesting occurs before sexual maturity in the transgenic trees. Such an approach would offer functional, while not physiological, sterility. Another strategy might be the use of sterile triploids.

Physiological approaches to reproductive sterility in genetically engineered trees have recently been reviewed (Strauss et al. 1995) and are not be treated extensively here. Basically, these include using floral promoter-cytotoxin to ablate floral tissues and disrupting expression of essential floral genes. In the former approach, cytotoxic genes regulated by reproductive-specific promoters kill all cells committed to reproductive development. The latter approach uses antisense RNA, sense suppression, or promoter-based suppression to impair the expression of genes required for fertility. These approaches can be deployed with varying levels of gender specificity, have relative advantages and disadvantages (Meilan and Strauss this volume; Strauss et al. 1995), and have yielded some successes (Mariani et al. 1990). The current obstacles relate to our lack of basic information about reproductive gene sequences and expression in clonally propagated species such as *Populus*.

Very little is known about the long-term stability of transgene expression in woody perennials. In 1 test examining the expression of a marker gene in field poplars, some level of seasonal variability in transgene expression was observed, but in general this variation was predictable, and relatively continuous expression levels occurred from year to year (Ellis et al. 1994). Of greater concern, however, is the variation in expression levels and patterns that occur between individual transformants containing the same construct. In addition to the variation in the overall levels and patterns of transgene expression, current molecular understanding of transgene regulation in plants is at a relatively elementary level. Genetic engineering for sterility requires very precise control over a transgene to interrupt and terminate flowering. There currently is no way to ensure that this transgene will function in all the plants over the 5 to 10 years a poplar plantation requires to mature. Additional research is needed to devise such capabilities.

Evolution of Resistant Pest Biotypes and Emergence of Secondary Pests

General Considerations

The evolution of insect and microbial biotypes in response to genetically engineered plants has been an area of concern since the early development of plant transformation technologies (Gould 1988). The same 3 questions posed for gene escape, with emphasis on insects, will be addressed here: 1) Is there assurance that resistant pests will not evolve? 2) Is there assurance that resistant pests will not cause environmental harm if they do arise? and 3) Are there ways for reducing the likelihood and impact of resistant biotypes?

The potential of pest-biotype evolution is well established by a strong mechanistic foundation and historical precedent (Brattsten et al. 1986; Forgash 1984; Georghiou

and Lagunes-Tejeda 1991; Georghiou and Saito 1983; McGaughey 1985; Roush and Tabashnik 1990; Tabashnik 1994). Extensive insecticide application, deployment of resistant cultivars, and even cultural practices have repeatedly selected for resistant insect biotypes (Via 1990). Moreover, the underlying mechanisms have been well characterized at the molecular, biochemical, physiological, and population levels (Cohan and Graf 1985; Eggers-Schumacher 1983; Flexon and Rodell 1982; Kulkarni and Hodgson 1984; Muggleton 1982; Mullin and Scott 1992; Oppenoorth 1984; Roush 1987; Ryan and Byrne 1988; Skylakakis 1982). Intraspecific differences among gypsy moths and forest tent caterpillars from different geographic sources to poplars transgenically expressing *Bt* have been observed (figure 1). Variation among these and other forest lepidopterans to exogenously applied *Bt* is also well documented (Rossiter et al. 1990; Van Frankenhuyzen et al. 1995). Adaptations by insects to altered sources of food plant quality, quantity, and distribution are well documented in natural and managed systems (Singer et al. 1993; Singer and Parmesan 1993; Via 1990). Thus, heritable variation required for gene frequency alteration in response to selection is sufficient.

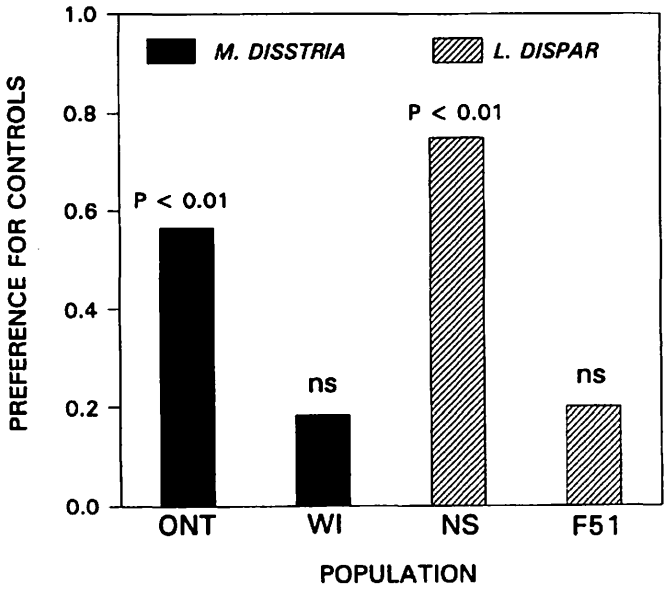


Figure 1. Intraspecific variation among gypsy moth and forest tent caterpillar populations to transgenic poplars expressing a *Bt* endotoxin gene. Two forest tent caterpillar, *Malacosoma disstria*, populations showed different levels of aversion from transgenic relative to control foliage. A similar difference was observed between 2 gypsy moth, *Lymantria dispar*, populations (Robinson et al. 1994). ONT=Ontario, Canada; WI=Wisconsin, USA; NS=Nova Scotia, Canada and USDA APHIS; F51=gypsy moths from laboratory culture at USDA Beneficial Insects Laboratory, Newark, DE, USA; ns=nonsignificant.

It does not matter whether an insect toxin is deployed through spray application, traditional breeding, or genetic transformation. For example, the introduction of new plant varieties is sometimes followed by a population increase of previously innocuous herbivores to pest levels. Attention should not be limited to plants specifically engineered for pest resistance, as development of presumably unrelated plant qualities can also alter selective pressures (Raffa 1989). For example, development of new rice varieties for various agronomic properties led to the emergence of new pest complexes (Oka and Bahagiaivani 1984). In addition, engineering plants for altered, increased, or novel secondary products could have multiple consequences. Even chemical groups that are typically considered "defensive" can directly benefit adapted herbivores (Bernays and Woodhead 1982), or indirectly benefit them by reducing the efficacy of beneficial predators (Codella and Raffa 1995), parasitoids (Campbell and Duffey 1979), and entomopathogens (Andrews et al. 1980).

Selective pressures imposed by transgenic trees could be higher than those resulting from insecticide treatments. Insecticide-resistant biotypes have been rare among forest pests, despite a rather extensive history of synthetic chemical and *Bt* application. Tree-feeding herbivores are not physiologically unique in this regard. Rather, tree-feeding insects are exposed to more variable exposure patterns, less complete spray deposition, and less frequent treatment, than are agronomic insects. These sources of variability could be lost using transgenics. Depending on migration rates, neighboring untreated forests could provide refugia for susceptible genotypes, when they are near to transgenic plantings. In this regard, transgenic poplars could pose less of a threat of biotype evolution than many transgenic agronomic crops. Where poplars are planted in isolation from native refugia, however, such as in irrigated deserts, this benefit would be minimal.

Usually, the impact of evolved resistance against pesticides, resistant cultivars, and genetically engineered trees, is limited to loss of efficacy (Raffa 1989). However, 2 general categories of adverse effects could extend beyond treated plantations: 1) induced insect emigration into neighboring non-transgenic stands; and 2) lost efficacy of previously useful tools in non-transgenic stands.

The possibility of between-stand movement raises the ethical concern that a grower who chooses not to plant genetically engineered trees may be subjected to immigrants from engineered stands (the same could be true of most traditionally bred forms of resistance and chemical pesticides). This risk may be greater in tree than agronomic crops, because airborne larval dispersion is on average more important in forest insect life histories. A critical need in assessing this impact is understanding the relationship

between toxicity and repellency, and in particular, whether repellency occurs pre- or post- ingestion (Hoy and Head 1995; Ramachandran et al. 1993a, 1993b). This could affect, for example, whether emigrating individuals possess a slightly higher level of physiological tolerance than the general population. The available evidence suggests that these parameters vary markedly with the insecticidal product, its interaction with plant allelochemicals, and insect species. We currently lack information on how best to balance the value of behavioral aversion as a resistance-delaying tactic versus its impact on non-treated stands (Gould 1988; Hoy and Head 1995; Johnson and Gould 1992).

The possibility of transgenic plants reducing the utility of an externally sprayed biopesticide, such as *Bt* endotoxin, to growers who use Integrated Pest Management (IPM) approaches based on economic injury levels, raises a similar ethical concern. This is analogous to neighboring growers applying insecticides on a calendar rather than density-activated basis. Likewise, integrated plant protection programs could be compromised when biotypes evolve against transgenic resistances that are based on elevated or altered allelochemicals (again, the same could be true of some traditional breeding).

Loss of efficacy can be compounded when the mechanism of evolved resistance confers cross-resistance to other insecticides or resistant-cultivar allelochemicals (Brattsten et al. 1986; Brattsten 1991). Cross-resistance is a widely occurring phenomenon that can arise by a number of well-characterized physiological mechanisms. For example, the introduction of the synthetic pyrethroids, derived analogues of *Chrysanthemum* spp. extracts, encountered rapid biotype evolution in regions where the synthetic organochlorine DDT had been widely used. Similarities in the pharmacological properties of these 2 groups provide a physiological explanation for cross-resistance, but *a priori* considerations based on the unrelatedness of their molecular structures failed to predict these consequences.

Mitigation Strategies

Although resistant biotypes are likely to evolve if transgenic poplars are deployed without preconceived resistance management programs, a variety of ameliorating strategies can be used. There are many examples of effective pest control tactics providing satisfactory control over many decades. Likewise, naturally evolved plant defenses provide many examples of long-term stability. Even among trees, in which host-generation times exceed those of insects and pathogenic microbes by orders of magnitude, most members of the host population are protected most of the time (e.g., Edmunds and Alstad 1978; Whitham 1983). A major principle to emerge independently from toxicology, plant breeding, and ecology is that the pattern and intensity of selection, more than the actual mode of toxicity, most strongly affect biotype evolution rates (Brattsten et al. 1986; Tabashnik and Croft 1982). The rate, impact, and extent can be greatly reduced by considering features of the target system and by incorporating heterogeneity at multiple levels of scale. Preconceived resistance management programs now accompany the introduction of many pesticides, as agrichemical corporations recognize the economic value of protecting their investments. Likewise, deployment of transgenic cotton and corn is now accompanied by guidelines prescribing inclusion of non-engineered seed.

Some features of the tree-insect system that can accelerate or retard biotype evolution include the availability of refugia for susceptible insect genotypes, attributes of the major pests' physiology, behavior, and ecology, and compatibility of the novel trait with other management tactics. Thus, intensively cultured, short-rotation *Populus* plantations pose less risk than forests of long-lived species such as *Pseudotsuga*. In the latter case, the enormous differences between pest and host generation times would greatly reduce the efficacy of any biotype-delaying tactics. Likewise, the defoliator guild that most strongly impacts *Populus* poses less risk than, for example, the scolytids associated with *Pinus* and *Picea*. In the latter case, beetle preference for stressed trees limits them to such hosts during lengthy nonoutbreak periods. Conferring a novel resistance that was expressed regardless of tree vigor would greatly alter the selective pressures on bark beetles and possibly result in more pestiferous behavior (Raffa 1989). No such relationship appears to regulate population dynamics of most folivores.

Diversifying tactics, such as mixed block plantings and crop rotation, are well suited for *Populus*. A wide range of hybrid poplar clones are available for deployment, including some that provide rapid growth and resistance against some key pests (Robison and Raffa 1990, 1994, 1997a, 1997b). Mixed block plantings can also incorporate host plant tolerance. For example, some of the less resistant clones against *Malacosoma disstria* feeding can withstand considerable defoliation without experiencing severe growth losses (figure 2).

Protection against multiple pest complexes can be achieved by integrating traditional and transgenic resistances. The need and potential for this approach are illustrated by the lepidopteran and coleopteran feeding guilds. Strong resistances against both groups have been identified, but no clones are highly resistant to both (Robison and Raffa 1994). Continued hybridization or characterization are unlikely to improve this relationship because the same allelochemicals, specific phenolic glycosides, which inhibit lepidopterans benefit coleopterans (Bingaman and Hart 1993; Lindroth and Bloomer 1991; Ramachandran et al. 1994; Smiley et al. 1985) (figure 3). Understanding these relationships can help guide molecular strategies. For example, inserting only a coleopteran-active *Bt* (*cryIIIA*, *cryIB*) into 'NM6' (*P. nigra* x *P. maximowiczii*), and only the

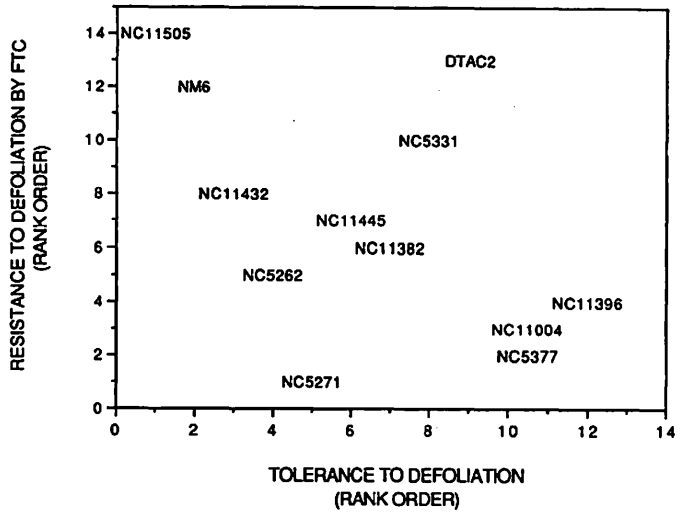


Figure 2. Variation in host resistance against defoliation by forest tent caterpillar (FTC) larvae and tolerance to a controlled level of artificial defoliation among hybrid poplar clones. Some clones are relatively unable to prevent defoliation but are highly tolerant if it occurs (from Robison & Raffa 1997a). cl. 'DTAC2' (*Populus deltoides* var. *angulata* x *P.* x *berolinensis*); cl. 'NC5262' (cl. 'NE387') (*P. balsamifera* var. *subcordata/ candicans* x *P.* x *berolinensis*); cl. 'NC5271 (cl. 'NE19') (*P. nigra* var. *charkowiensis* x *P. nigra* var. *caudina*); cl. 'NC5331' (cl. 'NE299') (*P. nigra* var. *betulifolia* x *P. trichocarpa*); cl. 'NC5377' (cl. 'Wisconsin #5') (*P. deltoides* x *P. nigra*); cl. 'NC11004' (*P. deltoides* cv. 'Siouxland'); cl. 'NC11382' (cl. 'NE27') (*P. nigra* var. *charkowiensis* x *P.* x *berolinensis*); cl. 'NC11396' (cl. 'NE49') (*P. maximowiczii* x *P.* x *berolinensis*); cl. 'NC11432' (cl. 'NE252') (*P. deltoides* var. *angulata* x *P. trichocarpa*); cl. 'NC11445' (cl. 'NE280'; cl. 'NE157') (*P. nigra* x *P. laurifolia*); cl. 'NC11505' (cl. 'NE388'; cl. 'NE88') (*P. maximowiczii* x *P. trichocarpa*); cl. 'NM6' (cl. 'Max-5') (*P. nigra* x *P. maximowiczii*).

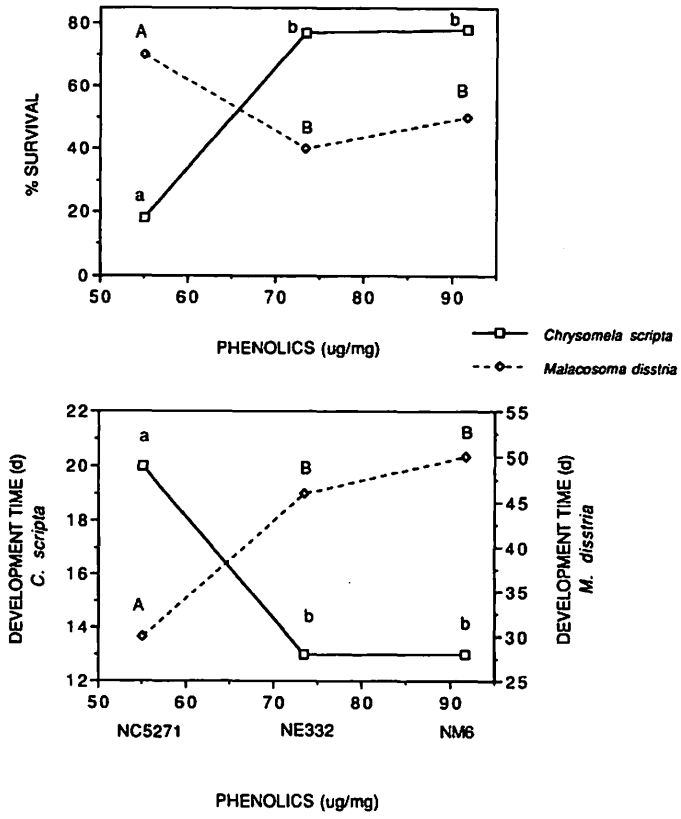


Figure 3. Opposing effects of foliar phenolics on 2 defoliating pests attacking hybrid poplars. The cottonwood leaf beetle, *Chrysomela scripta*, exhibits high survival and rapid development on clones, such as 'NM6' (*Populus nigra* x *P. maximowiczii*), which have high phenolic contents. Survival and development rates are poor on clones, such as 'NC5271' (*P. nigra* var. *charkowiensis* x *P. nigra* var. *caudina*), which have low foliar phenolic concentrations. Conversely, the forest tent caterpillar, *M. disstria*, experiences high survival and rapid growth on 'NC5271,' and poor survival and growth on 'NM6.' (Ramachandran et al. 1994).

lepidopteran-active *Bt* (*cryIA(a)*) into 'NC5271' (*P. nigra* var. *charkowiensis* x *P. nigra* var. *caudina*), can cut in half the number of genetically engineered trees needed to express any 1 trait, yet still provide full protection against both pests (table 1). Different forms of resistances can also be combined with transgenic traits. For example, the clones 'NE332' (*P. simonii* x *P.* x *berolinensis*) and 'NC11382' (*P. nigra* var. *charkowiensis* x *P.* x *berolinensis*) show resistances to *M. disstria*, but these defenses are based on foliar phenolic glycosides and bud resins, respectively (table 2) (Ramachandran et al. 1994; Robison and Raffa 1997a). Such combinations can increase heterogeneity because *Bt* interacts with different phytochemical groups differently (Appel and Schultz 1994; Hwang et al. 1995).

Heterogeneity can be further enhanced by linking expression to wound-inducible promoters. Wound-inducible expression of inserted genes could simulate the "economic injury levels" that trigger pesticide applications in Integrated Pest Management systems. That is, a certain level of insect feeding would be tolerated before expression was elicited. Opportunities for increasing heterogeneity at this level are supported by existing variation in inducibility among poplar clones (table 3). However, preliminary evidence suggests that the sensitivity of existing wound-in

Table 1. Proposed integrated use of traditionally bred and natural plant resistance with genetic engineering to confer protection against multiple pest complexes. An example using hybrid poplar.

Source of resistance	Example	Level of protection against pest group Lepidoptera	Coleoptera
Heritable plant defense	Clone		
(Current)	'NM6'[1]	High	Low
	'NC5271'[2]	Low	High
Transgenic trait	*Bt* Endotoxin		
(Current)	*cryIIIA*	Low	High
	cryIA(a)	High	Low
Integrated combination	Clone + *Bt*		
(Proposed)	'NC5271'[1] + *cryIA*(a)	High	High
	'NM6'[2] + *cryIIIA*	High	High

[1] 'NM6' = *Populus nigra* x *P. maximowiczii*
[2] 'NC5271' = *P. nigra* var. *charkowiensis* x *P. nigra* var. *caudina*

Table 2. Surces of resistance against forest tent caterpillar, *Malacosoma disstria*, in 2 hybrid poplar clones (Robison & Raffa 1997a). Foliage of 'NE332' is less suitable for forest tent caterpillar larvae than is foliage of 'NC11382.' Bud resins in 'NC11382' are more effective at immobilizing larvae and preventing access to foliage.

Insect parameter		Ratio of *M. disstria* performance in tissue 'NC11382'[1]/'NE332'[2]
Foliage	Growth rate (mg/day)	6.5
	Development time	1.5
	Feeding (2nd instar)	19.0
	Survival	2.3
	Weight (mg)	1.7
Buds	Larval mobility	0.4
	Weight (mg)	0.7

[1] 'NC11382' = *Populus nigra* var. *charkowiensis* x *P.* x *berolinensis*
[2] 'NE332' = *P. simonii* x *P.* x *berolinensis*

Table 3. Clonal variation in *Populus* inducibility in response to forest test caterpillar, *Malacosoma disstria*, feeding

Clone	Percent forest test caterpillar survival Constitutive tissue	Damaged tissue
'NC11382'	90	85
'NE332'	85	49

Source: Robison & Raffa (1997a)

ducible promoters may need to be increased before this strategy can provide field-level efficacy (Ellis et al. 1996).

Further heterogeneity could arise from tissue- and temporally-specific expression. Protecting only favored leaves can sometimes provide protection nearly equivalent to treating entire plants (table 4). This approach is most suitable when insects that prefer productive tissues can also tolerate other foliage. Within-plant spatial variation in allelochemistry occurs commonly among naturally coevolved plant defenses. For example, in *Populus*, phenolics are concentrated in the youngest leaves, with the result that lepidopteran defoliators feed mostly on older tissue. The underlying physiological basis for uneven phytochemical distribution is complex, but among the benefits incurred by the host are protection of the most photosynthetically active tissue, retained ability to translocate carbon to the growing tip, and reduced likelihood of complete defoliation (Coleman 1986; Meyer and Montgomery 1987). Likewise, pines allocate diterpene resin acids to new not old foliage. Thus, pine sawflies feed on the older foliage, a habit that only removes photosynthetically less valuable tissue. At first glance, this might suggest a high potential for these herbivores to overcome such defenses, but most herbivore species have not evolved this ability. The evolutionary "choice" in this case is not between overcoming a biochemical barrier and starvation. Rather, those larvae that did feed on new foliage would grow less and be less fecund than those on old foliage, and hence be less competitive.

Table 4. Performance of cottonweed leaf beetle, *Chrysomela scripta*, on trees completely or partially treated with *Bt*.

Treatment	No. egg masses produced
Control	10.25
All foliage	5.75
Young only	3.50
Mature only	9.75

Source: Ramachandran et al. (1994)

Alteration of Multi-Trophic Processes

Experiences with pesticides, introduced organisms, and other xenobiotic inputs suggest several means by which products of genetically engineered plants could affect ecosystem-level processes. Adverse impacts include reduced populations of predators, parasites, scavengers, pollinators, and endangered or aesthetically valued species. Both direct and indirect mechanisms have been delineated for impacts on each of the above, and strong historical data provides examples for each (e.g., see Caltigirone and Huffaker 1980; Ehler 1990; Findlay and Jones 1990; Holt 1977; Pimentel 1980, 1991; Pimentel and Warneke 1989).

Direct effects of xenobiotics on natural enemies can occur by acute toxicity or biomagnification. In general, genetically engineered trees should be less directly damaging to natural enemies than are traditional pesticides. One of the major advantages of transgenic plants is that toxins can be delivered directly to the herbivore, without broadcast application. The likelihood of biomagnification depends on the gene product. To date, most traits engineered into plants involve gene products that are rapidly broken down within the target insects. For example, we are unaware of any instances where predators were directly affected from ingesting prey killed by *Bt*. Other more stable gene products, however, could be problematic.

Evaluating potential effects on parasites is more difficult. A major concern is that parasitoids will oviposit in insects in which they cannot complete development before host death. This could drastically reduce parasite populations, and thereby release secondary pests. For example, negative effects of plant allelochemicals on parasitoid success are well documented (Barbosa and Saunders 1984; Campbell and Duffey 1979). However, xenobiotics can sometimes benefit parasitoids. For example, *Bt* application can enhance performance and population densities of the gypsy moth larval parasitoid, *Cotesia melanoscela* (Weseloh et al. 1983). In this example, the delayed growth rates caused by *Bt* apparently increase the period during which surviving early instars are vulnerable to parasitism. However, interpreting such results is complicated. For example, Johnson and Gould (1992) have argued that synergism between genetically engineered resistance and parasitoids could accelerate biotype evolution and its resultant hazards. That is, if exposure to a particular product increases the likelihood of ultimate mortality, selection will more strongly favor tolerance against the predisposing agent. But in some cases, increased parasitoid densities resulting from synergism could subsequently exert mortality independent of the xenobiotic. More research is required to better quantify and partition these multiple effects.

Some indirect effects following the introduction of any new organism are inevitable. In some cases, an introduced biological control agent may competitively displace other parasitoids, yet provide less overall control (Ehler and Hall 1982). This most commonly occurs where a non-host factor (e.g., nectar) is limiting. In the case of transgenic poplars, we see no readily apparent mechanism by which this could occur. Likewise, major effects on aesthetically valued species outside the genetically engineered plantations seem unlikely. Threats to endangered invertebrates arise primarily from habitat destruction, so environmental concerns would be better addressed if they included potential consequences of extensive *Populus* cultivation *per se*, rather than just the transgenic approaches taken to protect them.

Perhaps the least understood component of risk assessment concerns potential adverse affects on soil organisms. Risk assessment in this area is especially difficult because our basic understanding of nutrient cycling, detritivore taxonomy and ecology, and soil biochemical and biophysical processes is limited. Historical precedents of canopy inputs affecting soil processes include anthropogenic inputs, such as pesticides and other pollutants, and natural products such as foliar nutrients and lignins, allelochemicals from induced foliage, and insect frass (Aber et al. 1990; Cates et al. 1995; Horner et al. 1988; Mattson and Addy 1975; Melillo et al. 1982; Sugai and Schimel 1993).

Whether the effects of transgenic leaf litter are nonexistent, short- or long- term, and have point or non-point effects, depends on the gene product and soil properties. Products most commonly suggested for transformation into *Populus* have relatively high specificity, which reduces risk. For example, various *Bt* endotoxins are specific to Lepidoptera, Diptera, or Coleoptera. The first 2 groups do not appear to pose significant concerns; Lepidoptera exert a relatively minor role in litter decomposition, and genes conferring protection against Diptera are unnecessary in *Populus*. Beetles, however, are important components of the soil fauna, functioning as decomposers, vectors of beneficial microbes, and predators on a variety of potentially injurious arthropods and fungi. Thus, introduction of Coleoptera-active *Bt* or proteinase inhibitor poses some concern. Gene products with relatively general activity could be more problematic. For example, proteinase inhibitors can sometimes affect relatively diverse taxa and require more detailed evaluation.

Novel gene products could be altered by soil biochemical and biophysical processes, as occurs with synthetic materials (Angle 1994). Consideration of genetically engineered organisms must extend beyond the actions of the gene products themselves and include studies of breakdown products. The consequences of a stable gene product must also be considered. Although a protein may not be toxic to soil organisms at the levels present in a single leaf, buildup in the soil over a season or many years may pose a problem. Such residual effects are difficult to predict because stability is affected by factors such as soil pH, nutrient content, rainfall,

and temperature. The stability of proteins may also be altered within the plant and vary during the year.

Tactics for reducing potential risks to natural enemies and detritivores relate to the inherent properties of the gene products themselves and their expression. In general, environmental risk can be minimized when these products and their derivatives are specific to the target insect, of short duration, and exposed in a spatially and temporally limited pattern. Existing approaches to toxicological evaluation are available for such analyses. However, further theoretical development is required before optimal relationships between transgenic plants and parasitoids can be devised.

Conclusion

Some serious environmental concerns must be weighed against the potential benefits of genetically engineered *Populus*. Risk assessment can be improved by focusing on the most likely sources of environmental harm as opposed to generic listings of all hypothetical outcomes. As stated by previous authors from a broad range of backgrounds, emphasis should be placed on how gene products will interact with ecosystems not how these products arose. Conversely, the notion that genetic engineering has somehow been singled out for unique environmental scrutiny should be dispelled because there is a long history of guidelines and regulations limiting other insect control tactics including synthetic and naturally derived insecticides, biological control agents, insect growth regulators, antifeedants, and even cultural control (e.g., Charudattan and Browning 1992; Coulson and Soper 1989; Howarth 1991; Mcevoy 1996; Miller 1990; Samways 1988; Upholt 1985; USDA FS 1995).

A specific risk merits concern where its potential is supported by established mechanisms and relevant precedent. These criteria are met for several potential threats arising from 3 general categories of risk: 1) escape of engineered germplasm; 2) evolution of resistant biotypes; and 3) alteration of multi-trophic processes. Underlying mechanisms for each of these have been well established from multiple disciplines and across molecular through community levels. Risks can be prioritized as to whether: 1) they would be localized or affect adjacent ecosystems; 2) environmental harm would depend on continued deployment or be self-perpetuating; and 3) potential ameliorating tactics are available.

Table 5 summarizes the major anticipated risks, general mechanisms by which they might occur, historical precedents from which valuable lessons can be applied, and possible preventative strategies. Four points emerge from this overview. First, there is a need for proactive research on the likelihood of various environmental hazards and tactics for offsetting them. Second, interdisciplinary approaches are essential. Many of the challenges associated with plant genetic engineering may be identified from ecological perspectives, yet have fundamentally molecular solutions and vice versa (Raffa 1989). It is especially important that integrative collaborations function throughout the entire discovery and development process, rather than in sequential fashion. Sequential approaches fail to fully synergize the expertise that enhances efficacy and environmental safety and are likely to generate rivalries from differing vested interests. Third, none of the risks appears unmanageable if appropriate molecular, physiological, ecological, and management strategies are employed in a cohesive fashion. Fourth, *Populus* provides a particularly suitable model for research and deployment. There is a strong knowledge base from genetic, physiological, ecological, and production perspectives, and a need for traditional and emerging forest products that can be economically produced by this genus.

Table 5. Summary of environmental risks, mechanisms, precedents, and preventative strategies for genetically engineered insect resistant *Populus*.

Risk	Mechanisms	Precedents	Prevention
Escape	Pollen transfer; hybridization vegetative materials	Introduced pests	Sterility; site management early harvest
Resistant biotypes	Altered selection pressures; release from competitors, dispersion, cross resistance	Pesticides; resistant cultivars	Variable & opposing selective pressures; between- & within-plant mosaics, tissue-, temporal- & wound-specific expression
Altered tritrophic processes	Direct & indirect affects on beneficial species; effects of gene products on nutrient cycling	Introduced pests; pesticides; pollutants	Specificity of gene products and breakdown products; rapid environmental turnover of gene products; monitoring

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Chapter 33

Biotechnology Risk Assessment: A View From Plant Pathology[1]

X. B. Yang

Introduction

Agricultural science has established a framework for biological impact assessment through past work. Various risk assessments for diverse areas of agriculture, resulted in an accumulation of considerable information. Baseline information for biological impact assessment dates from the last century (Fulkerson 1987). One type of biological impact assessment is the environmental impact assessment on the release of transgenic poplar. Plant pathologists played a major role in an early experimental release of transgenic poplar (McNabb et al. 1991). Plant pathology has provided much of the information for developing biological impact assessment (Teng and Yang 1993) with a focus on complex microbe-related risk assessments. This paper discusses biological risk assessment for woody plants compared to herbaceous crops, specifically: 1) the need for and status of risk assessment; 2) risk assessment concepts; and 3) risk assessment methodology.

Need for and Status of Risk Assessment

Biotechnology has changed some sectors of agriculture into industries perceived as high risk and high return. The increasing ability to manipulate genetic components in biological systems elevates the potential for ecosystem impacts. Some propose that genetic engineering could create ecologically high-risk, super-competitive organisms that could alter ecosystem function. Biotechnology has generated new concepts and potentials for modifying biological systems. Biological scientists are asked to design predictable biological systems for human needs while sustaining natural resources (Fulkerson 1987). Many biological science disciplines assess potential ecosystem impacts from genetic engineering technology. Forestry initially focused on poplar, an agricultural crop grown worldwide for fiber, energy, and wood production, to assess the impact of biotechnology on the environment (Klopfenstein et al. 1991, 1993; McNabb et al. 1991).

Biological impact assessment estimates the potential or actual impact, including hazards and benefits, of the presence, introduction, or entrance of specific organisms into a biological system. Impacts can arise from the introduction of any new technology into a natural ecosystem, whether a physical product or knowledge-based process. All impacts should be assessed.

In microbial ecology, issues regarding testing and application of biotechnological products are frequently discussed (Gillett 1986; Lindow et al. 1989; Turgeon and Yoder 1985). Because of the nature of plant pathogens, and because microbes form the lower trophic levels of most ecosystems, genetic engineering of organisms has been considered a risk to ecosystem stability. This potential for environmental risk is associated with the: 1) creation of a new pest; 2) enhancement of existing pathogens through gene transformation; 3) harm to nontarget species; or 4) any other ecosystem disruption. Early environmental impact assessment of transgenic microbes involved the potential application of non-ice-nucleating bacteria to prevent frost injury in California (Andow et al 1989; Lindow et al 1983; Lindow and Panopoulus 1988). Currently, some epidemiological studies concern horizontal gene transfers in microbes such as those using *Colletotrichum* spp. for biological weed control (TeBeest et al. 1992).

The environmental impact of transgenic woody plants is a major issue in the development and application of bio-

[1] Klopfenstein, N.B.; Chun, Y. W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

technology. A concern of risk assessment is the potential for transgenic plants to displace native species (Duchesne 1993; Pimentel et al. 1990; Rogers and Parkes 1995; Teng and Yang 1993; Tiedje et al. 1989). Similarly, the potential of transgenic woody plants to replace wild flora is also a major concern. The need to assess the biotechnological impacts on nontargeted pests and nonpests, and the potential threat this poses to forest ecosystems has been discussed (Duchesne 1993). Unfortunately, assessing the environmental fate and impact of transgenic trees lags behind the ability to create them. Methods are needed to assess the environmental impact of transgenic trees to safely incorporate them into forestry research and silviculture.

Risks associated with transgenic plants have been extensively discussed (Gould 1988; Pimental et al. 1990; Tiedje et al. 1989). In 1987, transgenic tomato plants were the first field-tested, genetically engineered, food crop (Muench 1990). A gene encoding the coat protein of tobacco mosaic virus (TMV) was introduced into tomato plants for virus resistance. Risks associated with such a release are the potential formation of a virus with altered vectors and new host ranges and the possibility of new gene combinations (Zoeten 1991). Alternatively, such new combinations will likely occur less than that in nature (Falk and Bruening 1994). In other countries, various transgenic agricultural plants are at different stages leading up to field tests (e.g., tungro-resistant rice and virus X-resistant potato).

In the scientific community, a consensus is emerging on how much biosafety is needed. However, until a common policy is agreed upon, countries will continue to have differing guidelines. Transgenic hybrid poplar trees were used as an early first field test to assess the risk of transgenic woody plants (McNabb et al. 1991). The risk of harmful gene transfer via pollen dispersal was considered (McPartlan and Dale 1994; Sawahel 1994; Williamson 1993). A procedure to assess such risk was proposed in the Netherlands (Evenhuis and Zadoks 1991). However, methodology has not been developed. Teng and Yang (1993) proposed an outline for assessing the microbe-related risk on herbaceous crops. A conceptual risk assessment outline includes the common principles of impact assessment from different specializations, many of which are applicable to risk assessment for a woody crop such as poplar.

Concepts of Risk Assessment

The National Academy of Sciences (1983) defines "risk assessment" as the use of scientific methods, models, and data to develop information about specific risks. More recently, risk assessment was defined as a process to determine and evaluate potential risks, and the magnitude and probability of those risks occurring (Teng and Yang 1993). There are 2 steps in risk assessment: 1) risk determination, which is identifying and characterizing the risk source; and 2) risk estimation, which is estimating the probability and magnitude of adverse effects from an introduced organism in an ecosystem (figure 1). Biotechnology-related impact assessment is concerned with the estimation of potential or actual consequences after introduction of transgenic products into an ecosystem. Risk assessment before field testing may be premature unless the risk is known (Teng 1991).

Biotechnology-related risk concerns hazards that are negative acts or events in quantitative terms with the probability of risk. In this concept, risk assessment includes: 1) identifying the hazard; 2) characterizing the risk; and 3) managing the risk. The first 2 steps are similar to the risk determination step previously mentioned. Risk, in both of the above contexts, is used in plant pathogen-related assessments; however, the concept of risk changes with the discipline or situation. For example, risk is also considered the product of probability and the impact of a hazard, where hazard is any undesirable event (Evenhuis and Zadoks 1991).

"Monitoring," repetitive measurements made to specify the state of a system over time, does not include the data interpretation. Monitoring can provide a "time series,"

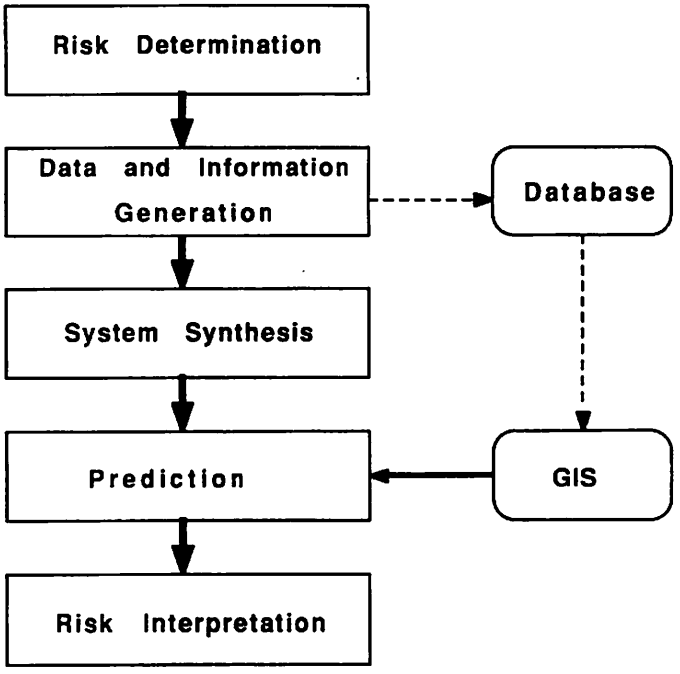


Figure 1. Schematic of risk assessment process in the context of biological impact assessment.

which is a collection of observations made sequentially in time to obtain "background" or "baseline information" (Duniker 1989). The background or baseline information is a description of conditions or dynamics existing in an ecosystem before an intervention, such as introducing a genetically engineered microbe (GEM), and serves as a check for any assessment. Statistically, a system baseline may be the inherent variability of an ecosystem. By using different statistical techniques, such as a time-series analysis, variability of system output can be partitioned into inherent variability (regular pattern) and noise. For example, Jacobi and Tainter (1988) reported a time series of drought affects on loblolly pine growth measured as reduced annual increment compared with the healthy status. The pine trees, measured annually for 60 years, showed a declining growth rate in the first 30 years, followed by a growth rate increase then a decrease again. Time-series analysis detected a 3-year periodic pattern in the data (Yang, unpublished). Patterns from such long-term data are baselines for comparison with predicted values, and the noise level provides information to estimate uncertainties of the system and its perturbations.

A "marker" is critical for quantitative assessment of recombinant DNA (recDNA) released into the environment. A good marker is sensitive, specific, efficient, and low cost. Several major techniques exist for DNA marker development: genetic-engineering, radioactivity, fluorescence, and immunology. Currently, antibiotic resistance is the primary marker for quantitative monitoring of GEM populations (Kluepfel et al. 1991). Markers of this type generally affect the competitive ability of an organism and therefore, are less useful for monitoring programs that are aimed at survival prediction. In the transgenic poplar, a chloramphenicol acetyltransferase (*CAT*) gene is used as reporter and marker gene (McNabb et al. 1991). When detected by a DNA probe, specific sequences of genetic code provide a sensitive marker, but associated cost and time limitations may restrict quantitative analysis.

The "endpoint" is a value used to characterize change at a particular level of interest (Suter 1990). Any change in an ecosystem arises from changes at the individual, population, community, or ecosystem level. To assess impact efficiently, an endpoint is defined to determine assessment output clearly. Suter (1990) proposed 2 types of endpoints in environmental risk assessment: 1) "assessment endpoint," which is a formal expression of the actual environmental values to be protected; and 2) "measurement endpoint," which is an expression of an observed or measured response to the hazard. Suter suggested that all risk assessments must have assessment endpoints based on assumptions; however, a measurement endpoint is not always possible because it must be based on observations. Suter (1990) proposed that a good assessment endpoint should be: 1) socially and biologically relevant; 2) have clear a operational definition, measurability and accessibility to prediction; and 3) be sensitive to hazard. The early field-test of transgenic poplars at Iowa State University had a well defined operational endpoint, which was to measure growth of transgenic poplars in comparison to nontransgenic poplars under field conditions (McNabb et al. 1991).

Assessment Methodology

Risk Determination

To efficiently assess a risk, ecosystem components at the individual, population, or community level must be outlined to define outputs and endpoints. Well-defined outputs and endpoints sharpen research objectives by allowing identification of knowledge gaps and data collection. There are direct and indirect effects from the introduction of transgenic plants into an environment. Direct effects are predictable by well-conducted risk assessment studies. Indirect effects, which are less predictable than direct effects, occur on nontargeted organisms feeding on the targeted organisms. For example, many lepidopteran (butterfly and moth) species were destroyed by parasites imported into Hawaii for moth control, causing the extinction of several important predators including wasps and insect-eating birds (Howarth 1980). Similarly, using fungi to control weeds can adversely affect animals that depend on the weeds. Fuxa (1988) estimated that about 8 percent of introductions for biological control, including insects and fungi, have caused extinction of native species. Sometimes, the physical environment could also be affected by introduced organisms. For example, large-scale application of non-ice-nucleating bacteria could alter normal precipitation patterns. Gene flow of an introduced transgene into nontransgenic populations is also a major concern because it could result in unwanted evolutionary consequences within an ecosystem (McPartlan and Dale 1994; Morris et al. 1994, 1994; Sawahel 1994). Assessing indirect effects from ecological and evolutionary consequences on the ecosystem is, however, difficult (Williamson 1993).

In plant pathology, 2 types of potential risk occur. If genetically modified crops are introduced, there is a risk that low durability resistance (Miller 1993; Rogers and Parkes 1995) or a change in pathogens, such as plant virus alteration will be introduced (Zoeten 1991). If modified microbes are introduced, there is a risk that they could become a new pathogen. Methods to identify this type of risk are available and commonly involve host-range testing of microbes before experimental release. Currently, to determine host range a close phylogenetic relationship between plants and their coevolved pathogens is assumed. For example, recombinant ice-nucleating bacteria were

tested on 75 plant species for pathogenicity and survival. Often, assessment results vary with the testing scale. Two pathogen-based biological control studies used the same fungus, *Colletotrichum gloeosporioides* f. sp. *aeschynomene* (TeBeest 1988; Weidemann and TeBeest 1990), but a different number of species to determine the host range. In these studies, the potential number of hosts increased as the number of tested species increased. Host range testing is helpful to avoid nontarget effects; however, Hollander (1991) considered that a pathogen's host range can change to permit exploitation of new food sources. For example, about 30 percent of pathogens attacking U.S. crops, originally fed on native vegetation before evolution allowed feeding on crop plants. While much has been learned about pathogen-host coevolution, our ability to predict potential genetic variability of many pathogens remains limited (Weidemann and TeBeest 1990).

Information is lacking on the general risks of transgenic woody plants because data on the environmental impacts of genetically modified, perennial crops, which are allowed to undergo reproductive processes in the field, is limited. Because woody plants are part of natural forests, their risk management is less controllable than annual/biannual crop systems. Studies are needed to directly address the environmental impacts of transgenic woody plants. Because of diversity in forest ecosystems, risk assessment of transgenic forestry crops is distinct from that of agronomic crops. Forest trees have undergone relatively little domestication and often have related wild relatives growing in the same area, which increases the likelihood of hybridization between introduced and native trees. For example, some researchers used genetic engineering to improve herbicide resistance of woody plants (Brasileiro et al. 1992; De Block 1990; Devillard 1992; Fillatti et al. 1987). Hybridization between transgenic and wild plants can produce a means of escape for engineered herbicide-resistance genes. Klinger and Ellstrand (1994) demonstrated with *Raphanus sativus* that an advantageous transgene introduced into natural populations tends to remain. Raybould and Gray (1994) suggested a possible invasion of hybrids produced by crossing genetically modified crops with natural communities.

Risk associated with transgenic trees involves the following questions: 1) Would the engineered gene be transmitted to other trees in the wild? 2) How does transgenic pollen compete with wild-type pollen in the environment? 3) What is the fitness of the developing progeny resulting from transgenic pollen compared with that of progeny resulting from wild-type pollen? 4) What is the stability and potential spread of the transgene in wild species? and 5) What are the overall effects of the transgene on the ecosystem? Another chapter (Raffa et al.) in this volume provides a detailed analysis of determining the potential risk associated with releasing transgenic poplar.

Information Collection and Generation

Data and information from previous research have been used in almost every study on impact assessment. MacKenzie and Henry (1991) stated, in the National Biological Impact Assessment Program (NBIAP), that analysis of existing knowledge would identify information gaps and determine the biosafety research needed. Before it is analyzed, data cannot provide the basis for a decision. Published results appropriately interpreted are considered information and are acceptable for use in decision-making. However, because information can be misleading, knowledge should be considered an abstraction of validated information. Knowledge is useful for reliable assessment, but assumptions are often required when knowledge is unavailable.

Because of the perceived or unknown nature of an introduced organism, genetically engineered organisms are tested in microcosms to collect data for a risk assessment project. A preliminary step is to use a microcosm analogous to field conditions for collecting data in the laboratory. A microcosm is a powerful tool for collecting primary data under relatively realistic, albeit restricted, conditions, which allows diverse plant pathogens to be safely and efficiently studied.

In plant pathology, microcosms are used to test an organism's response to specific environmental factors, which allows quantitative extrapolation of research results to the field environment for model development. There are 2 types of microcosms: 1) a simple design such as a growth chamber, which answers a few specific questions; and 2) a subunit of a natural system that contains selected sensitive abiotic and biotic components (Fournie et al. 1988; Pritchard et al. 1988). Data collection from a microcosm depends on the specific endpoint(s) of an assessment. For transgenic herbicide-resistant soybeans, many tests can be conducted within a microcosm. Plant pathologists can use microcosms to assess the survival of microbes under various simulated environments. Transgenic poplar plantlets were tested in a microcosm greenhouse at Iowa State University before field release (Klopfenstein et al. 1991).

Recent studies show that microcosm tests are useful to predict outcomes of many field trials of transgenic agronomic crops or microbes. Lindow and Panopoulos (1988) compared survival of non-ice-nucleating *Pseudomonas syringae* in microcosms to survival in field tests and concluded that microcosm results can closely predict field outputs. Work by Kluepfel et al. (1991) and Cook et al. (1991) provides additional evidence that microcosm studies can predict the spread and survival of genetically engineered bacteria in the field.

However, no microcosm study can replace field trials. A field test provides data on the population dynamics of an organism under natural conditions. After microcosm studies show that potential hazards are controllable in the field,

field tests of introduced organisms may be needed after approval by the appropriate government agencies. In a microcosm, simulating an entire system with all possible environmental conditions is impossible. Only field experiments provide data that comprehensively reflect the response of a released organism.

Most early field experiments examined the development of released transgenic plants. For example, the field test of transgenic poplar in Iowa determined if introduction posed an environmental risk (McNabb et al. 1991). Little information is available on competition between transgenic and wild-type woody plants. Information on competition between introduced and native organisms is urgently needed for regulatory agencies associated with risk assessment. Such information can only be confirmed in the field. An engineered trait without any adaptive advantage will probably not persist in the population. The selective advantage of an engineered trait must be great enough to improve the fitness of the transgenic plants in comparison to wild-type plants. Apart from their value in the impact assessment process, data from field studies also validate results from models and confirm conclusions from microcosm studies.

Synthesizing Information

Mechanisms for rationally synthesizing information are essential to the assessment process (Gillett 1986). This synthesis allows organization of data by ecological and statistical methods to reflect the behavior of the ecosystem after introduction of the genetically modified organism (GMO). By re-examining old data and synthesizing results, new information is often derived because the entire ecosystem is being considered. Models, expert panels, and expert systems synthesize information. Expert panels are useful when quantitative data is unavailable, while models are valuable when enough quantitative data exist to estimate the parameters of an assessed system. Models also provide information about the behavior of an introduced organism under ranging weather conditions, which expert panels and expert systems do not. These projections depend on a model's ability to reflect interactions among host, pathogens, and environment (Teng and Yuen 1991).

Expert panels, groups of subject matter authorities, assess risk. Experience with traditional unmodified organisms provides the basis for risk analysis of genetically modified organisms (MacKenzie and Henry 1991). The U.S. National Biological Impact Assessment Program (NBIAP) (MacKenzie 1991) used this approach to solicit consensus protocols for field testing GEMs and genetically altered plants. Expert opinion was used to estimate disease impact on crops and the potential of biotechnology to reduce impact (Herdt 1991).

An expert system, composed of a knowledge base and a procedure to infer an answer, is a computer program designed to simulate the problem-solving mechanism used by subject matter experts (Latin et al. 1987). Until general knowledge on the entire impact assessment process is available for field tests with GMOs, the only available system for impact assessment is the electronic bulletin board used by the National Biological Impact Assessment Program (MacKenzie and Henry 1991). In comparison with expert panels, expert systems are better for information delivery rather than improving assessment reliability.

When quantitative information is available, a model provides a positive or negative answer and an assessment of risk magnitude. Empirical and mechanistic models are available for prediction. Empirical models use statistical methods such as regression models, with limited predictive ability for biotechnology-related assessment. Mechanistic models use an understanding of underlying processes within an ecosystem. Most models used for risk assessment are simulations that, when coupled with databases and extrapolation algorithms, are useful to assess different ecosystem change strategies (Teng 1991). Simulation modeling, considered the most reliable technique for risk assessment (Duniker and Baskerville 1986; Teng 1991), has been used to assess chemical risks to the environment (Barnthouse and Suter 1984; O'Neill et al. 1982), impacts of plant pathogens in biocontrol (DeJont et al. 1990), plant quarantine programs (Yang et al. 1991), and biotechnology benefits (Andow et al. 1989). A simulation model was used with historical weather data to determine risk of several diseases to wheat (Luo and Zeng 1990; Luo et al. 1995). In global warming studies, simulation models are frequently used to assess the future impact on biodiversity under different climate scenarios.

The CLIMEX computer program developed in Australia exemplifies computer techniques for generating predictive models. The program, which compares climates in ecology, was applied to modeling microbes, arthropods, and plants (Sutherst and Maywald 1985; Worner 1988). Concerns about global climate change, have focused attention on the theory and methodology of climatic requirements of organisms as tools for predictive ecology. If transgenic poplar grows over large areas, the CLIMAX program may quantitatively assess potential damage from new insects, pathogens, and weeds.

Prediction and Evaluation

Risk prediction uses data-based predictive models to estimate chances of undesirable events. Prediction can be over time, which is assessing a future event or risk based on present information, or can be over space, which is regionally assessing the event or risk over a defined area based on information from multiple sites. Prediction over time is feasible if baseline information is available; how-

ever, accuracy decreases as the predictions extend further into the future.

Impact prediction over space and time relies on a quality database; prediction value is enhanced by data used in conjunction with geographic information systems (GIS) and geostatistical techniques. A database is critical for any regional assessment and should contain spatial data sets of vegetation, hosts, pathogens, weather, soil, and other variables that represent ecosystem attributes over time. Data on each variable is a descriptive information layer covering the region of interest. Because most ecosystem variables are interrelated, the population magnitude can be calculated if such relationships are available. Several computerized databases are being developed for impact assessment in plant pathology.

Geographic information systems, computer programs that use specific mathematical algorithms to enable input, management, analysis, and display of geographic point data (Berry 1987), are powerful tools for environmental risk assessment. A quantitative regional assessment requires analyses of extensive spatial data. In a GIS, each data set is a layer over a geographic area; layers are physically and biologically correlated. Knowing the relationships among layers allows assessment of regional impact. A regional impact map can be composed from spatially interrelated maps of individual layers. Because maps are visual evidence, they frequently help managers assess impacts and expedite the decision-making process.

GISs have been used in biological impact assessment in plant pathology. To predict the occurrence of potato-late blight in Pennsylvania, Royer et al. (1990) used a disease model with inputs from a relative moisture and temperature map. Royer and Yang (1991) used similar methods to generate a potential epidemic map of soybean rust in Pennsylvania and Maryland. GIS techniques allow generation of hazard maps in different forms depending on data set availability. However, if data are available from only limited locations, extrapolation across these locations may produce an overlay map that generates inaccurate conclusions (Berry 1987). An impact map with distinct symbols is appropriate when location number is limited. Using data from remote sensing for GIS analysis, Steven et al. (1991) predicted the potential invasion of a weed, Dyers woad (*Isatis tinctoria*), in northern Utah.

Although tools exist to project system behavior into the future, risk evaluation is still an art (Gillett 1986). Because risk is the probability of the magnitude of a hazard (Duniker and Baskerville 1986), some assessment uncertainty and subjectivity will always exist. Risk management depends on the extent that uncertain elements in an assessment have been identified. In microbe-related risk assessment, uncertainty can arise from every ecological process and failure becomes a function of these events (Alexander 1985). Prediction of survival and spatial dispersal produces the most uncertainty in microbe-related risk assessment because survival and dispersal are the least known aspects of epidemiology. For example, in DeJont et al.'s (1990) assessment for a mycoherbicide, the distance of spore dispersal was calculated by a Gaussian model with limited observations. Predictions with Gaussian models for long-distance dispersal (greater than 1,000 m) have not been validated for many pathosystems. Furthermore, socio-political factors exert a strong influence on risk interpretation for decision making. As recommended by the Gaussian model, it was decided that the safe distance between an applied area and an orchard was 5,000 m. However, the Dutch government determined that the risk of using *Chondrostereum purpureum* to control *Prunus serotina* is acceptable when an application area is less than 500 m from an area of commercial fruit production (DeJont et al. 1990). This illustrates that risk assessment is distinct from interpretation.

Conclusion

Hundreds of field tests with GMOs have been completed; the results demonstrate that behaviors of GMOs are predictable (Casper and Landsmann 1992; MacKenzie and Henry 1991). Currently, no generally acknowledged methodology for biological impact assessment exists; most research efforts use their own techniques. As discussed previously, biological risk assessment includes risk determination and risk estimation. Risk determination results in evaluation options, but often is the main assessment activity. This activity consists of system definition, risk identification, and determination of endpoints and knowledge gaps. Risk estimation generates data and information from microcosm and field studies, and synthesizes ecosystem-wide information into a prediction system capable of generating options. Results from risk determination and risk estimation are subject to risk evaluation.

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Chapter 34

Biotechnology, Biodiversity, and Bioethics[1]

M. Raj Ahuja

Introduction

Recent advances in plant biotechnology have enabled genetic improvement of forest trees. Basic techniques in tissue culture, genetic engineering, and molecular biology, developed in herbaceous crops, have been applied to forest tree species with varying successes. Although this suggests that biotechnology methods need further investigation and adaptation for application to long-lived and highly heterozygous forest trees, several recent advances are promising. These include rapid clonal propagation, germplasm preservation, gene transfer, molecular markers, genome mapping, and the isolation, cloning, and expression of genes (Ahuja 1991a, 1993a; Ahuja et al. 1996; Bonga and Durzan 1987). Although considerable progress was achieved in some of these areas during the past decade, others are in the embryonic stage.

Most of the advances mentioned above are discussed elsewhere in this book. I will address clonal propagation (*in vitro* regeneration) and gene transfer (genetic engineering) in relation to biodiversity and bioethics. Although somewhat philosophical, the pros and cons of new technologies should be examined in the framework of their impact on biodiversity, long-term adaptation, and evolutionary survival of forest trees. Pertinent questions include: What rules and ethics should people follow for long-term biodiversity conservation? Is the application of biotechnology, involving clonal propagation, likely to substantially reduce genetic variation in trees? Would the transfer of chimeric genes into forest trees increase the risk of undesirable genetic instability? Would new or foreign genes escape from commercial plantations, become established in natural populations, and later become maladapted? Would transgenic plants generate new diseases and pose a threat to the host ecosystem? These and related questions are discussed in this chapter.

Forest Biotechnology

Forest biotechnology development, although similar to that of herbaceous plants, has subtle differences including the longevity, heterozygous nature, life cycle, and environment of forest trees. Forest trees have long generation cycles, with the vegetative phase ranging from 1 to several decades. Once trees are germinated in nature or transplanted to plantations, they generally remain anchored in 1 location where they are exposed to changing environments and other vagaries of nature. Some of these factors may influence their physiology and alter complex morphogenetic processes. During the long life cycle of trees, many genetic and epigenetic changes are probably important to maintain within-tree and within-population genetic diversity, and to assure long-term survival of individuals and populations. Therefore, application of biotechnology to woody perennials should be considered long-term goals, as opposed to short-term annual benefits appropriate to crop plants that are harvested and replaced each year. This suggests a strategy shift for genetic improvement and modification, and for maintenance of forest biodiversity. Two promising methods in biotechnology, involving *in vitro* regeneration and genetic engineering, are discussed in relation to forest tree biodiversity.

In Vitro Regeneration and Genetic Diversity

In vitro regeneration of plants may be accomplished by exploiting one of the differentiation pathways, either from haploid or somatic tissues. The pathway from haploid tissues involves regeneration by organogenesis, which is the sequential differentiation (usually not simultaneously) of shoots and roots on tissues. The pathway from somatic tissues may proceed via embryogenesis, which is when

[1] Klopfenstein, N.B.; Chun, Y. W.; Kim, M.-S.; Ahuja, M.R., eds. Dillon, M.C.; Carman, R.C.; Eskew, L.G., tech. eds. 1997. Micropropagation, genetic engineering, and molecular biology of *Populus*. Gen. Tech. Rep. RM-GTR-297. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station. 326 p.

both shoot and root poles differentiate more or less simultaneously on the embryogenically competent cells. Somatic embryos are structurally similar to zygotic embryos and, following germination, produce somatic seedlings.

In vitro regeneration by organogenesis has been achieved with many woody plant species. With most, juvenile explants (e.g., embryos, cotyledons, or shoots from young plants) were used for clonal propagation (Ahuja 1988, 1991a, 1993a; Bonga and Durzan 1987; Pardos et al. 1994). Rejuvenation by tissue culture from mature tissues remains challenging in many forest tree species, although a degree of *in vitro*-conditioned rejuvenation was demonstrated in some species, including *Populus* (Ahuja 1983, 1986, 1987, 1993b; Ahuja et al. 1988; Chun 1993; Ernst 1993).

Since the first report by Winton (1968) on plantlet regeneration from callus cultures of triploid quaking aspen (*P. tremuloides*), several other species and hybrids of *Populus* were clonally propagated by tissue culture technology (Ahuja 1988, 1991a, 1993a; Bonga and Durzan 1987). By employing bud explants from 23- to 40-year-old trees of European aspen (*P. tremula*) and hybrid aspen (*P. tremula* x *P. tremuloides*), clonal propagules were regenerated for multiple genotypes (Ahuja 1983, 1986, 1993b). Micropropagation varied among the aspen trees. Bud explants from some mature aspens were unresponsive to tissue culture milieu, while in others they exhibited successful differentiation of microshoots for plant regeneration. Among other factors, regeneration potential of tissue depends on the explant source, tissue physiological state, time of year when tissue collection occurs, and age and genotype of the donor tree. In forest trees, maturation state and genotype largely determine the regeneration potential. In aspens, tissues from mature-tree buds apparently do not require special rejuvenation treatments because bud explants can grow and differentiate on relatively simple culture media. The genotype apparently is predominant in determining the *in vitro* morphogenetic response in aspens. In many other hardwoods, such as beech, oak, and most conifers, growth and differentiation from tissues of mature trees are strongly influenced by the maturation state. Theoretically, maturation state may also indirectly affect genetic diversity by modifying propagation qualities. In this situation, elite and mature genotypes may not contribute to the overall genetic diversity of the population.

Regeneration by somatic embryogenesis is another promising technique for large-scale, clonal multiplication of woody plant species. In most studies, somatic embryos were differentiated from the embryonal axes of zygotic embryos. Clonal propagation by somatic embryogenesis in several conifer and hardwood species was reported (Becwar 1993; Gupta et al. 1993; Gupta and Grob 1995; Redenbaugh and Ruzin 1989). Since somatic embryos can be grown in liquid media, it should be possible to increase their production in bioreactors for mass cloning of elite genotypes (Becwar 1993; Gupta et al. 1993; Gupta and Grob 1995). For an efficient delivery system to plant somatic embryos in soil, somatic embryos were encapsulated to produce "artificial seeds" (Redenbaugh and Ruzin 1989). This technology should be available for commercial forestry by the year 2,000 (Gupta et al. 1993).

In spite of optimism for somatic embryogenesis-mediated clonal forestry, several basic problems must be resolved (Chen and Ahuja 1993). Inducing somatic embryos is difficult for many commercially important forest tree species and genotypes, or the frequency of induced somatic embryos may be too low for practical applications. In several forest tree species, there are also problems with maturation and germination of somatic embryos and development of encapsulation protocols leading to somatic seedlings. Since somatic embryos usually go thorough a callus development phase, the relative genetic stability of somatic embryos, somatic seedlings, and somatic plants (Ahuja 1991b; Ahuja and Libby 1993) should be investigated at the morphologic and molecular levels before introduction into clonal forestry programs.

There are 2 main genetic concerns with tissue culture-derived plants, whether regenerated by organogenesis or somatic embryogenesis. The first relates to genetic instability in plant tissue cultures. Plant cells grown in an artificial environment of tissue culture are often under stress, and are likely to generate genetic and epigenetic mistakes; rapid mitotic divisions in the callus phase may contribute to this instability. Such genetic changes are manifested as single gene mutations, chromosome aberrations, and modifications of DNA methylation patterns (Kaeppler and Phillips 1993; Phillips et al. 1994). Callus cells are especially vulnerable to tissue-culture induced mutations. One possible cause of genetic variation in cultured tissues is the use of high concentrations of phytohormones. In particular, the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) induces a high frequency of changes in chromosome structure (Pavlica et al. 1991) and variation in DNA methylation patterns (Phillips et al. 1994). New genetic variation in micropropagated plants may be reduced by using only low concentrations of hormones and excluding 2,4-D. Our two-step micropropagation method (Ahuja 1984), using an aspen culture medium (ACM) (Ahuja 1983), produced over 10,000 regenerants from bud explants of more than 100 European aspen and hybrid aspen mature trees. In our cultures, minimal callus formation occurred, and microshoots differentiated directly on the bud explants. Very few gross phenotypic variants were observed in these micropropagated aspens; however, undetectable gene mutations may have been present. Likewise, new genetic variation could be minimized in other woody perennials by using organogenesis or somatic embryogenesis for regeneration. Although variability will always occur, it depends on how much and what types are acceptable for clonal forestry programs.

A second concern relates to the potential loss of genetic diversity by clonally propagating forests. A common belief is that clonal forestry leads to genetically uniform forests, which is a possibility but not a certainty (Libby 1982). Through use of sufficient numbers of pedigreed clones, genetic diversity can be effectively maintained, better controlled, and even enhanced in clonal forests, as compared with management options for zygotic or seedling forestry (Libby and Ahuja 1993). Through effective education and/or regulation, common management errors in testing and deployment can be avoided in a clonal forestry program.

Genetic Engineering and Biodiversity

Although genetic engineering and hybridization by conventional breeding can augment genetic variation in plants, there are important differences between these 2 processes. Not only are different techniques used, but the type of genes transferred and the time required also differ substantially. In terms of quick returns, the time needed to produce a new genotype can be a critical factor for its commercial exploitation. Producing and using genetically stable woody plants may require a long time span; therefore, a conflict exists between the basic research required to generate and test reliable new genotypes, and the commercial desire for quick returns.

A breeder uses a backcross program to transfer desirable genes from 1 species to another. Following hybridization, the hybrid and its progeny are backcrossed repeatedly for several generations to 1 parent species so that a single dominant gene or a small set of desirable genes is transferred. Essentially, this introgressive hybridization procedure selects for a single or a few gene(s) from 1 parent in the genetic background of the recurrent backcross parent. By using this approach, several useful genes for disease resistance or other traits were transferred in plants (Ahuja 1962; Ahuja and Hagen 1967; Knott 1961; Sears 1956). Transfer of such genes may involve intergeneric, interspecific, or intraspecific hybridizations. The F_1 intergeneric and interspecific hybrids are usually sterile; however, male sterility is more common than female. Some viable eggs may be formed in these hybrids between widely divergent species or genera; these can be used for backcrosses to the recurrent male parent. The hybrid and first backcross generations are highly heterozygous and exhibit considerable morphological and chromosomal variation. The backcross program is accompanied by selection of phenotypes with the desired trait(s) from the vast array of genetic variants in the backcross generations. Intraspecific hybridizations involving the transfer of a dominant or a recessive allele from the donor to the recurrent parent may similarly involve backcross or selfing cycles to achieve the desired goal. However, released genetic variability would be relatively less compared with the larger variation that accompanies transfer of genes by the interspecific or intergeneric hybrid route.

Gene transfer by genetic engineering is a one-step process that bypasses the time-consuming hybridization procedure. However, to establish the mode of inheritance and transmission of transgenes from male and female gametes, hybridization between transgenic and control plants is necessary. Genetic engineering uses chimeric or recombinant genes constructed by DNA recombinant technology. The chimeric genes are placed between the transferred DNA (T-DNA) borders of a disarmed tumor inducing (Ti) plasmid from *Agrobacterium* and transferred to plants mainly by an *Agrobacterium*-mediated gene transfer method or a biolistic DNA delivery system. Because a few genes are usually located between the T-DNA borders, at least 2 or more chimeric genes are transferred to plants during genetic transformation. In most studies, 1 selectable marker and 1 reporter gene are included. Other DNA sequences are usually included in the cassette but are typically ignored for the analysis. The reporter genes may be attached to promoters from viruses, bacteria, or plants. For example, the gene encoding neomycin phosphotransferase (*NPTII*), which confers kanamycin resistance from bacteria, is put under the regulatory control of a *NOS* (nopaline synthase) or *OCS* (octapine synthase) promoter from *Agrobacterium*. A proteinase inhibitor II (*PIN2*) gene from potato conferring pest tolerance under control of *35S*, a promoter region derived from the cauliflower mosaic virus, or *NOS* was expressed in transgenic hybrid poplar (Klopfenstein et al. 1993). As mentioned, besides the hybrid chimeric genes, other genetic sequences are often present between the T-DNA borders; these usually remain cryptic or uninvestigated. Therefore, an array of other "hitchhiking" DNA sequences in the T-DNA are transferred to the host plant along with the marker and reporter genes.

Transfer of genes by hybridization is a slow process that can require years for crop plants and perhaps decades for forest trees. Introgressive hybridization involving transfer of genes from 1 species to another has been important in plant speciation and evolution. Genes that displayed adaptive fitness were retained, while those with low fitness were gradually eliminated. Traditional breeding has been employed for genetic improvement between individuals in a species or between related species, where the genetic differences are minimal. Alternatively, genetic engineering can move functional genetic traits between widely divergent organisms in a relatively short time. Transgenic plants, including *Populus*, were produced with functional genes from insects (e.g., luciferase gene from the firefly) and bacteria (e.g., herbicide-resistance genes), and transgenic pigs and rodents were produced with functional human genes. This phylogenetic leapfrogging offers many unique opportunities to create populations with novel combinations of adaptive (Regal 1994) and nonadaptive genes. In ecologi-

cally competent organisms, such as forest tree species, introduced genes of low fitness may be eliminated by natural selection over the course of several generations. Most new trait combinations in transgenic plants do not seem ecologically adaptive, as with the firefly luciferase gene in tobacco or *Populus*. However, genes conferring resistance against diseases, pests, frost, or salinity might be more adaptive in selected ecosystems.

Although genetic engineering offers options for generating new gene combinations that may be adaptive, an inherent risk of genetic instability associated with transgenes exists in herbaceous annual plants and forest trees (see chapter titled "Transgenes and Genetic Instability" in this volume). Not only is transgene expression unpredictable, but *trans*inactivation of ectopic or endogenous genes are more frequent in transgenic plants than was anticipated. This implies that transgenic plants may be genetically unstable when they possess certain transgenes. Long-term testing of transgenic trees is recommended before widespread use.

Biodiversity is defined as variety and variability among living organisms and the ecosystems in which they exist (Woodruff and Gall 1992). Biodiversity may be considered at 3 hierarchial levels: 1) genetic diversity; 2) species diversity; and 3) ecosystem diversity (Smitinand 1995). In this chapter, biodiversity is the genetic variation within populations of genetically engineered or aggressively bred species in a plantation-generated ecosystem. In a changing environment, populations with diverse pools of genetic variation should be more ecologically competent, with increased response and survival during large and/or rapid environmental change. Tree species belong to this category and have survived the vagaries of nature over millions of years. Cross-hybridization between individuals within populations or between species and genera has enabled natural gene transfer, which has enriched gene pools and contributed to survival and evolution.

Transfer of genes between species and genera by introgressive hybridization is a slow process, but natural selection allows adequate time to incorporate adaptive gene combinations and reject those of inferior fitness. Phylogenetic leapfrogging by genetic engineering, which bypasses the traditional genetic tradeoffs (Regal 1994), presents a new dimension in biodiversity for long-lived forest trees. Whether genetic engineering-mediated gene transfers contribute to adaptive biodiversity in forest tree species or generate more genetic instability should be examined on a long-term global basis.

Bioethics in Biotechnology

Advances in biotechnology, and especially genetic engineering of plants and animals, inspire reflection on their short- and long-term affects on the global ecosystem. Bioethical concerns in forestry originated with conservationally minded foresters and environmentalists who had an interest in maintaining biodiversity and ecosystem stability (Coufal and Spuches 1995). Disturbances in 1 component of the forest ecosystem can cause devastating affects on other ecosystem elements. Certain ethical rules and biosafety regulations should be followed during the application of biotechnological research. Conservation of the genetic diversity of tree populations and the biodiversity of the ecosystems in which they grow is imperative. Because of differences in their value judgements, those interested in quick returns of biotechnological applications are often in conflict with "go-slow" traditionalists.

Forest trees are long-lived, usually well adapted, and have relatively long generation cycles with vegetative phases that can extend from 1 to several decades. Most genetic changes in their genome structure, whether by classical mutation or phylogenetic leapfrogging, may generate instability. However, some genetic changes may have adaptive value over time. According to Tiedje et al. (1989), evaluation and regulation of transgenic organisms should be based on their biological properties, including phenotypes, rather than on the genetic techniques used to produce them. Transgenic trees should be evaluated at different stages of their development by morphological, biochemical, and molecular methods to monitor transgene expression and other unexpected genetic changes. Since the potential risks of releasing transgenic poplars are discussed in chapters by Raffa et al. (this volume) and Yang (this volume), they are only briefly discussed here.

The release of transgenic plants into natural ecosystems raises several questions: 1) Will transgenic crops generate new viruses and new diseases? (Falk and Bruening 1994); 2) What are the potential risks of cross-fertilization between transgenic crops and their wild relatives? (Baranger et al. 1995; Kareiva et al. 1994; Paul et al. 1995); and 3) Will transgenic trees be genetically stable on a long-term basis under field conditions? Whether transgenic crops could create new viruses and diseases is a small concern for woody plants. Some viruses may infect forest trees, while others may exist as systemic entities. Unless trees are engineered for resistance against a specific virus, the risk of creating a virulent new virus by genetic recombination seems minimal. Alternatively, interactions between a transgene or its product with an endogenous tree virus might produce a mutant virus that may be harmful to the host. Therefore, it is difficult to predict if transgenic plants could become a source of new viruses.

The second concern, escape of transgenic pollen into the environment and cross-fertilization of wild relatives, poses serious biosafety and regulatory problems. In the worst scenario, the engineered plants or their hybrid derivatives may become invasive and eliminate original untransformed populations. Pollen can travel long distances or

can be spread by pollinating insects. Forest trees are generally cross pollinated; therefore, the spread of transgenic pollen to related species is considered a genuine biological concern. A recent study shows that containment of recombinant pollen or transgenes is unlikely if physical isolation is the only strategy (Kareiva et al. 1994). Genetic engineering of reproductive sterility in trees is another option (Strauss et al. 1995) for containing transgenes. Previously, it was argued that engineering of male sterility could stimulate faster tree growth and wood production, reduce production of allergic pollen in the atmosphere, and offer new options for hybrid breeding (Strauss et al. 1995). Currently, it is thought that some of these suggestions are based on speculation. Studies are needed to determine whether wood production can be increased by decreasing floral tissues through manipulating floral-specific genes, toxin encoding genes controlled by floral-specific promoters, or other approaches. However, manipulating floral-specific genes, or introducing the *rol*C gene from *Agrobacterium rhizogenes* with an appropriate promoter, may produce growth abnormalities in trees. The *rol*C gene under control of a *35S* promoter causes a degree of male sterility in tobacco and a multitude of morphological and physiological changes (Schmülling et al. 1993). The *rol*C gene under control of 2 different promoters, *35S* and *rbcS*, has also been transferred to aspen (Fladung et al. 1996). In aspen, *35S-rol*C transgenics exhibited much smaller, nonlanceolate, pale-green leaves than *rbcS-rol*C transgenic aspens, which produced pale-green leaves that were only slightly smaller than the controls. In tobacco, the *35S-rol*C transgene induces lanceolate and pale-green leaves. A transgene may act differently in an annual tobacco plant and a perennial *Populus* tree. The effects of *rol*C in tobacco or *Populus* have only been studied under greenhouse conditions. Therefore, whether this pleiotropic gene will behave similarly under the 2 sets of environments cannot be predicted. Nevertheless, all strategies toward containment of transgenic pollen or lessening of floral tissues in transgenic trees should be encouraged.

Gene silencing has developed into a challenging field of study that will hopefully contribute toward understanding transgenes and native gene stability. Recently, instances of transgene instability were reported (Finnegan and McElroy 1994; Jorgensen 1995; Matzke and Matzke 1995). The bioethic principle requires that all research in genetic engineering of plants or animals be reported for administrative authorities to conduct proper risk assessment for biosafety regulation of transgenic organisms (Giampierto 1994; Miller et al. 1995). More than 800 applications have been filed for testing genetically modified organisms in the environment, most of them in the United States (The Gene Exchange 1993). Nearly all of these tests were conducted on small plots, which may not reflect the entire spectrum of transgene-environment interactions. Since commercial application of transgenic crops would occur in large-scale field facilities, extrapolations from small plots may not provide adequate risk assessment information (Seidler and Levin 1994).

Value Judgement

Recent advances in biotechnology have ushered in a new era for the genetic improvement of forest tree species. Micropropagation through organogenesis and somatic embryogenesis has been achieved in many woody plants. Biotechnological procedures have also been effectively used to preserve woody plant germplasm (Ahuja 1989, 1994; Chun 1993). Various approaches have been used to transfer foreign genes to forest trees. Genetic engineering can provide exceptional genotypes for integration into clonal forestry and zygotic forestry programs. However, concerns have been raised regarding decreased biodiversity by clonal propagation, increased risk of genetic instability in genetically engineered plants, and the potential escape of transgenes into natural populations. Genetic variation can be maintained and better managed by employing appropriate numbers of genetically diverse pedigreed clones in clonal forestry programs (Ahuja and Libby 1993; Libby 1982). Multiclonal blocks of clones are recommended for conservation of ecosystem biodiversity.

The highest risk regarding transgenic crops is the escape of transgenic pollen into nature and hybridization of wild relatives (Abbot 1994; Angle 1994; Kareiva et al. 1994; Regal 1994; Seidler and Levin 1994; Williamson 1994). Since transgenic pollen would be difficult to contain under natural conditions, especially when transgenic crops are grown commercially, other strategies, including genetic manipulation of reproductive sterility (Strauss et al. 1995), should be explored for forest trees. Presently, more information is needed about dispersal, and the ability of transgenic pollen to preferentially pollinate wild relatives (Baranger et al. 1995; Lefol et al. 1995; Paul et al. 1995); or possibly transgenic pollen may pose no more risk than normal pollen from untransformed trees. Because of the relative ease of *in vitro* regeneration and genetic transformation, *Populus* can serve as a valuable model system to evaluate questions regarding the ecological risks of transgenic trees. Nevertheless, environmentally compatible applications of biotechnology must be developed (Frederick and Egan 1994), and biosafety regulations and directives must be followed until the risk factors associated with genetically engineered woody plants are assessed and the adequate means to address them are found.

The relevance of bioethics in forestry is emphasized in the following quote from a recent paper by Coufal and Spuches (1995). "The value of ethics in forestry is unlimited, although not always plain to see. At best, ethics com-

mands the power of moral authority, an authority that can often be flouted with few visible consequences, unlike the authority of the laws of nature. If we can believe that ethics allows us to view the world more clearly and not be limited by our scientific perceptions of it; that good forestry management will not be possible unless ethical dimensions are given the same consideration as scientific and economic factors; and thus that ethics should be an integral part of all forestry curricula . . . ."

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Section VI Appendices

Authors and Affiliations

M. Raj Ahuja
Federal Research Centre for Forestry and Forest Products,
Institute of Forest Genetics
Sieker Landstrasse 2
22927 Grosshansdorf
Federal Republic of Germany
Tel: 49 4102 696 156
Fax: 49 4102 696 200

Marie Baucher
Laboratorium voor Genetica
Department of Genetics
Flanders Interuniversity Institute for Biotechnology
Universiteit Gent
K.L. Ledeganckstraat 35
9000 Gent
Belgium

Tannis L. Beardmore
Natural Resources Canada
Canadian Forest Service
Hugh John Fleming Forestry Complex
Regions St. S.
P.O. Box 4000
Fredericton, New Brunswick
Canada E3B 5P7
Tel: 506 452 3881
Fax: 506 452 3525
E-mail: tbeardmore@fcmr.forestry.ca

Wout Boerjan
Laboratorium voor Genetica
Department of Genetics
Flanders Interuniversity Institute for Biotechnology
Universiteit Gent
K.L. Ledeganckstraat 35
9000 Gent
Belgium
Tel: 32 9 2645202
Fax: 32 9 2645349
E-mail: woboe@gengenp.rug.ac.be

Richard C. Carman
USDA Forest Service
Rocky Mountain Forest and Range Experiment Station
National Agroforestry Center
East Campus - University of Nebraska
Lincoln, NE 68583-0822
USA
Tel: 402 437 5178 ext 17
Fax: 402 437 5712

Maria Teresa Cervera
Laboratorium voor Genetica
Department of Genetics
Flanders Interuniversity Institute for Biotechnology
Universiteit Gent
K.L. Ledeganckstraat 35
9000 Gent
Belgium
Tel: 32 0 9 264 53 70/71
Fax: 32 0 9 264 53 49
E-mail: mamon@gengenp.rug.ac.be

Brigitte Chabbert
Laboratoire de Chimie Biologique
Institut National de la Recherche Agronomique (INRA)
F-78850 Thiverval-Grigon
France

Pierre J. Charest
Natural Resources Canada
Canadian Forest Service
580 Booth Street, 7th floor
Ottawa, Ontario
Canada K1A 0E4
Tel: 613 947 9011
Fax: 613 947 9090
E-mail: pcharest@am.ncr.forestry.ca

Vincent L. Chiang
Institute of Wood Research
Department of Forestry
Michigan Technological University
1400 Townsend Drive
Houghton, MI 49931-1295
USA
Tel: 906 487 2454/2959
Fax: 906 487 2915
E-mail: vchiang@mtu.edu

Young Woo Chun
College of Forestry
Kookmin University
861-1, Chongnung-dong, Songbuk-gu
Seoul, 136-702
Republic of Korea
Tel: 82 2 910 4812
Fax: 82 2 910 4809
E-mail: ychun@kmu.kookmin.ac.kr

Gary D. Coleman
Department of Horticulture and Landscape Architecture
122 Holzapfel Hall
University of Maryland at College Park
College Park, MD 20742-5611
USA
Tel: 301 405 4371
Fax: 301 314 9308
E-mail: GC76@umail.umd.edu

Daniel Cornu
Station d'Amélioration des Arbres Forestiers
Institut National de la Recherches Agronomique (INRA)
F-45160 Ardon
France

Yvonne Devantier
Molecular Genetics and Tissue Culture Group
Petawawa National Forestry Institute
Canadian Forest Service
Chalk River, Ontario
Canada K0J 1J0

Madelyn C. Dillon
USDA Forest Service
Rocky Mountain Forest and Range Experiment Station
3825 East Mulberry Road
Fort Collins, CO 80524
USA
Tel: 970 498 2344
Fax: 970 498 1660
E-mail: FSWA/S=M.Dillon/ou=S28A
@MHS.ATTMAIL.COM
DG address: M.Dillon:s28a

Ronald J. Dinus
Department of Wood Sciences
Faculty of Forestry
University of British Columbia
Vancouver, B.C.
Canada V6T 1Z4

Mary Ellen Dix
USDA Forest Service
Rocky Mountain Forest and Range Experiment Station
National Agroforestry Center
East Campus - University of Nebraska
Lincoln, NE 68583-0822
USA
Tel: 402 437 5178 ext 25
Fax: 402 437 5712
DG address: M.Dix:S28L04a

Gerry C. Douglas
Agricultural and Food Development Authority
Kinsealy Research Centre
Malahide Rd.
Dublin 17
Ireland

Hiroyasu Ebinuma
Central Research Laboratory
Nippon Paper Industries
Oji 5-21-1, Kita-ku
Tokyo 114
Japan
Fax: 81 3 3914 3350

LoriAnn E. Eils
Minn vitro, Inc.
15 West 36th Street
Minneaplois, MN 55408
USA

David D. Ellis
BC Research Inc.
Conifer Biotechnology Group
3650 Westbrook Mall
Vancouver, BC
Canada V6S 2L2
Tel: 604 224 5331
Fax: 604 224 0540
E-mail: Elvis@bcr.bc.ca

Saori Endo
Central Research Laboratory
Nippon Paper Industries
Oji 5-21-1, Kita-ku
Tokyo 114
Japan

Patricia Faivre-Rampant
Station d'Amélioration des Arbres Foretiers
Institut National de la Recherche Agronomique (INRA)
F-45160 Ardon
France

D. John Fry
Department of Environmental Resource Management
Faculty of Agriculture
University College, Belfield
Dublin 4
Ireland
Tel: 353 1706 7777
Fax: 353 1706 1102
Telex: 32693 EI

Marie-Christine Goué
Institut National de la Recherche Agronomique (INRA)
Station d'Amélioration des Arbres Foretiers
F-45160 Ardon
France

Stephen C. Grado
College of Forest Resources
Mississippi State University
Box 9681
Mississippi State, MS 39762-9681
USA

Richard B. Hall
Department of Forestry
235 Bessey Hall
Iowa State University
Ames, IA 50011
USA
Tel: 515 294 1453
Fax: 515 294 2995
E-mail: rbhall@iastate.edu

Simon Hawkins
Station d'Amélioration des Arbres Forestiers
Institut National de la Recherches Agronomique (INRA)
F-45160 Ardon
France

Scott Heuchelin
Department of Plant Pathology
Russell Laboratories
University of Wisconsin - Madison
1630 Linden Drive
Madison, WI 53706
USA
Tel: 608 263 2093
Fax: 608 263 2626
E-mail: sah@plantpath.wisc.edu

Martin Hubbes
University of Toronto
Faculty of Forestry
33 Willcocks Street
Earth Sciences Centre
Toronto, Ontario
Canada M5S 3B3
Tel: 416 978 6831
E-mail: hubbes@larva.forestry.utoron

Dirk Inzé
Laboratoire Associé de l'Institut National de la Recherche Agonomique
Universiteit Gent
K. L. Ledeganckstraat 35
B-9000 Gent
Belgium

Cathy Jones
Molecular Genetics and Tissue Culture Group
Petawawa National Forestry Institute
Canadian Forest Service
Chalk River, Ontario
Canada K0J 1J0

C. P. Joshi
Institute of Wood Research
Department of Forestry
Michigan Technological University
1400 Townsend Drive
Houghton, MI 49931-1295
USA

Lise Jouanin
Laboratoire de Biologie Cellulaire
Institut National de la Recherche Agronomique (INRA)
Centre de Versailles
F-78076 Versailles Cedex
France
Tel: 33 1 30 83 30 63
Fax: 33 1 30 83 30 99
E-mail: jouanin@versailles.inra.fr

Hoduck Kang
Tree Molecular Biology Laboratory
Division of Forest Microbiology
Forestry Research Institute
Chongryangri-Dong, Tongdaemun-Ku
Seoul 130-012
Republic of Korea
Tel: 82 2 961 2625
Fax: 82 2 967 5101

David F. Karnosky
Michigan Technological University
School of Forestry and Wood Products
1400 Townsend Drive
Houghton, MI 49931-1295
USA
Tel: 906 487 2898/2454
Fax: 906 487 2897/2915
E-mail: Karnosky@mtu.edu

Julie Russell Kikkert
Cornell University
New York State Agricultural Experiment Station
Department of Horticultural Sciences
Hendrick Hall
Geneva, NY 14456-0462
USA
Tel: 315 787 2221
Fax: 315 787 2216
E-mail: jrk2@cornell.edu

Mee-Sook Kim
Department of Forestry, Fisheries & Wildlife
101 Plant Industry
University of Nebraska -Lincoln
Lincoln, NE 68583-0814
USA
Tel: 402 437 5178 ext 28
Fax: 402 437 5712
E-mail: fofw047@unlvm.unl.edu

Karl W. Kleiner
Department of Entomology
University of Wisconsin
345 Russell Lab/ 1630 Linden Drive
Madison, WI 53706
USA
Tel: 608 262 4755
E-mail: kkleiner@facstaff.wisc.edu

Ned B. Klopfenstein
USDA Forest Service
Rocky Mountain Forest and Range Experiment Station
National Agroforestry Center
East Campus -University of Nebraska
Lincoln, NE 68583-0822
USA
Tel: 402 437 5178 ext 18
Fax: 402 437 5712
DG address: N.Klopfenstein:S28L04a
E-mail:FSWA/S=N.Klopfenstein/ou=S28L04A
@MHS.ATTMAIL.COM

Jae S. Lee
Biotechnology Division
Forest Genetics Research Institute
Forestry Administration
P. O. Box 24, Suwon
Kyonggido 441-350
Republic of Korea
Tel: 82 331 292 1187
Fax: 82 331 292 4458

Jean-Charles Leplé
Station d'Amélioration des Arbres Forestiers
Institut National de la Recherches Agronomique (INRA)
F-45160 Ardon
France

Dolly C. Lin
Faculty of Forestry
University of Toronto
Toronto, Ontario
Canada M5S 3B3
E-mail: j.lin@utoronto.ca

C. H. Anthony Little
Natural Resources Canada
Canadian Forest Service
P. O. Box 4000
Fredericton, New Brunswick
Canada E3B 5P7

Kathryn A. Louis
Sun Gro Horticulture
110-110th Ave. NE, #490
Bellevue, WA 98004
USA
Tel: 206 450 4447

Etsuko Matsunaga
Central Research Laboratory
Nippon Paper Industries
Oji 5-21-1, Kita-ku
Tokyo 114
Japan

Charles A. Maynard
Faculty of Forestry
State University of New York
College of Environmental Science and Forestry
1 Forestry Drive
Syracuse, NY 13210-2787
USA
Tel: 315 470 6560
Fax: 315 470 6956
E-mail: C.Maynard@Mailbox.syr.edu

Brent H. McCown
Department of Horticulture
University of Wisconsin
1575 Linden Drive
Madison, WI 53706
USA
Tel: 608 262 0574/0517
Fax: 608 262 4743
E-mail: bhmccown@facstaff.wisc.edu

Harold S. McNabb, Jr.
Departments of Plant Pathology and Forestry
251 Bessey Hall
Iowa State University
Ames, IA 50011
USA
Tel: 515 294 3120/2350
Fax: 515 294 9420/2995
E-mail: snabb18@iastate.edu

Richard Meilan
Department of Forest Science
A039E Peavy Hall
Oregon State University
Corvallis, OR 97331-7501
USA
Tel: 503 737 6097
Fax: 503 737 2668 or 541 737 5814
E-mail: meilanr@ccmail.orst.edu
meilan@fsl.orst.edu

Bernard Monties
Laboratoire de Chimie Biologique
Institut National de la Recherche Agronomique (INRA)
F-78850 Thiverval-Grignon
France

Thomas Moritz
Department of Forest Genetics and Plant Physiology
The Swedish University of Agricultural Sciences
S-90183 Umeå
Sweden

Ove Nilsson
The Salk Institute for Biological Studies
10010 North Torrey Pines Road
La Jolla, CA 92037
USA

Eun Woon Noh
Biotechnology Division
Forest Genetics Research Institute
Forestry Administration
P. O. Box 24, Suwon
Kyonggido 441-350
Republic of Korea
Tel: 82 331 292 1187
Fax: 82 331 292 4458

Olof Olsson
Department of Molecular Biology
Medicinaregatan 9C
Göteborg University
S-413 90 Göteborg
Sweden
Tel: 46 31 773 3268
Fax: 46 31 773 3801
E-mail: Olof.Olsson@genfys.slu.se

Michael E. Ostry
USDA Forest Service
North Central Forest Experiment Station
1992 Folwell Ave.
St.Paul, MN 55108
USA
Tel: 612 649 5231/5113
Fax: 612 649 5285
DG address: M.Ostry:s23a

Young Goo Park
Department of Forestry
College of Agriculture
Kyungpook National University
San Kyng-dong, Seo-gu
Daegu, 702-701
Republic of Korea
Tel: 82 53 950 5747
Fax: 82 53 950 6701
E-mail: ygpark@bh.kyungpook.ac.kr

Michel Petit-Conil
Centre Technique du Papier
F-38044 Grenoble Cedex
France

Gilles Pilate
Station d'Amélioration des Arbres Forestiers
Institut National de la Recherches Agronomique (INRA)
F-45160 Ardon
France
E-mail: pilate@orleans.inra.fr

Gopi K. Podila
Department of Biological Sciences
Michigan Technological University
1400 Townsend Drive
Houghton, MI 49931-1295
USA

William A. Powell
Faculty of Environmental and Forest Biology
State University of New York
College of Environmental Science and Forestry
1 Forestry Drive
Syracuse, NY 13210-2787
USA

Kenneth F. Raffa
Departments of Entomology and Forestry
University of Wisconsin
345 Russell Lab/1630 Linden Drive
Madison, WI 53706
USA
Tel: 608 262 1125/4775
Fax: 608 262 3322
E-mail: raffa@macc.wisc.edu

Sharon M. Regan
Department of Forest Genetics and Plant Physiology
Swedish University of Agricultural Sciences
S-90183 Umeå
Sweden

Don E. Riemenschneider
USDA Forest Service
North Central Forest Experiment Station
Forestry Sciences Laboratory
5985 Highway K
Rhinelander, WI 54501
USA
Tel: 715 362 7474
DG address:D.Riemenschneider:r09f06a

N.Th. Saieed
Department of Forestry
Mosul University
Mosul
Iraq

Göran Sandberg
Department of Forest Genetics and Plant Physiology
The Swedish University of Agricultural Sciences
S-90183 Umeå
Sweden

James C. Sellmer
University of Wisconsin-Extension
Racine County, 14200 Washington Avenue
Sturtevant, WI 53706
USA

Dongill Shin
Department of Plant Genetics & Breeding
Hyosung Catholic University
Hayang, Kyungsan, Kyungpook 7130-702
Republic of Korea

Sung-Ho Son
Research Institute
Forestry Administration
P. O. Box 24, Suwon
Kyonggido 441-350
Republic of Korea
Tel: 82 331 292 4458
Fax: 82 331 290 1188

Charles H. Strauss
School of Forest Resources
Pennsylvania State University
104 Ferguson Building
University Park, PA 16802-4302
USA
Tel: 814 865 7541
Fax: 814 865 4574

Steven H. Strauss
Department of Forest Science
020 Forestry Sciences Lab
Oregon State University
Corvallis, OR 97331-7501
USA
Tel: 541 737 6578
Fax: 541 737 1393
E-mail: strauss@fsl.orst.edu

Björn Sundberg
Department of Forest Genetics and Plant Physiology
The Swedish University of Agricultural Sciences
S-90183 Umeå
Sweden
Tel: 46 90 166303/166301
Fax: 46 90 165901
E-mail: Bjorn.Sundberg@genfys.slu.se

Girma Melesse Tabor
Department of Forestry
251 Bessey Hall
Iowa State University
Ames, IA 50011
USA
Tel: 515 294 1221
E-mail: gtabor@iastate.edu

Chung-Jui Tsai
Institute of Wood Research
Department of Forestry
Michigan Technological University
1400 Townsend Drive
Houghton, MI 49931-1295
USA
Tel: 906 487 2959

Hannele Tuominen
Department of Forest Genetics and Plant Physiology
The Swedish University of Agricultural Sciences
S-90183 Umeå
Sweden

Gerald A. Tuskan
Biofuel Feedstock Development Program
Building 1503
Oak Ridge National Laboratory
Bethal Valley Road
Oak Ridge, TN 37830-6352
USA
Tel: 423 576 8141
Fax: 423 576 8143
E-mail: gtk@ornl.gov

Jan Van Doorsselaere
Laboratorium voor Genetica
Department of Genetics
Flanders Interuniversity Institute for Biotechnology
Universiteit Gent
K.L. Ledeganckstraat 35
9000 Gent
Belgium
E-mail: jadoo@genwet1.rug.ac.be

Marc Van Montagu
Laboratorium voor Genetica
Department of Genetics
Flanders Interuniversity Institute for Biotechnology
Universiteit Gent
K.L. Ledeganckstraat 35
9000 Gent
Belgium

Marc Villar
Station d'Amélioration des Arbres Foretiers
Institut National de la Recherche Agronomique (INRA)
F-45160 Ardon
France
Tel: 33 38 41 78 74
Fax: 33 38 41 78 79
E-mail: villar@orleans.inra.fr

Suzanne Wetzel
Natural Resources Canada
Canadian Forest Service
P. O. Box 490, 1219 Queen St. E.
Sault Ste. Marie, Ontario
Canada P6A 5M7

Sarah W. Workman
Department of Forestry, Fisheries & Wildlife
University of Nebraska - Lincoln
National Agroforestry Center
Lincoln, NE 68583-0814
USA
Tel: 402 437 5178 ext 40
Fax: 402 437 5712

Keiko Yamada
Central Research Laboratory
Nippon Paper Industries
Oji 5-21-1, Kita-ku
Tokyo 114
Japan

Mikiko Yamakado
Central Research Laboratory
Nippon Paper Industries
Oji 5-21-1, Kita-ku
Tokyo 114
Japan

X. B. Yang
Department of Plant Pathology
351 Bessey Hall
Iowa State University
Ames, IA 50011
USA
Tel: 515 294 8826
Fax: 515 294 9420
E-mail: XBYang@iastate.edu

Jianwei Zhang
Environmental Resources Research Inst.
Pennsylvania State University
220 Forest Resource Building
University Park, PA 16802-4704
USA
Tel: 814 865 4092

Louis Zsuffa
Faculty of Forestry
University of Toronto
Toronto, Ontario
Canada M5S 3B3
ph: 416 978 6512
fax: 416 978 3834
E-mail: louis.zsuffa@utoronto.ca
zsuffa@larva.forestry.utoront

Reviewer acknowledgment

David P. Anderson
USDA-Natural Resources Conservation Service
Watershed Science Institute

Carlos M. Araya
University of Nebraska - Lincoln

Stefanie G. Aschmann
USDA-Natural Resources Conservation Service
Watershed Science Institute

J. Michael Bowker
USDA Forest Service, Southern Research Station

Harvey D. Bradshaw, Jr.
University of Washington

Zong Ming Cheng
North Dakota State University

Gary D. Coleman
University of Maryland at College Park

Joe P. Colletti
Iowa State University

Richard A. Cunningham
USDA-Agricultural Research Service (retired)

John M. Davis
University of Florida

Mary Ellen Dix
USDA Forest Service, Rocky Mountain Forest and Range Experiment Station, National Agroforestry Center

Raymon A. Donahue
Washington State University

Luc C. Duchesne
Forestry Canada, Great Lakes Forestry Centre

Craig S. Echt
USDA Forestry Service, North Central Station

Stephen G. Ernst
University of Nebraska - Lincoln

Glenn R. Furnier
University of Minnesota

Barry Goldfarb
North Carolina State University

Michael S. Greenwood
University of Maine

Richard B. Hall
Iowa State University

Kyung-Hwan Han
Oregon State University

Elwood R. Hart
Iowa State University

Patricia L. Herman
University of Nebraska - Lincoln

Glen T. Howe
University of Minnesota

Kyung-Moon Kim
University of Nebraska - Lincoln

Rudy M. King
USDA Forest Service, Rocky Mountain Forest and Range Experiment Station

Bohun B. Kinlock, Jr.
USDA Forest Service, Pacific Southwest Station

Gary A. Kuhn
USDA-Natural Resources Conservation Service

William J. Libby
New Zealand Forest Research Institute

Carol A. Loopstra
Texas A&M University

Charles A. Maynard
State University of New York - Syracuse, College of Environmental Science and Forestry

Amitava Mitra
University of Nebraska - Lincoln

Margaret T. Mmbaga
Tennessee State University

David Neale
USDA Forest Service, Pacific Southwest Station

Eun-Gyu No
Texas A&M University

Michael E. Ostry
USDA Forest Service, North Central Station

Soon Oh Park
University of Nebraska - Lincoln

Nagmani Rangaswamy
USDA Forest Service, Alabama A&M University

Paul E. Read
University of Nebraska - Lincoln

Don E. Riemenschneider
USDA Forest Service, North Central Station

Peter R. Schaefer
South Dakota State University

Michele M. Schoeneberger
USDA Forest Service, Rocky Mountain Forest and Range Experiment Station, National Agroforestry Center

Carol M. Schumann
USDA-Agricultural Research Service
Appalachian Soil & Water Conservation Laboratory

Ronald R. Sederoff
North Carolina State University

Robert M. Skirvin
University of Illinois

Paul E. Staswick
University of Nebraska - Lincoln

Loren G. Stephens
Iowa State University

Steven H. Strauss
Oregon State University

Charles G. Tauer
Oklahoma State University

Francine M. Tremblay
Université Laval

Anne K. Vidaver
University of Nebraska - Lincoln

James A. Walla
North Dakota State University

F. William Whitmore
Ohio State University

X.B. Yang
Iowa State University

Jianwei Zhang
University of Nebraska - Lincoln (subsequently: Pennsylvania State University)

Scientific and Common Names of Biological Organisms Associated With *Populus* Biotechnology

Madelyn C. Dillon, Richard C. Carman, and Mee-Sook Kim

Populus Sections

Abaso **section Eckenwalder** - poplar section containing *Populus mexicana* Wesmael, categorized by morphology, geographical localization, and crossability

Aigeiros **section Duby** - cottonwood and black poplar section, categorized by morphology, geographical localization, and crossability

Leuce **section Duby (currently termed *Populus* section)** - aspen and white poplar section, categorized by morphology, geographical localization, and crossability

Leucoides **section Spach** - Himalayan poplar, Wilson poplar, and swamp cottonwood section, categorized by morphology, geographical localization, and crossability

Populus **section (formerly *Leuce* section)** - aspen and white poplar section, categorized by morphology, geographical localization, and crossability

Tacamahaca **section Spach** - balsam poplar section, categorized by morphology, geographical localization, and crossability

Turanga **section Bunge** - Euphrates poplar section, categorized by morphology, geographical localization, and crossability

Populus Species and Varieties

Populus adenopoda **Maxim. (*P. suaveolens*, s.l.)** - Chinese aspen; *Populus* (formerly *Leuce*) section

Populus alba **L.** - white poplar; *Populus* (formerly *Leuce*) section

Populus angulata **Ait. (*P. deltoides* var. *angulata* Ait.)** - Eastern cottonwood, southern form; *Aigeiros* section

Populus angustifolia **James** - narrow-leaf or yellow cottonwood; *Tacamahaca* section

Populus balsamifera **L. (*P. tacamahaca*)** - balsam poplar; *Tacamahaca* section

Populus ciliata **Wall./Royal** - Himalayan poplar; *Tacamahaca* section

Populus davidiana **(Dode) Schneid. (*P. tremula*, s.l.)** - Korean aspen; *Populus* (formerly *Leuce*) section

Populus deltoides **Bartr. ex Marshall** - Eastern cottonwood; *Aigeiros* section

Populus euphratica **Oliver** - Euphrates poplar; *Turanga* section

Populus fremontii **S. Watson** - Fremont cottonwood; *Aigeiros* section

Populus glandulosa **Uyeki. (*P. davidiana* var. *glandulosa*)** - Suwon poplar; *Populus* (formerly *Leuce*) section

Populus grandidentata **Michaux** - bigtooth aspen; *Populus* (formerly *Leuce*) section

Populus koreana **Rehder (*P. suaveolens*, s.l.)** - Korean poplar; *Tacamahaca* section

Populus lasiocarpa **Oliver** - *Leucoides* section

Populus laurifolia **Ledebour** - laurel poplar; *Tacamahaca* section

Populus maximowiczii **A. Henry (*P. suaveolens*, s.l.)** - Japanese poplar; *Tacamahaca* section

Populus mexicana **Wesmael** - Mexican poplar; *Abaso* section

Populus nigra **L.** - black poplar; *Aigeiros* section

Populus nigra **var. *betulifolia* Torr.** - *Aigeiros* section

Populus nigra **var. *charkowiensis* Schroed. (*P. nigra* var. *plantierensis* Schneid.)** - *Aigeiros* section

Populus nigra **L. var. *italica* Duroi** - Lombardy poplar; *Aigeiros* section

Populus serotina **(*P.* x *canadensis* Moench) (*P.* x *euramericana* (Dode) Guinier)** - *Aigeiros* section

Populus sieboldii **Miquel** - Japanese aspen; *Populus* (formerly *Leuce*) section

Populus simonii **Carriére** - Simon poplar; *Tacamahaca* section

Populus suaveolens **Fisher** - *Tacamahaca* section

Populus szechuanica **Schneider** - *Tacamahaca* section

Populus tacamahaca **Mill. (*P. balsamifera*, s.l.)** - balsam poplar, collectively; *Tacamahaca* section

Populus tomentosa **Carr. (*P. alba* var. *tomentosa* Carriére) (*P.* x *tomentosa*) (*P. alba* x *P. adenopoda*) (*P. alba* x *P. davidiana*)** - Chinese white poplar; *Populus* (formerly *Leuce*) section

Populus tremula **L.** - European aspen; *Populus* (formerly *Leuce*) section

Populus tremuloides **Michaux** - quaking aspen; *Populus* (formerly *Leuce*) section

Populus trichocarpa **Torr. & Gary** - black cottonwood; *Tacamahaca* section

Populus tristis **Fisch.** - Himalayan balsam poplar; *Tacamahaca* section

Populus wislizenii **Sargent (*P. fremontii* var. *wislizenii* Wats.) (*P. deltoides*, s.l.)** - Rio Grande cottonwood; *Aigeiros* section

Populus xiaohei **T.S. Hwang ex C. Wang et Tung (*P.* x *xiaohei*)** - Chinese small black poplar

Populus yunnanensis **Dode** - species in the *Tacamahaca* section

Populus Hybrids

Populus x ***acuminata*** **Rydb. (*P.* x *andrewsii* Sarg.)** - *P. angustifolia* x *P. deltoides* hybrid, lanceleaf cottonwood

Populus x ***bernardii*** **Boivin** - *P. deltoides* x *P. tremuloides* hybrid, Bernard poplars

Populus x ***berolinensis*** **Dippel (*P.* x *rasumowskyana* Schr.) (*P.* x *petrowskyana* Schr.)** - *P. laurifolia* x *P. nigra* hybrid, Berlin or Russian poplars

Populus x ***canadensis*** **Moench (*P.* x *euramericana* (Dode) Guinier)** - *P. deltoides* x *P. nigra* hybrid

Populus x ***canescens*** **Sm.** - *P. alba* x *P. tremula* hybrid, gray poplars

Populus x ***dutillyi*** **Lepage** - *P. balsamifera* x *P. deltoides* hybrid

Populus x ***euramericana*** **(Dode) Guinier (*P.* x *canadensis* Moench)** - *P. deltoides* x *P. nigra* hybrid, Euramerican poplars

Populus x ***generosa*** **Henry (P.** x ***interamericana*** **Brockh.)** - *P. deltoides* x *P. trichocarpa* and reciprocal hybrid, interamerican poplars

Populus x ***heimburgeri*** **Boivin** - *P. alba* x *P. tremuloides* hybrid

Populus x ***interamericana*** **Brockh. (*P.* x *generosa* Henry)** - *P. deltoides* x *P. trichocarpa* and reciprocal hybrid, interamerican poplars

Populus x ***jackii*** **Sarg.** - *P. balsamifera* x *P. deltoides* hybrid, Jackii poplars

Populus x ***kitakamiensis*** - *P. sieboldii* x *P. grandidentata* hybrid

Populus x ***parryi*** **Sarg.** - *P. fremontii* x *P. trichocarpa* hybrid, Parry cottonwood

Populus x ***rouleauiana*** **Boivin** - *P. alba* x *P. grandidentata* hybrid

Populus x ***sennii*** **Boivin** - *P. angustifolia* x *P. tremuloides* hybrid

Populus x ***smithii*** **Boivin** - *P. grandidentata* x *P. tremuloides* hybrid

Other Trees and Plants

Abies **spp.** - genus of fir trees

Abies koreana **E.H. Wils.** - Korean fir tree

Acer saccharum **Marsh.** - sugar maple tree

Albizia lebbeck **(L.) Benth.** - siris tree

Amaranthus **spp.** - genus of several species characterized by a lasting red pigment in the stems and leaves

Antirrhinum **spp. L.** - genus of snapdragon

Arabidopsis thaliana **(L.) Heynh.** - mouse-ear cress

Astragalus **spp.** - genus of milk vetch (herbs)

Bambusa **spp.** - genus of bamboo

Betula papyrifera **Marsh.** - paper birch tree, white birch tree

Betula platyphylla **Sukachev** - birch tree

Betula verrucosa **Ehrh.** - European white birch tree

Biota orientalis **(L.) Endl.** - oriental arborvitae

Brassica **spp.** - genus of coles and mustards

Brassica napus **L.** - rapeseed

Calocedrus decurrens **(Torr.) Florin** - California incense cedar tree

Camellia japonica **L.** - common camellia

Carica **spp.** - genus of papaya

Castanea **spp.** - genus of chestnut trees

Castanea crenata **Sieb. & Zucc.** - Japanese chestnut tree

Castanea mollissima **Bl.** - Chinese chestnut tree

Castanea sativa **Mill.** - European chestnut tree

Cedrus atlantica **Manetti** - Atlas cedar tree

Cercis siliquastrum **L.** - redbud tree

Chamaecyparis lawsoniana **(A. Murr.) Parl.** - Port-Orford-cedar tree

Chrysanthemum **spp.** - genus of chrysanthemum

Citrus **spp.** - genus of citrus, including grapefruit, lemon, and orange

Citrus sinensis **(L.) Osbec** - sweet orange tree

Coffea arabica **L.** - arabic coffee, common coffee

Cupressaceae - Cypress family

Elaeis guineensis **Jacq.** - oil palm tree, American oil palm tree

Eranthis hyemalis **(L.) Salisb.** - winter aconite (for Eranthis)

Eucalyptus **spp.** - genus of several Australian tall trees with wood valued as timber and having aromatic leaves that yield oils used in medicine

Eucalyptus globulus **Labill.** - bluegum eucalyptus tree

Eucalyptus grandis **Hill ex Maiden.** - rosegum eucalyptus tree

Eucalyptus lansdowneana **F.J. Muell. & J.E. Br.** - crimson mallee box eucalyptus tree

Eucalyptus nitens **Maiden.** - shining eucalyptus tree

Eucalyptus occidentalis **Endl.** - flat-topped yate eucalyptus tree

Eucalyptus regnans **F. Muell.** - mountain-ash eucalyptus tree

Eucalyptus urophylla **S. T. Blake** - ampupu

Euglena **spp.** - genus of single-celled, freshwater organisms characterized by the presence of chlorophyll, a reddish eyespot, and a single anterior flagellum

Fabaceae - beech family

Ginkgoaceae - ginkgo family

Ginkgo biloba **L.** - ginkgo tree, maidenhair tree

Glycine max **(L.) Merr.** - sojabean, soybean

Hibiscus sabdariffa **L.** - Indian sorrel

Isatis tinctoria **L.** - dyers woad

Juglans **spp.** - genus of walnut

Larix decidua **Mill.** - European larch tree

Larix laricina **(Du Roi) K. Koch** - eastern larch tree, tamarack tree

Larix leptolepis **(Sieb. & Zucc.) Gord.** - Japanese larch tree

Larix occidentalis **Nutt.** - western larch tree

Liquidambar styraciflua **L.** - sweetgum tree

Liriodendron tulipifera **L.** - yellow poplar tree

Litchi chinensis **Sonn.** - lychee

Lycopersicon **spp.** - genus of tomatoes

Lycopersicon chilense **Dun.** - wild tomato

Lycopersicon esculentum **Miller** - goldapple, loveapple, tomato

Macrozamia communis **L.A.S. Johnson** - palmlike plant of Australia

Malus **spp.** - genus of apples

Malus domestica **Borkh.** - common apple tree

Malus hupehensis **(Pamp.) Rehd.** - tea crabapple tree

Medicago **spp.** - genus of alfalfa or lucern

Metasequoia glyptostroboides **Hu & Cheng** - dawn redwood tree

Musa **spp.** - genus of bananas

Nicotiana **spp.** - genus of tobacco

Olea **spp.** - genus of olive trees

Olea europaea **L.** - common olive trees

Oryza sativa **L.** - rice

Paulownia **spp.** - genus of several Chinese deciduous trees having large, heart shaped, opposite leaves and pyramidal panicles of purplish or white flowers with a spotted interior

Paulownia tomentosa **(Thunb.) Siev. & Zucc.** - foxglove tree

Petunia x *hybrida* **Hort. Vilm. -Andr.** - common garden petunia

Phaseolus vulgaris **L.** - kidney bean, French bean, snap bean

Picea **spp.** - genus of spruce trees

Picea abies **(L.) Karst** - Norway spruce tree

Picea engelmanii **Parry ex Engelm.** - Engelmann spruce tree

Picea glauca **(Moench) Voss** - white spruce tree

Pinaceae - pine family

Pinus **spp.** - genus of pine trees

Pinus lambertiana **Dougl.** - sugar pine tree

Pinus monticola **Dougl. ex D. Don** - western white pine tree

Pinus palustris **Mill.** - longleaf pine tree

Pinus pinaster **Ait.** - maritime pine tree

Pinus radiata **A.D. Don** - Monterey pine tree

Pinus rigida **Mill.** - pitch pine tree

Pinus strobus **L.** - eastern white pine tree

Pinus sylvestris **L.** - Scots' pine tree

Pinus taeda **L.** - loblolly pine tree

Pisum sativm **L.** - garden, common, English, and green pea

Prunus **spp.** - genus of cherry, peach, and plum trees

Prunus cerasus **L.** - pie cherry, sour cherry

Prunus japonica **Thunb.** - peach tree

Prunus persica **Batsch** - peach tree

Prunus serotina **Ehrh.** - black cherry tree

Pseudotsuga **spp.** - genus of Douglas-fir trees

Pseudotsuga menziesii **Franco.** - Rocky Mountain Douglas-fir tree, coast Douglas-fir tree

Pyrus **spp.** - genus of pear trees

Pyrus nivalis **Jacq.** - snow pear tree

Quercus accutissima **Carruthers** - sawtooth oak tree

Quercus ilex **L.** - holly oak tree, Holm oak tree

Quercus petraea **(Mattushka) Lieblein.** - durmast oak tree, sessile oak tree

Quercus robur **L.** - English oak tree

Raphanus sativus **L.** - radish plant

Ribes nigrum **L.** - black currant

Ricinus communis **L.** - castor bean

Robinia pseudoacacia **L.** - black locust tree

Rubus **spp.** - genus of raspberry and blackberry

Salicaceae - willow family

Salix **spp.** - genus of willow trees

Salix babylonica **L.** - weeping willow tree

Salix caprea **L.** - European willow tree, goat willow tree

Salix lucida **Muhl.** - shining willow tree

Salix x *smithiana* **Willd.** - hybrid of *S. caprea* and basket willow tree

Salix viminalis **L.** - basket willow tree

Sambucus nigra **L.** - European elder tree

Santalum album **L.** - sandal wood tree

Solanum **spp.** - genus of cosmopolitan herbs and shrubs, including potato, eggplant, and nightshade

Sophora japonica **L.** - Japanese pagoda-tree

Syzygium aromaticum **(L.) Merrill & L.M. Perry** - clove tree

Taxodium distichum **(L.) Rich** - bald cypress tree

Ulmus **spp.** - genus of elm trees

Vitis vinifra **L.** - European grape, wine grape

Zinnia **spp.** - genus of zinnia

Microorganisms and Insects

Agrobacterium rhizogenes **Conn** - bacterial pathogen; cause of hairy root disease

Agrobacterium tumefaciens **(Smith & Townsend) Conn** - bacterial pathogen; cause of crown gall disease

Alcaligenes eutrophus **Davis *in* Davis, Doudoroff, Stanier and Mandel** - Gram-negative eubacteria

Aspergillus **spp. P. Mich. ex Link: Fr.** - genus of fungi that causes black mold of cotton bolls, fruits, vegetables

Bacillus amyloliquefaciens **α (sic) Fukumato** - soil bacterium that breaks down proteins into polypeptides and resembles trypsin in its action

Bacillus thuringiensis **Berliner** - pathogenic bacteria for insects; produces a crystalline product that is toxic to larvae of *Lepidoptera*

Ceratocystis fimbriata **Ellis & Halst.** - fungal pathogen; cause of aspen canker

Chondrostereum purpureum **(Pets.: Fr.) Pouzar** - fungal pathogen; cause of silver-leaf disease of fruit trees

Choristoneura fumiferana **(Clemens)** - spruce budworm

Choristoneura occidentalis **Freeman** - western spruce budworm

Choristoneura pinus **Freeman** - jack pine budworm

Chrysomela populi **L.** - poplar leaf beetle

Chrysomela scripta **Fabr.** - cottonwood leaf beetle

Chrysomela tremulae **F.** - poplar leaf beetle

Colletotrichum **spp. Corda** - genus of broad-host-range, fungal pathogens; cause of anthracnose, root rot, and seedling blight; also used as a biocontrol agent

Colletotrichum gloeosporioides **(Penz.) Penz. & Sacc. in Penz.** - conidal state of *Glomerella cingulata;* fungal pathogen causing anthracnose of citrus, banana, and other plants

Colletotrichum lindemuthianum **(Sacc. & Magnus) Scribb.** - fungal pathogen causing anthracnose of bean and other plants

Corynebacterium diphtheriae **(Kruse) Lehmann and Neumann** - bacterial pathogen; gram-positive, rod-shaped cause of diphtheria

Cotesia melanoscela **Ratzeburg** - gypsy moth parasitoid

Cryphonectria parasitica **(Murrill) Barr** - fungal pathogen; cause of chestnut blight

Erwinia amylovora **(Burrill) Winslow et al.** - bacterial pathogen; cause of fire blight disease on cottoneaster, pear, and apple

Fusarium oxysporum **Schlechtend.: Fr. f. sp.** *lycopersici* **(Sacc.) W.C. Snyder & H.N. Hans** - fungal pathogen; cause of tomato and mimosa wilt

Fusarium solani **(Mart.) App. et Wr. emend. Snyd. et Hans.** - broad host range, fungal pathogen causing root and stem rot, damping off, and vascular wilt

Glomerella cingulata **(Stoneman) Spauld. & H. Schrenk** - broad-host-range, fungal pathogen; cause of blight, leaf spot, rot, cankers, and dieback

Heliothis virescens **(Fabricius)** - tobacco budworm

Heliothis zea **(Broddie)** - bollworm

Hyalophora cecropia **(Linnaeus)** - cecropia moth

Hypoxylon mammatum **(Wahl.) Miller** - fungal pathogen; cause of hypoxylon canker of aspen

Laccaria bicolor **(Maire) Orton.** - Ectomycorrhizal basidiomyceteous fungus

Lymantria dispar **(Linnaeus)** - gypsy moth

Malacosoma disstria **(Hübner)** - forest tent caterpillar

Manduca sexta **(Linnaeus)** - tobacco hornworm

Marssonina brunnea **(Ell. & Ev.) Sacc.** - fungal pathogen; cause of poplar and cottonwood leaf spot

Melampsora **spp.** - genus of rust fungus

Melampsora larici-populina **Kleb.** - rust fungus; cause of rust disease on *Populus* spp. and *Larix* spp.

Melampsora medusae **Thuem.** - fungal pathogen; cause of cottonwood leaf rust

Melampsora occidentalis **H. Jacks.** - fungal pathogen; cause of poplar rust

Mycosphaerella populorum **G.E. Thompson** - (anamorph: *Septoria musiva*) - fungal pathogen; cause of poplar leaf spot and canker

Ophiostoma ulmi **(Buisman) Nannf. (formerly** *Ceratocystis ulmi* **Busim. C. Moreau)** - fungal pathogen; cause of elm wilt disease

Paxillus involutus **(Fr.) Fr.** - ectomycorrhizal colonizer

Phaeolus schweinitzii **(Fr.: Fr.) Pat.** - fungal pathogen; cause of conifer butt rot

Plagiodera versicolora **(Laicharting)** - imported willow leaf beetle

Plodia interpunctella **(Hübner)** - Indianmeal moth

Plutella xylostella **(Linnaeus)** - diamondback moth

Pseudomonas aeruginosa **(Schroeter) Migula** - bacterial pathogen of animals and plants

Pseudomonas fluorescens **(Trevisan) Migula** - bacteria existing in soil and water that is the cause of soft rot disease

Pseudomonas solanacearum **Smith** - bacterial wilt pathogen

Pseudomonas syringae **van Hall** - bacterial spot and blight pathogen

Salmonella **spp.** - genus of bacterial pathogen; various rod-shaped bacteria, causes diseases in humans and animals, some are infectious

Septoria musiva **Peck (teleomorph:** *Mycosphaerella populorum*) - fungal pathogen; cause of poplar leaf spot and canker

Septoria populicola **Peck (teleomorph:** *Mycosphaerella populicola* **G.E. Thompson)** - fungal pathogen; causes leaf spot of poplar (e.g., *Tacamahaca* section)

Spodoptera exiqua **(Hübner)** - beet armyworm

Tobacco Mosaic Virus - positive sense, single strand RNA virus with a broad host range

Xanthomonas populi **(Ride) Ride & Ride** - bacterial pathogen; cause of bacterial canker and dieback of *Populus* spp.

Glossary of *Populus* Tissue Culture and Molecular Biology

Mee-Sook Kim, Madelyn C. Dillon, and Richard C. Carman

ABAXIAL - the side or on the side away from an axis such as on the lower surface of a leaf.

ABIOTIC FACTORS - nonliving factors such as light, temperature, wind, fire, soil, water, air.

ADAXIAL - the side or on the side toward an axis such as on the upper surface of a leaf.

ADDITIVE GENETIC VARIATION - the proportion of genetic variation that occurs in response to natural, mass, or "pick-the-winner" selection; the basis of a parent's breeding value or general combining ability.

ADVENTITIOUS - produced in an abnormal or unusual position, at an unusual time of development, or away from the natural habitat such as when plant organs develop on callus or when nonzygotic embryos develop without an ovary.

AFLP - *see* amplified fragment length polymorphism.

ALLELE - any of the alternative forms of a gene that may occur at a given locus.

ALTERNATE HOST - 1 of 2 kinds of plants on which a parasitic fungus must develop to complete its life cycle.

AMIDE - an inorganic compound derived from ammonia by replacement of a hydrogen atom with another element.

AMINO ACID - any of a class of organic molecules having an amino (-NH_2) group and a carboxyl (-COOH) group; 20 amino acids are the usual components of protein.

AMPHIDIPLOID - a polyploid from hybridization of 2 species or genera with resulting duplication of chromosome complement.

AMPLIFIED FRAGMENT LENGTH POLYMORPHISM - a powerful DNA fingerprinting technique based on selective polymerase chain reaction (PCR) amplification of restriction fragments from a complete digestion of genomic DNA; this technique shows the reliability of the RFLP technique but avoids Southern blotting by detecting restriction fragments with specific PCR amplification.

ANEUPLOIDY - a condition in which the chromosome number is not an exact multiple of the basic number in the genome.

ANGIOSPERM - a division of the plant kingdom that includes all flowering plants; vascular plants in which double fertilization occurs resulting in development of fruit containing seeds; composed of 2 classes, the Monocotyledonae and Dicotyledonae.

ANNUAL NET RETURN - the difference between gross annual income and annual expense.

ANTAGONISM - the opposition or counteraction that occurs between organisms such as a mold-inhibiting bacteria.

ANTHER - the part of a stamen in which pollen develops.

ANTHOCYANIN - one of a group of water soluble pigments (glycosides) that vary in color and are present in plant cell sap and flowers.

ANTIBIOTIC - a chemical compound produced by 1 microorganism that inhibits or kills other microorganism.

ANTIBODY - a protein produced in a warm-blooded animal in reaction to a foreign antigen that is capable of reacting specifically with that antigen.

ANTIGEN - foreign proteins, and occasionally complex lipids, carbohydrates, and some nucleotides, which induce the production of antibodies in warm-blooded animals.

ANTIMICROBIAL - destroying or inhibiting the growth of microorganisms.

ANTISENSE RNA - *see* antisense gene.

ANTISENSE GENE - a gene constructed *in vitro* by reversing the orientation of a portion of transcribed DNA, usually including the region encoding a protein, and placing this next to a transcription-control sequence (i.e., the promoter); this gene cassette can be delivered to the target cell, resulting in genetic transformation and inhibition of expression of the target gene (antisense RNA).

ANTISERUM (ANTISERA) - the blood serum of a warm-blooded animal that contains antibodies.

APEX - the most extreme point of growth of a plant; the apical shoot and root tips are located at the apexes (apices) and contain the apical meristem.

APICAL DOMINANCE - the terminal bud influence; a widespread phenomenon in the plant kingdom that is related to the auxin content of apical buds and is exerted on lateral buds resulting in their growth suppression.

ASCOSPORE - sexual spores produced in an ascus (sac) by ascomycetes (sac fungi).

AUG (Adenine, Uracil, Guanine) - *see* initiation codon.

AUTOTROPHIC - self-nourishing; organisms that are capable of synthesizing nutrition from inorganic compounds; all chlorophyll-containing plants.

AUXIN - one of a large class of plant growth regulators that regulates growth, especially one that induces root and shoot growth through cell elongation rather than through cell division; the commonly used auxins in plant tissue culture are indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), α-naphthaleneacetic acid (NAA), and 2,4-dichlorophenoxy acetic acid (2,4-D).

AVIRULENCE GENE - plant resistance (or incompatibility) is governed by the interaction of a single dominant host gene for resistance (R gene) and the corresponding pathogen genes for avirulence (*avr* gene).

AXIL - the angle between the stem axis and a leaf, branch, or other appendage attached to it; the site of axillary buds (shoots).

AXILLARY SHOOT - *see* axil.

BACILLUS THURENGIENSIS - soil bacteria that produces endotoxins that are highly toxic to insects; a type of biological control.

BACKCROSS - a cross between a progeny and either of its parents; usually used to incorporate a single desirable trait from 1 species or variety with several desirable traits from the species or variety used as the recurrent parent; repeated backcrossing is used to create near isogenic lines.

BACTERICIDE - a chemical compound that kills bacteria.

BASE PAIR (bp) - 2 nitrogenous bases that pair in double-stranded DNA or RNA molecules; the amount of adenine (A) - thymine (T) and guanine (G) - cytosine (C) pairs; in RNA, thymine is replaced by uracil (U); *see* nucleotide.

BASIPETAL - proceeding from the apex toward the base or from above downward.

BINARY VECTOR - T-DNA is located on a separate vector plasmid instead of the Ti-plasmid; T-DNA includes the gene(s) of interest and a selectable marker gene; *see* Ti-plasmid.

BIOASSAY - a biological assessment procedure performed on living cells or on a living organism that is sometimes used to detect minute amounts of substances that influence or are essential to growth.

BIOLISTICS - particle bombardment or microprojectile bombardment used to carry foreign DNA into living cells.

BIOLOGICAL CONTROL - pest control using agents of biological origin including parasites, predators, and pathogens.

BIOREACTOR - a container, such as a large fermentation chamber, for growing living organisms for industrial-scale production.

BIOTIC FACTORS - living factors such as those related to presence, behavior, and/or interactions of neighboring organisms (e.g., predators, parasites).

BIOTYPE - a subgroup within a species usually characterized by the possession of a single or few characteristics in common.

BRACT - a small, specialized leaf in the axil from which a flower or floral axis develop.

C - *see* C value.

C VALUE - the total amount of DNA in a haploid genome.

CAAT BOX - part of a conserved sequence located upstream of the startpoints of eukaryotic transcription units; *see* startpoint and leucine zipper.

CALLICLONES - clones derived from callus that may possess a high degree of genetic and phenotypic variability.

CALLOGENESIS - the process of callus formation.

CALLUS (CALLI) - disorganized tumor-like masses of plant cells that form in culture.

CALYX - the sepals of a flower considered a group.

CAMBIUM - a thin layer of longitudinally dividing cells between the xylem and phloem that produces secondary growth.

CANKER - a defined, relatively localized necrotic lesion primarily on the bark and cambium.

CANOPY - in a forest, the uppermost layer of branches, twigs, and leaves.

CAPITALIZED EXPENSES - expense on capital goods by firms, government agencies, households, etc.; may replace depreciated capital or create new capital.

CARPEL - a simple pistil or a division of a compound pistil; one of the innermost whorl of modified leaves that bear the megaspores.

CATKIN - a special type of spikelike inflorescence that bears either pistillate or staminate flowers and falls as a whole from the plant.

cDNA - *see* complementary DNA.

CECROPINS - a family of basic proteins with activity against a broad range of Gram-positive and Gram-negative bacteria.

CELLULASE - an enzyme that hydrolyzes cellulose.

CELLULOSE - a complex carbohydrate composed of glucose units that form the main constituent of the cell wall in most plants.

CENTIMORGAN (cM) - a measure of genetic distance; the distance that separates 2 genes between which there is a 1 percent chance of genetic recombination (1 cM).

CENTROMERE - the heterochromatic region of a eukaryotic chromosome associated with spindle fibers that participates in normal chromosome movement in mitosis and meiosis; it divides just before replicated chromosome separation and holds chromatids together.

CHAPERONE - *see* molecular chaperones.

CHIMERA - an individual, organ, or part consisting of components of diverse genetic constitution.

CHITIN - a polysaccharide (polymer of N-acetylglucosamine residues) that forms part of the hard outer integument especially of insects, arachnids, and crustaceans; also a component of some fungal cell walls.

CHITINASE - an enzyme that catalyzes the hydrolysis of chitin.

CHLOROPLAST DNA (cpDNA) - *see* organelle DNA.

CHLOROPLAST - a plastid that contains chlorophyll, is abundant in the cells of green plants, and functions in photosynthesis.

CHLOROSIS - yellowness in normally green plants resulting from a lack of chlorophyll.

CHROMATIDS - the longitudinal subunits produced by chromosome replication and joined at the centromere.

CHROMATIN - the fibrous aggregate of DNA and histone protein that makes up a eukaryotic chromosome.

CHROMOSOME - a discrete unit of the genome carrying many genes; each chromosome is a very long molecule of duplex DNA and an approximately equal mass of proteins; it is microscopically visible as a thread-like, morphological entity only during cell division.

CHROMOSOME LANDING - a technique based on the isolation/development of DNA marker(s) at a close physical distance to the genetic sequences (e.g., gene) of interest; the DNA marker(s) serves as a probe for screening genomic libraries allowing the isolation of the DNA clone containing the genetic sequences of interest; this technique eliminates the need for chromosome walking.

CHROMOSOME REARRANGEMENT - a chromosomal aberration in which chromosomal segments are rearranged by inversion and translocation.

CHROMOSOME WALKING - a procedure used for the systematic isolation and cloning of overlapping and contiguous regions of chromosomal DNA contained in a genomic library of cloned DNA; this procedure, which can be difficult to apply in plant species with complex genomes, allows the progressive development of genetic markers, each closer to the gene or genetic sequences of interest.

***CIS*-ACTING DNA SEQUENCE** - in eukaryotes, short (e.g., 10 to 12 bp) nucleotide sequences found in the 5'-flanking regions or within genes; they represent recognition elements that interact with specific transcription factors and are required for eukaryotic gene regulation.

CLADISTICS - a system of classification based on the phylogenetic relationships and evolutionary history of groups of organisms.

CLONAL PROPAGATION - *see* clone; *see* vegetative propagation.

CLONE - a group of vegetatively propagated organisms consisting of an ortet and its ramets; a cell line of single cell origin; a gene or piece of DNA replicated in a host bacterium; all definitions imply genetically identical material and reproduction by mitotic division.

CO-CULTIVATION - the joint culture of 2 or more types of cells such as a plant cell and a microorganism (includes most *Agrobacterium* spp.).

CO-INTEGRATION VECTOR - a transformation vector in which the T-DNA includes the gene(s) of interest with a selectable marker gene instead of oncogenes on the Ti-plasmid; *see* Ti-plasmid.

CODING REGION - *see* coding sequence.

CODING SEQUENCE - the part of a gene that directly specifies the amino acid sequence of its protein product; noncoding sequences of genes include control regions such as promoters, operators, and terminators, and intron sequences of certain eukaryotic genes.

CODOMINANT - the products of both genes at a locus that are expressed nearly equally.

CODON - a specific sequence of 3 consecutive nucleotides that is part of the genetic code and that specifies a particular amino acid in a protein or starts or stops protein synthesis.

COLLAR SHOOT - a shoot arising from a line of junction between a root and its stem.

COMPATIBILITY - the ability of pollen to affect fertilization; a susceptible plant and virulent pathogen capable of causing disease.

COMPETENCE - in development, the physiological state or condition of a tissue that enables it to react morphologically in a specific way to stimuli such as inducers.

COMPLEMENTARY DNA (cDNA) - a single-stranded DNA that is complementary to RNA and is synthesized from it by reverse transcription of RNA *in vitro*.

CONIDIOPHORE - a specialized fungal hypha-bearing conidia.

CONIDIUM (CONIDIA) - an asexual fungal spore usually at the tip of a special hypha called a conidiophore.

CONSTITUTIVE EXPRESSION - gene expression exemplified by any gene that encodes a product continuously required in the maintenance of basic cellular processes or cell architecture, as opposed to a regulated gene that typically encodes products required for growth under specific physiological conditions or in particular cell types.

COPPICE - a forest originating mainly from shoots or root suckers rather than from seed or tree cuttings; harvesting to promote new shoot growth.

COPY NUMBER - the number of genes per genome, plasmids, or genes contained in a cell.

COROLLA - the petals of a flower considered as a group or unit.

COSUPPRESSION - a process by which transgenes can inhibit their own expression and that of homologous (e.g., native) genes.

COTYLEDON - the seed leaf or leaves of an embryonic plant (i.e., 1 in a monocotyledon, 2 in a dicotyledon, and a variable number in gymnosperms).

CROSS-POLLINATION - the transfer of pollen from one plant to a female flower of another plant or clone.

CROSS-RESISTANCE - protection given to a plant by a pathogen (e.g., virus) infection to subsequent infection by strains of related pathogens.

CROSSING OVER - a process of exchange between nonsister chromatids of a pair of homologous chromosomes that results in the recombination of linked genes.

CRYOPRESERVATION - storage of cells, tissues, seeds, etc. at extremely low temperatures.

CULTIVAR - an assemblage of cultivated individuals distinguished by any useful reproducible character and designated by 'cv.'

CYTOKININ - a large class of plant growth regulators that promote cell division and enlargement *in vitro* and *in vivo* in the presence of auxins and have other effects such as control of organ differentiation, influence of auxin transport, breaking of dormancy, and apical dominance in buds; commonly used cytokinins in plant tissue culture are 6-benzyladenine (BA), 2-isopentyladenine (2-iP), zeatin, and kinetin; thidiazuron (TDZ) has cytokinin-like activity.

CYTOLOGY - a branch of biology dealing with the structure, function, and life history of cells.

CYTOPLASM - the protoplasm (or "cell sap") of a cell surrounding the nucleus; it includes the cytoplymph, organelles, inclusions, and the plasma membrane.

CYTOTOXIN - a substance having a specific toxic affect on certain cells or cellular components.

DE NOVO - anew; once more; again.

DEAMINATION - the removal of an amino ($-NH_2$) group from an organic compound.

DEFENSIN - one class of antimicrobial peptide like cecropins and magainins; *see* cecropins and magainins.

DEHYDROGENATE - the removal of hydrogen (H).

DEMETHYLATION - the removal of a methyl ($-CH_3$) group.

DENATURATION - the loss of the native configuration of a macromolecule from specific treatment (e.g., heat treatment) that usually results in loss of biological activity.

DEOXYRIBONUCLEIC ACID (DNA) - the macromolecule, usually composed of 2 polynucleotide chains in a double helix, which is the carrier of genetic information in all cells and many viruses; *see* nucleotide.

DEPOLYMERIZE - the degradation of a polymer to oligomers and/or monomers.

DICOT (DICOTYLEDON) - *see* cotyledon.

DIHAPLOID - possessing a diploid number of chromosomes from a doubling of the haploid genome.

DIMER - a compound formed from 2 molecular subunits (i.e., monomers).

DIOECIOUS - producing male and female flowers on different plants.

DIPLOID - a cell or organism with 2 complete sets of homologous chromosomes (i.e., 2N).

DISCOUNTED CASH FLOW - appraising projects based on discounting future costs and benefits to their present values.

DNA - *see* deoxyribonucleic acid.

DNA FINGERPRINTING - electrophoretic identification of individuals using DNA probes for highly polymorphic regions of the genome so that the genome of virtually every individual exhibits a unique pattern of bands.

DNA MICROINJECTION - *see* microinjection.

DNA POLYMERASE - an enzyme that synthesizes a daughter (complementary) strand(s) of DNA under the direction of a DNA template strand.

DOMINANT - an allele or the corresponding phenotypic traits that are expressed in a heterozygote.

DORMANT - in a resting or nongrowing state such as buds, seeds, spores.

DOWN-REGULATED - causing reduced gene expression.

DOWNSTREAM - the direction in which a nucleic acid or protein molecule is synthesized or on the C-terminal side of any site within a polypeptide.

EARLYWOOD - the wood formed early in the growing season (earlywood) that has larger cells with thinner walls and a lower density than the wood formed later in the season (latewood).

ECOSYSTEM - an ecological system; a natural unit of living and nonliving components that interact to form a system in which cyclic interchange of materials takes place between living and nonliving units such as in an aquarium, lake, or forest.

ECOTYPE - within a species having a wide geographical distribution, a subgroup that has developed specific adaptions to local conditions such as temperature, light, and humidity.

ECTOMYCORRHIZAE - *see* mycorrhizae.

ECTOPIC - occurring in an abnormal position or in an unusual manner or form.

ELECTROPHORESIS - a technique used to separate molecules on the basis of their molecular size and charge by differential movement through a liquid or a gel in response to an applied electric field.

ELECTROPORATION - introduction of DNA fragments into a cell by means of an electric migration.

ELISA - *see* enzyme-linked immunosorbent assay.

ELONGATION FACTOR - proteins that associate with ribosomes cyclically during addition of each amino acid to the polypeptide chain during protein synthesis.

EMBRYO - that portion of the seed resulting from the union of male and female gametes that can develop into a mature plant.

EMBRYOGENESIS - the process of embryo or embryoid formation, whether by sexual (zygotic) or asexual (somatic) means.

ENDOGENOUS - produced or synthesized within the organism or system.

ENDOMYCORRHIZAE - *see* mycorrhizae.

ENDOPLASMIC RETICULUM (ER) - a complex set of membrane lamellae and tubules in eukaryotic cells that is the site of major biosynthetic processes such as protein secretion and membrane assembly.

ENDOSPERM - food storage tissue contained in the seed and surrounding the embryo.

ENDOTOXIN - a poisonous substance present in bacteria but separable from the cell body only on its disintegration.

ENERGY CROP - a crop (e.g., poplar, willow, or switch grass) grown and used as a renewable energy source.

ENHANCER - a *cis*-acting sequence that increases the activity of some eukaryotic promoters and can function in either orientation and in any location relative to the promoter.

ENZYME - a protein or ordered aggregate of proteins that catalyzes a specific biochemical reaction and is unaltered after the process.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) - a serological test in which one antibody carries with it an enzyme that catalyzes a colorimetric reaction.

EPIDEMIOLOGY - a branch of plant and animal pathology that studies the incidence, distribution, and control of disease in a population.

EPIGENETIC - environmentally induced variations in the phenotype perpetuated by cloning but not involving permanent changes in the genotype resulting from the interaction of genetic factors that occurs during the normal development of an individual plant; epigenetic phenomena including habituation, determination, and juvenile/adult phase changes.

EPISTASIS - the interaction between nonallelic genes such that one gene interferes with or prevents expression of the other.

ESTER - any class of often fragrant compounds formed by the reaction between an acid and an alcohol.

ETIOLOGY - the assigned underlying cause or reason; the predisposing causes of either abiotically or biotically induced events, especially disease.

EUCHROMATIN - a region of a chromosome having normal staining properties and undergoing the normal cycle of condensation; relatively uncoiled in the interphase nucleus and apparently containing most of the structural genes.

EUKARYOTE - a cell or an organism composed of cells with true nuclei (chromosomal DNA enclosed in nuclear envelopes) and membrane-bounded cytoplasmic organelles (e.g., chloroplasts and/or mitochondria) in which nuclear division is by mitosis or meiosis.

EX SITU - somewhere other than the natural or original position or place.

EX VITRO - organisms removed from culture and transplanted, usually to soil or potting mixture.

EXOGENOUS - introduced from or produced outside the organism or system.

EXON - the sequences in a gene that are retained in the mRNA after the introns are removed from the primary transcripts.

EXOTOXIN - a toxic substance excreted by organisms.

EXPLANTS - the excised plant portion used to initiate a tissue culture.

F_1 - *see* F_1 generation.

F_1 GENERATION - the first (filial) generation produced by crossing 2 parental lines.

5'-P GROUP - the end of a DNA or RNA strand where the phosphate is attached to the number 5 carbon of the nucleotide sugar.

FLANKING DNA SEQUENCE - segments of DNA immediately adjacent to the 2 ends of a DNA region under consideration; any DNA sequence flanking close to the 5' (upstream) end or 3' (downstream) end of a gene.

FLANKING REGION - *see* flanking DNA sequence.

FLUOROMETRY - measuring fluorescence and related phenomena (e.g., intensity of radiation).

FOREIGN DNA - foreign gene; *see* transgenic plant.

FOREIGN GENE - transgene, chimeric gene, and alien gene; *see* transgenic plant.

FULL-SIBS - progeny (sibling) with both parents in common.

FUNGICIDE - a compound toxic to fungi.

FUNGISTATIC - a compound that prevents fungal growth without killing the fungus.

G-BOX - a ubiquitous, *cis*-acting DNA regulatory element (G-box motif: CACGTG or CCACGTGG) found in plant genomes; proteins known as G-box factors bind to G-boxes in a context-specific manner, mediating a wide variety of gene expression patterns.

GAMETE - a haploid generation male sperm cell or female egg cell capable of developing into a zygote (embryo) after fusion with a germ cell of the opposite sex.

GAMETOCLONE - a plant regenerated from cell cultures originating from gametic culture.

GAMETOPHYTE - the few-celled, haploid generation portion of a seed plant from meiotic division that through mitosis produces male or female gametes.

GAS CHROMATOGRAPHY - a chromatographic technique in which an inert gas is run through a column to separate materials.

GENE - the hereditary unit containing genetic information that is transcribed into RNA and processed into an RNA molecule that functions directly or is translated into a polypeptide chain.

GENE EXPRESSION - the multistep process by which a gene is regulated and its product synthesized.

GENE FREQUENCY - the proportion of one type of allele to the total of all alleles at this genetic locus in a breeding population; the probability of finding the specific gene under consideration when a gene is randomly chosen from the population.

GENE SILENCING - alteration of a structural gene or regulatory region in a DNA sequence so that the gene is not expressed.

GENE TRANSFER - *see* genetic transformation.

GENERATION TIME - the average time between 2 successive generations; the time required for a cell population to double.

GENETIC GAIN - the increase in productivity following a change in gene frequency.

GENETIC MAP - a chromosome map showing the relative locations of the known genes or markers on the chromosomes of a given species.

GENETIC MARKER - any region used as an experimental probe to mark a nucleus, chromosome, gene, or DNA region.

GENETIC TRANSFORMATION - the unidirectional gene transfer and incorporation of foreign DNA by prokaryotic and eukaryotic cells, and the subsequent recombination of part or all of that DNA into the cell's genome.

GENOME - the total complement of genes contained in a cell or virus; commonly used to refer to all genes present in 1 complete set of chromosomes in eukaryotes.

GENOMIC LIBRARY - a gene bank consisting of a set of independent clones that statistically contains the entire genome of an organism among the recombinant DNA molecules; construction and screening of a complete library of clones for any genome is a starting point for isolating specific genes.

GENOTYPE - the genetic constitution of an organism or virus as distinguished from its appearance or phenotype; the allelic composition of 1 or more genes of interest.

GEOGRAPHIC INFORMATION SYSTEMS (GIS) - computer software programs that use mathematical algorithms to enable input, management, analysis, and display of geographic point data.

GERMPLASM - the genetic material that forms the physical basis of inherited qualities that are transmitted from generation to generation by the germ cells.

GLUCOSIDE - a glycoside that yields glucose on hydrolysis.

GLYCOSIDE - a compound in which 1 or more sugars are involved in a linkage to another molecule.

GOLGI APPARATUS (DICTYOSOME) - a subcellular organelle consisting of individual stacks of membranes near the endoplasmic reticulum; it is involved in glycosylating proteins and sorting them for transport to different cellular locations.

GRAM-POSITIVE/GRAM-NEGATIVE BACTERIA - *see* Gram's staining reaction.

GRAM'S STAINING REACTION - the specific staining techniques used to differentiate bacteria into Gram-positive and Gram-negative based on cell wall characteristics.

GYMNOSPERMS - any class or subdivision of woody vascular seed plants that produces naked seeds not enclosed in an ovary that sometimes have motile spermatozoid (e.g., conifers).

HAPLOID - a cell or organism having only 1 set of chromosomes (e.g., 1N).

HERITABILITY - the ratio of genetic variance to phenotypic variance; narrow-sense heritability: the ratio of additive genetic variance to phenotypic variance that is useful in predicting the response of a population to natural selection or to "pick-the-winner" selection; broad-sense heritability: the ratio of total genetic variance to phenotypic variance that is used to estimate the degree of genetic control of a trait in a population and to predict response to clonal selection.

HETEROCHROMATIN - chromatin that remains condensed, is not transcribed into mRNA, and can be heavily stained during interphase; usually adjacent to the centromere and in the telomeres of chromosomes.

HETERODIMER - a chemical structure consisting of 2 dissimilar subunits.

HETEROGENEITY - a preparation of macromolecules in which the macromolecules differ in size, charge, structure, or other properties.

HETEROGENEOUS - dissimilar of diverse components or constituents.

HETEROPOLYMER - a high molecular weight chemical compound consisting of different repeated molecules (monomers).

HETEROSIS - the marked vigor or capacity for growth often exhibited by crossbred animals or plants; hybrid vigor.

HETEROTROPHIC - designating a type of nutrition in which an organism requires preformed carbohydrates or other organic compounds for energy.

HETEROZYGOUS - having dissimilar alleles of 1 or more genes; not homozygous.

HISTOCHEMISTRY - a science that combines the techniques of biochemistry and histology in the study of the chemical constitution of cells and tissues.

HOMEOSTASIS - the tendency of a system to be specified by context (individual, population, process, etc.) to maintain a dynamic equilibrium and, in case of disturbance, to restore the equilibrium by its own regulatory mechanisms.

HOMEOTIC GENES - genes that determine fundamental patterns of development.

HOMOLOGOUS - in reference to DNA, having the same or similar nucleotide sequence; in reference to chromosomes, those that pair in meiosis, have the same morphology, and contain genes that govern the same traits.

HOMOZYGOUS - having the same allele of a gene in homologous chromosome.

HOST - an organism (e.g., host plant) that is invaded by a parasite and from which the parasite obtains its nutrition.

HYBRIDIZATION - the crossing of different races or species, also sometimes used to denote the crossing of individuals; the pairing of complementary RNA and DNA strands to give an RNA-DNA hybrid.

HYDROXYLATION - the introduction of a hydroxyl (-OH) group into an organic compound.

HYPHA (HYPHAE) - one of the structural threads that make up the mycelium of a fungus.

HYPOCOTYL - the part of the embryo or embryonic seedling stem between the cotyledons and radicle.

ICE-NUCLEATING BACTERIA - certain bacteria (e.g., *Pseudomonas syringae*, *P. fluorescens*, and *Erwinia herbicola*) whose presence on the surface of plant tissue may result in increased sensitivity to frost injury at temperatures that would not otherwise cause damage.

IN PLANTA - occurring within the plant.

IN SITU - in the natural or original position or place.

IN VITRO - experimentation on whole organisms or portions thereof, in glassware or culture; growing under artificial conditions as in tissue culture.

IN VIVO - in the living body of a plant or animal.

INBREEDING - mating between genetically related individual organisms.

INCOMPATIBILITY - the inability of pollen to affect fertilization because growth of the pollen tube is arrested in the style; *also see* avirulence gene.

INITIATION CODON - any codon in mRNA that directs initiation of genetic translation by stimulating the binding of initiator tRNA and by phasing the translation of mRNA; in eukaryotes, AUG is the usual initiator and is translated as methionine.

INSTAR - the stage of development or the form assumed by an insect between successive molts.

INTERCELLULAR SPACE - the space between or among cells.

INTERGENIC SPACER (IGS) - any of the DNA sequences (sequence, region, spacer, intercistronic region) flanking the eukaryotic genes on the 3' and/or 5' side.

INTERNAL TRANSCRIBED SPACERS (ITS) - a variable, noncoding region between the highly conserved nuclear small and large subunit rDNA that is useful for distinguishing closely related species.

INTROGRESSION - the incorporation of genes from 1 species into the gene pool of another species by hybridization and backcrossing.

INTRON - a noncoding segment of DNA that is transcribed but removed from the transcript by splicing together the sequences (exons) on either side of it.

INVERTED REPEAT - a pair of base sequences present in the same molecule that are identical or nearly identical but oriented in opposite directions; often found at the ends of transposable elements.

ISOENZYME - *see* isozyme.

ISOGENIC - any group of individuals that possess the same genotype, irrespective of their being homozygous or heterozygous

ISOLATE - a culture or subpopulation of microorganisms separated from its parent population and maintained under controlled conditions; to affect such separation and control such as isolating a pathogen from diseased plant tissue.

ISOMER - any of 2 or more substances that are composed of the same elements in the same proportions but differ in properties because of differences in the arrangement of atoms (structure).

ISOTONIC - having the same osmotic potential; the same molar concentration of a solution.

ISOZYME - any of 2 or more chemically distinct but functionally similar enzymes.

KB - *see* kilobase.

KILOBASE - 1,000 bases (nucleotides) of DNA or 1,000 bases of RNA.

LARVA (LARVAE) - the wormlike, wingless, immature, feeding form that hatches from the egg of insects and undergoes complete metamorphosis; called caterpillar, maggot, grub, or wiggler.

LATEWOOD - *see* earlywood.

LEADER SEQUENCE - a segment of nucleotides at the 5'-end of mRNA that precedes the AUG initiation codon where translation begins; the leader is an untranslated segment of mRNA that varies in length but always contains part or all of a unique nucleotide sequence (e.g., Shine-Dalgano sequence).

LEAF PLASTOCHRON INDEX - stages of vegetative (physiological) development of leaves in relation to the shoot apex.

LENTICEL - an opening on the stem of a plant through which air is admitted to underlying tissues.

LEUCINE ZIPPER - in DNA-binding proteins, an amino acid sequence (30 residues with leucine at every seventh amino acid in the region of similarity) that binds specifically to the CAAT box and transcriptional enhancer.

LIGAND - a small molecule (e.g., activators, substrates, and inhibitors of enzyme activity) bound to a protein by noncovalent forces; an ion, a molecule, or a macromolecule group that binds to another chemical entity to form a larger complex.

LIGNIFICATION - the process of deposition of lignin in cells walls.

LIGNIN - a complex substance present in the cell walls of woody plants that, with cellulose, forms wood.

LINKAGE - any association of genes in inheritance that exceeds what is expected from independent assortment and is due to their location on the same chromosome; linkage is assessed by the tendency of 2 markers to remain together during recombination.

LOCUS (LOCI) - the site or position of a particular gene on a chromosome.

MAGAININS - 1 of 2 closely related peptides (23 amino acids each) isolated from frog skin that kill a variety of bacteria, fungi, and protozoa; believed to be part of a defense system in vertebrates that is different from the immune system.

MARKER ASSISTED SELECTION (MAS) - an efficient approach to identify linkage between genetic markers and quantitative trait loci (QTL); an indirect, early selection based on the DNA markers.

MARKER GENE - a gene, usually dominant, with a large, recognizable effect that can identify recombinant organisms or 1 parent in offspring from open or mixed pollination.

MATING PAIR - a male and female brought together for breeding and reproduction.

MATRIX ATTACHMENT REGIONS (MAR) - a region of DNA that attaches to the nuclear matrix; known as SAR for scaffold attachment site.

MEGAGAMETOPHYTE - the female gametophyte; the plant that develops from a megaspore.

MEGASPORE - the larger of 2 types of spores that produce a female gametophyte; also called macrospore.

MEIOSIS - the process of nuclear division in gametogenesis or sporogenesis in which 1 replication of the chromosomes is followed by 2 successive divisions of the nucleus to produce 4 haploid nuclei.

MERISTEM - plant tissue composed of dividing cells and producing organs such as leaves, flowers, roots, etc.

MESSENGER RNA (mRNA) - RNA produced by transcription that carries the code for a particular protein from the nuclear DNA to a ribosome in the cytoplasm and acts as a template for the formation of that protein.

METHYLATION - the modification of a molecule (e.g., DNA or RNA base) by the addition of a methyl ($-CH_3$) group.

MICROMOLAR (μM) - one millionth of a mole.

MICROBE - a microscopic, possibly pathogenic, organism or microorganism.

MICROCOSM - a community, institution, or other unit serving as a small representation of a larger unit.

MICROINJECTION - direct physical injection into individual cells, particularly for DNA transfer.

MICROPARTICLE BOMBARDMENT - *see* biolistics.

MICROPROJECTILE BOMBARDMENT - *see* biolistics.

MICROPROPAGATION - refers to propagation in culture by axillary or adventitious means; a general term for vegetative (asexual) *in vitro* propagation.

MICROSHOOT - a shoot that is produced and grown under *in vitro* conditions such as for micropropagation.

MICROSPORE - a haploid male cell that ripens into a pollen grain; immature pollen grain.

MITOCHONDRIAL DNA (mtDNA) - *see* organelle DNA.

MITOSIS - the process of nuclear division in which the replicated chromosomes divide and the daughter nuclei have the same chromosome number and genetic composition as the parent nucleus.

MOLAR - of or relating to a mole of a substance; containing 1 mole of solute in 1 liter of solution.

MOLE - in the International System of Units, the base unit amount of pure substance that contains the same number of elementary entities as atoms in exactly 12 grams of the isotope carbon 12.

MOLECULAR CHAPERONES - a family of unrelated classes of protein that mediate the correct assembly and conformation of other polypeptides but are not components of the functional assembled structures; one important class of molecular chaperones is the 70-KDa family of heat shock proteins or stress-related proteins.

MONOCOTS (MONOCOTYLEDON) - *see* cotyledon.

MONOLIGNOLS - the building blocks of lignin (e.g., *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol).

MONOMER - an individual structural unit that undergoes polymerization.

MORPHOGENESIS - the formation and differentiation of tissues and organs.

mRNA - *see* messenger RNA.

MUTANT - any variant strain, individual, cell, or gene resulting from mutation.

MUTATION - any heritable alteration, whether a single base change in DNA (or RNA) or a large-scale rearrangement, in the genetic material of a living cell or virus.

MYCELIUM (MYCELIA) - the mass of interwoven filamentous threads (hyphae) that forms the vegetative body of a fungus.

MYCORRHIZAE - the symbiotic association of the mycelium of a fungus with the roots of a seed plant in which the mycelium forms a thick web over and between the outer surface of roots (ectomycorrhizae) or the mycelium hyphae colonize the root cells (endomycorrhizae or vesicular arbuscular mycorrhizae).

NECROSIS - the death of cells usually resulting in tissue darkening.

NODULE - independent, spherical, dense cell clusters that form a cohesive unit and display consistent internal cell/tissue diffentiation, derived from plant cell suspension culture.

NONADDITIVE GENETIC VARIATION - the proportion of genetic variation that does not respond to simple mass selection and that causes specific pairwise crosses to depart from performance values predicted by the breeding values of the parents.

NORTHERN ANALYSIS - *see* northern blotting.

NORTHERN BLOTTING - a technique, analogous to Southern blotting, used for transferring RNA from agarose gel to a membrane where it can be hybridized to a labeled, complementary, single-stranded DNA or RNA probe.

NUCLEAR SCAFFOLD ATTACHMENT REGIONS (SAR) - *see* matrix attachment site.

NUCLEOTIDE - any of several compounds that consist of a ribose or deoxyribose sugar joined to a purine or pyrimidine base and to a phosphate group; the basic structural units of nucleic acids.

OLIGOMER - a chemical compound composed of a few to several monomers (structural units).

OLIGONUCLEOTIDE - a short polymeric chain of multiple (e.g., 2 to 20) nucleotides.

ONCOGENE - a gene whose product has the ability to transform eukaryotic cells so that they grow in a manner analogous to tumor cells.

OPEN READING FRAME (ORF) - a stretch of triplet codons with an initiator codon at one end and termination codon at the other, identifiable by nucleotide sequences coding for protein.

OPERATOR - a regulatory region in DNA that interacts with a specific repressor protein in controlling the transcription of adjacent structural genes.

OPERON - a collection of adjacent structural genes regulated by an operator and a repressor.

OPINE - a family of amino acids (e.g., octopine, nopaline, agropine, and agrocinopine) that are catabolized by *Agrobacterium* strains.

OPPORTUNITY COSTS - the value of the foregone alternative action; the most favorable price that a factor of production (land, labor, or capital) can command.

ORGANELLE DNA - extranuclear DNA (cpDNA, mtDNA) of eukaryotes localized in cellular organelles other than the nucleus and usually inherited uniparentally (frequently maternal) in plants and animals.

ORGANELLE GENOME - *see* organelle DNA.

ORGANOGENESIS - the initiation and growth of organs, usually roots and shoots, from cells or tissue; organs may form on the surface of explants (direct organogenesis) or upon an intervening callus phase (indirect organogenesis).

ORTET - the initial individual that is vegetatively propagated to produce a clone; *see* ramet.

OSMOTIC POTENTIAL (OSMOTIC PRESSURE) - the pressure exerted by the flow of water through a semipermeable membrane separating 2 solutions with different concentrations of solute.

OSMOTICUM - an agent such as mannitol, sugars, polyethylene glycol, and salts used to maintain the osmotic potential of the surrounding medium.

OVIPOSIT - to discharge eggs from the body.

OVULE - an oval body in the ovary of a flower that develops into a seed.

PALINDROME - a sequence of DNA that is the same when a strand is read left to right and the other complementary strand is read right to left; consists of adjacent inverted repeats.

PALISADE PARENCHYMA - the undulating lower epidermis of plant leaves with meandering vascular bundles; larger cells of the lower epidermis.

PARAMUTATION - directed genetic change caused by 1 allele on the other.

PARASITE - an organism living on or in another organism (host) from which it obtains nutrition.

PARENCHYMA - a tissue of higher plants that consists of thin-walled, living photosynthetic or storage cells capable of division that, even when mature, makes up much of the substance of leaves and roots, pulp of fruits, and parts of stems and supporting structures.

PARTICLE ACCELERATION DNA DELIVERY SYSTEM - *see* biolistics.

PARTICLE BOMBARDMENT - *see* biolistics.

PATHOGEN - an agent (parasite) capable of causing disease.

PATHOGENESIS - the phase of the pathogen life cycle when it is associated with the host.

PATHOSYSTEM - a particular host and pathogen interaction.

PCR - *see* polymerase chain reaction.

PECTINASES - an enzyme that degrades pectin (the adhesive material that cements cells together) and is used alone or with other enzymes to digest the polygalacturonic acid component of plant cell walls for protoplast isolation.

PEPTIDE - a compound formed by the union of 2 or more amino acids such as a dipeptide or polypeptide.

PETAL - a modified leaf, usually colored, forming a part of the corolla of a flower.

PETIOLE - the slender stalk that supports the blade of a leaf.

PHASE CHANGE - the developmental change from one maturation state to another.

PHENOTYPE - the observable properties of a cell or an organism resulting from the interaction of the genotype and the environment.

PHENYLPROPANOID PATHWAY - a metabolic pathway that produces phenolic compounds (e.g, phytoalexins) with a significant range of biological functions including defense of plants against pests; *see* phytoalexin.

PHLOEM - food-conducting tissue in vascular plants consisting principally of sieve tubes and companion cells.

PHOTO LABILE - unstable in light.

PHOTOPERIOD - the duration of the light portion in an alternating light-dark sequence.

PHOTOSYNTHESIS - the process by which plant chloroplasts make carbohydrates from carbon dioxide and water in the presence of chlorophyll by using light energy and releasing oxygen.

PHOTOSYSTEM I - a photochemical reaction system in photosynthesis that produces $NADPH^+$ but does not emit oxygen.

PHYLLOTAXY - the leaf arrangement around the stem of a plant that is genetically determined and species characteristic.

PHYLOGENETIC - of or relating to the evolution of a genetically related group of organisms as distinguished from the development of the individual organism.

PHYLOGENY - the evolutionary history of an organism or taxonomic group.

PHYTOALEXIN - a substance that inhibits the development of fungi on hypersensitive tissue that is formed when host plant cells come in contact with the parasite.

PHYTOHORMONE - *see* plant growth regulator.

PHYTOREMEDIATION - the use of specially selected and engineered plants for environmental remediation.

PHYTOTOXICITY - poisonous to plants.

PISTIL - a gynoecium or a unit of a gynoecium; the female structure of a flower consisting of an ovary, style, and stigma; a female organ composed of 1 or more carpels.

PLANT GROWTH REGULATOR - any organic substance, other than a nutrient, which in small amounts induces a physiological, developmental, timing, or size change response in plant cell, tissues, or organs; this body of substances includes plant hormones, growth regulators, many herbicides, substances from nonplant sources, and laboratory-synthesized substances; *see* auxin and cytokinin.

PLANT GROWTH HORMONE - *see* plant growth regulator.

PLANT GROWTH SUBSTANCE - *see* plant growth regulator.

PLANT HORMONE - *see* plant growth regulator.

PLANT TRANSFORMATION - *see* genetic transformation.

PLASMA MEMBRANE - the cell membrane that surrounds the cellular organelles and cytoplasm.

PLASMID - an autonomous, self-replicating, extrachromosomal, circular DNA.

PLEIOTROPY - the condition in which a single mutant gene affects 2 or more distinct and seemingly unrelated traits.

PLOIDY - the number of chromosome sets per cell (e.g., haploid, diploid, or polyploid).

POINT MUTATION - a mutation caused by the substitution, deletion, or addition of a single nucleotide pair.

POLAR TRANSPORT - directional transport within a plant (e.g., movement of an endogenous plant growth regulator).

POLLEN - the partly developed male gametophyte produced by the anthers of seed plants, a germinated microspore; these male spores often appear as yellow dust.

POLYADENYLATION SEQUENCES - the 3' ends of most eukaryotic mRNAs containing long stretches of polyadenine (A) that are enzymatically added after transcription.

POLYCISTRONIC - a prokaryotic mRNA carrying the information for the synthesis of more than 1 protein.

POLYETHYLENE-GLYCOL-MEDIATED GENE TRANSFER - introduction of DNA fragments into a cell by the chemical substance polyethylene-glycol (PEG); treatment of a plant protoplast with PEG promotes absorption of DNA.

POLYMER - a chemical compound or mixture of compounds formed by polymerization and consisting essentially of repeating structural units.

POLYMERASE - an enzyme that catalyzes covalent joining of nucleotides.

POLYMERASE CHAIN REACTION PRIMERS - *see* polymerase chain reaction; *see* primer.

POLYMERASE CHAIN REACTION - repeated cycles of DNA denaturation and renaturation with primer oligonucleotide sequences, and replication that results in exponential growth in the copy number of the target DNA sequence located between the primers.

POLYMORPHISM - the presence in a population of 2 or more relatively common forms of a gene, chromosome, or genetically determined trait.

POLYPEPTIDE - a polymer of amino acids linked together by peptide bonds (e.g., protein).

POLYSOME - a multiribosomal structure representing a linear array of ribosomes held together by mRNA.

POSITION EFFECT - a change in the expression of a gene depending on its position within the genome.

PREDATOR - any organism that preys on other organisms.

PRIMER - a short sequence (e.g., RNA or DNA oligonucleotide) that is paired with 1 strand of nucleic acid and provides a free 3'-OH end at which a polymerase starts synthesis of a complementary strand.

PRIMORDIUM (PRIMORDIA) - the earliest, rudimentary developmental stage of an organ or cell such as leaf primodia, which are barely visible on a dissected shoot tip.

PROBE - a defined (labeled) DNA or RNA sequence used to detect complementary sequences by hybridization techniques (e.g., Southern blotting, northern blotting).

PROGENY - the offspring of a particular parent or a particular combination of 1 female and 1 male organism.

PROKARYOTE - an organism in which a nuclear envelope is lacking and the genetic material does not divide by mitosis or meiosis (e.g., bacteria and blue-algae).

PROMOTER - a specific DNA sequence where RNA polymerase binds and initiates transcription of a gene or operon; in an operon, the promoter is usually located at the operator end, adjacent but external to the operator; the nucleotide sequence of the promoter determines the nature of the enzyme that attaches to it and the rate of RNA synthesis.

PROTEASE - any of numerous enzymes that hydrolyze proteins and are classified according to the most prominent functional group at the active site.

PROTEIN - a molecule composed of 1 or more polypeptide chains.

PROTEINASE - an enzyme the catalyzes the hydrolytic breakdown of internal (peptide) bonds of protein (polypeptide).

PROTOCLONE - distinct phenotypic regenerants from a plant protoplast; a clone initiated from a protoplast or protoplast-fusion.

PROTOPLAST - a cell without a cell wall but with a plasma membrane.

PROTOPLAST FUSION - the uniting of 2 protoplasts.

PUPA (PUPAE) - in insects with complete metamorphosis, a dormant, inactive stage between the larva and the adult.

PURINE - an organic base in nucleic acids; the predominant purines are adenine (A) and guanine (G).

PYRIMIDINE - an organic base in nucleic acids; the predominant pyrimidines are cytosine (C), uracil (U, in RNA only), and thymine (T, in DNA only).

QTLs - *see* quantitative trait loci.

QUALITATIVE RESISTANCE (VERTICAL RESISTANCE) - many plant varieties are resistant to some pathogen races while susceptible to other races of the same pathogen; such resistance is sometimes called specific, qualitative, or differential resistance but is usually referred to as vertical resistance; vertical resistance is usually controlled by 1 or a few genes.

QUANTITATIVE RESISTANCE (HORIZONTAL RESISTANCE) - all plant have a certain level of unspecific resistance against each of their pathogens; such resistance is sometimes called nonspecific, general, quantitative, adult plant, field, or durable resistance but is usually referred to as horizontal resistance; horizontal resistance is usually controlled by many genes.

QUANTITATIVE TRAIT - a trait typically measured on a continuous scale, such as height or weight, which results from the combined action of multiple genes in conjunction with environmental factors.

QUANTITATIVE TRAIT LOCI - multiple discrete chromosomal sites (loci) that control quantitatively inherited polygenic traits.

RACE - a genetically and often geographically distinct mating group within a species; a group of pathogens that infect a given set of plant varieties.

RADICLE - the lower part of the axis of a plant embryo or seedling.

RAMET - all vegetative propagules of an ortet; a clone is composed of the ortet and its ramets.

RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) - random amplified polymorphic DNA markers are generated by polymerase chain reaction (PCR) amplification using arbitrary primers (e.g., oligonucleotides or 10mers); amplified DNA fragments are electrophoretically separated on agarose gel and can be viewed under UV light when stained with ethidium bromide.

RAPD - *see* random amplified polymorphic DNA.

RAY CELL - a radiating, linear group of cells, as a pith ray.

rDNA - *see* ribosomal DNA.

REACTIONWOOD - tilting of trees by wind results in redistribution of the amount and nature of cambial growth on the leeward and windward sides of the stem and in

formation of abnormal reactionwood that occurs on the lower sides of tilted stems.

RECALCITRANT - resistant to directed change (e.g., tissue that is difficult to manipulate *in vitro*).

RECEPTOR - transmembrane protein located in the plasma membrane that binds a ligand in a domain on the extracellular side and has a change in activity of the cytoplasmic domain.

RECESSIVE - obscured in the phenotype of a heterozygote by the dominant gene, often due to inactivity or absence of the product of the recessive gene.

RECOMBINANT DNA - *see* recombinant DNA technology.

RECOMBINANT DNA TECHNOLOGY - the techniques by which genetic recombination is carried out *in vitro*; the breakage and rejoining of DNA molecules from different organisms, and the production and isolation of the modified DNA or fragments thereof.

RECOMBINANT GENES - genes produced by recombinant DNA technology.

RECOMBINASE - any enzymes that recognize specific DNA sequences, introduce single-strand breaks, and are involved in general recombination.

RECOMBINATION FREQUENCY - a measure of the frequency of exchange (crossing over) occurring between 2 specific loci.

REFUGIA - an area that provides protection/escape from ecological consequences occurring elsewhere.

REGENERATION - to give or gain new life or to renew by a new growth of tissue such as regenerants (e.g., shoots, roots, or plantlets), which form from explants in plant tissue cultures.

REGULATORY GENE - a gene with the primary function of controlling the rate of synthesis of the products of 1 or more other genes.

REJUVENATION - a change in a tissue or an organism from a more mature state to a more juvenile one.

REMEDIATION - to improve the ecosystem by removing, detoxifying, or absorbing harmful substances.

REPEAT-INDUCED POINT MUTATION (RIP) - point mutations that occur frequently in the newly methylated sequences resulting from deamination of methylated cytosines.

REPORTER GENE - a coding unit whose product is easily assayed such as chloramphenicol acetyltransferase (CAT), β-glucuronidase (GUS), and luciferase (*luxF2*); it may be connected to any promoter of interest so that gene expression can be used to assay promoter function.

REPRESSOR - a protein that binds specifically to a regulatory sequence adjacent to a gene and that blocks transcription of the gene.

RESTRICTION ENDONUCLEASE - a nuclease that recognizes a short specific nucleotide sequence (restriction site) in a DNA molecule and cleaves the molecule relative to that site; also called restriction enzyme.

RESTRICTION ENZYME - *see* restriction endonuclease.

RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) - genetic variation in a population associated with the size of restriction fragments containing sequences homologous to a particular probe DNA; the polymorphism results from the positions of restriction sites flanking the probed fragment, each variant is essentially a different allele.

REVERSE TRANSCRIPTASE - *see* reverse transcription.

REVERSE TRANSCRIPTION - synthesis of DNA on a template of RNA accomplished by reverse transcriptase enzyme.

RFLP - *see* restriction fragment length polymorphism.

RHIZOSPHERE - the soil region that surrounds and is influenced by plant roots.

Ri-PLASMID - in *Agrobacterium rhizogenes*, a large root-inducing plasmid that transfers a specific segment of DNA (the T-DNA) into plant cells where it integrates into the nuclear genome and induces hairy root disease.

RIBONUCLEASE (RNase) - any enzyme that cleaves phosphodiester bonds in RNA.

RIBONUCLEIC ACID (RNA) - a nucleic acid in which the sugar constituent is ribose.

RIBONUCLEOPROTEIN PARTICLE (RNP) - any of the ribonucleoprotein complexes involved in gene expression of eukaryotes.

RIBOSOMAL DNA (rDNA) - the family of chromosomal or extrachromosomal DNA sequences encoding ribosomal RNA.

RIBOSOMAL PROTEIN - any of the essential proteins that are synthesized individually and assembled into ribosome.

RIBOSOMAL RNA (rRNA) - RNA that is linked noncovalently to the ribosomal proteins in the 2 ribosomal subunits and that constitutes about 80 percent of the total cellular RNA; the transcription product of ribosomal DNA.

RIBOSOME - a subcellular structure consisting of 2 subunits each composed of RNA and proteins on which the codons of mRNA are translated into amino acids in protein synthesis.

RIBOSOME BINDING SITE - in mRNA, a site for binding ribosomes that is required for initiation of genetic translation; in *E. coli*, 3 basic elements constitute the ribosome binding site: the Shine-Dalgarno sequence, the initiator codon (usually AUG), and an intervening spacer region, normally 5 to 9 nucleotides long.

RNA - *see* ribonucleic acid.

RNA BLOT ANALYSIS - *see* northern blotting.

RNA POLYMERASE - an enzyme that makes RNA by copying the base sequence of a complementary DNA strand.

RNase - *see* ribonuclease.

RNP - *see* ribonucleoprotein particle.

rRNA - *see* ribosomal RNA.

SCAFFOLD ATTACHMENT REGION (SAR) - *see* matrix attachment region.

SDS-PAGE ANALYSIS - *see* sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

SEGREGATION - separation of the members of a pair of alleles into different gametes during meiosis.

SELECTABLE MARKER - used in marker-assisted selection or to select a transformed genotype from a mixture of transformed and nontransformed cells; antibiotic-resistance and herbicide-resistance genes are examples, where cells that lack the marker are killed on a selective medium.

SELF-INCOMPATIBILITY - in diverse angiosperm families, the failure of self-fertilization; the inability to produce seed following self-pollination.

SEPAL - a modified leaf forming one of the components of the calyx of a flower.

SEQUENCE DIVERGENCE - the percent difference in a nucleotide sequence between 2 related nucleic acid sequences or, in an amino acid sequence, between 2 proteins.

SHIKIMATE - a hydroxylated, unsaturated acid that is a key intermediate in the biosynthesis of the aromatic amino acids.

SHIKIMATE PATHWAY - a pathway for the synthesis of shikimate and its conversion to phenylalanine, tyrosine, and tryptophan.

SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) - electrophoresis in polyacrylamide gels containing SDS; this electrophoretic technique separates denatured macromolecules (e.g., proteins) and provides estimates of molecular weights by reference to the mobility of marker proteins of known molecular weight.

SOMACLONAL VARIATION - variation occurring in plants regenerated from cell cultures that originated from somatic tissue.

SOMATIC HYBRID - a cell or plant product of somatic cell fusion that results from cell or protoplast fusion and implies genomic integration.

SOMATIC EMBRYOGENESIS - a nonsexual developmental process leading to the differentiation of zygotic embryo-like structures from somatic cells.

SOUTHERN BLOTTING - transfer of single-stranded, restriction-cut DNA fragments, separated by agarose gel electrophoresis to a membrane that is then analyzed by hybridization to labelled, single-stranded DNA or RNA probes.

SOUTHERN HYBRIDIZATION - *see* Southern blotting.

SPACER SEQUENCE - any DNA sequence separating neighboring genes; spacer sequences may or may not be transcribed.

SPONGY MESOPHYLL - a leaf tissue consisting of loosely arranged, chloroplast-bearing usually lobed cells; also called spongy parenchyma.

SPONTANEOUS MUTATION - a mutation occurring in the absence of any known mutagenic agents.

STALK - the main supporting axis or stem of a plant or embryo.

STAMEN - the pollen-producing structure of a flower consisting of an anther on the tip of a filament.

STARTPOINT - the position on DNA corresponding to the first base incorporated into RNA.

STERILITY - any complete or partial failure of an individual to produce functional gametes or viable zygotes under a given set of environmental conditions.

STIGMA - the portion of the pistil that receives the pollen; the expanded apex of the style.

STOMATE (STOMATA) - a minute opening in a leaf or stem through which gases pass.

STORAGE PROTEIN - a protein stored in specific tissue under specific environmental conditions that can be metabolized at a later time.

STRUCTURAL GENE - a gene that encodes the amino acid sequence of a polypeptide chain (protein).

STYLE - the slender, elongated portion of a pistil with the stigma at its tip.

SUBCULTURE - a culture derived from another culture or the aseptic division and transfer of a culture or a portion of that culture to fresh nutrient medium.

SUCKER - a shoot originating from the basal stem region or roots of a plant.

T-DNA - transferred DNA; *see* Ti-plasmid.

TANDEM ARRAY - multiple copies of the same DNA sequence lying in series.

TATA BOX - a conserved A-T rich septamer about 25 bp before the startpoint of each eukaryotic RNA polymerase II transcription unit.

TELOMERES - the tips of a chromosome that contain DNA sequences required for stability of chromosome ends.

TEMPLATE - a nucleic acid strand whose base sequence is copied in a polymerization reaction.

TERATOMA - a tumor derived from embryonic tissue consisting of a disorganized mass of many tissue types; many cells representing a variety of differentiated tissues.

TERMINATION CODON - 1 of the 3 mRNA codons (UAG, UAA, and UCG) at which polypeptide synthesis stops.

3'-OH (hydroxy) GROUP - the end of a DNA or RNA strand that terminates in a sugar and has a free hydroxyl group on the number 3 carbon.

Ti-PLASMID - in *Agrobacterium tumefaciens*, a large tumor-inducing plasmid that transfers a specific segment of DNA (the T-DNA) into plant cells where it is integrated into the nuclear genome and induces crown gall tumors.

TOTIPOTENCY - the capacity of a cell to express all of its genetic information under appropriate conditions and to develop into a complete and fully differentiated organism.

TRACHEID - in vascular plants, an elongated, dead cell with a pronounced cavity or lumen forming an element of xylem.

***TRANS*-ACTING FACTOR** - any of the protein factors that mediate transcription of eukaryotic genes by transcriptional activation and bind to *cis*-acting DNA sequence elements.

TRANSCRIPTION - the process by which the information contained in the coding strand of DNA is copied into a single-stranded RNA molecule with a complementary base sequence.

TRANSCRIPTION FACTOR - any of the multiple ancillary DNA-binding proteins that interact with the *cis*-regulatory DNA sequences to control gene expression.

TRANSFER RNA (tRNA) - a small RNA molecule that translates a codon into an amino acid in protein synthesis.

TRANSFORMANT - any cell that has either stably integrated foreign DNA into its chromosomal DNA or transiently harbors foreign DNA.

TRANSGENE - *see* transgenic plant.

TRANSGENE EXPRESSION - the multistep process by which a transgene is regulated and its encoded product synthesized.

TRANSGENIC PLANT - an individual plant containing a foreign gene (a transgene) that was introduced by a gene transfer technique.

TRANSIT PEPTIDE - in the case of post-translationally imported mitochondrial and chloroplast protein, an amino terminal sequence that contains targeting information and is removed during or shortly after import into the organelles.

TRANSLATION - the process of forming a protein molecule at a ribosomal site of protein synthesis from information contained in mRNA.

TRANSLATION INITIATION SITE - *see* ribosome binding site.

TRANSLOCATION - the interchange of parts between chromosomes (e.g., nonhomologous chromosomes); the movement of mRNA with respect to a ribosome in protein synthesis.

TRANSPOSABLE ELEMENT - a DNA sequence capable of moving from 1 location to another in a genome.

TRANSPOSON - a transposable element especially when it contains genetic material controlling functions other than those related to its relocation.

TRIPLOID - a cell or individual organism with 3 complete sets of chromosomes.

tRNA - *see* transfer RNA.

UBIQUITIN - a small protein (76 amino acid residues) in nearly all prokaryotic and eukaryotic cells with a high degree of amino acid conservation between groups of organisms.

UPSTREAM - on the 5' side of any given site in DNA or RNA and on the N-terminal side within a polypeptide; it identifies sequences proceeding in the opposite direction from expression.

UREDIOSPORES - binucleate resting spores produced in the life cycle of rust fungi that are capable of infecting the host species on which they were produced.

UREDIUM (UREDIA) - a fruiting structure formed by the rust fungi in which urediospores are produced.

VACUOLE - a space or cavity within a cell such as the cavity of a plant cell containing cell sap.

VASCULAR CAMBIUM - the cambium of a vascular cylinder that produces secondary xylem and phloem.

VECTOR - an organism that carries and transmits a pathogen to another organism (e.g., plant) particularly inoculum and especially a fungus, insect, mite, or nematode that transmits a mollicute or a virus; the plasmid or nucleic acid vector used in the insertion of genes into cells in genetic engineering; *see* binary vector and co-integration vector.

VEGETATIVE PROPAGATION - the propagation or multiplication of plants by asexual means.

VITRIFICATION - a condition of cultured tissue with leaves and sometimes stems that have a glassy, transparent, or wet and often swollen appearance; eventually shoot tip and leaf necrosis occurs.

WESTERN BLOTTING - the transfer of proteins from a polyacrylamide gel onto a suitable immobilizing matrix where the proteins may be probed with a specific antibody to identify particular proteins.

XENOBIOTIC - components of the chemical environment that are foreign to the metabolic network of an organism.

XYLEM - 1 of 2 components of vascular tissue; the wood portion of woody plants; complex tissue made up of fibers, vessel members, tracheids, and parenchyma cells; it conducts water and mineral salts and lends structural support to plant organs.

ZYGOTE - the product of the fusion of a female and a male gamete in sexual reproduction; a fertilized egg.

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Index of *Populus*

Populus sections

Abaso 290
Aigeiros 8, 13, 35-37, 106, 107, 112, 114, 121, 290, 291
Leuce 7, 10, 13, 35-38, 53, 106, 107, 114, 121, 175, 290, 291
Leucoides 13, 106, 107, 121, 290
Tacamahaca 8, 13, 35-37, 53, 106, 107, 112, 114, 121, 290, 291, 294
Turanga 13, 106, 290

Populus species and hybrids

Populus
alba 6, 14, 26-29, 31, 32, 35, 38, 41, 42, 106, 107, 112, 114, 123, 137, 144-148, 159, 166, 290, 291
'5972-S38' 35
alba x *P. glandulosa* 26-29, 31, 32, 55, 57, 144-148, 171, 207, 210
alba x *P. grandidentata* 8, 10, 14, 15, 18, 20-23, 25-29, 31, 35, 43, 45, 46, 49, 52-56, 61, 62, 70, 85, 91, 94, 166, 174, 179, 200, 202, 207, 291
'Crandon' ('NC5339') 10, 15, 18, 20, 21, 35, 61, 62, 70, 72, 73, 85-88, 91, 93, 94, 179, 182, 183
'Favorit' 35
'Hansen' 15, 20, 21, 91, 174, 175

alba x *P. tremula* 52, 55, 166, 208, 291
alba x *P. tremuloides* 35, 291
'802-36-7' 35
angulata 1, 255, 290
angustifolia 34, 35, 106, 107, 290
angustifolia x *P. fremontii* 35
balsamifera (*P. tacamahaca*) 106, 107, 117-122, 125, 290, 291
'TAC8' 118
'TAC21' 118
'TAC51' 118
balsamifera var. *subcordata/candicans* x *P.* x *berolinensis* 255
'NC5262' ('NE397') 255
ciliata 10, 12, 14, 21, 23, 26, 31, 37, 40, 48, 106, 107, 290
davidiana 15, 22, 26, 55, 58, 106, 107, 144-148, 171, 207, 210, 290
deltoides 1, 14, 15, 17, 19, 22, 23, 26, 32, 35, 55, 57, 66, 106-110, 112, 114, 117-123, 125, 126, 128-130, 135, 144, 148, 157, 160, 221, 223, 233, 235, 255, 290, 291
'D35' ('W-1-2') ('Larsson') ('D39') 118
'D196' ('Northwest') 118
'D391' (Mixoploid) (Brockville#C136) 118
'K417' 35
'NC11004' ('Siouxland') 255
deltoides var. *angulata* x *P.* x *berolinesis* 255
'DTAC2' 255
deltoides var. *angulata* x *P. trichocarpa* 255
'NC11432' ('NE252') 255
deltoides x *P. maximowiczii* 107
deltoides x *P. trichocarpa* (*P. interamericana*) 66, 108, 109, 117-122 291
'DTAC9' ('Donk') 118
'DTAC10' ('Barn') 118
fremontii 34, 35, 106, 107, 290, 291
glandulosa 14, 15, 22, 26-29, 31, 46, 47, 144-148, 171, 290
grandidentata 105, 106, 133, 135-137, 161, 166, 290
koreana 106, 166, 171, 290
koreana x *P. nigra* 26-31, 144, 146, 147, 159
lasiocarpa 14, 106, 107, 290
laurifolia 26, 35, 106, 107, 255, 290, 291
maximowiczii 15, 19, 22, 23, 49, 106, 107, 112, 114, 117-123, 144, 146-148, 156, 290
'M900' 118
'M901' 118
'M908' 118
maximowiczii x *P.* x *berolinensis* 255
'NC11396' ('NE49') 255
maximowiczii x *P. nigra* 144
maximowiczii x *P. trichocarpa* 14, 35, 144, 255
'NC11390' 35
'NC11505' ('NE88') ('NE388') 255
'Androscoggin' 35
nigra 1, 26, 28, 29, 31, 34, 35, 48, 55, 57, 98, 106, 107, 112, 114, 117-123, 144-148, 166, 172, 290
'N75' ('#113') 118
'N84' ('Italica') ('#555/50) 118
'N91' ('Purkersdorf') (#44/62(10)) 118
'N100' ('Kunovice')(312/65(002/66)) 118
'R1029' 35
'Italic' 31, 34, 35, 118, 290
'Italica' 31, 34, 35, 118, 290
var. *caudina* 14, 26, 30, 35, 255, 256
var. *charkowiensis* 14, 26, 30, 35, 255, 256, 290
var. *italica* 31, 34, 35, 118, 290
nigra x *P. deltoides* (*P.* x *interamericana*) 66
nigra x *P. laurifolia* 26, 35, 255
'NC5272' 35
'NC11445' ('NE157') ('NE280') 255
nigra x *P. maximowiczii* 8, 10, 12, 14, 15, 19, 23, 26-29, 32, 49, 55, 62, 63, 85, 88, 94, 117-122, 144, 146, 147, 180, 185, 207, 254-256
'NM1' 63, 118
'NM2' ('Max-1') 118
'NM6' ('Max-5') 8, 62, 63, 85, 88, 94, 180, 182, 185, 254-256
'NM8' ('#62-7') 118
nigra x *P. trichocarpa* 26-29, 35, 62, 255
'NC5331' 35, 62, 255
nigra var. *betulifolia* x *P. nigra* 14
nigra var. *betulifolia* x *P. nigra* cv. 'Volga' 35
nigra var. *betulifolia* x *P. trichocarpa* 14, 35, 37, 255
'NC5331' 35, 255
'NE299' 35, 37, 255
nigra var. *charkowiensis* x *P.* x *berolinensis* 255, 256
'NC11382' ('NE27') 255, 256
nigra var. *charkowiensis* x *P. deltoides* 14, 35
nigra var. *charkowiensis* x *P. nigra* var. *caudina* 14, 26, 30, 31, 35, 255, 256
'NC5271' ('NE19') 35, 255, 256
serotina 1, 290
sieboldii 26, 106, 161, 290
sieboldii x *P. grandidentata* (*P. kitakamiensis*) (*P.* x *kitakamiensis*) 55, 66, 68, 161, 163, 164, 188, 194, 195, 204, 205, 207, 291
'Kitakami Hakuyo' 161
'Y63' 163, 188
simonii 26, 28, 29, 32, 38, 43, 106, 107, 291
simonii x *P. nigra* 36
simonii x *P.* x *berolinensis* 255, 256
'NE332' 255, 256
szechuanica 106, 107, 291
tacamahaca (*P. balsamifera*) 106
tacamahaca x *P. trichocarpa* 26
tomentosa (*P.* x *tomentosa*) 26-29, 55, 106, 107, 166, 172, 291
tremula 5, 8, 14, 15, 26-29, 35, 43, 55, 92, 93, 106, 107, 166, 171, 274, 290, 291
'Erecta' 5
tremula x *P. alba* 26-31, 35, 36, 42, 52-55, 57, 61, 63, 64, 84, 98, 171, 175, 176, 198, 200, 202, 207, 208, 210, 216, 270
'706-8' 35
'7171-B4' 35, 63, 84, 85
'INRA 717 1B4' 35, 63, 84, 85
tremula x *P. tremuloides* 37, 55, 74, 86, 92-94, 100, 166, 175, 176, 179, 274
'333-4' 37
'INRA 353-38' ('353-38') 86, 175
tremuloides 14, 15, 19, 23, 26, 35, 43, 54, 55, 58, 59, 66, 69, 73, 100, 105-107, 111, 129, 156, 158-160, 194, 195, 197, 199, 205, 222, 233, 238, 239, 251, 274, 291
tremuloides x *P. tremula* 239
trichocarpa 1, 14, 19, 49, 106-110, 112, 121, 128, 135, 144, 148, 150, 195, 213, 222, 291
trichocarpa x *P. balsamifera* 35
'TT32' 35, 37, 39, 41
trichocarpa x *P. deltoides* (*P.* x *interamericana*) 14, 35, 41, 53, 55, 66, 69, 108, 117-122, 194, 195, 199, 200, 203, 205, 208, 291
'DTAC7' ('Unal') 118
'DTAC8' ('Beaupre') 118
'DTAC26' ('Boelare') 118
'Hunnegem' 35, 41
trichocarpa x *P. tacamahaca* 15, 26
tristis 291
tristis x *P. balsamifera* 125

'Tristis #1' 125, 248
wislizenii 106, 107, 291
xiaohei 36, 42, 291
yunnanensis 106, 107, 291
x *balsamifera* 117
x *berolinensis* 36, 255, 256, 291
x *canadensis* 114, 122, 123, 130, 207, 290, 291
x *euramericana* (*P. deltoides* x *P. nigra*) 1, 3, 14-16, 19, 21, 22, 26-31, 34, 35, 37, 42, 55, 58, 63, 107-109, 117-122, 125, 126, 156, 174, 204, 207, 255, 290
'DN5' ('Gelrica') 118
'DN14' ('Harff') 118
'DN16' ('Batarde d'Hauterive') 118
'DN17' ('Robusta') 118
'DN21' ('I-78B') ('LW42') 118
'DN30' ('Canada Blanc') 15, 118
'DN93' ('Triplo') ('I-37/61') 118
'DN106' 63
'DN173' ('Dorskamp') ('Koster 925') 118
'DN177' ('Spijk') ('Koster 2195') 118
'I-45/51' 15, 118
'I-214' 31, 118
'I-455' 118
'NC5377' 255
'Beaupré' 37
'Carpaccio' 37
'Eugenei' 15, 125
'Guariento' 37
'L Avanzo' 37
'Ogy' 3, 16, 35, 58, 174-177
'Robusta' 35, 42, 118, 130
'Wisconsin #5' 15, 255
x *generosa* (*P. generosa*) 1, 291
x *interamericana* (*P. deltoides* x *P. trichocarpa*) (*P. trichocarpa* x *P. deltoides*) 35, 112, 194, 291
'Barn' 35, 118
'Rap' 35
x *jackii* 117-122, 291
'JAC4' 118
'JAC7' 118
'JAC28' 118
x *kitakamiensis* (*P. kitakameiensis*) (*P. sieboldii* x *P. grandidentata*) 55, 66, 68, 161, 163, 164, 188, 194, 195, 204, 205, 207, 291

General Index

A

10-mer (*see:* oligonucleotide)
35S (CaMV) (*see:* CaMV*35S* promoter)
70S (double enhancer sequence) (*see:* CaMV) 63, 67
A1 95
A4a 66
abaxial 15, 40, 295
Abies 33, 34, 98, 291
Abies koreana (*A. koreana*) 134, 291
ablate 252
abscisic acid (ABA) 156
abscission 77, 124, 125
Ac (Activator) 53, 55
Ac-AMP1 (*Amaranthus* defensin-like protein) 167
  Ac-AMP1.2 167, 168
ACC (1-aminocyclo-propane-1-carboxylic acid) 21
accelerated flowering 212, 215, 216
acclimation (acclimate) (acclimatization) 7, 75, 237, 239
Acer 129
  saccharum 126, 291
acetolactate synthase gene (*ALS*) 55, 61, 67, 207
acetosyringone (AS) 56
active oxygen 187-189
adaptive advantage 86, 268
adaxial 20, 295
additive genetic variation (additive and nonadditive) 224, 228
adenine 214, 296, 306
S-adenosyl-L-methionine synthase (SAM) 66
ADP ribosyl transferase 214
adult phase 5, 299
adventitious 5, 7, 13-15, 33, 47, 62, 156, 163, 221, 231, 237-239, 295, 303
AFLP (amplified fragment length polymorphism) 102, 104-106, 108, 109, 119, 222, 295
AGAMOUS (*AG*) 213
agarose 27, 103, 104, 116, 117, 152, 304, 306, 308
Agrobacterium 55, 156, 165, 175, 275, 293, 297, 305
  Agrobacterium-mediated transformation (gene transfer) 24, 51-57, 68, 70, 90, 91, 97, 162, 169, 175, 197, 201, 275
  rhizogenes 51, 55, 76, 78, 91, 93, 207, 209, 277, 293, 307
  tumefaciens 51, 53-56, 67, 70, 74, 76-78, 85, 150, 161-163, 167, 197, 198, 214, 293, 309
    A281 52, 53, 56
    A348 53, 56
    C58 53, 56, 74
    EHA101 56
    LBA4404 56, 161, 163
    PO22 161, 163
air pollution 44, 187, 207
  air-pollutant resistance 187
albinism 35
Albizzia lebbeck 34
albumin 132-135, 137
Alcaligenes eutrophus 208, 293
alfalfa mosaic virus 63, 198
allele 96, 102, 104, 110, 184, 275, 295, 299-301, 304, 307
allelochemical 183
allergen 165, 212
allozyme 102, 105, 107, 108
ALS (acetolactate synthase) 55, 61, 67, 207
alternate host 295
Amaranthus 167, 291
amide 295
1-aminocyclo-propane-1-carboxylic acid (ACC) 21
amphidiploid 95, 295
amplification 101-104, 116, 117, 143, 145, 147, 148, 151, 152, 197, 295, 306
amplified fragment length polymorphism (AFLP) (*see:* AFLP)
amplified polymorphic DNA (RAPD) 103, 116, 143, 148, 150, 151, 222, 306 (*see:* RAPD)
amplified sequence polymorphism (ASP) 105
analog 167
aneuploid 38
angiosperm 65, 67, 125, 133, 194, 196, 197, 215, 295, 308
anionic peroxidase 66, 197
annealing 103, 104, 117, 148, 152
annual net return 242, 295
annual yield 247
anther 14, 15, 33, 165, 213, 295, 308
  anther culture 14, 15, 33, 36, 221
  anther tapetum-specific gene 213
anthocyanin 96, 295
antibiotic-resistance gene 91
antifeedant 258
antigen 295
antimicrobial 295
  antimicrobial peptide 298
Antirrhinum 212, 213, 291
antisense 67, 75, 87, 145, 197-203, 215
  antisense DNA 161-163
  antisense gene 232, 295
  antisense orientation 67, 87, 197-200, 214
  antisense RNA 67, 200, 252, 295
  antisense technology 223
antiserum (antisera) 295
AP1 212, 213, 216
ap24 leader 168
APETALA1 (*AP1*) 212, 213, 216
APETALA3 214
aphidicolin 62
apical dominance 7, 77, 296, 298
apical meristem 13, 76, 78, 295
Arabidopsis 74-78, 80, 81, 103, 138, 182, 212-214, 216
  thaliana 52, 67, 194, 291
aroA (modified EPSP synthase) 70-72, 91, 92, 207
ASCAD (antisense CAD) 87
ascorbate peroxidase 187, 188
ascorbate-glutathione cycle 187
ascospore 296
aspartic proteinase 173
Aspergillus 214, 293
assessment endpoint 266
Astragalus (Fabaceae) 145, 146, 291
AtALS (*see also:* acetolactate synthase) 67
ats1A (Arabidopsis RUBISCO small subunit promoter) 71, 182
AUG 296, 302, 308
autoradiography 104
auxin (*see also*: 2,4-D; IAA; IBA; and NAA) 10-12, 30, 47, 51, 77-80, 274, 296, 298, 305
avirulence 150, 296, 302
axillary bud 7, 13, 15, 16, 18, 76, 236, 237, 239
axillary shoot 13, 22, 296

B

b-zip (leucine zipper protein binding site) 126
BA (benzyladenine) 11, 14-16, 19, 29, 30, 38, 39, 46, 62, 92, 298
Bacillus
  amyloliquefaciens 214, 293
  thuringiensis (*see: Bt*)
  thuringiensis -endotoxin (*see: Bt* endotoxin, *Bt* endotoxin genes)
backcross 103, 110, 222, 274, 296
  backcross breeding 103, 110, 275
  backcross population 222
bacterial canker 108, 294
bacterial wilt 294
bactericide 296
Bambusa 34, 291
bar 52, 55, 56, 208
bark cambium 13
bark phloem parenchyma 125
bark storage protein (BSP) 66, 124-128
Barnase 214
Barstar 214
basipetal 296
beetle 86, 241, 254-256, 294
benzaldehyde 202
benzyladenine (6-benzyladenine) (BA) (*see:* BA)
6-benzylaminopurine (*see:* BA)
Betula 43, 83
  papyrifera 126, 291
  platyphylla 291
  populifolia 5
  verrucosa 216, 291
binary vector 56, 70, 93, 296, 310
bioassay 158, 296
biochemistry 124, 138, 221, 301
biocontrol 268, 294
biodiversity 44, 206, 252, 268, 273, 275-277
bioethics 273, 276, 277
biofuel 251
biolistic (microparticle bombardment) (microprojectile-mediated DNA delivery)(particle bombardment/

acceleration DNA delivery system) 7, 12, 51, 60-63, 85, 90, 91, 208, 275, 296, 303, 304
biological control 250, 257, 258, 266, 267, 296
biological impact assessment 264, 265, 269
biological risk assessment 264, 269
biomagnification 257
biomass 13, 40, 116, 193, 203, 209, 212, 232, 241-247
biopesticide 254
bioreactor 12, 296
biosafety 265, 267, 270, 276, 277
Biota
orientalis 134, 291
biotype 250, 252-254, 257, 296
bm (brown-midrib) 196, 197, 200
bmr (brown-midrib) 196, 197, 200, 202
bole form 224
bombardment (*see:* biolistic)
Bowman-Birk Inhibitor (BBI) (*see also*: proteinase inhibitor) 175
bract 212, 296
Brassica napus 214, 291
BSP (bark storage protein) 66, 124-128
Bt (*Bacillus thuringiensis*) 9, 52, 55, 62, 86, 87, 172, 178-184, 252, 253, 257, 259, 293
endotoxin 9, 177-184
endotoxin genes (endotoxin) 2, 55, 252, 253
cryIA 9, 87, 178, 179, 181-183, 255, 256, 261
cryIB 178, 180-182, 253, 254
cryIC 181
cryII 178
cryIIA 181
cryIII 178
cryIIIA 87, 179, 184, 254, 256
cryIV 178
bud resin 255, 256
bud set 5, 77
budworm 293, 294

C

C value 296
C-function 213, 214
CAAT box 302
CAD (cinnamyl alcohol dehydrogenase) 65-67, 86-88, 194-197, 200-203
cadmium 207
caffeic acid 194, 199
caffeic acid/5-hydroxyferulic acid-*O*-methyltransferase (COMT) (*omt*) 65-67, 87, 194-203
caffeoyl-CoA-*O*-methyltransferase (CCoAOMT) 194, 195, 200, 203
CAL 213
calliclone 35
callogenesis 12, 296
callus (calli) 7, 10-12, 14, 19, 23, 25-27, 29-34, 36-39, 41-43, 45-49, 53, 54, 56, 61-63, 67, 92, 156-160, 163, 234, 274, 279, 295, 296, 304
Calocedrus 134
decurrens 134, 292
calyx 296, 308
cambium 13, 15, 16, 74, 77, 79, 94, 165, 296
cambial tissue 46, 174
Camellia japonica 216, 292
cAMP response element (CRE) 126
CaMV *35S* promoter (*35S*) (CaMV promoter) (cauliflower mosaic virus) 62, 63, 67, 70-72, 76-78, 85, 86, 88, 91, 93-95, 161, 162, 168, 175, 179, 182, 188, 197, 198, 200, 208, 216, 275, 277
CaMV *70S* promoter (*70S*) 63, 67
CaMV promoter (*35S*) (*70S*) (*see:* CaMV 35S promoter)
canker 108, 150, 157, 158, 241, 293, 294, 296
capitalized expense 245, 296
capitalized net value 242
carbenicillin 56, 163
carbon metabolism 81
carbon sequestration 249
Carica 33, 34, 292
carpel 213, 296
casamino acids 28
Castanea 133, 167, 292
crenata 133, 292
mollissima 167, 292
sativa (*C. sativa*) 133, 292
CAT (chloramphenicol acetyltransferase) 52, 54-56, 67, 175, 182, 266, 307
catkin 13, 19
cauliflower mosaic virus (CaMV)(*see:* CaMV *35S* promoter)
CCoAOMT (caffeoyl-CoA-*O*-methyltransferase) 194, 195, 200, 203
CCR (cinnamoyl-CoA reductase) 194, 195, 203, 205
cDNA (complementary DNA) 65, 66, 68, 103, 126, 129, 137, 175, 194, 195, 199, 200, 204, 296, 298, 308
cecropin 166, 167, 171
Cedrus
atlantica 134, 292
cefotaxime 56, 237
cell culture 10, 61, 92, 156, 236
cell division 28, 30, 61-63, 77, 78, 156, 157, 296-298
cell fusion 229, 308
cell suspension 10-12, 14, 19-26, 61-63, 156, 303
cell synchronization 61
cellulase 26, 27, 297
cellulose 193, 194, 297
centiMorgan (cM) (*see:* cM)
centromere 297, 301
Ceratocystis fimbriata (aspen canker) 156, 293
Cercis
siliquastrum 133, 292
chalcone synthase (CHS) 65, 96
Chamaecyparis 134, 292
lawsoniana 134, 292
chaperone 297
chaperone-mediated folding 133
chemical composition 247
chemical tolerance 207
chestnut blight (*Cryphonectria parasitica*) (*C. parasitica*) 166, 167, 233, 294
chimera 297
chimeric gene 55, 60, 67, 90-92, 94, 96, 97,
chitin 167, 297
chitin-binding protein 167
chitinase 66, 297
chloramphenicol acetyltransferase (*see: CAT*)
chlormequat 215
chloro-*cis*-*cis*-muconate 208
3-chloroaniline 208
chlorocatechol 208
chlorophenol hydroxylase 208
chlorophyll content 19, 35, 92
chloroplast 21, 103, 116, 144, 145, 147-149, 187-191, 297, 299, 305
chloroplast DNA (*see:* cpDNA)
chloroplast rDNA spacer region 145
chloroplastic transit peptide 188
chlorosis (chlorotic) 45, 189, 297
chlorsulfuron 30, 52, 56, 61, 68, 170, 207, 211, 270
Chondrostereum purpureum 269, 270, 293
Choristoneura 178
Choristoneura fumiferana (*C. fumiferana*) (spruce budworm) 178, 293
Choristoneura occidentalis(*C. occidentalis*) (western spruce budworm) 293
Choristoneura pinus (*C. pinus*) (jack pine budworm) 294
chromatin 279, 297, 299, 301
chromosome 34, 36, 49, 60, 92, 101, 151, 221-223, 229, 274, 279, 295, 297
chromosome damage 47
chromosome doubling 221
chromosome landing 109, 235, 297
chromosome rearrangement 229, 297
chromosome transplantation 220
chromosome walking 297
Chrysanthemum spp. 254, 292
Chrysomela
populi (poplar leaf beetle) 175, 294
scripta (cottonwood leaf beetle) 174, 175, 178, 241, 255, 256, 259, 294
tremulae (aspen leaf beetle) 58, 86, 89,175, 176, 179, 294
CHS (chalcone synthase) 65, 96
chymotrypsin 174
chymotrypsin inhibitor (*see also*: proteinase inhibitor) 52
cinnamaldehyde 197, 200, 204
cinnamate-4-hydroxylase (C4H) 194
cinnamic acid 194, 197
cinnamoyl-CoA reductase (CCR) 194, 195, 203, 205
cinnamyl alcohol 66, 68, 86, 194, 195, 200, 202, 205
dehydrogenase (CAD)(*POPCAD*) (*EuCAD*) 65-67, 86-88, 194-197, 200-203
cis-acting DNA sequence 297, 309
Citrus 33, 34, 292, 294
sinensis 216, 218, 292
cladistic 106, 297
classical breeding 155, 220, 222, 224-232
classical genetic 220, 224, 228, 229
climate change 44, 138, 268
clonal deployment 224
clonal identification 105

clonal planting 236
clonal propagation 8, 15, 48, 97, 116, 273, 274, 277, 278, 297
clonal trial 101, 109, 224, 228
cM (centiMorgan) 107, 222, 297
co-cultivated explant 75
co-cultivated leaf disc 70
co-cultivation 54, 56, 175, 297
co-integrate vector (co-integration vector) 297, 310
co-suppression 94, 96, 97, 214, 279, 298
CO (*CONSTANS*) 212, 216
CO_2 sequestration 251
cobalt 207
coding region 76, 126, 144-146, 148, 151, 170, 297
coding sequence 70, 71, 86-88, 102, 151, 198, 297
codling moth 185, 186
codominant 102-105, 108, 143, 222, 297
codon 167, 168, 170, 179, 296, 297, 302, 309
  codon preference 165, 170
Coffea
  arabica 34, 292
cold storage 7, 36, 44-46, 49
Coleoptera 3, 58, 87, 89, 174, 178, 179, 254, 256, 257, 259-261
collar shoot 7, 297
Colletotrichum 264, 294
  gloeosporioides 267, 271, 294
  lindemuthianum 174, 294
commercial field yield 247
commercial plant production 236
commercial production 19, 44, 179, 236
commercial-scale 14
commercial-sized plantation 242
compatibility 106, 214, 254, 259, 296, 297, 302, 308
competence 61-63, 79, 141, 298
competition 2, 86, 103, 114, 260, 261, 263, 268
complementary DNA (*see:* cDNA)
COMT (caffeic acid/5-hydroxyferulic acid-*O*-methyltransferase)(OMT) 67, 194-203
confirmation 52, 113, 215, 263
conidiophore 298
conidium (conidia) 157, 167, 298
coniferaldehyde 202
coniferin 196
coniferyl alcohol 194-196, 200, 303
  coniferyl alcohol oxidase 195
consensus sequence 168
conservation 3, 44, 47, 48, 67, 76, 106, 132, 170, 208, 209, 233, 236-243, 273, 276, 277, 309
conserved 67, 76, 80, 110, 130, 132, 134, 143-148, 151, 296, 302, 309
CONSTANS (*CO*) 212, 216
constitutive expression 86, 168, 198, 216, 298
constitutive promoter 86, 168, 170, 208
conventional breeding 51, 165, 166, 275
copper 173, 215
coppice (coppicing) 13, 193, 206, 247, 298
copy number 52, 57, 85, 90, 93, 94, 96, 161, 175, 198, 298, 305
corolla 298, 304
cortex 94
Corynebacterium
  diphtheriae 214, 294
Cotesia
  melanoscela 257, 294
cottonwood leaf beetle (*Chrysomela scripta*) (*C. scripta*) 174, 175, 178, 241, 255, 256, 259, 294
cotyledon 132, 134, 274, 298, 301
4-coumarate-3-hydroxylase (C3H) 194
p-coumaric acid 194, 200
p-coumaroyl-CoA-3-hydroxylase (CCoA3H) 195
p-coumaryl alcohol 194, 196, 303
cpDNA (chloroplast DNA) 103, 107, 116, 119, 143-148, 297, 304
CRE (cAMP response element) 126
crop protection 251
cross-hybridization 276
cross-pollination 220, 298
cross-resistance 254, 298
crossing over 222, 298, 307
crown gall 1, 51, 52, 150, 161-163, 293, 309
crs1-1 52, 55, 56, 207
cry (*Bt* endotoxin gene) 2, 55, 252, 253
  cryIA 9, 87, 178, 179, 181-183 255, 256, 261
  cryIB 178, 180-182, 253, 254
  cryIC 181
  cryII 178
  cryIIA 181
  cryIII 178
  cryIIIA 87, 179, 184, 254, 256
  cryIV 178
cryopreserve (cryopreservation) 41, 44-47, 298
cryoprotection 45
Cryphonectria parasitica (*C. parasitica*) (chestnut blight) 166, 167, 233, 294
CTAB (hexadecyltrimethylammonium bromide) 117
cultivar 53, 157, 253, 254, 258, 298
culture condition 11, 15, 24, 36, 75, 92, 138, 229
culture filtrate 158
Cupressaceae 134, 292
cysteine 167, 170, 173, 174, 176
  cysteine protease inhibitor (*OCI*) 52, 55, 85-87, 174-176
  cysteine proteinase 173-175
cytokinin (*see also*: BA; kinetin; and zeatin) 7, 8, 11, 14, 19, 30, 38, 51, 53, 62, 75, 78, 79, 83, 128, 161, 298, 305
cytology 298
cytoplasm 63, 71, 96, 103, 106, 188, 207, 221, 229, 298, 299, 303, 305, 307
cytoplasmic DNA (*see also:* cpDNA, mtDNA) 103, 106
cytosol 70, 71, 188, 190
cytotoxin 213-215, 252, 298
  cytotoxic gene 252

D

2,4-D (2,4-dichlorophenoxyacetic acid) 10-12, 15, 19, 29, 30, 38, 46, 52, 56, 92, 208, 274, 296
daminozide 215
de novo 133, 225, 298
deamination 194, 298
defense 3, 131, 156, 158, 173, 187, 254-256, 302, 305
defensin 167, 298
DEFICIENS (*PTD*) 213
deglycosylation 125
degree of branching 35
dehydrogenate 195, 298
deletion 102, 103, 105, 143, 305
delivered cost 243, 245
demethylation 96, 298
denaturation 117, 152, 298, 305
deoxyribonucleic acid (DNA) 298
deployment 176, 181, 223, 224, 234, 249, 251-254, 258, 261, 275
depolymerize 193, 202, 298
detoxification 187, 188, 206-208
detritivore 257, 258
developmental influence 52, 84
developmental process 10, 11, 77
developmental stage 10, 11, 85, 110, 141, 155, 178, 214, 239, 306
dextran 45
DHDPS (dihydrodipicolinate synthase) 66
diamondback moth (*Plutella xylostella*) 180, 294
diazoacetyl-compound 173
2,4-dichlorophenol 208
2,4-dichlorophenoxyacetic acid (*see:* 2,4-D)
dicotyledon (dicot) 33, 65-67, 76, 142, 145, 168, 298
DIFP (diisopropylphosphofluoridate) 174
dihaploid 221, 229, 298
diisopropylphosphofluoridate (DIFP) 174
dimer 125, 128, 135, 174, 196, 298
dimethyl sulfoxide (DMSO) 45
dioecious 220, 229, 298
diphtheria toxin subunit A (DTA) 214
diploid 36-38, 65, 97, 221, 298, 305
Diptera 178, 257
direct gene transfer (*see also:* biolistics, electroporation, DNA microinjection) 12, 60-63, 165
discounted cash-flow 241
disease 1, 3, 23, 42, 44, 51, 55, 78, 96, 108, 150, 155, 157-158, 161, 163, 164, 174, 209, 268, 269, 273, 276, 293, 294, 304
  disease rating 157
  disease resistance 3, 113, 150, 155-158, 161-164, 168, 174, 193, 223, 224, 275
disulfide bond 132, 133, 135, 137, 167
dithiothreitol (DTT) 31, 136, 175
DMSO (dimethyl sulfoxide) 45
DNA-banding pattern 119
DNA extraction 7, 117
DNA fingerprint 103, 104, 112, 115, 116, 118-121, 123, 222, 228-231, 295, 298
DNA hybridization 103
DNA marker (genetic marker) 2, 79, 101-111, 115-121, 143, 145-148, 220-223, 228-231, 266, 267, 297, 300, 302, 306
DNA methylation 38, 92, 95, 98, 99, 274

DNA microinjection 24, 299, 303
DNA polymerase 101, 114, 116, 117, 123, 222, 299
dominant 77, 93, 95, 102, 104, 110, 130, 134, 143, 180, 183, 184, 222, 275, 296, 299
dormant (dormancy) 5, 9, 11, 58, 78, 82, 83, 94, 100, 116, 125, 127, 129, 130, 179, 221, 236, 299, 306
Douglas-fir (*Pseudotsuga menziesii*) (*P. menziesii*) 76, 82, 134, 183, 254, 293
down-regulated (down-regulation) 88, 196, 197, 200-202, 213, 299
Driselase 27
Drosophila 113
DTA (diphtheria toxin subunit A) 214
DTT (dithiothreitol) 31, 136, 175
durability 168, 169, 266
durable resistance 109, 168, 169
Dutch elm disease (*Ophiostoma ulmi*) (*O. ulmi*) 41, 166, 294

E

early flowering 74, 76, 78, 81, 215-217
early selection 1, 105, 158, 221, 231, 302
earlywood 79-81, 299, 302
ecological interaction 150, 176
economic 1-3, 10, 13, 33, 34, 44, 69, 74, 84, 86-88, 104, 108, 109, 116, 150, 161, 165, 176, 180, 181, 193, 203, 205, 206, 209, 223, 224, 229, 231, 232, 241, 245, 247-249, 251, 254, 255, 258, 272, 278, 279
  economic evaluation 245, 247, 248
  economic injury level 254, 255
ecophysiological 251
ectomycorrhiza 155, 160, 208, 210, 211, 294, 299, 303
ectopic 77, 96, 215, 217, 276, 299
  ectopic expression 77, 215, 217
  ectopic gene expression 77
EDTA (ethylenediaminetetraacetic acid) 117, 174
Elaeis
  guineensis 292
electric discharge method 61, 63
electron transport 187, 190
electrophoresis (electrophorese) (electrophoretic) 64, 102-105, 113, 114, 117, 120, 133, 135-137, 139, 140, 148, 152, 235, 298, 299, 306, 308
electroporation 24, 30, 41, 51, 60, 61, 63, 64, 74, 91, 98, 210, 299
ELISA (enzyme-linked immunosorbent assay) 52, 175, 299
elongation factor 214, 299
embryo 11, 13-15, 17, 19, 23, 41, 64, 132-135, 139-141, 217, 220, 221, 229, 231, 232, 235, 299-301, 306, 308
  embryo culture 14, 15, 23, 217, 235
  embryo rescue 220, 221, 229, 231, 232
embryogenesis 2, 10-12, 19, 21, 23, 40, 48, 49, 64, 130-133, 138-141, 221, 222, 230, 231 273, 274, 277, 299, 308
embryogenic 10-12, 19, 23, 24, 49, 133, 141, 274
  embryogenic callus 11, 23, 49
  embryogenic cell culture 24
embryoid 11, 12, 299
endogene (endogenous gene) 78, 90, 93-96, 276
endomycorrhiza (VA mycorrhiza) 208, 299, 303
endopeptidase 173
endoplasmic reticulum (ER) 126, 132, 133, 299, 301
endoprotease 128
endosperm 132, 139, 141, 142, 299
endotoxin (-endotoxin) 2, 9, 55, 62, 86, 87, 89, 177-186, 252-254, 256, 257, 260, 262, 296, 299
endpoint 266, 267, 269, 271
energy cost 241-245
energy crop 44, 186, 193, 299
enhancer 63, 67, 176, 177, 198, 299, 302
5-enolpyruylshikimate-3-phosphate synthase (EPSP) (*see:* EPSP)
ent-kaurene acid 215
entomopathogen 253, 270
environmental consequence 251
environmental factor 77, 94, 95, 100, 109, 127-129, 155, 215, 249, 267, 306
environmental impact 86, 87, 209, 212, 217, 259, 260, 263-265, 267, 270
environmental risk 249, 250, 258, 261, 264, 266, 268-270
environmental safety 212, 249, 251, 258
environmental stress 113, 187-189, 191
enzyme 26, 27, 30, 34, 52, 66, 67, 69, 72, 73, 81, 82, 87, 102-106, 113, 126, 131, 142-147, 151, 152, 173-175, 177, 187, 188, 194, 197, 198, 200-205, 208, 222, 260, 297, 299, 302, 304-308
  enzyme activity assay 52
  enzyme assay 197, 198
enzyme-linked immunosorbent assay (ELISA) (*see:* ELISA)
epicuticular wax 19, 23
epidemiology (epidemiological) 150, 264, 269, 299
epigenetic 97, 100, 273, 274, 299
  epigenetic change 36, 92, 93, 95, 96, 99, 223, 273
  epigenetic effect 98, 229
epimutation 97
epistasis (epistatic) 96, 99, 299
epoxy propane 173
EPSP 52, 70-72
  EPSP synthase 70-72, 207
Eranthis hyemalis 137, 140, 292
Erwinia amylovora 167, 294
escape of engineered germplasm 258
escape of transgene 251, 277
Escherichia coli (*E. coli*) 91, 113, 188-191, 207, 218, 308
ESF12 (synthetic analogue of PGLa) 167-169
EST markers (expressed sequence tags) 105
establishment cost 206, 242, 243
ester 173, 299
ethanol 152, 199, 201, 202, 241, 245-247, 259
ethylene 21, 79
ethylenediaminetetraacetic acid (EDTA) 117, 174
etiology 150
EuCAD (Eucalyptus cinnamyl alcohol dehydrogenase) 67, 86, 87
Eucalyptus 33, 34, 65-67, 74, 82, 86, 101, 103, 104, 108, 110-113, 203, 215-218, 235, 292
Eucalyptus globulus (*E. globulus*) 215, 292
Eucalyptus grandis (*E. grandis*) 82, 104, 112, 235, 292
Eucalyptus lansdowneana (*E. lansdowneana*) 216, 218, 292
Eucalyptus nitens (*E. nitens*) 103, 111, 215, 218, 292
Eucalyptus occidentalis (*E. occidentalis*) 215, 217, 292
Eucalyptus regnans (*E. regnans*) 103, 292
Eucalyptus urophylla (*E. urophylla*) 112, 292
euchromatin 299
Euglena gracilis 144
eukaryote 99, 100, 168, 297, 299, 300, 302, 304, 307
evolution 3, 86, 92, 96, 107, 110, 112, 114, 121, 123, 140, 143, 149, 179-186, 250, 252-254, 256-263, 266, 267, 270, 272, 273, 275, 276, 278, 297, 305
  evolutionary consequences 266
ex situ 43, 44, 48, 300
ex vitro 6, 7, 70, 156, 237, 300
  ex *vitro* growth 7
  ex vitro rooting 237
exogenous 15, 92, 156, 253, 300
exon 253, 260, 300, 302
exopeptidase 173
exotic introduction 251
exotoxin 214, 218, 300
explant 5-7, 10-16, 19, 21, 22, 31, 37, 41, 45, 52-54, 56, 60, 62, 63, 92, 156-159, 197, 222, 236-238, 274, 300, 304, 307
  explant source 7, 15, 37, 92
expression pattern 67, 78, 80, 85, 86, 138, 181, 217, 300
extension 104, 105, 117, 152, 185, 232, 286

F

F_1 10, 32, 34, 95, 107-111, 144, 164, 227, 275, 300
F_2 74, 108-110, 222
Fabaceae 133, 134, 140, 145, 149, 292
fasciated stem 78
feedback inhibition 77
feeding assay 175, 180, 183
feeding test 176
female catkin 19, 21
feral population 251
fertile (fertility) 54, 64, 76, 78, 129, 166, 208, 213, 214, 252, 279
fertilizer 183, 206, 207, 209, 216, 237, 243
ferulate-5-hydroxylase (F5H) 194
ferulic acid 67-69, 83, 194, 197-200
fiber 2, 77-79, 87, 116, 155, 183, 193, 206, 220, 241, 247, 264, 297, 310
  fiber length 247
field-grown 7, 11, 24, 39, 46, 84-88, 130, 179, 236
field resistance 157, 180
field screening 157

field test 155, 157, 158, 166, 179, 182, 231, 265, 267-269
field trial 3, 39, 84-88, 97, 111, 166, 176, 202, 209, 224, 229, 231, 232, 267
financial cost 242-244, 246
financial impact 245
fingerprint analysis 104
fitness 90, 97, 153, 180, 267, 268, 270, 275, 276
flanking DNA 300
FLO (*FLORICAULA*) 212, 213, 217
FLO/LFY (*PTFL*) 213, 214
floral bud 216
floral development 82, 212, 216
floral differentiation 213
floral gene 212, 213, 252
floral homeotic gene 82, 212-214
floral meristem 212-214
floral meristem identity gene 212, 214
floral organ 212-214
floral organ identity gene 212, 214
floral promoter-cytotoxin 252
FLORICAULA (*FLO*) 212, 213, 217
flower (flowering) 1, 2, 5, 19, 22, 55, 58-60, 74-76, 78, 79, 81-83, 87, 95, 96, 98-100, 125, 133, 141, 142, 161, 162, 165, 168, 172, 175, 179, 188, 197, 198, 202, 208, 212-216, 252, 275, 279, 292, 295, 296, 298, 303-305, 308
  flower bud 19, 22, 216
  flower color 95, 96, 99, 100
  flower development 212, 213
  flower initiation 59, 75, 83, 216
  flower primordium 75
  flower production 212
fluorescence 112, 266, 300
fluorometer (fluorometry) 85, 152, 300
folding of mRNA 168
folivore 254
force 40, 87, 236, 247, 261, 302
foreign DNA 54, 58, 60, 64, 100, 296, 300, 309
foreign gene 3, 49, 52, 84, 90, 92-94, 273, 277, 300, 309
forest tent caterpillar (*Malacosoma disstria*) (*M. disstria*) 9, 62, 89, 178-183, 253-256, 294
frost hardiness 77
frost tolerance 108
frosty spot (*Frs*) 95
fruit 33, 40-43, 95, 110, 124, 165, 215, 217, 269, 293, 295
fuel 1, 193, 243, 245, 247
full-sib progeny (full-sib offspring) 229, 300
fungicide 174, 241, 242, 300
fungistatic 156, 174, 300
fungus (fungi) 24, 115, 151-153, 155-158, 167, 170, 173, 174, 208, 257, 266, 267, 293-296, 300-303, 305, 309, 310
Fusarium 41, 167, 174, 294
  oxysporum 167, 294
  solani 174, 294

G

G-box 126, 300
GA (Gibberellic acid) (Gibberellin) 78, 79, 128, 215
galactose-(1-3)-N-acetylgalactosamine 125
galactose-(1-4)-N-acetylglucosamine 125
gallic acid 184
gamete 213, 275, 299, 300, 308, 310
gametoclonal (gametoclone) 33, 34, 36, 300
  gametoclonal variation 34, 36, 92, 98
gametophyte (gametophytic) 104, 134, 214, 300, 303, 305
gas exchange 34, 39, 40
gas chromatography 105, 198, 300
Gaussian model 269
gelling agent 19
GEM (genetically engineered microbe) 266
gene construct 62, 93, 94, 168-170, 197, 198
gene containment 212, 214
gene escape 250-252, 276, 277
gene expression 11, 13, 56, 60-63, 65-68, 70-72, 75, 77, 79-81, 85-87, 93-95, 127, 132, 134, 138, 175, 179, 180, 196, 197, 200, 213, 223, 230, 252, 276, 298-300, 307, 309
gene flow 251, 266
gene frequency 253, 300
gene introgression program 110
gene mapping 2
gene mutation 274
gene regulation 52, 67, 127, 131, 132, 252, 297
gene silencing 85, 87, 90, 94-97, 214, 277, 300
gene transfer 24, 30, 51, 52, 60-62, 90-93, 97, 165, 169, 223, 228, 230, 264, 265, 273, 275, 276, 300, 305
generation time 78, 79, 105, 155, 254, 273, 300
genetic code 168, 266, 297
genetic contamination 230
genetic diversity 13, 105, 106, 224, 230, 273-277
genetic engineering 2, 5, 7, 24, 30, 51, 71, 74, 77-81, 84, 90-94, 116, 138, 161-164, 165-170, 173-176, 178-184, 187-191, 193-203, 206-209, 212-216, 223, 228, 249-259, 264-269, 273-278, 310
genetic fidelity 223
genetic fingerprint (*see also:* DNA fingerprint) 105, 221
genetic gain 220, 221, 229-232, 300
genetic improvement 116, 119, 273, 275, 277
genetic instability 90-92, 95, 97, 273, 274, 276, 277
genetic loci 109
genetic manipulation 21, 74, 92, 151, 277
genetic map 74, 79, 91, 101, 103-109, 116, 206, 222, 223, 228, 229, 231, 232, 273, 300
genetic marker (*see:* DNA marker)
genetic pollution 212
genetic resource 48, 231, 233
genetic selection 208, 209
genetic stability 33, 48, 92, 93, 274
genetic transformation 13, 34, 48, 53, 63, 67, 84, 87, 90, 91, 93, 94, 161-163, 220-223, 228, 230-232, 253, 275, 277, 295, 300
genetic variability 221, 228, 229, 231, 232, 267, 275
genetic variance 110, 301
genetic variation 33, 44, 47, 92, 101, 143, 148, 220, 222, 224, 228, 230, 231, 273-277, 295, 303, 307
genetically engineered microbe (GEM) 266
genetically engineered organism (GEO) 250-252, 257, 266, 267
genetically modified tree (GMT) 87
genome map (*see*: genetic map)
genome size 51, 65, 67
genome structure 110
genomic DNA 65, 104, 144, 152, 161, 222, 295
genomic library (libraries) 65, 103, 104, 297, 300
genotype 7, 8, 10, 13, 24, 27, 29, 30, 37, 44, 52-54, 60, 62, 63, 65, 68, 92, 94, 95, 101, 105, 108-110, 132, 155, 156, 165, 166, 180, 206, 207, 216, 220-223, 228-232, 236-239, 253, 254, 274, 275, 277, 299, 300, 302, 305, 308
genotype x environment interaction 110, 216, 305
genotypic fidelity 47, 221, 222, 231
Geographic Information Systems (*see:* GIS)
germination 11, 131-133, 167, 237, 274
germplasm 1, 2, 12, 44, 45, 47, 48, 103-105, 113, 220, 230-232, 250, 258, 273, 277, 301
germplasm analysis 104, 105
germplasm preservation 2, 12, 44, 45, 273, 277
GFP (Green Fluorescent Protein) 86
Gibberellic acid (Gibberellin) (*see:* GA)
Ginkgo biloba 134, 292
Ginkgoaceae 134, 292
girdling 215
GIS (Geographic Information System) 269, 300
globulin 132-134
Glomerella 294
  cingulata 294
ß-1,3-glucanase 96
p-glucocoumaryl alcohol 196
glucose 28, 29, 297, 301
glucoside 196, 301
ß-glucuronidase (glucuronidase) (*GUS*) 7, 55, 56, 61-63, 67, 85, 86, 88, 91, 94, 95, 137, 162, 182, 198, 208, 307
glutamine 11, 30, 128
glutathione 55, 187-191, 207
glutathione reductase (GR) 55, 187-191, 207
glutelin 132, 133
glycerol 45, 198, 200
Glycine max 70, 292
glycoside 183, 254, 255, 295, 301
glycosylation (glycosylated) 125, 126, 135, 137, 138
glyphosate 52, 70-72, 93, 207
  glyphosate tolerance 52, 70-72

gn1 (*see also*: ß-1,3-glucanase) 96
Golgi apparatus (dictyosome) 133, 301
GR (glutathione reductase) 55, 187-191, 207
Gram-negative bacteria 167, 297, 301
Gram-positive bacteria 167, 294, 297, 301
Gram's staining reaction 301
gravity 79, 80
Green Fluorescent Protein (*GFP*) 86
growth and development 74, 75, 77-80, 92, 175, 201
growth and yield 40, 74, 228, 247
growth medium 26
growth rate 34, 36, 51, 62, 63, 85, 247, 256, 257, 266
growth regulator (*see:* plant growth regulator)
guaiacyl 194, 196, 199, 201
guideline 166, 209, 254, 258, 265
Guinier 1, 290, 291
gunpowder method 61, 63
GUS (ß-glucuronidase) 7, 55, 56, 61-63, 67, 85, 86, 88, 91, 94, 95, 137, 162, 182, 198, 208, 307
gymnosperm 33, 51, 65, 125, 126, 132-134, 139-142, 194, 196, 298, 301
gypsy moth (*Lymantria dispar*) (*L. dispar*) 62, 178, 179, 253, 257, 294

H

hairpin loop 168
hairy root disease (*see also*: *Agrobacterium rhizogenes)* 51, 55, 78, 293, 307
halogenated phenolics 206, 208
haploid 15, 33, 36, 38, 92, 221, 222, 229, 273, 296, 298, 300, 301, 303, 305
  haploid culture 33
  haploid plantlet 15
  haploid tissue culture 221
heat stress 165
heavy metal 174, 206-209
hedging 236-238
height and diameter growth 223
height/diameter 110
height growth 2, 45, 110, 183
Heimburger 1, 291
Heliothis 174, 181, 294
  virescens 181, 294
  zea 174, 294
helium gas 61
helium propulsion method 63
hemicellulase 27
herbicide 2, 34, 36, 37, 52, 70, 72, 84, 91-93, 96, 146, 187, 189, 207-209, 222, 223, 241-243, 247, 252, 267, 269, 275, 305, 308
  herbicide-binding protein (Qb) 146
  herbicide resistance (herbicide-resistant) 52, 84, 212, 267
  herbicide tolerance 2, 34, 36, 37, 70, 84,91, 93, 96, 207, 223
herbivore 251, 253, 256, 257
heritability (heritabilities) (heritable) 38, 80, 92, 93, 109, 207, 221, 223, 229-231, 253, 256, 301, 303
heterochromatin (heterochromatized) 96, 301
heterodimer 125, 301
heterogeneous (heterogeneity) 60, 125, 254-256, 301
heterokaryon 30
heterologous 65, 67, 71, 106, 181, 200, 222
heteroploid 37
heteropolymer 193, 195, 301
heterosis 10, 110, 161, 224, 228, 301
heterotrophic 131, 301
heterozygote 183, 299, 307
heterozygous (heterozygosity) 95, 96, 101, 102, 104, 105, 220, 221, 273, 275, 301, 302
heterozygous loci 102, 104
hexadecyltrimethylammonium bromide (CTAB) 117
hexanucleotide 145
Hibiscus sabdariffa 26, 30, 292
high-density linkage map 105
histochemistry 85, 301
homeostasis 79, 301
homeotic gene 212-214, 301
homologous 65, 93, 94, 96, 97, 103-105, 134, 135, 143, 152, 167, 212, 214, 222, 298, 301, 307, 309
homology 65, 67, 76, 92, 94-96, 135, 137,
homozygote 183
homozygous 95, 102, 104, 105, 221, 229, 301, 302
homt1 (OMT) 66
homt2 (OMT) 66
horizontal gene transfer 264
horizontal resistance 109, 306
hormone (*see*: plant growth regulator)
host-parasite interaction 155, 156, 175
host-pathogen interaction 158
host range 265-267, 294
host resistance 1, 150, 158, 176, 255
host-specific toxin 155, 156, 158
HPLC (High Pressure Liquid Chromatography) 202, 203
HPT (hygromycin phosphotransferase) 55, 56, 208
human serum response factor (*SRF*) 213
Hyalophora cecropia 168, 294
hybridization 10, 30, 52, 67, 90, 94-96, 102, 104, 106, 144, 197, 220-222, 230, 231, 251, 254, 258, 267, 275-277, 295, 301, 302, 306, 308
hybrids spontanés 1
hydrogen bond 45, 135
hydrogen peroxide 187
hydroxamic acid derivative 174
3'-hydroxy (3'-OH) 306, 309
hydroxy-acetosyringone (OH-AS) 56
4-hydroxycinnamate-CoA ligase (4CL) 194
hydroxycinnamyl alcohol 194
5-hydroxyconiferyl alcohol 200
5-hydroxyferulic acid 194, 197-200
hydroxylation 194-196, 301
p-hydroxyphenyl 194
hygromycin 55, 56, 63, 96, 208
hygromycin phosphotransferase (*HPT*) 55, 56, 208
hypermethylation 95
hypertonic 24
hypha (hyphae) 298, 301, 303
hypocotyl 158, 301
Hypoxylon canker 157, 158, 294
Hypoxylon mammatum (*H. mammatum*) 156-158, 294

I

IAA (indole-3-acetic acid) 21, 77, 296
IAA-biosynthesis gene 76-79
  iaaH (indoleacetamide hydrolase) 51, 55, 77, 161-163
  iaaM (tryptophan monooxygenase) 51, 55, 77, 161-163
IBA (indole-3-butyric acid) 19, 46, 163, 296
ice-nucleating bacteria 264, 266, 301
IGS (intergenic spacer) 144-146, 151, 302
illumination 45, 189
immunochemical 125, 188
  immunochemical analysis 188
  immunochemical localization 125
immunogenic analysis 137
immunology 266
impact assessment 264-270
impact prediction 269
imported willow leaf beetle (*Plagiodera versicolora*) (*P. versicolora*) 174, 175, 294
in planta 175, 301
in situ 44, 48, 80, 236, 238, 301
in vitro culture 2, 5, 11, 13, 15, 19, 21, 44, 45, 47, 53, 85, 94, 221, 230, 231, 236-239, 278
in vitro feeding assay 175
in vitro propagation 13, 44, 53, 221, 222, 230, 231, 303
in vitro roots 237-239
in vitro screening 155, 158, 167
in vitro selection 85, 155, 156, 207, 209, 222, 229
in vivo 14, 34, 56, 131, 166, 170, 174, 199, 298, 301
inbreeding (inbred) 151, 224, 230, 301
incompatibility 106, 214, 296, 302, 308
indirect selection 108, 223
indole-3-acetic acid (IAA) 21, 77, 296
indole-3-butyric acid (IBA) 19, 46, 163, 296
infect (infection) 51-54, 108, 155, 157, 158, 161, 163, 165, 167, 174, 276, 294, 298, 306, 309
inflorescence meristem 212
initiation codon 168, 296, 302
inoculum 56, 155, 157, 310
  inoculum titer 56
insect 1, 62, 84, 86, 87, 155, 165, 168, 174, 175, 178-184, 209, 212, 241, 244, 249, 252-258, 266, 302, 310
  insect attack 165, 182, 183
  insect biotype 86, 180, 183, 253
  insect-eating bird 266
  insect resistance 86, 181-184
  insect-resistant 249
  insect tolerance 84, 182
insecticide 180, 181, 183, 241, 242, 253, 254, 258
insertion 97, 223, 310
instability 38, 56, 87, 90-92, 94-97, 179, 214, 273, 274, 276, 277

instar 175, 176, 178, 256, 302
Institute of Poplar Culture 1
Integrated Pest Management (IPM) 254, 255
integration 30, 54, 56, 60, 63, 79, 90, 92-94, 96, 197, 198, 208, 209, 220, 228, 231-233, 277, 297, 308, 310
intercellular space 19, 20, 156, 157, 302
intergenic spacer (IGS) 144-146, 151, 302
internal transcribed spacer (ITS) 151, 152, 302
internode (internodal) 13-16, 53, 54, 75, 77, 156, 163
interspecific 10, 15, 101, 106-109, 152, 161, 220, 221, 224, 229-231, 275
intracellular 45, 70
intraspecies differentiation 116, 253
intraspecific variation 103-107, 253
introgression (introgressive) 41, 43, 94, 103, 105, 106, 109, 110, 112-114, 123, 276, 275, 302
intron 65, 145, 297, 302
inventory control 245, 246
inverted repeat (inverted-repeated) 146, 302, 304
  inverted-repeat region 144
investment 232, 233, 241, 243, 254
iodoacetamide 174
iodoacetate 174
IPM (integrated pest management) 254, 255
IPT (isopentenyltransferase) 51, 55, 161
Isatis 269, 292
  tinctoria 269, 292
isoenzyme (*see:* isozyme)
isogenic 151, 296, 302
isolate 11, 24, 25, 30-32, 46, 52, 65, 66, 71, 74, 75, 80, 81, 95, 126, 137, 138, 148, 151, 155, 157, 302
isolation medium 27, 30
isomer (isomerization) 133, 135, 302
isopentenyltransferase (*IPT*) 51, 55, 161
isotonic 24
isozyme 34, 36, 102, 103, 105, 108, 116, 222, 228, 302
ITS (internal transcribed spacer) 151, 152, 302

J

Juglans (walnut) 125, 292
juvenile characteristic 80, 238
juvenile-mature correlation 155, 158
juvenile-mature trait relationship 220
juvenile phase 5, 138
juvenile tissue 34
juvenilewood 80

K

kanamycin 54, 56, 62, 63, 67, 91, 93, 96, 163, 175, 275
kinetin 19, 29, 298
KM8P (Kao and Michayluk media) 28, 30
Kunitz inhibitors (*see also*: proteinase inhibitor) 131

L

l'Union Allumettiere, S.A. (UNAL) 1
Laccaria bicolor (*L. bicolor*) 155, 294
laccase 195, 203
Larix 103, 126, 134, 216, 292, 294
Larix decidua (*L. decidua*) 126, 292
Larix laricina (*L. laricina*) 103, 292
Larix leptolepis (*L. leptolepis*) 216, 292
Larix occidentalis (*L. occidentalis*) 132, 292
larva (larvae) (larval) 62, 175, 176, 178-180, 182, 183, 253, 255-257, 293, 302, 306
latewood 79-81, 299, 302
leader sequence 70, 71, 168, 302
leaf area 110, 175, 179
leaf disc 14, 70, 92, 162, 183, 184, 189, 190
leaf disk 11, 54, 157
leaf enlargement 77
leaf explant 11, 12, 15, 54, 62, 237
leaf phenology 108, 110
leaf plastochron index (LPI) 85, 302
leaf primordia (leaf primordium) 75
leaf micro-cross section technology (MCS) 236-240
leaf rust 150-152, 157, 294
leaf spatial position 85
leaf spot 108, 150, 157, 166, 167, 294
leaf thickness 39
leaf-tissue explants 11
LEAFY (*LFY*) 74-76, 78, 79, 81, 138, 212, 214, 216
least-cost basis 241, 243
lectin 131, 137
legumin 132
Leguminosae 173
lenticel 16, 302
Lepidoptera 256, 257, 293
leucine zipper 126, 296, 302
library screening 74
ligand 302, 307
light 11, 29, 46, 56, 75, 77, 87, 118, 156, 157, 187-191, 295, 299, 305
lignification 65, 67, 68, 79, 193-203, 302
lignin 67, 79, 81, 84, 86-87, 185, 193-203, 223, 230, 252, 302, 303
  lignin biosynthesis 81, 87, 193-203, 223
  lignin metabolism 84, 86, 87, 193-203
lime 241
linkage 101, 103-105, 107-110, 116, 196, 202, 222, 223, 245, 302
  linkage analysis 103, 222
lipoxygenase 131
liquid culture 21, 28
liquid (nonpersistent) endosperm 132
Liquidambar styraciflua (*L. styraciflua*) 208, 292
Liriodendron tulipifera (*L. tulipifera*) 135, 292
Litchi chinensis (*L. chinensis*) 216, 292
loblolly pine (*Pinus taeda*) (*P. taeda*) 103, 104, 215, 266, 293
locus (loci) 79, 102-105, 109-110, 116, 126, 165, 212, 214, 222, 223, 295, 297, 300, 302, 306, 307
LOD scores 110
long-day condition 76, 127
long-lived species 84, 251, 254
long-term strategies (long-term stratagy) 224, 228, 231
long-term stability 38, 87, 252, 254
low durability resistance 266
low-temperature storage 45, 46
luciferase (*luxF2*) 55, 56, 275, 276, 307
LUMIDEPENDENS 212
luxF2 (luciferase) 55, 56, 275, 276, 307
Lycopersicon 93, 95, 167, 292
  chilense 95, 292
  esculentum (tomato) 167, 292
Lymantria dispar (*L. dispar*) (gypsy moth) 62, 178, 179, 253, 257, 294
lytic peptides 167

M

M13 universal primer 118
macerozyme 26, 27
macro-propagation
Macrozamia 134, 292
  communis 134, 292
MADS box 212, 213, 216
magainins 167, 298, 302
maintenance cost 242, 245, 247
maize (*Zea mays*) 53, 55, 66, 95, 97, 144-146, 196-198, 200, 252
Malacosoma 178, 253, 254, 256, 294
  disstria (*M. disstria*) (forest tent caterpillar) 9, 62, 89, 178-183, 253-256, 294
malt extract 11
Malus 33, 34, 167, 215, 216, 292
  domestica 167, 292
  hupehensis 216, 292
Manduca sexta (*M. sexta*) (tobacco hornworm) 174, 294
mannitol 28, 29, 304
mannopine synthetase (*MAS*) 70-72, 77, 91
mannose 125
map-based gene cloning 223
MAR (matrix attachment region) (nuclear scaffold attachment region) (SAR) 97, 303, 304, 308
marker 56, 67, 79, 91, 93, 101-110, 116, 119, 138, 143-148, 150, 175, 207, 208, 220, 222, 223, 229-231, 252, 266, 275, 296, 297, 300, 302, 303, 308
  marker-assisted selection (marker-aided selection) 110, 223, 229, 302, 308
  marker gene 56, 91, 252, 266, 296, 297, 303
Marssonina 108, 157, 294
  brunnea 108, 157, 294
Marssonina leaf spot 157
MAS (mannopine synthase) 70-72, 77, 91
mass clonal propagation 48
mass production 10, 12
material input 243
maternal inheritance 144
mating 108, 109, 220, 224, 228-231, 301, 303, 306
matrix attachment region (MAR) (matrix-attachment region) (nuclear scaffold attachment region) (SAR) 97, 303, 304, 308

maturation (mature) 10, 11, 38, 39, 91, 93, 132-134, 202, 274, 304
mature tissue 156, 274
MCM1 213
measurement endpoint 266
Medicago 144, 292
megagametophyte 134, 303
megaspore 303
meiosis 10, 297, 299, 301, 303, 306, 308
Melampsora leaf rust 157
Melampsora 38, 108, 109, 150, 152, 157, 294
Melampsora larici-populina (*M. larici-populina*) 109, 152, 157, 294
Melampsora medusae (*M. medusae*) 152, 157, 294
Melampsora occidentalis (*M. occidentalis*) 152, 294
membrane 24, 61, 103, 166, 189, 190, 298, 299, 304-308
  membrane-active peptide 166
  membrane disruption 189, 190
Mendelian inheritance 103, 104, 229
mercaptoacetyldipeptide 174
2-mercaptoethanol (ß-mercaptoethanol) 117, 175
meristem 5, 7, 13, 55, 62, 74, 76, 78, 80, 212-214, 221, 222, 231, 239, 295, 303
mesophyll cell 19, 21, 80, 168
messenger RNA (*see:* mRNA)
metabolic pathway 68, 81, 206, 305
metal 174, 191, 207, 208
metal-chelating agent 174
metallo-proteinase 173, 174
Metasequoia 126, 292
  glyptostroboides 292
methoxylation 194, 196
methyl 66, 173, 189, 207, 208, 298, 303
O-methyl transferase (OMT) (COMT) (*see also:* caffeic acid/5-hydroxyferulic acid-*O*-methyltransferase) 65-67, 87
methylation 92, 94-97, 195, 196, 198, 274, 303
micro-cross section 236-239
micro-propagation 2, 7,13, 30, 33, 37, 44, 230, 236-239, 273, 274, 277, 303
microbe 254, 257, 264-269, 303
microbial biotype 252
microcalli (microcallus) 62
microcosm 267-268, 303
microculture 5
microcutting 6, 7, 238
microinjection 24, 299, 303
microorganism 47, 155, 169, 207-209, 251, 293, 297, 302, 303
microparticle bombardment (*see:* biolistic)
microprojectile (*see:* biolistic)
  microprojectile-bombardment (*see:* biolistic)
  microprojectile-mediated DNA delivery (*see:* biolistic)
microsatellite 104, 118, 122
microsatellite repeat 118
microscopy 12, 15, 21
microshoot 37, 237-239, 274, 303
microspore 213, 303, 305
mineral 30, 45, 75, 80, 310
mini-cutting 238
minisatellite marker 222
mitochondria 103, 106, 116, 143, 147, 148, 299, 303, 309
mitochondrial DNA (mtDNA) (*see:* mtDNA)
mitosis 297, 299, 300, 303, 306
mode of toxicity 254
model species 5, 48, 80, 84, 220, 233
model system 1, 13, 44, 51, 67, 74, 75, 80, 81, 91, 93, 96, 101, 138, 166, 277
modeling 168, 183, 268
moisture stress 158, 216
molecular biology 51, 101, 116, 128, 165, 273, 295
molecular breeding 105
molecular chaperone 297, 303
molecular mapping 74, 251
molecular marker(*see also:* DNA marker) 78, 79, 101, 102, 105, 106, 108-110, 119, 120, 150, 273
molecular polymorphism 150, 151
Mönch 1
Monet 1
monitoring 56, 81, 87, 92-94, 138, 157, 183, 188, 230, 258, 265, 266
monocot (monocotyledon) 33, 65, 66, 132, 145, 295, 298, 303
monogenic 105, 109, 110
monolignol 196, 200, 203, 303
monomer 67, 174, 303
morphogenesis (morphogenetic) 10, 13, 15, 19, 90, 274, 303
  morphogenetic response 274
morphological marker 108
morphology 11, 12, 36, 38, 61, 77, 85, 106, 156, 179, 199, 290, 301
mortality 45, 60, 87, 175, 178-180, 183, 184, 249, 257
motif 126, 300
mRNA (messenger RNA) 66-69, 71, 72, 86, 96-98, 127-129, 132, 138-142, 165, 168, 171, 176, 179, 182, 183, 197, 198, 201, 205, 223, 300-303, 305-309
  mRNA secondary structure 66, 71, 86, 127, 132, 165, 168, 179, 223, 303
mRNP (ribonucleoparticle) 132, 307, 308
MS (Murashige and Skoog) medium 7, 8, 11, 14, 18, 19, 28, 30, 37, 46, 47, 54, 75, 163, 237, 238
mtDNA (mitochondrial DNA) 103, 106, 116, 119, 120, 143, 148, 303, 304
Muhle Larsen 1
multi-trophic process 250, 257, 258
multigene family (multigene families) 126, 127
multiple resistances 181
Murashige and Skoog (MS) (*see:* MS medium)
Musa 33, 34, 37, 292
mutant 12, 52, 55, 56, 70, 72, 80, 81, 91, 95, 196, 197, 200-202, 207, 276, 303, 305
mutant acetolactate synthase (*crs1-1*) 52, 55, 56, 207
mutation 7, 34, 36, 45, 75, 80, 92, 94-97, 105, 143, 163, 169, 178, 180, 181, 196, 212, 213, 229, 274, 276, 303-305, 307, 308
mycelium (mycelia) 301, 303
mycoherbicide 269
mycorrhiza (mycorrhizae) 155, 208, 294, 299, 303
Mycosphaerella (*Septoria*) 150, 151, 157, 166, 167, 174, 294
  populicola 150, 294
  populorum 150, 157, 166, 167, 174, 222, 294

N

NAA (α-napthaleneacetic acid) (napthaleneacetic acid) 11, 15, 19, 29, 38, 56, 62, 92, 157, 238, 296
N-ethyl maleimide 174
N-glycoslyation 133
NADPH 188, 305
naphthaleneacetic acid (*see:* NAA)
necrosis 8, 157, 189, 190, 303, 310
nematode 251, 310
neomycin phosphotransferase (*NPTII*) 54-56, 63, 67, 91, 94-96, 162, 175, 188, 197, 198, 275
NH_2-terminal amino acid 126
Nicotiana 93, 95, 96, 144-147, 197, 292
  debneyi-tabacum 95
  glauca-langsdorffii 95
  longiflora 95
  plumbaginifolia 96
  tabacum 144-147, 197
nicotine 183
nitrate 15, 28, 30, 163, 207, 209, 216
nitrogen 15, 30, 45, 47, 71, 117, 124, 125, 127, 128, 138, 163, 209, 215, 216, 237, 241, 242
  nitrogen availability 124, 127
  nitrogen cycling 124, 125
  nitrogen deposition 124
  nitrogen metabolism 138
  nitrogen redistribution 124
  nitrogen translocation 124
node 5, 11, 14-16
nodule 7, 19, 62, 303
nonallelic 299
nonallergenic 165
nonadditive genetic variation 224, 303
nondenaturing polyacrylamide gel (ndPAGE) 105
nonhomologous 94, 96, 309
nontarget effect 266, 267
nontarget organism 174, 249, 266
nontarget species 251, 264
nopaline 54, 67, 91, 162, 175, 188, 198, 275, 304
nopaline synthase (*NOS*) 54, 67, 91, 94, 162, 175, 188, 198, 275
normalwood 81
northern analysis (*see:* northern blot) (*see also*: RNA blot)
northern blot (northern analysis) (northern hybridization) (*see also:* RNA blot) 52, 67, 71, 72, 161, 175, 304, 306, 308
northern hybridization (*see:* northern blot)
NOS (*see:* nopaline synthase)
NPTII (neomycin phosphotransferase) 54-56, 63, 67, 91, 94-96, 162, 175, 188, 197, 198, 275
nuclear DNA 51, 103, 107, 144, 303
nuclear genome 101, 144, 307, 309
nuclear genomic DNA 144

nuclear scaffold attachment region (SAR) (matrix attachment region) (MAR) 97, 303, 304, 308
nucleotide 102-105, 126, 143, 296-298, 301, 302, 304-308
nutrient cycling 258
nutritional status 216

O

O-methyltransferase (OMT) (COMT) (*see also:* caffeic acid/5-hydroxyferulic acid-*O*-methyltransferase) 65-67, 87
oat 76, 77
OCI (oryzacystatin) (*see also*: proteinase inhibitor) 52, 55, 85-87, 174-176
octapine synthase (OCS) 91, 275
oil palm 33, 34, 216, 292
Olea europaea 33, 34, 216, 292
oligogenic trait 110
oligomer 132, 148, 298, 304
oligonucleotide (10-mer) 104, 148, 304-306
omt (*see:* COMT)
oncogene 51, 56, 161, 297, 304
open reading frame (ORF) 126, 168, 170, 304
operating demand 245
operator 297, 304, 306
operon 108, 144, 151, 304, 306
Ophiostoma ulmi (*O. ulmi*) (Dutch elm disease) 41, 166, 294
opine 304
opportunity cost 242, 243, 304
optimum rotation. 241, 243
ORF (*see:* open reading frame)
organ 33, 47, 85, 212-214, 298, 305
organelle 36, 72, 143, 297, 301, 303, 304
  organelle DNA (*see also*: cpDNA, mtDNA) 36, 297, 303, 304
  organelle genome 143, 304
organic 28, 30, 193, 298, 301, 305, 306
  organic acid 28
organochlorine (DDT) 254
organogenesis 10-12, 15, 19, 21, 26, 30, 38, 70, 221, 222, 230, 273, 274, 277, 304
orientation 20, 67, 75, 87, 103, 161-163, 197-200, 214, 295, 299
ortet 297, 304, 306
Oryza sativa (rice) 146, 174, 216, 253, 265, 292
oryzacystatin (OCI) (*see also*: proteinase inhibitor) 52, 55, 85-87, 174-176
OsMADS1 216
osmotic 134, 156, 302, 304
  osmotic potential 156, 302, 304
  osmotic pressure 304
osmoticum (osmotica) 11, 27-30, 45, 304
outcrossing 220
over-expression 67, 179, 187, 189, 190, 216
oviposit 182, 257, 304
ovule 15, 304
Oxford Paper Co. 1
ozone 70-72, 157, 187, 190, 191

P

pA2 (*see also*: anionic peroxidase) 66
pA3 (*see also*: anionic peroxidase) 66
paclobutrazol 215
PAGE (*see:* polyacrylamide gel electrophoresis)
PAL (phenylalanine ammonia-lyase) 65, 66, 194, 195, 197
palindrome (palindromic) 126, 304
palisade parenchyma 20, 21, 304
paramutation 94, 96, 97, 304
paraquat 189, 208
parasite 150, 155, 156, 176, 257, 301, 304, 305
parasitoid 257, 294
parenchyma 20, 21, 79, 125, 127, 132, 304, 308, 310
parental 33, 36-39, 76, 109, 118, 144, 226, 228, 300
  parental origin 118
paromomycin 30
particle acceleration (*see:* biolistic)
particle acceleration DNA delivery system (*see:* biolistic)
particle bombardment (*see:* biolistic)
PAT (phosphinotricin acetyl transferase) 52, 61, 208
p*AtALS* (*Arabidopsis thaliana* acetolactate synthase promoter) 67
paternity analysis 105
pathogen 78, 84, 108, 150-152, 155-158, 165-170, 173, 174, 176, 265-267, 293, 294, 296-298, 302, 304, 306, 310
  pathogen pest 173, 174, 176
  pathogen resistance 84, 165-167, 170
  pathogen variation 150-152, 157
pathogenesis 150, 174, 304
pathogenic fungi 167, 173, 174
pathology 150, 165, 264, 266, 267, 269, 299
pathosystem 158, 304
Pauley 1, 128
Paulownia taiwaniana (*P. taiwaniana*) 30
Paulownia tomentosa 33, 34, 292
Paxillus 208, 294
  involutus 208, 294
PCR (polymerase chain reaction) 52, 65, 67, 81, 93, 101, 103-105, 116-119, 143, 144, 150-152, 175, 179, 197, 222, 223, 295, 304, 306
PCR-RFLP 143, 144, 147-149
pectinase 24, 304
pectolyase 27
pedigree 108, 110
PEG (*see:* polyethylene glycol)
pepstatin (*see also:* proteinase inhibitor) 173
peptide 70-72, 126, 133, 165-170, 188, 298, 304, 306, 309
  peptide bond 173, 306
  peptide stability 165, 168, 170
  peptide target 165
peroxidase (*PRX*) 21, 23, 55, 58, 65-69, 187, 188, 195, 197, 203
pest-biotype evolution 249-259
pest resistance 2, 51, 52, 96, 209, 228, 251, 253
pest-resistance gene 2
pesticide 2, 167, 178-180, 206, 209, 247, 249, 250, 253-255, 257, 258
petiole 15, 62, 304
Petunia hybrida (*P. hybrida*) (*Petunia* x *hybrida*) (*P.* x *hybrida*) 95, 96
PGLa 167
Phaeolus 156, 294
  schweinitzii 156, 294
Phaeolus vulgaris 133, 292
pharmaceutical 21
phase change 5, 84, 299, 304
phenolic glycoside 181-184, 254, 255
phenolic 7, 183, 184, 200-202, 206, 208, 254-256, 305
phenotype 33, 36, 74-79, 101, 168, 200, 212, 222, 224, 229, 250, 275, 276, 299, 300, 305, 307
phenotypic 21, 33, 36, 37, 45, 47, 74, 77, 78, 87, 91-93, 95, 96, 101, 109, 166, 197, 201, 221, 223, 231, 252, 274, 296, 299, 301, 306
  phenotypic fidelity 36, 231
  phenotypic variance 101, 301
  phenotypic variation 33, 45, 47
phenylalanine ammonia-lyase (PAL) 65, 66, 194, 195, 197
phenylmethylsulfonyl fluoride (PMSF) 173
phenylpropanoid pathway 66, 194, 305
phloem 74, 94, 125, 198, 215, 296, 305, 309
5'-phosphate (5'-P) 300
phosphatase 126, 129
phosphinotricin (glufosinate) 52, 55, 56, 61, 208
  phosphinotricin acetyl transferase (PAT) 52, 61, 208
phosphoramidon 174
phosphorus 241
photo labile 179, 305
photoinhibition 189, 190
photoperiod 11, 47, 77, 79, 80, 81, 127, 215, 216, 236, 237, 305
photosynthesis (photosynthetic) 24, 40, 46, 70, 131, 182, 187, 189, 190, 256, 297, 304, 305
Photosystem I 189, 305
phyllotaxy (phyllotaxis) 305
phylogenetic 101, 131-133, 138, 151, 152, 266, 275, 276, 297, 305
phylogeny 106, 305
physiology (physiological) 7, 11, 21, 24, 48, 57, 70, 85, 90, 124, 128, 135, 138, 156, 180, 181, 252, 254, 256, 277
phytoalexin 305
phytochemical 254-256
phytochrome 2, 55, 77
  phytochrome A (*phyA*) 76-78
  phytochrome B (*phyB*) 77
phytohemaglutinin 131
phytohormone (*see*: plant growth regulator)
phytoremediation 206-209, 305
phytotoxic 8, 167, 305
PIC (Polymorphism Information Content) 101, 104
Piccarolo 1
Picea (spruce) 33, 34, 85, 94, 134, 144, 215, 254, 292, 293
  engelmanii 293
  glauca 85, 94, 134, 215, 293
  glauca x *engelmanii* 134

Picea abies (*P. abies*) 134, 293
PIN2 (proteinase inhibitor II) 2, 52, 54, 55, 67, 91, 92, 173-175, 182, 275
Pinaceae 134, 293
Pinus (pine) 33, 34, 103, 254, 293
lambertiana 293
monticola 134, 293
palustris 293
pinaster 134, 293
radiata 103, 293
rigida 103, 293
strobus 103, 126, 293
sylvestris 126, 293
taeda 103, 104, 215, 266, 293
pistil 213, 214, 296, 305, 308
Pisum sativum (*P. sativum*) 70, 293
pith cells 94
Plagiodera versicolora (*P. versicolora*) (imported willow leaf beetle) 174, 175, 294
plant breeding 101, 103, 105, 108, 250, 254
plant defense 131, 173, 254, 256
plant development 78, 95, 97, 193
plant growth 11, 12, 15, 19, 29, 30, 44, 45, 47, 56, 75, 77, 79, 80, 202
plant growth regulator (hormone) (phytohormone) (plant growth substance) (*see also*: abscisic acid, auxin, cytokinin, ethylene, gibberellic acid, thidiazuron) 11, 12, 15, 19, 21, 27, 29, 30, 37, 38, 44-47, 56, 61, 63, 75, 77-79, 81, 92, 96, 128, 134, 156, 157, 161, 274, 296, 298, 305
plant-herbivore interaction 251
plant/parasite interaction 150
plant pathology 150, 170, 264, 266, 267, 269
plant regeneration 10, 15, 19, 21, 24, 25, 27, 30, 197, 274
plant tissue culture 48, 92, 165, 274, 296, 298, 307
plant transformation 252, 305
plantation cost 243
plantation site 241, 246, 247
plantlet 11, 13, 15, 19, 21, 31, 39, 54, 62, 63, 75, 158, 163, 236-238, 267, 274, 307
plasma membrane 24, 298, 305, 307
plasmid 51, 54, 56, 70, 91, 94, 161-163, 175, 188, 198, 275, 296-298, 305, 307, 309, 310
plating density 29, 30
pleiotropy (pleiotropic) 93, 110, 277, 305
Plodia interpunctella (*P. interpunctella*) 294
ploidy 36, 156, 166, 295, 305
Plutella xylostella (*P. xylostella*) 180, 294
PMSF (phenylmethylsulfonyl fluoride) 173
point mutation 36, 45, 92, 105, 305, 307
polar transport 77, 305
pollen (pollination) 1, 2, 13, 19, 60, 105, 167, 170, 212-214, 220, 230, 251, 252, 258, 265, 267, 276, 277, 295, 297, 298, 302, 303, 305, 308
pollen dispersal 265
pollution (pollutant) 2, 44, 187, 189, 190, 206-209, 212, 257, 258
polyacrylamide gel electrophoresis (PAGE) (SDS-PAGE) 30, 34, 134-136, 308
polyadenylation 162, 179, 305
polycistronic 151, 305
polyethylene glycol (PEG) 24, 156
polyethylene-glycol-mediated gene transfer 305
polygenic 105, 109, 110, 306
polygenic trait 105, 109, 110, 306
polymer 193-197, 201, 202, 297, 298, 301, 304-306
polymerase 52, 65, 67, 81, 93, 101, 103, 116, 145, 150-152, 175, 179, 197, 222, 295, 299, 304-306, 308
polymerase chain reaction (*see*: PCR)
polymorphic DNA 101-110, 116-120, 143-148, 150, 151, 222, 306
polymorphism 34, 35, 101-105, 107, 119, 132, 143, 144, 150-152, 222, 295, 305, 307
Polymorphism Information Content (PIC) 101, 104
polypeptide 128, 132-135, 137, 173, 293, 299, 300, 303, 304, 306, 308, 309
polyploid 36, 295, 305
polysaccharide 193, 202, 297
polysome 132, 306
polyvinylpyrrolidone (PVP) 45
POPCAD 66
poplar mosaic virus 108
Populus - *see*: Index of *Populus*
population genetic 101, 150, 273
position effect 90, 93, 306
post-transcriptional 94, 96, 97, 127, 133
post-transcriptional regulation 127, 133
post-translational 127, 132, 137, 138, 168, 309
post-translational processing 132, 138
potassium 178, 241
potato 55, 65, 67, 86, 91, 97, 137, 174, 182, 252, 265, 275, 293
PR-1a (tobacco leader sequence) 168, 182
precocious flowering 75, 212, 215, 216
preculture 56
predator 2, 183, 253, 257, 266, 296, 306
prediction 266, 268, 269
preformed meristem 5, 7, 239
prey 257, 306
primer 65, 103-105, 108, 117-122, 143-145, 147, 148, 151, 152, 222, 305, 306
primordium (primordia) 75, 306
probe 65, 66, 71, 103-106, 108, 143, 144, 197, 222, 266, 297, 298, 300, 304, 306, 307, 308, 310
production cost 13, 158, 241, 246, 247
progeny 1, 34, 88, 95, 108-110, 144, 158, 169, 224, 226-228, 267, 275, 296, 300, 306
progeny test 224, 228
prokaryote 306
prolamin 132
promoter 62, 63, 67, 68, 70-72, 74, 76, 77-79, 85-88, 91, 93-97, 132, 137, 138, 161-163, 168-170, 175, 179, 182, 183, 188, 197, 198, 200, 208, 213, 214, 216, 252, 275, 277, 295, 299, 306, 307
promoter-based suppression 252
propagation 11, 33, 34, 36-38, 40, 44, 51, 101, 109, 128, 169, 206, 220, 221, 224, 228-232, 236, 238, 239, 273, 274, 277, 297, 303, 310
propeptide 132
protease 87, 128, 133, 170, 173, 178, 306
protein bodies (protein body) 133, 134
protein hydrolysis 124, 128, 133
protein storage vacuole 125
proteinase 173-175, 306
aspartic proteinase 173
cysteine proteinase 173-175
metallo-proteinase 173, 174
serine proteinase 173-175
proteinase inhibitor 2, 52, 55, 66, 67, 91, 173-176, 181, 257, 275
Bowman-Birk Inhibitor (BBI) 175
Kunitz inhibitor 131
pepstatin 173
proteinase inhibitor I (*PIN1*) 173
proteinase inhibitor II (*PIN2*) 2, 52, 54, 55, 67, 91, 92, 173-175, 182, 275
oryzacystatin (OCI) 52, 55, 85-87, 174-176
protoclonal variation 33
protoclone 33, 35, 38, 92, 306
protoplast 12, 24, 29, 38, 60-62, 91, 92, 221, 229, 305, 306
protoplast culture 2, 7, 24, 27, 28, 30, 33, 44, 60, 61
protoplast fusion 24, 30, 221, 230, 306, 308
protoplast isolation 24-26, 30, 304
provenance test 228
Prunus 33, 34, 126, 293
cerasus 215, 293
japonica 216, 293
persica 126, 215, 293
serotina 269, 293
PRX (peroxidase) 21, 23, 55, 58, 65-69, 187, 188, 195, 197, 203
prxA1 55, 66
prxA3a 66
prxA4a 66
psbA 144, 146-148
pseudo-testcross 108
Pseudomonas
aeruginosa 214, 294
fluorescens 179, 294
solanacearum 167, 294
syringae 167, 267, 294, 301
Pseudotsuga menziesii (*P. menziesii*) (Douglas-fir) 76, 82, 134, 183, 254, 293
PTAG 213, 214
PTD 213
PTFL 213, 214
PTOMT 66
pupa (pupae) 175, 176, 183, 306
purine 304, 306
PVP (polyinylpyrrolidone) 45
pyrethroid 254
pyrimidine 304, 306
Pyrus nivalis 34, 215, 293

Q

QTL (*see:* quantitative trait loci)
quantitative resistance (horizontal resistance) 109, 181, 306
qualitative resistance (vertical resistance) 109, 306

quantitative trait 79, 105, 108, 109, 223, 302, 306
quantitative trait loci (QTL) 79, 105, 110 223, 229, 302, 306
Quercus 105, 133, 144, 147, 293
accutissima 144, 147, 293
ilex 133, 293
petraea 105, 293
robur 105, 133, 293

R

R_1 94
R_2 94
race 157, 306
race-specific resistance 157
radical 2, 11, 189, 190, 195
radicle 301, 306
radioactive (radioactivity) 63, 102, 196
ramet 304, 306
random amplified polymorphic DNA (RAPD) 103, 116, 143, 148, 150, 151, 222, 306
RAPD analysis (RAPD analyses) 104, 118
RAPD banding pattern (RAPD band) 116, 143
RAPD fingerprint 105, 148
RAPD marker 103-105, 108, 109, 116, 118, 119, 151, 222
Raphanus sativus 267, 293
rate of return 242, 243
raw material 193, 241, 245-247
ray cell 66, 78, 125, 127, 306
*rbc*L 144
*rbc*S 91, 93, 94, 277
rDNA (ribosomal DNA) 106, 116, 119, 143-145, 148, 302, 306, 307
rDNA spacers 144
reactionwood 79-81, 306, 307
recalcitrant 19, 33, 60, 231, 307
receptor 12, 178, 180, 307
recessive 180, 183, 184, 229, 275, 307
reciprocal recurrent selection 224, 227
recognition site 168
recombinant DNA (recDNA) 222, 266, 300, 307
recombinant gene 90, 94, 96, 275, 307
recombinase 214, 307
recombination 10, 56, 109, 222, 223, 229, 276, 297, 298, 300, 302, 307
recombination frequency 109, 222, 223, 307
recombination value 222
redundant (redundancy) 167, 214
refugia 180-184, 253, 307
regenerant 33, 34, 36-39, 274, 306, 307
regeneration 10, 13-15, 19, 21-26, 27, 29, 30, 37-39, 44-47, 52, 54, 56-60, 70, 73, 75, 88, 91, 94, 97-100, 155, 162, 163, 197, 221, 231, 273, 274, 277, 307
regression model 268
regulations 87, 230, 232, 258, 268, 276, 277
regulatory gene 97, 212, 213, 215, 216, 307
regulatory sequence motif 126
rejuvenation 7, 34, 36, 38, 231, 274, 307
remediate (remediation) (remediatory) 206-209, 305, 307
repeat-induced point mutation (RIP) 92, 307
repellency 254
reporter gene (*see also:* CAT, GFP, GUS, LUXF2) 7, 56, 67, 85-87, 91, 175, 208, 275, 307
repressor 304, 307
reproductive development 216, 252
reproductive potential 224, 229
reproductive-specific promoter 252
reproductive sterility 212, 214, 216, 252, 277
reproductive tissue 212, 213
rescue cloning 74
resistance (resistant) 1, 2, 34, 36-38, 52, 56, 62, 67, 84, 86, 87, 91, 96, 101, 108-110, 150, 155-158, 161-163, 165, 166, 168, 170, 173-176, 178-184, 186-190, 193, 196, 207-209, 222-224, 228, 249-254, 258, 265-267, 275, 276, 296, 298, 306-308
resistance management program 254
resistance to abiotic stress 221, 223
resistant allele 182-184
resistant clone 108, 155, 163, 254
resistant (R) gene 2
resistant insect biotype 86, 180, 253
resorb 124, 125, 128
resorcinol 183
restriction digestion 145-148, 152
restriction endonuclease (restriction enzyme) 101, 103-106, 143, 144, 152, 168, 222, 307
restriction fragment length polymorphism (*see:* RFLP)
reverse transcriptase 307
reverse transcriptase-polymerase chain reaction (RT-PCR) 67, 179
reverse transcription 298, 307
RFLP (restriction fragment length polymorphism) 101-108, 111, 116, 119, 143, 144, 147, 148, 152, 222, 223, 295, 307
rhizosphere 208, 209, 307
ribosomal DNA (rDNA) 106, 116, 119, 143-145, 148, 302, 306, 307
Ri (root-inducing) 51, 307
Ri-plasmid 51, 307
Ribes nigrum 216, 293
ribonuclease (RNase) 214, 307, 308
ribonucleic acid (RNA) (*see:* RNA, mRNA, rRNA, tRNA)
ribonucleoprotein particle (RNP) 132, 307, 308
ribosomal 106, 146, 308, 309
ribosomal DNA (rDNA) 106, 143, 144, 306, 307
ribosomal DNA polymorphism (rDNA polymorphism) 116
ribosomal protein 307
ribosomal RNA (rRNA) 143, 144, 151, 152, 214, 307, 308
ribosome 299, 303, 306, 307, 309
ribosome binding site 308
ribosome inactivating protein (RIP) 214
ribulose
ribulose *bis*phosphate carboxylase 131
ribulose 1,5-*bis*phosphate carboxylase/oxygenase 124
ribulose-1,5-*bis*phosphate carboxylase (Rubisco) small subunit promoter 182
rice (*Oryza sativa*) 146, 174, 216, 253, 265, 292,
ricin 214
Ricinus communis 214, 293
RIP (*see:* repeat-induced point mutation, ribsome inactivating protein)
riparian (area, buffer, zone) 206, 209
risk assessment 87, 180, 249-258, 264-269, 277
risk determination 265, 266, 267, 269
risk estimation 265, 269
risk evaluation 250, 269
risk magnitude 268
RNA 66, 67, 71, 72, 94, 96, 127, 132, 143, 144, 151, 165, 168, 175, 179, 197, 198, 200, 201, 214, 223, 252, 294-296, 298, 300-304, 306-309
RNA blot (*see also:* northern blot) 77, 201, 308
RNA polymerase 145, 306, 308, 309
RNase 214, 307, 308
RNase gene 86
RNP (ribonucleoprotein particle) 132, 307, 308
Robinia pseudoacacia (*R. pseudoacacia*) 126, 208, 293
rol gene (*rol*)
rolB 96
*rol*C 55, 76, 78, 79, 91-94, 277
root (rooted) 10, 13-15, 19, 34, 46, 51-53, 77, 79, 87, 127, 155, 165, 174, 206-209, 215, 236-239, 251, 252, 274, 293-298, 303, 307
root cutting 13
root form 35, 51
root radical 11
root segment 14, 15, 92, 238
root sprout 251, 252
root sucker 10, 236, 238, 298
root-inducing (Ri) 51, 307
Ri plasmid (Ri-plasmid) 51, 307
rooting 25, 54, 163, 168, 221, 224, 231, 237, 238
rotation 34, 51, 84, 91, 116, 181, 206, 229, 231, 241, 243-247, 251, 252, 254
rpl2 gene (*rpl2*) 146, 147
*rpo*C1 144-146, 148
*rpo*C2 144-146
rrn operon 144
rRNA (ribosomal RNA) 143, 144, 151, 152, 214, 307, 308
Rubisco small subunit (*ats1A*) 71, 182
RUBISCO 124, 182
RUBISCO small subunit transit peptide 70
RUBISCO transit peptide 70, 72
Rubus 33, 34, 161, 293
rust 108, 109, 150-152, 157, 269, 294
rust fungi (rust fungus) 150-152, 157, 294, 309

S

S (Svedburg unit) 132
S1 nuclease protection assay 127
S_1 38, 127

S_2 stage 38
S-adenosyl-L-methionine synthase (SAM) 66
safety 178, 212, 230, 249, 251, 258, 265, 267, 276, 277
Salicaceae 1, 13, 33, 34, 133-135, 138, 293
Salix 33, 34, 146, 147, 293
babylonica 34, 293
caprea 293
lucida 167, 293
pseudolasiogyne 144-147
smithiana (x *smithiana*) 126, 293
viminalis 223, 293
Salmonella 93, 294
salt 8, 27, 28, 125, 132, 156, 304, 310
SAM (S-adenosyl-L-methionine synthase) 66
Sambucus nigra (*S. nigra*) 126, 293
Santalum album 33, 34, 293
sarcin loop 214
scaffold attachment region (SAR) (matrix attachment region) (MAR) 97, 303, 304, 308
SCAR (sequence characterized amplified region) 103, 104
sCEC 168
Schreiner 1
scolytid 254
score (scoring) 117, 215
screen (screening) 25, 28, 38, 54, 74, 81, 86, 105, 108, 155-158, 200, 207, 221, 224, 297, 300
SDS-PAGE (sodium dodecyl sulfate-polyacryl amide gel electrophoresis) (PAGE) 30, 34, 135-137, 308
seasonal growth cycle 5
seasonal nitrogen cycling (seasonal nitrogen cycle) 124, 125, 128
secondary metabolite 21
secondary structure 165, 168, 170
seed 2, 131-138, 167, 220, 221, 230, 237, 251, 252, 254
seed dispersal 252
seed production 212
seed storage protein (SSP) 124, 130-138
segregation 96, 101, 104, 157, 222, 308
selectable marker 56, 67, 91, 96, 175, 208, 275, 296, 297, 308
selection pressure 2, 132, 180-184, 258
selective pressure 180, 253, 254
self-cleaving protease 170
self-incompatibility 214
sense 75, 98, 161-163, 197, 198, 200, 201, 223, 230, 294
sense strand 145, 147
sense suppression 252
sepal 213, 296, 308
Septoria 150, 151, 157, 167
Septoria canker 150, 167, 241
Septoria leaf spot 150, 157, 167
Septoria (*Mycosphaerella*) 150, 151, 157, 166, 167, 174, 222, 241, 294
musiva 150, 157, 166, 167, 174, 222, 294
populicola 150, 294
sequence characterized amplified region (SCAR) 103, 104
sequence divergence 145, 308
sequence tagged site (STS) 105, 107, 108, 116
serine 173
serine proteinase 173-175
shikimate 52, 55, 72, 91, 194, 207, 308
shikimate pathway 194, 308
shoot culture 5-8, 24-30, 44-48, 70, 156, 158, 236-239
shoot multiplication 25, 46
shoot primordia (shoot primordium) 13
shoot regeneration 13, 15, 19, 29, 33, 47, 162, 163
shoot tip 7, 156, 237, 239, 306, 310
shoot training 215
short rotation 51, 116, 241, 251
short-term strategies (short-term strategy) 224, 225, 228
sialic acid 125
sieve tube (companion cells) 305
signal polypeptide 132
silviculture (silvicultural) 2, 34, 39, 206, 230, 232, 245, 265
simple recurrent selection 224, 226
simple sequence repeat (SSR) 102, 104, 108, 118, 222
sinapyl alcohol 194, 196, 303
single strand conformation polymorphism (SSCP) 105
site-directed PCR 150, 151
site preparation 241, 243, 247
site-specific amplication 143
SLG (S-locus glycoprotein) 214
SLR (S-locus-related) 214
SOD (superoxide dismutase) 187, 188, 190
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 30, 34, 135-137, 308
soil pH 208, 257
Solanaceae 173
Solanum 144, 293
solute leakage 189, 190
somaclonal 34, 35, 37-40
somaclonal selection 36
somaclonal variation 2, 33, 34, 36-38, 40, 92, 93, 157, 207, 221, 222, 228-230, 232, 308
somaclone 34, 36-40
somatic 10, 30, 34, 60, 92, 97, 273, 274, 299, 308
somatic embryo 11, 48, 133, 274
somatic embryogenesis 2, 10-12, 19, 48, 138, 221, 222, 230, 231, 274, 277, 308
somatic hybrid 24-30, 221, 308
somatic mutation 34
Sophora japonica 126, 293
sorghum 196-198, 200
Southern blot (Southern hybridization) (Southern analysis) 52, 94, 103, 104, 144, 295, 304, 306, 308
soybean 126, 127, 132, 137, 173, 175, 252, 267, 269, 292
spacer 143-148, 151, 302, 308
spacer sequence 308
spatial 80, 180, 181, 250, 269
spatial refugia 181-184
spatial regulator 212, 213
SPL-3 216
Spodoptera exiqua 174, 294
spongy mesophyll 19, 20, 308
spontaneous mutation 92, 181 308
spore 152, 157, 167, 178, 179, 182, 236, 296, 298, 299, 303, 305, 309
spore dispersal 269
spring flush 5
spruce (*see: Picea*)
spruce budworm (*see: Choristoneura*)
spur leaf 125
SQUAMOSA 216
SSCP (single strand conformation polymorphism) 105
SSP (seed storage protein) 124, 130-138
SSR (simple sequence repeat) 102, 104, 108, 118, 222
stability 1, 5, 7, 33, 34, 38-40, 84, 86, 90, 93, 95-97, 110, 132, 138, 165, 168, 170, 176, 223, 229, 230, 254, 257, 258, 264, 267, 274, 276, 277, 309
stability of transgene expression 70, 84, 85, 87, 97, 252
stabilization 7, 208
stabilization phase 7
stable integration 198, 208
stalk 19, 304, 308
stamen 213, 214, 295, 308
startpoint 296, 308, 309
Steenackers 1, 176
stem cutting 37, 38, 236
stem diameter 39, 110
stem girth 35
stem height 35, 40, 110
stem segment 75, 92, 162, 163
stem volume 110
sterile 75, 87, 166, 212-216, 232, 275
sterile triploid 252
sterility 86, 166, 184, 212-216, 230, 232, 250, 252, 258, 275, 277, 308
sterility-inducing gene 166
steroidal glyco-alkaloid 183
stigma 214, 252, 305, 308
stigma-specific promoter (STIG1)214
stock plant 46, 236, 238, 239
stomate (stomata) 18, 308
stop codon 168
storage protein 65, 66, 124-128, 131-138, 308
Stout 1
stress 21, 70-72, 86, 87, 95, 155, 156, 158, 165, 183, 187-191, 208, 216, 220, 221, 223, 224, 228, 252, 254, 274, 303
structural gene 175, 300, 308
STS (sequence tagged site) 105, 107, 108, 116
stylar transmitting tissue-specific gene 213
style 305, 308
subculture (subculturing) 5-7, 10, 11, 34, 37, 38, 45-47, 61, 63, 163, 308
subline 224, 230
sucker 309
sucrose 28, 29, 163, 237-239
sugar 28, 125, 247, 300, 301, 304, 307, 309
sugar alcohol 28
sulfometuron methyl 207
sulfur dioxide (SO_2) 189, 190, 208
sulfur trioxide radical 190

superoxide dismutase (SOD) 187, 188, 190
suppression 75, 95, 96, 201, 213, 214, 223, 252, 296, 298
survival 19, 45, 47, 62, 90, 97, 155, 175, 179, 182, 224, 247, 255, 256, 266, 267, 269, 273, 276
susceptible (susceptibility) 1, 2, 51-57, 108, 156-158, 165, 168, 174, 178, 180-183, 189, 222, 253, 254, 297, 306
suspension culture 10-12, 37, 38, 61, 156, 303
suspensor 132
sustainability (sustainable) 2, 250
Svedburg unit (S) 132
sylleptic branch 110
symbiont 249
symbiotic 207, 208, 303
synchronization 45, 61, 62
syntenic map 105, 108
synthetic peptide sequence 165
syringaldehyde 197, 202
syringin 196
syringyl 194, 196, 199-201
systematics 106, 116, 131
Syzygium aromaticum (clove tree) 215, 293

T

T-DNA 51, 52, 54-56, 70, 74, 76-78, 94-97, 161, 175, 188, 197, 198, 201, 275, 296, 297, 307, 309
  T-DNA border 51, 60, 198, 275
TA29 (male reproductive tissue-specific promoter) 213
tag (tagging) 109, 116
tandem array 151, 309
tandem repeat 104
tannin 7, 183
tapetum 213
  tapetum-specific promoter (tapetal-specific promoter) 86, 214
Taq (*Taq* DNA polymerase) (*Taq* polymerase) 117, 152
target insect 87, 178, 184, 257, 258
targeting 71, 79, 126, 131, 138, 165, 168, 181, 214, 309
TATA box 126, 309
Taxodium
  distichum 126, 293
taxonomy (taxonomic) 35-37, 104-106, 116, 134, 143, 150, 222, 257, 305
TDZ (*see*: thidiazuron)
telomere 301, 309
temperature 45-48, 75, 77, 80, 87, 94, 127, 128, 148, 157, 187, 189, 190, 214, 216, 269, 295, 298, 299, 301
template DNA 116, 151, 152
temporal 86, 134, 180, 181, 250, 258
  temporal refugia 180, 182, 184
temporally specific expression 256, 258
teratoma (teratomatous) 162, 163, 309
terminal (terminus) 76, 105, 126, 133, 167, 173, 179, 202, 236, 239, 296, 299, 309
  terminal amino residue 173
  terminal carboxyl residue 173
termination 168, 270
  termination codon 168, 304, 309
  termination sequence 165, 170
tetranucleotide 144, 145, 168
tetraploid 36-38
tfdB 208
tfdC 208
thidiazuron (TDZ) 15, 29, 30, 56, 62, 203, 238, 298
thioacidolysis 198-203
thioglycolic acid 202
α-thionin (thionin) 166
thymidine 61, 62
Ti (tumor-inducing) plasmid 51, 56, 161, 175, 275, 297, 309
timber 33, 86, 87, 166, 251, 292
time series analysis 266
tissue ablation 213
tissue culture 10, 13, 24, 33, 47, 48, 51, 56, 61, 62, 76, 80, 84, 85, 92, 138, 155-157, 163, 165, 206, 220, 221, 223, 228, 229, 236, 238, 239, 251, 273, 274, 295, 296, 298, 300, 301, 307, 310
tissue specific expression 66, 127, 138
tissue-specific promoter 79, 87, 213
TMV (*see*: tobacco mosaic virus)
tobacco 13, 24, 65-68, 70, 86, 93, 94, 96, 137, 138, 144-146, 161, 162, 164-168, 179, 182, 188, 190, 196-198, 200-202, 213, 214, 216, 276, 277, 292, 294
  tobacco hornworm (*Manduca sexta*) 174, 294
  tobacco mosaic virus (TMV) 154, 265, 294
  tobacco *rbc*L gene (*rbc*L) 144
totipotent (totipotency) 48, 239
toxA 214
toxic (toxicity) 27-30, 167, 168, 178-181, 183, 187, 206-209, 214, 250, 257, 293, 296, 298, 300
toxin 155, 158, 178-184, 214, 218, 253, 277
  toxin resistance 158
tracheid 79, 309, 310
traditional breeding 78, 90, 101, 108, 253, 275
trans-acting factor 309
trans-inactivation 94, 96, 97, 276
transcription 70, 71, 75, 86, 127, 132, 147, 295, 298, 303, 305-307, 309
  transcription factor 203, 212, 213, 216, 297, 309
  transcription unit 151, 296, 309
transcriptional 67, 71, 72, 103, 127, 132, 133, 137, 161, 214, 302, 309
  transcriptional start site 126
transfer DNA (*see:* T-DNA)
transfer RNA (tRNA) (*see:* tRNA)
transformant 7, 54, 56, 60, 74-79, 85-87, 90, 93, 94, 96, 97, 138, 168, 182, 197-199, 201, 230, 252, 309
transformation 12, 13, 34, 48, 51-57, 67, 70, 74, 75, 78, 84, 86-88, 90-95, 97, 161-163, 166, 169, 170, 174, 175, 179, 188, 197, 198, 201, 207-209, 214, 220-223, 231, 232, 253, 257, 264, 275, 277, 295, 297, 300, 305
  transformation frequency (transformation frequencies) 54, 56, 61,75
transgene escape 251
transgene expression 2, 7, 56, 61, 70, 72, 75, 84-88, 90, 93, 95-97, 179, 180, 214, 252, 276, 309
transgene inactivation 90, 93-97
transgene instability 87, 94, 97, 277
transgene regulation 52, 252
transgenic organism 166, 250, 276, 277
transgenic plant 24, 30, 52, 60, 63, 67, 70, 74, 75, 77, 90-97, 161, 163, 165-168, 170, 175, 181, 187-190, 197, 199, 207, 214, 216, 223, 230, 231, 253, 254, 257, 258, 265, 266, 273, 275, 276, 300, 309
transgenic pollen 251, 267, 276, 277
transgenic *Populus* 60, 74, 75, 207, 252-254, 257, 264, 266,
transient expression (transient gene expression) 60-63
transit
  transit peptide 70-72, 182, 188, 309
translation 70-72, 126, 132, 168, 170, 302, 308, 309
  translation initiation 165, 168, 170, 309
translational enhancer 63
translocate carbon 127, 256
translocation 70-72, 124, 127, 207, 297
transport 63, 77, 79-81, 91, 124-126, 128, 132, 138, 206, 208, 209, 298, 301, 305
transposable element 36, 302, 309
  Ac (Activator) 53, 55
transposon 94, 153, 309
tree breeder 101
tree breeding 1, 74, 78, 79, 101, 220, 221, 230
tree improvement 34, 51, 78, 220, 232, 233
triazole growth retardant 215
trichloroethylene (TCE) 208
triploid 19, 36, 252, 274, 309
tRNA (transfer RNA) 144, 145, 168, 309
*trn*H 147
trophic level 249, 264
tropism 77
trypsin (*see also*: proteinase) 52, 131, 173, 174, 293
trypsin inhibitor (*see also*: proteinase inhibitor) 131, 173
tumor 51, 54, 93, 95, 161-163, 175, 275, 296, 304, 309
tumor-inducing (Ti) plasmid (*see:* Ti-plasmid)

U

ubiquitin (*UBQ-1*) 168, 182, 183, 309
uidA 85, 86, 94, 208
Ulmus (elm) 33, 34, 293
uniconazole 215
unit cost 243-247
untranslated leader 182
urease 131
urediospore 152, 309
uredium (uredia) 309

V

vacuole 45, 125, 133, 138, 141, 309
vanillic acid 197
vanillin 197, 202

variability 33, 34, 36, 37, 79-81, 93, 96, 106, 110, 147, 150, 163, 221, 228-232, 241, 252, 253, 266, 267, 274-276, 296
variegation 35, 95
vascular cambium 15, 16, 74, 77, 79, 94, 309
vascular system 10, 193
vascular tissue 94, 310
vector 7, 52, 54, 56, 60, 63, 66, 67, 70, 74, 91, 161, 163, 168, 169, 174-176, 188, 203, 214, 257, 265, 296, 297, 310
vegetative propagation 221, 297, 310
vegetative storage protein (VSP) 124, 125, 131, 135, 137, 138
vertebrate 206, 251, 302
vesicular arbuscular mycorrhiza (VA mycorrhiza) (endomycorrhiza) 208, 303
viability 24, 27, 45, 135, 138
vicilin type 132
virulence (*vir*) 51
  vir gene 51
  vir inducer 56
virus 37, 63, 70, 76, 85, 86, 91-93, 108, 197, 198, 208, 216, 265, 266, 275, 276, 294, 298, 303, 310
  virus X-resistant potato 265
vitamin 19, 28
Vitis vinifra 215, 293
vitrification (vitrified) 18, 19, 21, 310
VSP (*see:* vegetative storage protein)
VSP promoter 137

W

walnut (*Juglans*) 125, 292
wasp 266
water status 80
water stress 216
watershed 251
weed 86, 96, 207, 241, 252, 264, 266, 268, 269
weediness 166
western blot (western blotting) 52, 71, 72, 175, 310
wild flora 265
WIN (wound inducible) 65
  WIN3 66, 67
  WIN3.12 168
  WIN4 126
  WIN6 (chitinase) 66, 67
  WIN6.39b 168
  WIN8 (chitinase) 66
wood chip 193, 202, 246
wood density 79-81, 108
wood propertiy (wood properties) 2, 77, 79-81, 223, 224
wood quality 86, 108, 110, 116, 220
wood structure 74, 79-81
Woody Plant Media (WPM) 8, 14, 15, 62
wound (wounded) (wounding) 12, 27, 51, 52, 56, 66, 67, 86, 95, 127, 156, 157, 163, 165, 167, 168, 170, 175, 182, 255, 258
wound-inducible (WIN) 2, 55, 65, 66, 67, 86, 126, 168, 170, 175, 182, 255
  wound-inducible promoter 168, 170, 255
wounding response 65
WS1 (xylem ray storage protein) 66

X

Xanthomonas populi (X. *populi*) 108, 157, 294
xenobiotic 257, 310
Xenopus laevis 167
xylem 74, 77-81, 86, 87, 94, 95, 128, 193, 197-202, 296, 309, 310
  xylem ray 66, 125, 127
  xylem sap 125, 128

Z

Zea mays (maize) 53, 55, 66, 95, 97, 144-146, 196-198, 200, 252
zeatin 8, 14-16, 19, 29, 30, 47, 163, 298
  zeatin riboside ([9R]Z) 78
zinc 174
  zinc finger 216
Zinnia 80, 81, 195, 200, 293
Zygote 183, 300, 308, 310
  zygotic embryo 10, 11, 48, 60, 135, 274, 300, 308
zymogram 103

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