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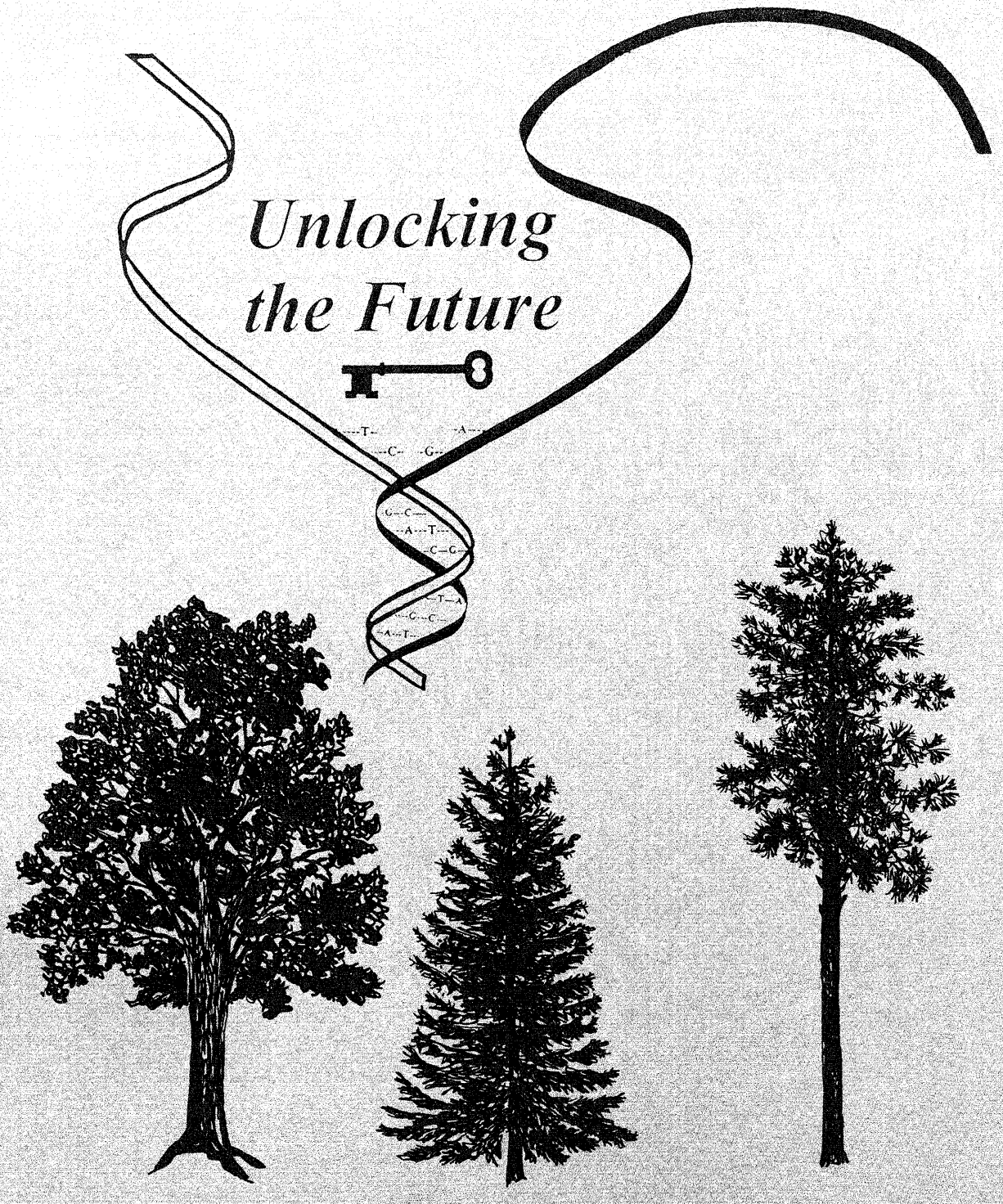

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Papers and Abstracts Presented
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on the

APPLICATIONS OF BIOTECHNOLOGY TO TREE CULTURE, PROTECTION, AND UTILIZATION

Bloomington, Minnesota October 2-6, 1994

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1994 SECOND INTERNATIONAL SYMPOSIUM ON THE APPLICATIONS OF BIOTECHNOLOGY TO TREE CULTURE, PROTECTION, AND UTILIZATION

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ABSTRACTS

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INVITED SPEAKERS

AND

CONTRIBUTED TALKS

THE ROLE OF CARBOHYDRATE METABOLISM IN LIGNINOLYSIS

PHILIP J. KERSTEN. USDA-Forest Service, Forest Products Laboratory, One Gifford Pinchot Drive, Madison, WI, USA 53705

Extracellular enzymes of the lignin-degrading system of *Phanerochaete chrysosporium* include lignin peroxidase (LiP), manganese peroxidase (MnP) and glyoxal oxidase (GLOX). Peroxide generated by GLOX is an oxidant required in the peroxidase catalytic cycles. Subsequent to the discovery of GLOX, the metabolites glyoxal and methylglyoxal were found to be produced by cultures of *Phanerochaete* during secondary metabolism, thus the name glyoxal oxidase. The derivation of these metabolites from the carbohydrate growth substrate is not known and can not be readily explained from established pathways of intracellular metabolism. Our recent efforts to elucidate the metabolic pathway for the conversion of wood-derived carbohydrates have identified additional substrates for GLOX. Importantly, further metabolism of these substrates may directly involve catalysis by the extracellular peroxidases. Literature on the chemistry of these compounds shows them to be reactive, susceptible to autoxidation with the generation of active oxygen species, and in some cases, to support oxidative reactions with peroxidases without exogenous peroxide. This suggests that the peroxidases of *P. chrysosporium*, studied almost exclusively in the context of lignin degradation, may also have an important role in the breakdown of carbohydrate-derived metabolites.

HOW DO WHITE-ROT FUNGI REALLY DEGRADE LIGNIN?

KENNETH E. HAMMEL. USDA Forest Products Laboratory, One Gifford Pinchot Drive, Madison, WI 53705 USA

The primary catalysts of fungal lignin degradation are thought to be lignin peroxidases (LiPs), extracellular enzymes that have been extensively characterized both structurally and genetically. The basis for this view is that LiPs are the only fungal enzymes known that can directly oxidize the recalcitrant nonphenolic units that make up the bulk of lignin. However, previous attempts to depolymerize lignins with these enzymes in vitro have been unsuccessful.

Our recent results show that LiPs are, in fact, ligninolytic. Molecular weight analysis by gel permeation chromatography of enzyme-treated ^{14}C-labeled synthetic lignins showed that they were extensively degraded. Moreover, ^{13}C NMR experiments with enzyme treated synthetic lignin showed that it was cleaved between C_a and C_b of the propyl side chain, in agreement with previous work on LiP mechanisms.

However, we also find that LiPs are not essential for fungal attack on nonphenolic lignin structures in wood. A LiP-negative ligninolytic fungus, *Ceriporiopsis subvermispora*, degraded both an exhaustively methylated (nonphenolic) lignin and a nonphenolic ß-0-4-linked lignin model dimer in solid wood culture as readily as the LiP producer *Phanerochaete chrysosporium* did. Product analysis of the dimer experiments showed that less than 10% of the mineralization of this compound by *C. subvermispora* is attributable to a LiP-dependent pathway.

REMEDIATION OF PCP- AND CREOSOTE-CONTAMINATED SOILS USING WOOD-DEGRADING FUNGI

RICHARD T. LAMAR. Institute for Microbial and Biochemical Technology, USDA Forest Service, Forest Products Laboratory, One Gifford Pinchot Drive, Madison, WI 53705 USA

Microbiological treatment of hazardous wastes has generally been associated with the use of bacteria. During the past decade a significant body of evidence has accumulated that demonstrates that fungi, in particular white-rot fungi, have the ability to degrade a wide range of hazardous organic compounds (xenobiotics) and thus might be used for treatment of materials contaminated with these compounds. Our work has focused on the development of a soil remediation technology that is based on the xenobiotic-degrading abilities of these fungi. This work has demonstrated that the technology is useful for remediation of pentachlorophenol-contaminated soils and may also be useful for creosote-contaminated soils. In this presentation a summary of applications of this technology to remediation of PCP and creosote-contaminated sites and a discussion of technological developments necessary for commercialization of the technology will be given.

KINETIC ANALYSIS OF MN PEROXIDASE: THE REACTION WITH MN COMPLEXES

I-Ching Kuan, Kenneth A. Johnson and MING TIEN. Department of Biochemistry and Molecular Biology and Center for Biomolecular Structure and Function, Pennsylvania State University, University Park, PA 16802 USA

Mn peroxidase from the lignin-degrading fungus *Phanerochaete chrysosporium* catalyzes the H_2O_2-dependent oxidation of Mn^{2+} to Mn^{3+}. Presteady-state methods were employed to characterize the reactions of free and chelated Mn^{2+} with the two-electron and one-electron oxidized forms of the enzyme, compound I and II, respectively. At pH 4.5, the optimum pH for steady state turnover, the reaction of compound I with Mn^{2+}, either free or complexed, is too rapid to measure by stopped flow methods. The reactions of compound I with Mn2+ can only be monitored under non-optimal conditions of pH 2.5. The reaction of compound II with Mn2+ is much slower than compound I. Chelators such as oxalate, lactate and malonate facilitated the reaction of Mn^{2+} with compound II. In contrast, succinate which does not readily form a complex with Mn^{2+}, and polyglutamate, which is polymeric, were ineffective in stimulating the reaction of Mn^{2+} with compound II. The 1:1 chelator/Mn^{2+} complex is the preferred substrate for compound II; this conclusion is based on known formation constants for the various Mn^{2+} complexes. The ubiquity of oxalate in wood degrading fungi would suggest that oxalate plays a major role in Mn peroxidase-dependent oxidation of lignin.

THE MOLECULAR CLONING AND EXPRESSION OF LACCASES FROM THE WHITE-ROT BASIDIOMYCETE *POLYPORUS PINSITUS*

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Laccase is a multi-copper enzyme which catalyzes the oxidation of phenolic compounds. Extracellular laccase activity has been detected in cultures of many fungi including several white-rot fungi. In most fungi laccase activity is constitutively low, but can be induced by a variety of compounds. The white-rot basidiomycete *Polyporus pinsitus* produces several extracellular laccase activities. The production of at least one laccase by *P. pinsitus* is induced by the addition of 2,5-xylidine to the culture. We report here the purification of laccases from *P. pinsitus* and the subsequent cloning of the genes coding for several laccases. Two laccases have been purified and are glycoproteins with apparent molecular weights of 65 kDa on SDS-PAGE. Further biochemical characterization is in progress. Partial N-terminal sequence of one of the purified laccases was determined, and degenerate oligonucleotides based on this sequence were used to isolate a partial cDNA clone. Using the partial cDNA as a probe both a cDNA library and genomic bank were screened. Five unique genomic clones have been isolated. We are currently characterizing these clones at the molecular level and dettermining the number and location of the laccase genes within the genome.

APPLICATION OF GENETIC DIAGNOSTICS TO PLANT GENOME ANALYSIS AND PLANT BREEDING

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New developments in molecular biology are leading to a continual expansion of the repertoire of genetic diagnostic tools available for DNA fingerprinting, mapping, and plant breeding. Both RFLP and RAPD are now well established methods for genome analysis. The codominance of RFLP markers is often sacrified in favor of the technical ease and simplicity of the RAPD dominant marker assay. More recently, simple sequence repeats (SSRs) or microsatellites were shown to be abundant and polymorphic in many plant genomes, thus affording a new codominant and highly informative method for genome diagnostics. Although initially laborious, the construction of SSR marker based maps of many crop plants is now underway; once established, each map will provide highly polymorphic codominant markers that can be applied to evolutionarily related species. In addition, multiplexed genome assay methods that reveal multiple polymorphisms simultaneously, such as amplified fragment length polymorphism (AFLP) and inter-repeat amplification, hold great promise for genome fingerprinting, germplasm analysis and marker discovery. Although all of these methods have been applied separately towards analysis of individual genomes, we know of no case whereby all have been directly compared. Our objective, therefore, was to compare the utility of these various marker systems on a common set of plant genotypes, and to determine the relative efficiency of each for estimating polymorphism and genetic similarity. An initial RFLP survey of soybean germplasm, together with co-ancestry analysis, identified 12 soybean genotypes which collectively account for 92% of the allelic variation in the gene pool of North American soybean cultivars. Polymorphisms for 110 RFLP loci, 37 RAPD bands, 36 SSR loci, and 136 AFLP bands were scored in all 12 lines.

These molecular data, along with morphological information, were used to calculate the expected heterozygosity, effective multiplex ratio, and relative informativeness of each marker system. In addition, the ability of each set of data to estimate genetic similarities among these cultivars was compared. In general, the expected heterozygosity was the greatest (on a per locus basis) for SSR markers, and lowest for the AFLP assay. However, AFLP carries the highest effective multiplex ratio, which is at least 10-20-fold higher than that for RFLP, RAPD, or single-locus SSR. Combining these two types of information, the AFLP assay appears to have great utility for genome applications involving polymorphic marker discovery. Esimates of genetic similarities among the 12 soybean cultivars differed somewhat for the different marker systems, consistent with the fact that each assay measures genomic polymorphism in a distinct way. Finally, since each of these marker systems differs considerably in the type of genetic information revealed as well as in the degree of technical expertise required, the final choice for a genome assay method must take into consideration not only the informativity, but also the convenience and relative technical difficulty inherent to the assay.

CUSTOMIZING LIGNIN THROUGH BIOTECHNOLOGY

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As one of the primary determinants of wood quality, lignin has become the focus of numerous biotechnology research efforts within the last three years. From mutation studies and from initial genetic engineering results it is clear that, at least in herbaceous species, it is feasible to alter both the quality and quantity of lignin deposition during plant development. The range of lignin variation occurring naturally in woody species suggests that it will be possible to accomplish the same end in conifers and hardwoods once efficient gene transfer systems are developed for those species.

Current efforts at lignin modification are focussed on altering the flux through different steps in the biosynthetic pathway (phenylalanine ammonia-lyase, 4-coumarate CoA ligase, catechol O-methyl transferase, cinnamyl alcohol dehydrogenase, coniferin *beta*-glucosidase, peroxidase), and progress with each of these targets will be reviewed. A number of ambiguities still remain in the canonical pathway of lignin biosynthesis, but they are likely to be resolved through these directed gene manipulation studies. Other aspects of lignin deposition may provide alternative targets in efforts to customize lignin. These include the mechanisms of pathway organization and substrate transport, and the regulatory circuits that control patterns such as the spatial orientation and timing of secondary/tertiary cell wall formation, or the number and size of xylem fibres.

Ultimately, detailed information will be needed on the structure/function relationships for each protein that controls a rate-determining step in lignin formation, so that the power of protein engineering can be employed to "fine tune" this process to meet the various demands of the marketplace.

THE APPLICATION OF TARGETED AND RANDOMLY GENERATED MARKERS (RFLP, RAPD, SCAR, AFLP AND SSR) TO CROP BREEDING PROGRAMS AND THE USES OF THESE MARKERS FOR FOREST GENETIC RESEARCH

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We have been using a variety of genetic markers to investigate the genome organization of resistance genes in *Lactuca sativa* (lettuce) and its wild relatives. The variety of markers used reflects the breadth and the limitations of each type of marker. Together these markers have allowed us to detect the genomic clustering of disease resistance genes specific for a diverse array of pathogens, to construct a series of overlapping composite genetic maps generated from a variety of intra- and inter-specific crosses, to generate comparative maps for related agronomic species and to accelerate breeding programs designed to incorporate different resistance genes into popular cultivars. This work has also laid the foundation for the cloning of resistance genes and the understanding of their evolution.

Recently we have been generating the Simple Sequence Repeat (SSRs or microsatellites) markers for a companion project involving tropical trees. This work demonstrates the versatility of this type of marker for work with predominantly outcrossed, long lived species. Because of the high variability detected at these loci, constructing comparative intra-specific genetic maps from a standard set of markers becomes trivial. Also, breeding program or population genetic problems such as determining paternity, monitoring introgression and detecting inbreeding are greatly simplified by these markers. These markers in conjunction with other more easily targeted markers should accelerate analyses of both simply inherited and quantitative traits.

PROPERTIES OF TWO ENZYMES OF LIGNIFICATION IN CONIFERS.

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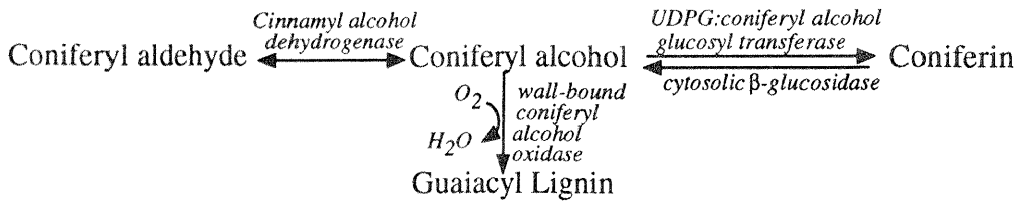
Coniferyl alcohol oxidase (CAO) and coniferin-specific β-glucosidase (CSBG) are cell-wall bound and cytosolic enzymes, respectively, associated with lignification [1,2]. Our studies indicate that CAO and CSBG participate in lignification as follows:



Gradient gel electrophoretic methods enabled a distinction to be made between CAO and a p*I* ~ 9 pine "laccase" recently implicated in lignification (*Science* **260**, 672, 1993). Proteins obtained from lignifying cambial derivatives of conifers (family *Pinaceae*) were compared with CAO and the p*I* ~ 9 pine "laccase" following Western blotting. For *Abies balsamea*, *Larix laricina*, *Picea rubens*, *Pinus banksiana*, and *Pinus taeda*, the isoelectric points of oxidatively active bands were identical to those of purified CAO. For all species, only the p*I* 7.6 band was immunoreactive with antibodies against periodate-deglycosylated CAO.

Purified CAO, p*I* 7.6, was determined to be a glycoprotein (38% glycan), M_r 107 500, pH and temperature optima 6.3 and 30 °C, respectively. By graphite-furnace atomic-absorption analysis, CAO contained one copper atom per protein. By substrate specificity and effector studies, CAO is not a monophenol monooxygenase. CAO utilizes several laccase substrates; however, monoclonal antibodies against both native and deglycosylated CAO did not recognize laccases. The copper in CAO is not detectable by either UV/Vis or electron paramagnetic resonance spectroscopy. CAO activity is inhibited by phenylhydrazine and tropolone and enhanced in the presence of sodium dodecyl sulphate.

In *Pinus* spp., CSBG from lignifying xylem comprised two cytosolic, acidic (p*I* 3.8) glycoproteins (M_r 110 000 and 90 000). By combined gas chromatography - mass spectrometry, both isoenzymes were confirmed to produce *trans*-coniferyl alcohol from *E*-coniferin. To our knowledge, CSBG is the first highly acidic β-glucosidase found in vascular plants.

1. Leinhos V, Udagama-Randeniya PV, Savidge RA (*in press*) Purification of an acidic coniferin-hydrolyzing β-glucosidase from developing xylem of the conifer *Pinus banksiana* Lamb. *Phytochemistry.*
2. Udagama-Randeniya PV, Savidge RA (*in press*) Electrophoretic analysis of coniferyl alcohol oxidase and related laccases. *Electrophoresis.*

MOLECULAR CHARACTERIZATION OF HARDWOOD LACCASE GENES AND THEIR APPLICATION IN LIGNIN REDUCTION VIA ANTISENSE TECHNOLOGY

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Our laboratory is interested in modifying the lignin content of forest trees to lower the energy cost and chemical input required to manufacture pulp and paper. Recent studies suggest that laccase (p-diphenol:O_2 oxidoreductase; EC .10.3.2) may play a significant role in the deposition of lignin in vascular plants. We have isolated and characterized laccase cDNAs from sycamore maple (*Acer psuedoplantanus*) and yellow-poplar (*Liriodendron tulipifera*) in an effort to more fully define the role played by laccase in lignification. We will report the organ-level expression of our cloned laccase genes and the complexity of the laccase gene family in both species. We will also report the effects of various environmental conditions (light, Cu2+ levels, and nitrogen source) on the expression of laccase in the sycamore maple tissue culture system.

To test the hypothesis that laccase is involved in the deposition of lignin in trees we have prepared antisense constructs of the yellow poplar laccase gene. Transformation of yellow-poplar with antisense laccase constructs may block the synthesis of laccase *in vivo*, and thereby lead to reduced lignin content in transformants. Two antisense constructs were made: one encompassed 75% of the coding region and all four conserved copper-binding domains, while the second contained the 5'-untranslated region and 12% of the amino-terminal coding sequence. Expression of each antisense laccase sequence is dependent upon the nominally constituitive CaMV35S viral promoter. Constructs were introduced into embryogenic cultures of yellow-poplar by microprojectile bombardment, and kanamycin-resistant cells were subjected to regeneration conditions. We will provide preliminary data on the expression of the antisense laccase gene constructs, as well as the laccase and lignin contens of transformed yellow-poplar.

STRUCTURE AND EXPRESSION OF PHENYLALANINE-AMMONIA LYASE-ENCODING GENES IN HYBRID POPLAR

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Phenylpropanoid metabolism is a secondary metabolic pathway in plants which diverts aromatic amino-acids away from primary metabolism to form a variety of biologically significant molecules such as lignin and flavonoids. Phenylalanine-ammonia lyase (PAL) is the enzyme catalyzing the initial conversion of phenylalanine to cinnamic acid. Because of its position between primary and phenylpropanoid metabolism, the regulation of this enzyme at both physiological and transcriptional levels is of great interest.

In a pedigree of hybrid poplar we have anlyzed a small gene family encoding PAL. The genomic sequences of one allele from each of two loci have been isolated and characterized by restriction analysis; the 5' and 3' noncoding regions have been sequenced for comparison to each other and to those of other phenylpropanoid-associated genes.

The expression patterns of the two isolated PAL genes are being characterized and compared, using gene specific probes to test for differential expression The PAL II promoter has been fused to the GUS reporter gene and introduced into transgenic tobacco. Histochemical analysis of these tobacco plants has demonstrated vascular specific expression of the PAL II promoter :: GUS reporter fusion. Poplar suspension cell cultures transformed with the above construct have also been generated to test the ability of the PAL II promoter to direct elicitor-activated gene expression. Expression of the two gene family members is also being examined by differential Northern analysis of RNA extracted from tissue samples collected monthly from buds/leaves, bark, and stem. Preliminary data show that high expression is associated with annual environmental and phenological changes.

FUNCTIONAL EXPRESSION OF THE MYB-RELATED C1 GENE FROM CORN AND OF A C1 HOMOLOGUE FROM BLACK SPRUCE IN LARCH AND SPRUCE TISSUES

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The MYB-related genes in black spruce (*Picea mariana*) are part of a large and complex multigene family. One of the subfamily share extensive homology to the C1 MYB-related gene from maize. At least two of these spruce C1-like genes are predominately expressed in developing female strobilli, consistent with the highly pigmented nature of these reproductive structures. Screening of a female strobilli cDNA library resulted in the isolation of several putative C1-related clones. DNA sequence analysis confirmed that one of these (MBF1) encodes a polypeptide that has extensive amino acid homology to the MYB DNA binding domain of C1 from maize. Using a vector carrying the luciferase gene under the control of the Bz2 promoter (bronze 2 gene involved in anthocyanin synthesis in corn) in combination with two vectors carrying the maize C1 and Lc activator genes, we demonstrated by particle bombardment into larch (*Larix laricina*) and white spruce (*Picea glauca*) embryonal cell masses that this system is functional in conifers. Furthermore, by replacing the corn C1 gene with the spruce MBF1 gene, the maize Bz2 promoter was effectively transactivated to similar levels to the ones obtained with C1. In black spruce female strobilli bombarded with MBF1 and Lc, anthocyanin production was observed. This provides the first direct evidence that angiosperm transactivating factors can function in gymnosperms and that this mechanism is evolutionary conserved.

GENETIC ANALYSIS OF GROWTH AND DEVELOPMENT IN TREES

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Analysis of mutations affecting growth and development has contributed greatly to our current understanding of the genetic control of these processes in animals and herbaceous plants. Much that is known of the genetic control of herbaceous plant growth is also true of trees, but there are aspects of tree physiology that are unique. Among these unique features are the annual entry into and emergence from dormancy, and the deposition of large amounts of secondary xylem. We have initiated a genetic analysis of tree growth and development, using peach as a model system. Peach has many attributes desirable for classical and molecular genetic analysis: the generation time is two years or less, the trees are small and can be grown at close spacing, both controlled crosses and self-pollination are straightforward, and the genome is very small (about 2x the size of the Arabidopsis genome). We are screening for phenotypic variation in three populations. One is a set of M2 families derived by selfing plants grown from chemically-mutagenized seed, another is an F1 family from selfing a tree with a putative active transposable element, and the third is a set of trees grafted from γ-irradiated haploid budstock. We have obtained preliminary evidence regarding the efficiency of chemical and γ-ray mutagenesis and the frequency of excision of the putative transposon. Effort is now focused on cloning the transposon for use in future gene tagging experiments, and on identifying mutations that affect wood formation. These experiments should provide an experimental approach to aspects of tree growth and development for which no suitable model exists in herbaceous plants.

REGULATORY EVENTS IN SOMATIC EMBRYOGENESIS - TRANSCRIPTIONAL AND TRANSLATIONAL

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Synthesis of the major 11S, 7S and 2S storage proteins in the zygotic embryos of alfalfa (*Medicago sativa*) reaches a peak at the cotyledonary stage of development. It is quantitatively related to the amount of mRNA present in the embryo and its recruitment into polysomes. Thus the regulation of storage protein synthesis is primarily at the transcriptional level. In contrast, somatic embryos are quantitatively poor in storage proteins, especially the 2S and 11S, and mature somatic embryos appear to be arrested developmentally at a stage equivalent to early-cotyledonary zygotic embryos. During early somatic embryogenesis the storage protein mRNAs are undertranslated and are sequestered in the cytoplasm as free mRNP complexes—the repressed fraction. However, at later stages the storage protein messages from both somatic and zygotic embryos are translated *in vivo*. mRNA-associated proteins have been identified in both the polysomes and repressed fractions of the developing embryos by uv-cross-linking. Both polysomes and the repressed fraction contain a similar set of proteins during the cotyledonary stage of somatic embryogenesis, but at earlier stages they are different, with some proteins being enriched in, or exclusive to, the repressed fraction. Proteins from the repressed fraction at the early developmental stages of somatic embryogenesis inhibit *in vitro* protein synthesis, but at later stages enhance it. The regulation of storage protein synthesis at early stages of embryo development thus appears to be post-transcriptionally regulated, whereas at later stages it depends upon appropriate mRNA synthesis. An understanding of the reasons for the limitations to protein synthesis in somatic embryos would appear to be an essential prelude to developing strategies to overcome them.

CONIFER SYNTHETIC SEEDS

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Plant somatic embryos typically suffer from poor vigour which limits their value for large scale propagation. We have generated large numbers of high quality conifer somatic embryos, which survived desiccation and grew to plantlets more vigorously than excised zygotic embryos cultured *in vitro*. Poyethylene glycols (PEGs) and dextrans of molecular weights greater than 1000 and 6000, respectively, produced a non-permeating water stress which enhanced embryo development and storage lipid accumulation in white spruce (*Picea glauca* [Moench] Voss). Somatic embryos converted to plantlets at frequencies of 76-84% following drying and frozen storage (-20 °C) for one year, similar to the 77% recorded for control embryos. Somatic embryos cultured for 7 to 8 weeks survived rapid drying to 5% moisture content at ambient relative humidity, and convereted to plantlets at frequencies of 60-70%. Water stress during development promoted desiccation tolerance. Thus, tolerance to rapid drying coincided with moisture contents below about 55%, which occurred after 5 weeks culture in the presence of PEG 4000 and abscisic acid. White spruce somatic embryos were cultured in a simple bioreactor. Over 6,300 mature white spruce somatic embryos were recovered from a single culture chamber without manual manipulation. After desiccation and imbibition the somatic embryos converted to normal plantlets at a frequency of 92%, compared to 80% for embryos grown in petri dishes. Somatic embryos cultured within the bioreactor developed to plantlets that were 20% longer than zygotic embryos excised from mature seed and grown *in vitro*, and were 38% longer than somatic embryos cultured upon agar medium in petri dishes. Plantlets underwent continued growth following transfer to soil. Methods have been transferred to black and Norway spruce. The high vigour of the somatic embryos compared to excised zygotic embryos suggests

that the increased level of storage lipid within somatic embryos has in part compensated for the absence of a nutritive megagametophyte. This should be useful for creating synthetic seeds of conifers capable of germinating *ex vitro*.

HARDWOOD PROPAGATION AND GENE TRANSFER VIA SOMATIC EMBRYOGENESIS

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Embryogenic cultures of hardwood forest trees promise to be highly useful for propagation and gene transfer applications, if some basic limitations characterizing these systems can be overcome. Even given these limitations, some applications of embryogenic hardwood cultures can be realized now. Over the past 10 years, we have initiated embryogenic cultures of a number of hardwood forest trees, which we have in turn tested for a number of applications, including propagation of rare species and rare hybrids, protoplast culture, artificial seeds, cryopreservation and microprojectile-mediated gene transfer. Embryogenic cultures of yellow-poplar (*Liriodendron tulipifera*) have been scaled-up for mass production of clonal plantlets by growing suspension cultures of proembryogenic masses (PEMs) which can be size-fractionated to produce synchronous populations of somatic embryos. Plantlets from these cultures are currently in their third year of field testing. Embryogenic yellow-poplar cultures have also been used as sources of highly-regenerable protoplasts and have proven to be ameanable to microprojectile-mediated gene transfer. Transgenic yellow-poplar plantlets obtained via somatic embryos continue to express inserted genes over 3 years following transformation. We have applied a procedure similar to that used with yellow-poplar to produce somatic embryo-defived plantlets of rare magnolias, such as pyramid magnolia (*Magnolia pyramidata*) and a rare hybrid between yellow-poplar and the Chinese tuliptree (*Liriodendron chinense*). Black locust (*Robinia pseudoacacia*) somatic embryos have proven to be excellent subjects for encapsulation to generate artificial seeds. A low percentage of these embryos converted to plantlets directly on potting mix. While American chestnut (*Castanea dentata*) embryogenic cultures have proven to be the most challenging for plantlet regeneration, they may eventually provide a route for production of transfenic trees for testing of genes that may confer resistance to the chestnut blight fungus.

DIPLOIDIZATION IN HAPLOID EMBRYOGENIC TISSUE CULTURES

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Haploid embryogenic lines of *Larix decidua* initiated from megagametophyte tissue were maintained on half-strength LM medium without growth regulators. The cultures were analyzed for ploidy level at various times. All lines tested were found to have eventually doubled their chromosome number (2n=24), although there were a few lines which still gave occasional haploid counts. Flow cytometric data confirmed these results. Protoplasts were stained in ethidium bromide and cultured human leucocytes and chicken erythrocytes were used a internal standards. Haploid megagametophytes from immature seeds of *L. decidua* and known diploid culture lines of a related hybrid (*L.* x *eurolepis*) were also analyzed by flow cytometry. Haploid reference material had 12.3-13.6 pg DNA/cell, whereas formerly haploid callus lines had different values according to clone which were around the 24 pg DNA/cell level. The one exception was a known genetically unstable line of *L. decidua* which had 34 pg DNA/cell. The diploid cell line of L. x eurolepis had 27.6 pg DNA/cell. The results show that spontaneous diploidization of megagametophyte lines is relatively rapid and both haploid and dihaploid lines are embryogenic in larch.

SOMATIC EMBRYOGENESIS IN *PICEA ABIES* (NORWAY SPRUCE) AND *PICEA RUBENS* (RED SPRUCE): GROWTH RATES AND POLYAMINES

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Zygotic embryos of Norway spruce (*Picea abies*) and red spruce (*Picea rubens*) were used to initiate embryogenic cultures on proliferation medium (PM). The tissues were either subcultured bi-weekly on PM in order to maintain early stage proembryos or grown on maturation medium (MM) to develop fully mature embryos. Whereas proembryogenic calli growing on PM more than doubled in fresh weight during the two week subculture period, the calli containing maturing embryos on MM grew only 10% per two weeks following an initial burst of growth after transfer to MM. .Analysis of polyamines in calli grown on these two media for a 12 day period showed that: 1) Putrescine and spermidine levels were always higher in cultures grown on PM as compared to those on MM; 2) Spermidine levels declined with time in calli of both species grown on MM; 3) Spermine was present in minute quantities and showed only a small change with time. Arginine decarboxylase (ADC) was found to be the major putrescine biosynthetic enzyme in both species. Ornithine decarboxylase was barely detectable. Difluoromethylornithine had little effect on polyamine content. Difluoromethylarginine inhibited putrescine in calli of both the species grown on PM as well as MM. An inverse relationship was observed between polyamine levels and ADC activity for tissues growing on MM. In order to further understand the biochemistry of developing embryos without any interference from the surrounding tissues, eight-week-old embryos were collected and compared with calli growing on PM and MM for polyamine levels and ADC activities. The results indicate that the embryos had, in general, much higher polyamine levels and ADC activity compared to the callus.

CLONING FAMOUS TREES

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Propagation via tissue culture has been used as a means preserving rare or endangered plant species. An extension of this application of tissue culture is in the cloning of famous trees. In this country, as well as throughout the world, many individual trees have achieved a degree of recognition by virtue of their size, longevity, or association with historic events. Cloning these "famous" trees not only preserves a piece of history, but also affords an investigation of the limits of tissue culture-based rejuvenation. In addition, famous trees may represent outstanding genotypes. The size and longevity of many of these trees may be due to exceptional growth characteristics, disease resistance or stress resistance rather than mere fortuitous circumstances.

Somatic embryogenesis was initiated from pollen catkin explants on a modified Brown and Lawrence medium containing 2,4-D and BA. Three trees were examined; one *Quercus veluntina* and two *Q. rubra*. All three trees were in excess of 200 years-old and were associated with events surrounding the American Revolution. Embryogenic tissue was maintained on media containing either BA or TDZ. Well-formed, early stage embryos were transferred to a half-strength WPM media containing 0.5% activated charcoal for germination. Embryogenic tissue produced from these trees is indistinguishable from that produced from immature embryo explants, suggesting that rejuvenation has occurred.

AGROBACTERIUM TUMEFACIENS-MEDIATED TRANSFORMATION AND REGENERATION OF *POPULUS*

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A genetic transformation protocol using *Agrobacterium tumefaciens*-based system has been developed in our laboratory for hybrid poplar `53-246' *(Populus trichocarpa* x *P. deltoides*). Several factors, including selection of plant tissues, the effect of wounding, and kanamycin (Km) levels, were tested to optimize the transformation system. Stem segments of `53-246' expressing high tissue culture competence were inoculated with *A. tumefaciens* strain `LGA 4404' carrying a Ti plasmic p35SGUS-INT with the *NPT* II gene for kanamycin resistance. Various parameters affecting *Agrobacterium*-mediated transformation of hybrid poplar were further tested using histochemical staining for the transient expression of ß-glucuronidase (*GUS*) gene. The optimal procedure involved inoculation of stem explants with a bacterial suspension ($5x10^8$ cells ml^{-1}) for 4 hr, followed by a 3-d co-cultivation on an antibiotics-free, callus-inducing medium. On a shoot-inducing medium, containing 50 mg l^{-1} of kanamycin, putatively transformed shoots were successfully developed within 4 wk; excised shoots were rooted on the phytohormone-free medium containing 10 mg l^{-1} kanamycin. The presence of T-DNA in transformants was demonstrated by polymerase chain reaction (PCR) and slot blot analysis.

AGROBACTERIUM MEDIATED TRANSFORMATION OF SILVER BIRCH; A CASE STUDY OF CO-INOCULATION TECHNIQUE

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Agrobacterium mediated gene transfer is especially suitable for dicots, but it has also been used successfully with many deciduous tree species. There are several *Agrobacterium* mediated transformation techniques available; inoculation, cocultivation, and co-inoculation. Inoculation technique can be used to study pathogenity of bacterial strains and cocultivation to produce *in vitro* transgenic tissues and further on whole plants through organogenesis. By using co-inoculation method shoots are formed spontaneously in tumours induced by *Agrobacterium* in host plant. The aim of the present work was to study possibilities to use co-inoculation method in silver birch (*Betula pendula* Roth.) transformation.

Two-month-old silver birch seedlings at greenhouse were used for co-inoculation experiments. Seed material originated from an open-pollinated seed orchard as well as from a local seed collection stand. *Agrobacterium tumefaciens* strains used for co-inoculation were the wild type strain 82.139 and the disarmed strain carrying 35S CamV-intron-ß-glucuronidase (GUS) construct. The following co-inoculation techniques were performed:

1) The original methods with bacterial suspensions mixed 1:10, respectively (Brasileiro et al. 1991, Plant Mol. Biol. 17:441-452).
2) 1:10 mixture of bacterial colonies from corresponding strains.
3) 1:1 mixture of bacterial colonies from corresponding strains.
4) Engineered strain 82.139 carrying 35S CaMV-intron-GUS construct.

Similar experiments were performed *in vitro* with micropropagated plantlets. After co-inoculation tumour formation and shoot induction in tumourous tissues were followed. Transgenic nature of produced shoots was tested by using

histochemical detection of GUS and by polymerase chain reaction (PCR).

All of the co-inoculation methods used in the experiments had potential to induce tumour formation in silver birch seedlings and plantlets. The growth of tumourous tissues and their ability to produce shoots spontaneously varied significantly depending on co-inoculation technique used. In the greenhouse seedlings 81% of the tumours induced by engineered strain (treatment # 4) was proved to be transgenic in histochemical GUS test. The other treatments (# 1, 2, and 3) were less effective, the percentage of transgenic tumours being 48, 53, and 33, respectively. *In vitro* the tumour formation was not as prominent as in the greenhouse conditions. The shoots formed in *in vitro* plantlets were rooted more easily than those ones produced in the greenhouse seedlings. On the other hand, the tumours in the greenhouse seedlings were able to continue shoot production for several growing seasons. When compared with conventional cocultivation techniques, the co-inoculation method seems to be less laborous and it offers an alternative way for silver birch transformation.

ENGINEERING RESISTANCE TO THE INSECT PEST CODLING MOTH IN APPLE AND WALNUT

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The main objective of our program is to engineer resistance to *Cydia pomonella* (codling moth, CM), the key insect pest in both apple and walnut, through the production of transgenic trees expressing the insecticidal crystal protein(s) (ICP) of *Bacillus thuringiensis* (Bt). The ICP's of Bt are synthesized as a full length non-toxic protoxin (ICP) that is truncated in the midgut of the insect via protease action resulting in a fragment (ICPF) that is toxic to the insect. We have shown that 1) purified ICPFs of *Bacillus thuringiensis* encoded by *cry*IA(c) or *cry*IA(b) were lethal at low concentrations to codling moth. 2) Expression of ICPF from DNA fragments containing the *cry*IA(c) sequences from Bt were low in both transgenic walnut and apple tissues and were found to be at insufficient levels to reveal any significant mortality of CM. To correct this problem we have used vectors that contain either full length or truncated synthetic versions of this gene. We have qualitatively analyzed 61 independent transgenic walnut embryo lines transformed with the full length construct. We observed that in 34% of these lines, designated as "class A", expression was high enough to kill almost all of the codling moth larvae (98% mortality). Twelve clones (20%) were designated "class B" and these showed a marked retardation of larval development and significant mortality between 40-79%. Insect mortality and development from the remaining 28 lines (46%), although transformed, were indistinguishable from the control. A detailed quantitative analysis of these lines will be presented along with a qualitative analysis of transformed tissue expressing the truncated synthetic version of the *cry*IA(c) gene.

USE OF SYNTHETIC HYPOVIRUS TRANSCRIPTS TO ESTABLISH TRANSMISSIBLE HYPOVIRULENCE IN PATHOGENIC FUNGI

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Strains of the chestnut blight fungus *Cryphonectria parasitica* that contain viral double-stranded (ds) RNA belonging to the genus *Hypovirus* exhibit reduced levels of virulence (hypovirulence). These elements are transmissible to vegetatively compatible strains of *C. parasitica* by anastomosis, providing the basis for biological control. This paper reviews the recent development of a hypovirus transfection system and the expansion of hypovirus-mediated virulence attenuation to other fungal pathogens. Synthetic transcripts corresponding to a full-length hypovirus RNA coding strand were found to be infectious when introduced into fungal spheroplasts via electroporation. Hypovirus infections were readily established in *C. parasitica* and in several fungal species not previously reported to harbor virus. Significantly, infection of the pin oak blight fungus, *Endothia gyrosa*, was shown to result in reduced virulence, thus extending hypovirus-mediated virulence-attenuation to a new fungal taxonomic family. These results predict expanded application of hypovirus-mediated virulence attenuation for understanding and controlling fungal pathogenesis.

THE USE OF TRANSGENIC BACULOVIRUSES FOR CONTROL OF FOREST PEST INSECTS

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Although baculoviruses have been employed for controlling certain insect populations for a number of years, one factor for their limited use is the length of time it takes for the virus to kill the target insect. This often results in extensive damage to host plants caused by continued feeding of the infected insect. With the advent of recombinant DNA technology, it has become possible to isolate a variety of genes that express proteins with insecticidal properties. However the application of this technology to insect control has been limited because of the lack of efficient methods for the delivery and expression of such genes within insects. Baculoviruses offer an attractive method for the resolution of these limitations. Research is currently underway to investigate the ability of genetically altered baculoviruses expressing insect-specific neuropeptides or toxins to control insect populations more efficiently than wild-type virus.

ISOLATION AND CHARACTERIZATION OF XYLEM PROTEINS FROM AMERICAN ELM (*ULMUS AMERICANA* L.) INOCULATED WITH THE DUTCH ELM DISEASE FUNGUS *OPHIOSTOMA ULMI*

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We are investigating the biochemical responses of American elms to the Dutch elm disease fungus *Ophiostoma ulmi*. A pilot experiment was carried out in 1993 to examine extractable proteins from American elm xylem and to determine if observable changes in protein expression correlated with fungal inoculation, disease development, or the date of tissue harvest.

Rooted cuttings of American elm selection 8630 were inoculated with *O. ulmi* on June 9 or July 15. Branches were harvested at 23 days and/or 29 days post-inoculation, respectively. Disease symptoms were estimated visually at harvest dates as the percentage of total canopy. Harvested branches were debarked and current year xylem was shaved using vegetable peelers, frozen, and ground into a fine powder. Proteins were extracted from the powder using a homogenization buffer containing 0.1 M Tris, pH 7.0; 15 mM *B*-mercaptoethanol; 1.5% polyvinylpolypyrrolidone; 10 mM dithiothreitol; and a variety of protease inhibitors. The proteins were resolved by two-dimensional gel electrophoresis using an isoelectric focusing range of pH 4-8 in the first dimension and 10% acrylamide in the second dimension. Gels were stained with silver or Coomassie Blue. Selected spots from Coomassie-stained gels were blotted to polyvinylidene difluoride and subjected to Edman sequencing.

Detected proteins from fungus-inoculated trees were distinguishable from uninoculated controls by a decrease in higher molecular weight proteins and increases in lower molecular weight proteins. Several lower molecular weight proteins appeared to be present in the uninoculated controls, but at much lower abundance levels. The changes in protein expression correlated with fungal

inoculation, but the degree of change did not correlate with the percentage of canopy wilt, which ranged from 5% to 75%. Total protein yields were lower from uninoculated and inoculated trees from the second harvest date, but the change in protein expression with fungal inoculation was observed for both dates. The amino terminal sequences of two 16 kDa proteins which increased with fungal inoculation were different. The sequence information from proteins will be used to isolate genes expressed in elm xylem in response to infection with *O. ulmi*.

PROTEINASE INHIBITOR II GENE EXPRESSION AND PEST RESISTANCE OF TRANSGENIC POPLAR

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The hybrid *Populus alba* L. X *P. grandidentata* Michx. clone 'Hansen' was transformed with chimeric genes to study the roles of a proteinase inhibitor and defense gene expression in pest resistance of woody plants. Transferred chimeric genes contain the potato proteinase inhibitor II (*pin2*) coding region under the control of either a bacterial nopaline synthase (*nos*) or cauliflower mosaic virus (*35S*) promoter. Transfer DNA also contains a selectable marker gene in the form of a *nos* promoter linked to a neomycin phosphotransferase II (*NPT II*) structural gene. Polymerase chain reaction confirms the presence of transferred *pin2* and *NPT II* sequences in transgenic, greenhouse-grown poplars. Enzyme-linked immunosorbant assays demonstrate variable leaf expression of *pin2* among different lines of transgenic poplar, and Western blotting indicates that *pin2* is translated and processed appropriately. *Pin2* expressing lines of transgenic poplar were used in leaf feeding bioassays with imported willow leaf beetle, *Plagiodera versicolora* (Laicharting). Significant differences in insect development and leaf consumption were observed among transgenic poplar lines relative to untransformed control poplar. The role of *pin2* in resistance to fungal pathogens is also under investigation. *Pin2* is postulated to inhibit nutrition deriving processes of pests, and thereby delay pest growth, development, and reproduction. By avoiding a strong selective pressure on the pests, we believe *pin2* will contribute toward a pest resistance that is stable and compatible with other mechanisms of biological control.

MOLECULAR BIOLOGY OF RESISTANCE TO INSECTS AND DISEASES IN BIRCH AND PINE

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Two resistance mechanisms of plants against insects and diseases, proteinase inhibitors and chitinases, respectively, were studied. The production of native chitinases in birch (*Betula pendula*) was assayed and similar work on proteinase inhibitors is in progress. The corresponding foreign genes (potato proteinase inhibitor 2, *pin 2*, and sugarbeet chitinase, *Ch4*) were used in gene transfer to birch in order to study their effect on resistance. A native putative protease inhibitor gene from birch is being cloned.

The potential of the *pin 2* gene to increase the resistance was defined by characterizing the gut proteases in seven common forest insect pests and extracellular fungal proteases in four pathogens of trees, and then measuring the effect of the potato proteinase inhibitor on the enzyme activities. Also, the sugarbeet chitinase 4, coded by the gene (*Ch4*) that we used in gene transfer, was shown to effectively inhibit the growth of the tree pathogen *Heterobasidion annosum*.

The function of the native wound-inducible promoter (win) of the potato proteinase-inhibitor gene was tested in transgenic birch carrying the construct win - *uidA* (GUS). When a leaf was wounded, the promoter had a systemic activity. The induction was stronger in transgenic older birch leaves and in older parts of the stem and roots than in younger ones. Comparisons of several promoters transferred by bombardment into cells of birch and pine (*Pinus sylvestris*), suggested that the CaMV 35S promoter had a weaker effect on the *uidA*-gene than, e.g., the win promoter. The pine cells had competence for somatic embryogenesis before bomardement but seem to loose it during kanamycin selection.

The *Agrobacterium tumefacience*-mediated gene transfer system to birch was optimized and the resulting transgenic lines are now multiplied for resistance testing. Preliminary resistance testing in the laboratory with pathogens and insects on a few transgenic birch lines have so far given inconclusive results.

IN SITU HYBRIDIZATION AND GENOME ORGANIZATION IN FOREST TREES

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The technology of *in situ* hybridization is leading to improved understanding of plant genome organization. Gymnosperms, for which so little is known about genome organization, are particularly promising candidates for application of this technology, especially with those conifer species for which there are established tree genetics programs and for which genetic linkage maps are being constructed. We have optimized a fluorescence *in situ* hybridization (FISH) technique that we are using to determine the distribution of repetitive DNA elements in the nuclear genome of white spruce (*Picea glauca)* (2n = 24) and other related *Picea* species. We will present our results on the chromosomal location of several repetitive DNAs in white spruce, Sitka spruce and Engelmann spruce including the 5S ribosomal RNA genes, the nucleolar organizer regions (18S/5.8S/26S rRNA genes), rDNA intergenic spacer sequences, a centromeric tandem array, telomere sequences, and total genomic DNA probes. These molecular cytogenetics studies using FISH are revealing a remarkable and unique genome organization of repetitive DNAs in conifers. Such information may spark renewed interest in chromosome evolution.

GENETIC MAPPING AND TRAIT IMPROVEMENT IN FOREST TREES

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Traditional tree improvement programs have treated most traits of economic importance, such as volume growth, stem form, frost hardiness, drought tolerance, and disease resistance, as quantitative traits under the control of many genes each with a small effect on the phenotype. This is in part due to the relatively poor resolution offered by testing designs where the minimum replicated genetic unit is the family (full- or half-sib related), and is exacerbated by the generally large environmental component of phenotypic variance for such integrative traits as growth. Even when the minimum replicated genetic unit is reduced to the individual tree (clone), statistical partitioning of phenotypic variance components does not permit a detailed understanding of quantitative trait expression, which occurs at the level of single genes.

The tools of molecular biology and genetics have recently been used to bridge the gap between classical genetics (which has had limited application in forest trees with their long generation intervals and outcrossing mating systems) and quantitative genetics. Relatively detailed linkage maps of the chromosomes of *Pinus*, *Eucalyptus*, *Populus*, and other forest tree genera have been constructed and used to locate quantitative trait loci (QTLs) with significant effects on such traits as wood specific gravity, growth, stem taper, leaf phenology, and disease resistance. Each trait can be modeled as the sum of effects of QTLs of known number, chromosomal position, magnitude of effect, and mode of action. This increased precision has the potential to accelerate genetic gain by suggesting improved mating designs and propagation systems to maximize the frequency of favorable QTL combinations, by permitting indirect (marker-assisted) selection, and by providing a genetic understanding of the physiological and biochemical processes underlying complex phenotypes.

PINE GENE EXPRESSION: A TRANSGENIC APPROACH

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Transgenic approaches have been used to alter the location and/or abundance of specific gene products in higher plants. This approach allows one to ask questions about the function of specific genes *in vivo*, since the phenotypes of transgenic individuals with altered gene product levels (either up- or down-regulated) can be measured relative to normal individuals. In some cases, transgenic plants have exhibited increased agronomic value. Of equal or greater value, however, is the insight into fundamental processes that have been gained by altering gene function in transgenic systems.

In order to complement ongoing tree improvement activities, it is essential that we obtain the fundamental knowledge necessary to understand and subsequently manipulate gene expression in a meaningful, predictable way in pines. Because it is not yet possible to routinely produce transgenic pines for research purposes, we are developing alternative experimental systems to ask questions about the cellular machinery that controls gene expression in pine trees. We cloned an active (wound- and chitosan-inducible) gene from pine and are currently studying its regulation in a transgenic system, using an herbaceous plant species as a host. We hope to use this system as a model to understand the factors that activate the transcription of genes in pines compared to those of herbaceous plants. These studies should help determine the similarities and possible differences between gene regulation in pines and herbaceous plants.

GENETIC DIVERSITY AMONG ASPEN POPULATIONS IN YELLOWSTONE NATIONAL PARK

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In 1988 fires burned ca. 45% of YNP, eliminating many of the mature stands of aspen (*Populus tremuloides* Michx.) and creating an environment which permitted aspen seed germination and seedling establishment. The objectives of this study were to characterize the degree of genetic variability among and within the new aspen seedling populations and to estimate the phylogenetic relationship between older mature aspen stands and the new seedling populations. Leaf disks were collected from each of 20 individual stems in 10 mature stands and 4 seedling populations. DNA was obtained from each sample using a modified 2X CTAB extraction procedure containing 1% PVPP. A PCR protocol for RAPD markers was used to survey genetic variability among samples. Four arbitrary 10-mer primers were used in the PCR reaction to obtain 64 RAPD markers. Approximately 1/3 of the RAPD markers were polymorphic in any given population. Nei's band sharing index was used to determine the degree of relatedness among populations and stands. Neighbor joining analysis was then used to determine phylogenetic relationships among the sampled populations. On the average, across all genotypes, the paired samples shared 72% of the scored markers. All mature stands were clonal in nature, yet no stand was monoclonal. Each sampled stand generally clustered as a discrete clade, though individual mature stands did display greater similarity to the seedling stands than to the other mature stands. Genetic distance was related to geographic distance in the seedling stands, but not in the mature stands. From these results it is speculated that seedlings within a stand share a common parent, probably the maternal parent. In conclusion, (1) genetic diversity within aspen populations in YNP has increased and (2) there is little evidence to indicate that the seedling stands are the direct progeny of the mature stands that pre-existed the 1988 fires.

RELATIONSHIP BETWEEN NUCLEAR DNA MARKERS AND SEASONAL PHYSIOLOGICAL PARAMETERS OF SEEDLINGS FROM THE SITKA X INTERIOR SPRUCE INTROGRESSION ZONE IN BRITISH COLUMBIA

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There is a large introgression zone that occurs between Sitka spruce (*Picea sitchensis* (Bong.) Carr.) and interior spruce (*Picea glauca* (Moench) Voss *x Picea engelmannii* Parry ex. Engelm.) in a region called the Nass Skeena transition in British Columbia, Canada. Given the lack of information about the extent of introgression and the adaptive traits of the hybrid genotypes, information is required to define seed transfer guidelines within this area. Restriction fragment length polymorphisms of the nuclear ribosomal RNA genes (rDNA) were used to define species specific patterns for interior and Sitka spruce populations. Hybrid fraction was estimated from an index based on the relative abundance of rDNA bands and expressed as fraction interior spruce (Si rDNA). Seasonal patterns of drought tolerance, measured by pressure-volume analysis, and freezing tolerance, measured by freeze-induced electrolyte leakage, were also measured on these interior and Sitka spruce populations. All sources of Sitka *x* interior hybrid seedlings had typical seasonal patterns of osmotic potential at saturation (Ψ_{sat}) and turgor loss point (Ψ_{tlp}), relative water content turgor loss point (RWC_{tlp}) and total turgor (Ψ_{PTotal}). However, during all times of the year, there were linear relationships between Si rDNA values of the seedlings and shoot water relations parameters which showed that drought tolerance increased as their Si rDNA values increased. During the fall, seedlings having higher Si rDNA values exhibited greater freezing tolerance based on LT_{50} (the temperature causing 50% needle electrolyte leakage). By knowing the Si rDNA values of a seed source, the degree of drought and freezing tolerance could be predicted throughout most of the year.

DNA LEVEL VARIABILITY IN EMBRYOS OF SEVERAL FAMILIES OF JACK PINE (*PINUS BANKSIANA* LAMB.) AS ESTABLISHED WITH FLOW CYTOMETRY

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Variability in nuclear DNA levels has been documented in many plant species. From this data it has become evident that nuclear DNA levels vary considerably not only between species, but also amongst and within populations or cultivars of a single species. This plasticity, which appears to be related to an adaptational function in plants, may also have a potential use in selection, breeding and biotechnology. In order to understand the basic processes of DNA variation and its potential roles, we examined DNA levels of dormant embryos and germinating seedlings of *P. banksiana.* Because a method for isolating nuclei from small amounts of fresh tissue (100mg) was developed (Wyman *et. al.* 1993), minimal pooling of embryos was necessary, thus improving the statistical probability of detecting variation in DNA levels. Ten open pollinated half-sib families classified as superior on the basis of trunk straightness, height, diameter at breast height, number of branches, and wood density in field trials, and ten classified as inferior on the same basis were selected for the study. Nuclei from twenty embryos from each family were extracted, stained with propidium iodide and DNA levels were determined using flow cytometry. Preliminary studies indicated that an imbibition period of 144 hrs. was optimal. Between 2,000 and 10,000 nuclei were analysed and the frequencies of nuclei in classes ranging from 2C to 6C were calculated. A large amount of variability in the percentage of nuclei in the classes greater than 4C was noted amongst families, however this variability was not correlated with the growth status of the mature trees. The DNA data was also analysed in relation to a variety of morphological characteristics including the number of cotyledons per embryo and the percentage of embryos that underwent greening. A correlation between the percentage of nuclei in classes greater than 4C and the percentage of

embryos that underwent greening was noted in several families. The correlation of DNA levels in the dormant embryos to morphological traits may provide new avenues of preselection.

DISTINGUISHING INDIVIDUALS AND CLONES OF NORWAY SPRUCE USING RAPD FINGERPRINTING

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Data are presented on the ease of distinguishing between genotypes in a collection of Norway spruce (Picea abies L. Karst) graftings, cuttings, somatic embryo-derived plants, and seedlings via DNA analysis. Quick preparations of DNA and screening with a few primers in a random amplified polymorphic DNA (RAPD) assay usually results in the assignments of individuals to clones with high probabilities. For instance, uncertain pedigree information for a set of morphologically distinct plants could be confirmed. Controlled pollinations can be checked for plausibility of recorded parents.

Taken together, RAPD provides an efficient means for the management of genotype collections in Norway spruce.

APPLICATION OF HYPERVARIABLE DOUGLAS-FIR CHLOROPLAST DNA IN PATERNITY ANALYSIS

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We describe a method to distinguish seed orchard pollen parents based on chloroplast DNA (cpDNA) polymorphism in Douglas-fir. In the testing of seed orchard progeny, total genomic DNA (ca. 1 ng), isolated from single embryos, is amplified by PCR using oligonucleotide primers flanking a hypervariable cpDNA region. The amplified DNA is analyzed by gel electrophoresis in order to distinguish sizes of amplified product ranging from 600 to 1,400bp to a resolution of as little as 1bp. The level of discrimination is sufficient to identify, unambiguously, 80% of unrelated clones in an experimental orchard. The technique has been applied to determine contributions of individual pollen parents within the orchard and the level of contaminating pollen from outside the orchard. In addition, the efficacy of supplemental mass pollination relative to the flowering phenology of individual females has been measured. The method provides an opportunity for the seed orchard manager to assess various operational practices in a cost effective manner.

TOWARDS COMMERCIALISATION OF CONIFER EMBRYOGENESIS

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Progress in conifer embryogenesis over the past 5 years has been promising with the establishment of large field trials of somatic seedlings, particularly with spruce. Spruce embryogenesis is somewhat of a "model system" for conifer embryogenesis and it is expected that applications on a commercial scale will occur within 5 years. Pine embryogenesis has been more problematical, but is being tested on a pre-commercial scale by a number of forestry companies worldwide. Carter Holt Harvey Ltd is one of the Southern Hemisphere's largest plantation forest owners with 327,000 hectares of pine forest in New Zealand. It's Forest Biotechnology Centre is currently applying and scaling-up the techniques initially developed at the New Zealand Forest Research Institute with the aim of developing a commercially viable process. Radiata pine (*Pinus radiata* D. Don) somatic seedlings have been produced from the 1992/3 cell lines and over 2,000 embryogenic cell lines have recently been established from identical control pollinated crosses from different seed orchards. The best mature-embryo forming clones will be cryopreserved and will be established in clonal trials in 1996.

Constraints to the successful introduction of commercial embryogenesis are perceived by Carter Holt Harvey to be: application of new techniques in a new laboratory; decisions on the fundamentals of production; improving embryo quality, efficiency and the number of responding genotypes; selection of the best delivery system from "lab to forest"; and development of a cost effective system. Through all stages of embryogenesis, physiological and biochemical profiles, molecular biology and total management information systems are used to monitor media, growth and progress in the lab and the forest. Continued advances in cryopreservation, automation, liquid culture, encapsulation, rejuvenation, genetic

fingerprinting and gene transformation are expected to further enhance the process.

Application of somatic embryogenesis techniques to a range of conifers on a commercial scale is now a real possibility.

PILOT PRODUCTION AND SCALE-UP DELIVERY OF SPRUCE SOMATIC SEEDLINGS

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Somatic embryogenesis (SE) in forestry involves the rapid, high-volume cloning of elite conifer seeds. Clonal lines can be preserved indefinitely by storage in liquid nitrogen and bulked up for production at any time. Biochemical, physiological and field tests indicate that the plantlets produced by this process are identical to true seedlings. The SE process offers distinct advantages over seed orchards or the production of rooted cuttings in delivery of improved reforestation stock.

A pilot production system for Spruce (Picea spp.) SE has been developed using high-volume tissue culture techniques and semi-automation, and is being scaled-up for commercial application. In this production system, suspension culture and other bulk handling techniques developed for SE callus bulk-up, maturation, desiccation and germination have significantly reduced production costs, since no per-unit handling of embryos or germinants is required. Quality assurance is performed at the time of ex vitro planting of 4-6 week old germinants through the use of a patent pending, semi-automated sorting and planting machine. The production system provides greater than 95% uniform crop establishment in the greenhouse in commercial volumes.

TAXOL - THE PRODUCTION OF AN ANTI-CANCER DRUG FROM TREES

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The dynamics of the production of taxol, a valuable anti-cancer drug, have changed rapidly within the past five years. In the late 1980's and early 1990's a lack of taxol supply was a major limiting factor not only in the chemotherapeutic use of taxol for the treatment of patients with ovarian cancer, but also in the testing of taxol in clinical trials against other forms of cancer. In these phase I and Phase II clinical trials, taxol consistently showed activity against a wide range of cancer cells and tumors types. Although the primary commercial source of taxol has been the bark of *Taxus brevifolia* (Pacific yew), all *Taxus* spp. and all parts of the plants thus far investigated contain taxol. This widespread productin of taxol within the genus has provided an opportunity for the renewable production of taxol from faster growing horticultural *Taxus* spp., as well as the manipulation of tissue and cell cultures for the production of taxol and related taxanes. Taxol and taxane concentrations in these different species and horticultural cultivars can vary widely, for example *T. x media* contains a relatively high taxol concentration while *T. baccata* contains a relatively low taxol concentration and a relatively high level of the precursor taxane, 10-D baccatin. The production of taxol in shoots of 3 year-old field grown Taxus plants varies only 3-5 fold from bud break in the spring until bud set in the fall, suggesting that actively growing tissues can tolerate the presence of this potentially toxic compound. In contrast to the shoots however, taxol concentration in the roots is more developmentally regulated with young actively growing roots producing low levels of taxol compared to developmentally older roots.

The chemical synthesis of taxol has been reported, but considerable work is still needed prior to total synthesis becoming a commercial option. Therefore, in the foreseeable future the sole source of taxol will continue to be the plant. The biosynthetic regulation of taxol, a complex diterpene amide, within the plant is

complex and poorly understood. Using shoot cultures and cambium sections numerous putative precursors to taxol, such as phenylalanine, have been shown to be incorporated into taxol. These compounds have also been used in tissue culture to increase taxol production. Although the site of taxol synthesis is still unknown, taxol has been localized to the cell walls using immunocytochemistry. This site of taxol deposition in the plant might account for the ability of rapidly growing tissues to contain relatively high levels of taxol. In tissue culture, taxol has been produced in suspension, callus, seedling, shoot, nodule, hairy root, transformed tumor, and embryogenic cultures.

Recent advances in the semi-synthesis of taxol from precursors, such as 10-D baccatin, and the use of cultivated plants have contributed to the supply of taxol, such that supply is no longer limiting either therapeutic use of clinical trials. The dynamics of supply could rapidly change however with the approval of taxol for the use in other forms of cancer such as breast cancer. To offset the wider demand of taxol the use of tissue culture and cultivated plants will certainly play a role in the supply of taxol and the precursors needed for the semi-synthesis of taxol. In addition, the development of active taxol analogues, such as Taxotere, and the use of peptides which mimic the action of taxol will also play a role in the future use and demand of taxol.

POSTER ABSTRACTS

MICROPROPAGATION OF *EUCALYPTUS* HYBRID FRI4 BY SHOOT TIP CULTURE

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Rapid mass clonal propagation of trees using tissue culture technology is envisioned to meet the ever-increasing demand for pulp, timber, and firewood. Micropropagation methods for tree species, including some *Eucalyptus*, have been achieved, but species-specific requirements necessitates separate investigations. Therefore, this study was conducted to establish a micropropagation system for *Eucalyptus* hybrid FRI4 (*E. tereticornis* X *E. camaldulensis*). Our objectives were to test the response of shoot apex explants to combinations of growth regulators and to the concentration of basal salts in the culture medium.

Shoot apices (3 mm long) of aseptically-germinated seedlings were isolated and cultured on a medium consisting of Murashige and Skoog (MS) salts (half- or full-strength), 100 mg L^{-1} *myo*-inositol, 1 mg L^{-1} thiamine-HCl, 1 mg L^{-1} nicotinic acid, 1 mg L^{-1} pyridoxine-HCl, 2 mg L^{-1} glycine, 2% sucrose, 0.8% agar, and combinations of three growth regulators. The growth regulator treatments were kinetin (1, 5, or 10 mg L^{-1}) combined with 6-benzylaminopurine (0, 0.5, or 1 mg L^{-1}) and 1-naphthaleneacetic acid at 0, 0.01, or 0.1 mg L^{-1}. The cultures were maintained at 23C under 12-h photoperiods.

Multiple shoot formation was observed within 3 to 4 weeks after culturing. There was variation in explant response depending upon the hormonal supplement. Information gathered from this system could be useful in developing protocols for the in vitro vegetative propagation of *Eucalyptus* hybrid FRI4 from mature sources.

CALLUS INDUCTION AND REDIFFERENTIATION IN *EUCALYPTUS* HYBRID FRI4

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Eucalyptus is a fast-growing source of wood for timber and paper industries. Gains from clonal propagation of superior individuals makes micropropagation an attractive alternative. Callus-based regeneration systems are not only a prerequisite for improvement through genetic transformation, but also offer a source for somaclonal variants. Our aim was to develop a regeneration system for *Eucalyptus* hybrid FRI4 (*E. tereticornis* X *E. camaldulensis*). The objective of this study was to evaluate the effect of growth regulators on callus induction and subsequent plant regeneration.

Leaf explants isolated from seedlings were induced to callus on MS medium containing (in mg L^{-1}) 100 *myo*-inositol, 1 thiamine-HCl, 1 nicotinic acid, 1 pyridoxine-HCl, 2 glycine, 2% sucrose, 0.8% agar, and growth regulators. The growth regulator treatments were furfurylaminopurine (kinetin) or 6-benzylaminopurine (BAP) at 0.1, 0.5, and 2.5 mg L^{-1} combined with 2,4-dichlophenoxyacetic acid (2,4-D) at 0.25, 0.5, and 1 mg L^{-1}. Callus induction cultures were maintained at 23C under 11-h photoperiods. After 4 weeks, callus fresh weights were measured and the callus was transferred to regeneration medium. Calli induced on medium containing kinetin and 2,4-D were transferred to regeneration medium with 0.2 mg L^{-1} kinetin and 0 or 1 mg L^{-1} NAA. Calli that were induced on medium containing BA and 2,4-D were transferred to regeneration medium with 0.2 mg L^{-1} BA and 0 or 1 mg L^{-1} NAA. Callus proliferation was significantly influenced by the growth regulators. The redifferentiation response of the callus will be discussed.

INFLUENCE OF PRECULTURE, ACETOSYRINGONE, AND SELECTION METHOD ON THE TRANSFORMATION OF *EUCALYPTUS* HYBRID FRI4 WITH *AGROBACTERIUM TUMEFACIENS*

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In *Agrobacterium*-mediated transformation systems, the efficiency of transformation is dependent upon an interaction between the plant genotype and the bacterial strain, and the outcome may be influenced by other physical and chemical factors. In this study we investigated the influence of explant preculture, acetosyringone (3',5'-dimethoxy-4'-hydroxyacetophenone) treatment, and the selection approach on the transformation of *Eucalyptus* hybrid FRI4 (*E. tereticornis* x *E. camaldulensis*). Leaf sections isolated from seedlings were either immediately cocultured with bacterial suspension or precultured on a medium for 2 and 3 days prior to being cocultured with *A. tumefaciens* LBA4404 harboring the pBI121 plasmid which carries neomycin phosphotransferase II and β-glucuronidase (GUS) genes. Overnight-grown bacterial suspensions were diluted at 1:10 and treated with acetosyringone at 0, 100, and 200 μM. Explants were cocultured with bacteria for 2 days, after which they were washed and transferred to selection medium, either directly or following a 1-week incubation on kanamycin-free selection medium. The selection medium consisted of MS medium with 0.5 mg L^{-1} 2,4-D, 0.5 mg L^{-1} 6-BAP, 3% sucrose, and 0.8% agar, augmented with 50 mg L^{-1} kanamycin, 100 mg L^{-1} cefotaxime, and 200 mg L^{-1} carbenicillin. The cultures were incubated at 23C and 11-h photoperiods. Callus formation from the transformed explants was observed within four weeks. The percentage of transformation of *Eucalyptus* hybrid FRI4 in response to these three factors will be discussed.

EXPRESSION OF GUS GENE IN *EUCALYPTUS* CALLUS DERIVED FROM *AGROBACTERIUM TUMEFACIENS*-TRANSFORMED EXPLANTS

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Genetic engineering offers a biotechnological approach for the improvement of trees to meet the challenge of the world's increasing demand for wood. As a means of incorporating foreign genes into plant genomes, *Agrobacterium tumefaciens* has been successfully used to mediate the transformation of numerous plant species. The objective of the present study was to examine the effectiveness of *A. tumefaciens* in transforming the GUS gene into *Eucalyptus* hybrid FRI4 (*E. tereticornis* X *E. camaldulensis*).

Shoot tip, leaf, cotyledon and hypocotyl explants were cocultured for 2 days with *A. tumefaciens* strain ASE208 harboring pMON9749 which carries neomycin phosphotransferase II and β-glucuronidase (GUS) genes. The culture medium consisted of Murashige and Skoog (MS) medium, 0.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid, 0.5 mg L^{-1} 6-benzylaminopurine, 3% sucrose, and 0.8% agar. The explants were subsequently transferred to selection medium supplemented with (in mg L^{-1}) 50 kanamycin, 100 cefotaxime, and 200 carbenicillin. The cultures were incubated at 23C and 11-h photoperiods.

Based upon the X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronic acid) assay, all four explant types produced callus expressing GUS activity, but at variable transformation rates. This study demonstrates the effectiveness of *A. tumefaciens* in transforming *Eucalyptus* hybrid FRI4.

DEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBONS IN WOOD BY WOOD ROT FUNGI

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Polycyclic aromatic hydrocarbons (PAHs), are major, toxic organopollutants. Their toxicity underlies their use in the wood preservative creosote, but also makes it difficult to dispose of creosote-treated wood wastes. White-rot fungi that delignify wood have emerged as one possible biotreatment agent for the remediation of creosote-contaminated wood. These fungi use ligninolytic enzymes to oxidize a wide variety of PAHs in defined culture media, and in some cases are able to detoxify or even mineralize the starting material. However, it has not been shown that white-rot fungi are able to oxidize PAHs in wood, the environment in which creosote pollutants must be treated.

In this study, we show that phenanthrene, the major PAH in creosote, was degraded in wood by the white-rot fungi *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora*. Wood blocks infiltrated with [14]C-labeled phenanthrene were incubated over agar cultures of the fungi. The culture headspaces were flushed at intervals and vented CO_2 was trapped for scintillation counting. After incubation, the wood blocks were pulverized and extracted with methanol, and the resulting extract was subjected to gel permeation chromatography. The low molecular weight fractions were then analyzed by reversed phase HPLC and scintillation counting.

A variety of poplar phenanthrene metabolites were formed, including 2,2[1]-diphenic acid, which is a major phenanthrene metabolite formed by *P. chrysosporium* on defined medium. In earlier work , we showed that diphenic acid results from an unsaturated lipid-dependent cooxidation of phenanthrene that is promoted by manganese-dependent peroxidases of these fungi. Therefore, we conclude that one or more components of the ligninolytic system in white-rot fungi functions to oxidize phenanthrene in wood.

Mineralization was a minor fate of phenanthrene in wood, amounting to about 2% in 30 days. It is noteworthy that a structurally related PAH, pyrene, was mineralized to a significantly greater extent, about 10% in 30 days. These results parallel those obtained previously in defined medium.

Phenanthrene and pyrene were also polymerized in wood blocks inoculated with both white rot fungi, as shown by the finding that high molecular weight radiolabeled compounds were detected by gel permeation chromatography. The polymerized products were not further characterized, but probably resulted from peroxidase-mediated crosslinking with phenolic wood components. Crosslinking reactions of this type may be beneficial, in that they immobilize potentially toxic PAHs. However, they obviously have a negative effect on total biodegradation, the most desirable outcome.

RECIPROCAL PARENTAL EFFECT ON SOMATIC EMBRYOGENESIS INDUCTION FROM IMMATURE ZYGOTIC EMBRYOS OF *Pinus taeda* L.

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Genetic control and effect of explant type on induction of somatic embryogenesis (SE) was investigated using immature zygotic embryos derived from controlled crosses, including reciprocals, of highly, moderately and poorly responsive parent trees of loblolly pine. Two types of explants, megagametophytes containing immature zygotic embryos, and immature zygotic embryos isolated from the megagametophyte, were cultured on DCR medium supplemented with 2,4-D and BA. Higher induction frequencies and increased growth of embryogenic tissue, as measured by culture fresh weight, were obtained when the maternal parent was more embryogenic than in the reciprocal cross when the paternal parent was more embryogenic. The fact that the reciprocal effect was observed from immature zygotic embryos with or without the megagametophyte tissue suggests that the controlling mechanism resides in the zygotic embryos, rather than in the megagametophyte tissue. Our results are consistent with an extra-nuclear (mtDNA) genetic component influencing SE induction, or a genomic imprinting mechanism of nuclear genes involved in SE induction resulting in the expression of maternally and repression of paternally inherited SE induction genes.

IDENTIFICATION OF THE *25K* GENE OF THE *LYMANTRIA DISPAR* NUCLEAR POLYHEDROSIS VIRUS

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The baculovirus life cycle has two morphologically distinct forms: a non-occluded budded virus that infects cells within the same larva, and an occluded form termed a polyhedron that ensures transmission from one larva to another. Passage of multinucleocapsid nuclear polyhedrosis viruses (MNPV) in cell culture results in the rapid generation of few polyhedra (FP) mutants with increased budded virus titre and reduced numbers of polyhedra. Polyhedra produced from FP mutants are virtually devoid of nucleocapsids. The phenotype associated with FP mutants of *Autographa californica* MNPV (AcMNPV) and *Galleria mellonella* MNPV (GmMNPV) have been shown to be due to the inactivation of a 25 kDa protein. Often these mutants have deletions or large (0.8 to 2.8 kB) insertions of host DNA into the *25K* gene. This loci appears to a "hot spot" for DNA mutation as these insertions and deletions occur at a higher frequency than mutations in other regions of the viral genome. The function of the 25K protein is unknown. It is not required for viral replication but is required for the production of wild type numbers of polyhedra. One possibility is that the 25K protein is a nucleocapsid envelope protein that interacts with the polyhedrin and initiates nucleation. FP mutants are also generated upon serial transfer of the *Lymantria dispar* MNPV (LdMNPV) in cell culture. Unlike AcMNPV and GmMNPV FP mutants, LdMNPV mutants do not appear to contain large deletions or insertions as determined by DNA restriction analysis. While mapping the genomic location of a group of FP mutations in LdMNPV, we have identified, sequenced, and characterized a gene encoding the LdMNPV 25K homologue.

SEQUENCE CHARACTERIZATION AND TEMPORAL EXPRESSION OF THE *LYMANTRIA DISPAR* NUCLEAR POLYHEDROSIS VIRUS IMMEDIATE-EARLY GENE G22

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Baculovirus expression studies indicate that viral genes are expressed in a temporal cascade in which immediate-early genes encode regulatory proteins that control the transcription of all other viral genes. Identification of these regulatory proteins is an important step in understanding the molecular aspects that govern viral potency. Many of the *Autographa californica* multinucleocapsid nuclear polyhedrosis virus (AcMNPV) immediate-early genes have been identified and sequenced. These genes are transcribed by the host RNA polymerase and do not require other viral proteins for their expression; although, transcription of these genes may be enhanced by transactivation with other viral factors.

Temporal analysis of transcripts in the *Lymantria dispar* multinucleocapsid nuclear polyhedrosis virus (LdMNPV) had identified three putative immediate-early genes which were localized between 6.0 and 13.7 map units on the viral genome. One of the genes, designated *G22* because it is located within the *Eco*RV-G fragment and is predicted to encode a 22 kDa protein, was chosen for further study. This region has been cloned and sequenced. A 24 kDa protein is seen when *G22* is expressed in an *in vitro* translation and transcription system, which is in close agreement to the 22 kDa protein predicted from the DNA sequence. Comparison of the predicted amino acid sequence of G22 with other proteins in GenBank reveals no strong homology to any other known protein. Temporal expression studies indicate that *G22* codes for a 850 kB transcript that can be detected immediately following the adsorption period of the virus. This transcript continues to be expressed throughout the time-course of infection with an increase in transcription that may be dependent on the expression of other viral proteins from

4 to 8 hr post-infection. The *G22* gene transcript is expressed in the presence of cycloheximide and alphidicolin. Consequently, this gene is classified as an immediate-early gene.

AN EVALUATION OF IN VITRO ADVENTITIOUS SHOOT AND ROOT FORMATION FROM COTYLEDON EXPLANTS OF JACK PINE (PINUS BANKSIANA LAMB.)

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Adventitious buds were induced from cotyledon explants of jack pine (*Pinus banksiana* Lamb.) when cultured for 4 weeks in Gresshoff and Doy (GD) medium supplemented with 5 mg/L N^6-benzyladenine (BA), 1% sucrose and 0.7% agar. Variations in methods of BA treatment did not improve bud production. Optimal bud induction occurred from explants excised at 6-7 days after placement on germination medium. Primary multiplication rates were as high as 230 buds per seedling, which had an average of four cotyledons; response was highly variable. After a 4 week exposure to BA, cotyledons were transferred to half-strength GD medium with 1% sucrose and 0.7% agar for subsequent shoot growth (elongation medium). Proliferating shoot 'mother cultures' were subcultured monthly on elongation medium for up to 1 year, with an overall survival rate of 45%. Individual shoots were excised and grown on elongation medium for up to 6 months, with monthly subcultures. Spontaneous rooting occurred at a rate of 15.5%. Rooted shoots were successfully transferred to peat-based media. Further studies are aimed at improving rooting response of shoots.

TISSUE CULTURE OF ELMS, HACKBERRY, BIRCH AND PONDEROSA PINE

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Elms, hackberry, birch and ponderosa pine are among the few tree species which have adapted to the northern Great Plains states. Diseases, insects and herbicides have been the major threats to these species. The goal of our research is to apply genetic engineering technology to improve these species. The program is currently focusing on developing tissue culture systems.

Both American and Siberian elms have been micropropagated and regenerated from leaf tissues of in vitro germinated seedlings. The best proliferation system to culture shoots in MS medium with 1 μM BA solidified with gelrite and to transfer weekly. The best regeneration media were MS media with 10-20 μM BA without auxin. Transformation of both elms have been attempted with *Agrobacterium* system and the biolistic process. Kanamycin resistant calli with β-glucuronidase activity was obtained with both systems. Further optimization of these systems is under progress to regenerate transgenic plants.

Shoots have been regenerated from cotyledons, young leaf tissues using elm regeneration media without auxin. Shoots have also been regenerated from cell suspension culture in MS medium with 17.5 μM BA and 5 μM IBA. Rooting from these regenerated shoots have proven very difficult.

A simple and efficient micropropagation system has been developed for an elite Asian white birch clones, *B. platyphylla*, selected at North Dakota State University. Morphology and rooting capability showed typical transition between juvenile and mature phases. Plant regeneration has also been obtained in Woody Plant Medium with 20 μM BA. An initial 2-week dark treatment was essential for shoot induction. Kanamycin resistant shoots have been obtained with *Agrobacterium* infection. Confirmation of transformation is under progress.

The main objective for ponderosa pine is to develop a micropropagation system to produce clonal pines for a gene-for-gene study (in cooperation with Dr. C. Schumann of USDA-FS in Lincoln, NE). Shoots have been regenerated from mature embryos in the 3/4MS medium with 10 μM BA, and been elongated in 1/2MS medium without hormones. Roots formed, at 5-10%, in medium with 5.0 μM either IAA or IBA.

USING MOLECULAR BIOLOGY TO STUDY FUSIFORM RUST

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Fusiform rust is the most costly tree disease in the southern United States. It is characterized by the formation of galls on loblolly (*Pinus taeda* L.) and slash pines (*Pinus elliottii* Engelm. var. *elliottii*) and is caused by the rust fungus *Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme*. Despite the economic significance of fusiform rust, the pathogenic mechanisms underlying this disease remain poorly understood. In recent years, molecular biology has contributed significantly to our understanding of the interactions between other plant pathogens and their hosts. Our long-term goal is to apply a variety of molecular biological techniques to the study of fusiform rust, and thus improve our understanding of this costly tree disease.

Our immediate objective is to develop several experimental tools for the analysis of fusiform rust. Current work focuses on the identification of an appropriate CHEF protocol for the separation of *C. q. fusiforme* chromosomes, the development of a biolistic transformation system for *C. q. fusiforme*, the construction of a *C. q. fusiforme* genomic library in pMOcosX, and the characterization of urediniospore and basidiospore germination behavior on different hosts. Progress towards these goals will be presented, as will approaches for the analysis of gall induction in pines.

EFFECTS OF CONDITIONED MEDIA FROM EMBRYOGENIC SUSPENSION CULTURES DERIVED FROM JUVENILE MATERIAL ON NON-EMBRYOGENIC SUSPENSION CULTURES OF *LARIX* X *EUROLEPIS* (HENRY) DERIVED FROM MATURE MATERIAL

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The effects of conditioned media from embryogenic suspension cultures were tested on non-embryogenic suspension cultures of hybrid larch (*Larix* x *eurolepis* Henry). The non-embryogenic suspension cultures were established with calli derived from short shoot buds of cuttings from an eight year old tree, while the embryogenic suspension cultures were established with calli from immature larch embryos. The suspensions were cultured in modified MS media (MSG) containing 11.0 mgl^{-1} 2,4-D and 0.5 mgl^{-1} BAP. Conditioned media was collected from the embryogenic suspension cultures on the seventh day of culture, and then filtered to eliminate any cells or cell debris. Non-embryogenic suspension cultures were subcultured weekly for 4 weeks with various concentrations of conditioned media (0, 20, 40, 60, 80 and 100%; made up to volume with fresh media). Conditioned media had a negative effect on the growth rate of the non-embryogenic suspension cultures, and growth rates gradually diminished as the concentration of conditioned media increased. The suspensions with 100% conditioned media were barely able to sustain themselves. In the suspension cultures containing 0% conditioned media, most of the cells were organized into micro-calli with few small clusters of cells. As the percentage of conditioned media increased, the number and size of the micro-calli diminished, and the number of single cells and small cell clusters increased. The suspension cultures with 0% conditioned media contained a few small cell clusters which appeared similar to early stages of embryogenic development. The presence of conditioned media appeared to increase the number of cell clusters which resembled early stages of embryogenic development.

OPTIMIZATION OF ELM SUSPENSION CULTURES FOR ELICITOR/PHYTOALEXIN EXPERIMENTS

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Suspension cultures of five elm selections (*U. americana* A, 680, 8630, Del 2 and *U. pumila* S.) exhibiting a range of susceptibility responses to the Dutch Elm Disease fungus (*(Ophiostoma ulmi*) have been successfully established for future elicitor/phytoalexin studies. Calli were initiated from foliar tissues of greenhouse-grown plants cultured on a solid modified MS medium containing either NAA and BA (1 mg /l each) or IAA and BA (1 mg/l each). Minute clumps of calli (10-25 mg) were placed in 1 ml of liquid media (described above) in 22 mm well culture plates. After 3 weeks, each 1 ml suspension was added to 3 ml of liquid media in 35 mm well culture plates. After 1 week, each 4 ml suspension was transferred to a 125 ml baffled culture flask containing 30 ml of media. Cells were grown in either the presence or absence of light with continuous agitation.

The maintenance of uniform, stable and rapidly dividing cells through repeated subculturing was highly dependent on the quality of initial calli. White or tan friable calli produced from young, expanding leaves grown on NAA and BA in darkness yielded optimal cultures. Calli cultured on IAA and/or in light exhibited increased phenolic production and resulted in decreased cell uniformity and proliferation.

Growth curves of the five selections varied as determined by cell counts, conductivity measurements of the media and visible light absorbance at 700 nm. Maintenance of culture quality was highly influenced by punctual subculturing at the late log phase. Cells in the stationary phase produced increased phenolics and subcultures of these cells did not result in successful, sustainable suspensions.

TRANSGENIC TOBACCO PLANTS WITH ALTERED LIGNIN COMPOSITION HAVE IMPROVED PULPING CHARACTERISTICS

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The composition of lignin in tobacco, a model woody plant, has been altered by genetic engineering. Antisense expression of sequences encoding cinnamyl alcohol dehydrogenase (CAD), the enzyme catalysing the final step in lignin precursor synthesis, leads to the production of a modified lignin in otherwise normal plants. While the total lignin content is apparently unchanged in CAD antisense plants, a number of qualitative changes have been identified. Consistent with a block in CAD activity, decreased levels of cinnamyl alcohols and increased levels of cinnamyl aldehydes are found in the lignin of antisense plants. The structure of the lignin is also altered, making it more susceptible to chemical extraction by alkali and thioglycolic acid. These changes may have utility to the pulp and papermaking industry. Simulated kraft pulping experiments on wild-type and CAD-antisense tobacco plants show an improved selective delignification in the antisense plant pulp. This improves both the yield of antisense pulp and the amount of chemical required for subsequent bleaching. Down-regulation of CAD activity by expression of antisense RNA may therefore present a mechanism for the genetic improvement of trees used for papermaking.

ADVENTITIOUS SHOOT FORMATION FROM BROADLEAVED TEMPERATE FOREST TREES

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Transformation is becoming an effective tool not only for practical plant improvement, but also for investigating gene function in relation to plant development. Shoot regeneration is an essential prerequisite to transformation and also for producing somatic hybrid plants following protoplast fusion. There are still few tree species for which regeneration and transformation methods are now routine. Based upon our success in producing, by shoot micropropagation, leaves for use as explants, we have embarked upon the development of shoot regeneration methods for *Prunus avium* L. (wild cherry) and *P. padus* L. (bird cherry) distributed through Eurasia and N. Africa, *P. serotina* Ehrh. (black cherry) from Eastern N. America and *Fraxinus excelsior* L. (common ash) found in Europe, N.Africa and W.Asia.

Wild cherry leaves produced shoots on media containing relatively high concentrations of benzyladenine (BA) or 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea (thidiazuron, TDZ) and low concentrations of 1-naphthaleneacetic acid (NAA), regeneration occurring most often on media containing TDZ rather than BA. Regeneration was further enhanced by using the salts and organics of woody plant medium (WPM) rather than those of Murashige and Skoog (MS) or Driver and Kuniyuki walnut (DKW) media. If the concentration of NAA was high in relation to that of BA, roots instead of shoots were produced. In individual experiments, regeneration was achieved, at best, from 35% of leaves, with a maximum of three independent regeneration events per explant. During a four month period, when shoot production by the donor shoot cultures was reduced on micropropagation medium, regeneration from the leaves was also lower; however, regeneration was restored to its previous mean frequency following addition of silver nitrate to the medium.

Unlike *P. avium*, leaves of a mature fastigiate form of *Prunus padus* did not require TDZ to induce efficient shoot formation, a high proportion (79%) of leaves producing shoots on media containing BA. Regeneration was significantly enhanced by adding NAA to the medium, using DKW instead of MS medium, and sucrose rather than glucose or fructose.

Incorporation of 1mM of 1,3,5-trihydroxybenzene (phloroglucinol) to regeneration media strongly inhibited callus and shoot formation from leaves of both *P. avium* and *P. padus*, in direct contrast to its beneficial effects on root formation by shoots of these species.

P. serotina leaves placed onto WPM with TDZ and NAA also readily produced adventitious shoots, the frequency of regeneration being dependent upon the genotype used. The most responsive clone originated from a mature tree from a mountainside in West Virginia, 70% of leaves producing shoots. Shoots were usually produced from the cut surfaces of the explants; however, in some explants, shoots also developed along the full length of the midrib.

Juvenile hypocotyls and cotyledons of *F. excelsior* produced shoots on MS medium, shoots developing more frequently on medium with TDZ rather than BA. Shoots have also been obtained from the cut surfaces of isolated petioles from mature ash clones. Regeneration from petioles was stimulated by culture on WPM rather than MS or DKW media. Supplementing WPM with NH_4NO_3 suppressed shoot initiation. Whole leaves from micropropagated shoots of both juvenile and mature ash clones also produced shoots on medium with TDZ, these developing from the axils of the leaflets rather than from the cut surfaces of the petioles.

In all four species, adventitious shoots excised from the initial explants, could be micropropagated and rooted. Based on these successes, we are proceeding to develop transformation and somatic hybridisation techniques for these species.

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PHENOTYPIC VARIATION IN THE EMBRYOGENIC CULTURE #2086 OF *LARIX X EUROLEPIS*

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The production of thousands of somatic embryos from one zygotic embryo can alleviate the shortage of plant material for reforestation resulting from low seed set in species such as larch, including the hybrid *Larix x eurolepis*. One embryogenic culture line of *L. eurolepis*, #2086, is unique in that it requires neither plant growth regulators nor osmoticum treatments to initiate and mature somatic embryos. This feature enables the study of somatic embryo development without manipulation. The developmental pathways of #2086 have been traced from the embryonal mass stage to over 10 developmental fates. The ability to produce normal roots and shoots is preserved even in some embryos which initially display morphological abnormalities. Embryonal masses and suspensors in this embryogenic tissue can be categorized into six groups according to the morphology of the suspensor (long, short, compact loose) and the embryonal mass (smooth or rough surface). These six categories of embryonal masses plus suspensors may explain the differing developmental fates of the somatic embryos and the low rate of maturation from embryonal mass to mature somatic embryo.

BIOLOGICAL CONTROL OF WHITE GRUBS BY GENETICALLY-ENGINEERED ECTOMYCORRHIZAL FUNGI

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A novel strategy of utilizing genetically-engineered ectomycorrhizal fungi as biological control agents against insect pests is being pursued. Many trees are attacked by insect pests both in the soil and in aerial parts. Because of harmful effects associated with chemical insecticides, there is a great need for developing new environmentally-safe biological control mechanisms. In addition, it is desirable that the control agents are specific for each pest to maintain a healthy forest without affecting the ecosystem.

The larvae of June beetles of the genus *Phyllophaga* (Coleoptera: Scarabaedae), commonly known as white grubs, are widespread in North America and other parts of the world. While generally considered to be pests of field crops and turf, they can become major pests of conifer seedlings, including several economically important species of pines and firs. The larvae irreversibly damage roots of conifers resulting in inadequate nutrient uptake, which predisposes seedlings to other fungal pathogens such as species of *Fusarium*. The use of chemical insecticides to control the white grub population has proven costly and environmentally unsafe.

Nearly all conifer species are known to establish mutually beneficial interactions with ectomycorrhizal fungi. A particle gun-mediated technique was developed to transform *Laccaria bicolor*, an aggressive colonizer of red pine roots. The method was used for simultaneous introduction of the hygromycin resistance, GUS (beta glucuronidase), and BtCryIIIA (coleopteran-specific bacterial insecticidal protein) genes into fungal genome. The presence and expression of introduced genes was confirmed by Southern and Western analyses. Transformants were shown to form mycorrhizae on roots of red pine seedlings.

Bioassays are being carried out to test the effectiveness of the recombinant fungus against white grubs. Since the transformants express BtCryIIIA and form a mantle around roots, it is expected that larvae will not damage the roots. An important advantage of using engineered mycorrhizae is that the insecticide is localized only to the specific area where it is required. The system can be further improved by expressing the BtCryIIIA gene through symbiosis-associated gene promoters. The transformation and use of genetically-engineered ectomycorrhizal fungi as biological control agents could have several potential applications in biocontrol of other insect and fungal pests associated with forest trees.

REJUVENATION OF TREES BY SHOOT-TIP GRAFTING IN VITRO AND EVIDENCE SUGGESTIVE OF A MITOCHONDRIAL DNA BASIS

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Maturation of woody trees is usually accompanied by decline of organogenic competence and vigor. Thus, tissue cultures that must undergo plant regeneration, e.g., in genetic engineering, have relied on embryo and immature seedlings for explants. Fortunately, the developmental phase of shoot apical meristems is frequently reversible, enabling emergence of rejuvenated shoots. Using coastal redwood, *Sequoia sempervirens*, as model we have developed a rejuvenation protocol that involves repeated grafting of adult shoot tip onto juvenile rootstocks in vitro. The rejuvenation, evident as restored rooting competence and vigor, was sustainable indefinitely in vitro. Analyses of restriction fragments by DNA cloning, double restriction, cross hybridization, and sequencing disclosed distinct nucleotide sequences between juvenile and adult shoots; rejuvenated shoots displayed sequences identical to juvenile. The graft transmissibility and sustainability are consistent with a hypothesis that phase change is regulated by mtDNA.

GENETIC DIVERSITY IN AMERICAN ELMS (*ULMUS AMERICANA* L.)

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American elms (*Ulmus americana* L.) have been under extensive selection pressure during the latter half of the twentieth century following the accidental introduction of both the fungus which causes Dutch elm disease (DED), *Ophiostoma ulmi*, and the smaller European bark beetle *Scolytus multistriatus*, an insect vector which causes infection while feeding on healthy elm trees. Screening programs to determine responses to artificial inoculation utilized surviving elms and were based on the idea that these genotypes may have escaped DED infection because of natural resistance. The ARS - National Arboretum screening programs have identified several individuals with some level of resistance to the disease.

The sample population for this study included rooted cuttings from individual elms which appear to possess limited resistance to *O. ulmi*, cuttings from DED-susceptible elms, and randomly collected seedlings which have not been assayed for DED-resistance. Leaves were taken from greenhouse or field grown rooted cuttings, greenhouse grown seedlings, or were randomly collected from wild seedlings. We have attempted to estimate the extent of genetic differences among selected and non-selected individual American elms by contrasting isozyme markers in total leaf proteins and by contrasting genomic DNA sequences using two different DNA fingerprinting strategies, RFLPs and PCR-RAPDs. We have used the number of detectable polymorphisms as a measure of the level of diversity within the species, and have compared the diversity estimates obtained by the three approaches. Even with a limited sample size, a significant number of genetic polymorphisms have been identified indicating that there is a relatively high level of genetic diversity in the tetraploid *U. americana*.

By conducting controlled breeding experiments using the DED-resistant cuttings as parents, we hope to assess the level of DED-resistance in progeny trees and identify co-segregating polymorphic DNA and/or isozyme markers. Recognizable molecular markers which are closely linked to genetic loci which code for fungus resistance would greatly assist tree breeding efforts. Several crosses have been completed and progeny plants propagated in the greenhouse. Such genetic studies will also allow us to determine several genetic characteristics of the American elm, including the level of heterozygocity within the species.

INFLUENCE OF CARBON SOURCE IN THE MEDIUM ON GROWTH OF EMBRYOGENIC TISSUE CULTURES OF *PINUS STROBUS* L.

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Embryogenic tissue cultures of *Pinus strobus* L. were established using female gametophytes, approximately 25 days after fertilization, as explants. A DCR basal medium supplemented with 50mg/l glutamine, 500 mg/l casein hydrolysate, 3% sucrose, 2mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), and 1 mg/l benzyl adenine (BA) was used for induction of embryogenic tissue proliferation. Isolated embryogenic tissue lines were maintained on the induction medium by subculturing every three to four weeks. Influence of carbon source on growth of and somatic embryo development in three selected lines was investigated. Tissue was put on DCR basal media supplemented with 50mg/l glutamine, 500 mg/l casein hydrolysate, 2mg/l 2,4-D, 1 mg/l BA, and 1 to 6% glucose, fructose, galactose, sucrose, maltose, cellobiose, or lactose. Increase in dry weight of tissue and the extent of somatic embryo development were determined after four weeks of culture. Maltose was found to be the best carbon source.

MICROPROPAGATION AND REGENERATION SYSTEMS FOR GREEN ASH (*FRAXINUS PENNSYLVANICA* MARSH.)

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Green ash (*Fraxinus pennsylvanica* Marsh.) trees provide environmental protection in the form of wind breaks and watershed buffers in urban and agricultural ecosystems of the Great Plains. Since this species survives and grows well on widely ranging sites, some green ash genotypes may have adaptations that confer tolerance to stressful environments. However, the genetic bases of pest and stress resistance are poorly understood. Mechanistic studies of stress and pest resistance in green ash are hampered because the species is difficult to propagate vegetatively. Because these problems can potentially be addressed with *in vitro* and molecular techniques, it is desirable to develop tissue culture and other biotechnological methods for green ash. Toward this goal, green ash seed sources from different geographic areas within the western range of this species are being used to develop micropropagation and regeneration systems. Mature seeds from seven sources of green ash were cultured on agar-solified Murashige and Skoog (MS) medium. Axillary shoot proliferation from seedlings was best on MS medium supplemented with a combination of 10 μM thidiazuron (TDZ) and 10 μM benzyladenine (BA). Regeneration of adventitious shoots from hypocotyl derived callus was effective using treatments of 20 μM TDZ or 10 μM TDZ with 10 μM BA. Microshoots (<10mm) originating from seedling explants were elongated by adding a combination of 0.5 μM indole-3-butyric acid (IBA), 5 μM TDZ, and 5 μM BA. Optimal rooting of axillary shoots occurred using half-strength MS medium supplemented with 2 or 5 μM IBA. Continued development of micropropagation and regeneration systems for green ash should supplement conventional propagation methods and supply plant material for molecular biological studies of pest and stress resistance.

DEVELOPMENT OF PCR-BASED ASSAYS TO DETECT *CERATOCYSTIS FAGACEARUM* AT THE SPECIES AND STRAIN LEVELS

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Oak wilt disease is caused by the fungal pathogen *Ceratocystis fagacearum* (Bretz) Hunt. To monitor spreading populations of the oak wilt fungus and investigate epidemiology of the disease, it is necessary to distinguish between isolates (strains) of the fungus. In addition, a sensitive species-specific assay is needed to study potential vectors of the pathogen and detect early tree infections.

The ability of the polymerase chain reaction (PCR) to amplify minute quantities of selected DNAs from crude preparations makes PCR a useful tool for diagnostic assays. By using arbitrary 10-mer primers, randomly amplified polymorphic DNAs (RAPDs) can be assessed. However, each amplification may detect several loci with possibly only one being polymorphic and of interest as a distinguishing marker. Further, the RAPD technique is sensitive to slight changes in reaction conditions and can be difficult to reproduce. To overcome these problems, the RAPD markers of interest will be cloned and sequenced. Sequence-specific primer pairs then will be designed to reliably amplify a single polymorphic locus that is unique to each fungal strain.

Eight fungal strains (seven from five counties in Texas and one from Ohio) were tested with 144 RAPD primers. The results indicated that the strains were almost entirely monomorphic. One primer identified two subgroups among the strains and another primer appeared to distinguish individual strains. The polymorphic bands generated by these primers are being cloned and sequenced for use in developing a strain-specific PCR assay.

A second PCR-based assay is being developed to detect the fungus in or on insects to evaluate their potential role as vectors. This assay will take advantage

of sequence information on the large subunit ribosomal DNA gene (LSrDNA) of *C. fagacearum* and many other Ascomycetes (Hausner et al., Can. J. Bot. 71:52-63, 1993). The species *C. fagacearum* differs from other members of the genus *Ceratocystis* by a single nucleotide in this highly conserved region. Only *C. fagacearum* LSrDNA will be amplified utilizing a primer that contains this specific nucleotide at the 3' position. The specificity of the amplified band can be confirmed through restriction analysis, providing a powerful diagnostic technique for detection of the oak wilt fungus.

CAN AUXIN-INDUCED GENE EXPRESSION BE CORRELATED TO COMPETENCE FOR ADVENTITIOUS ROOT INITIATION IN *NICOTIANA TABACUM* V. XANTHII?

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A major obstacle to the implementation of efficient cloning systems which could be used to enhance forest tree breeding is the incompetence of cuttings from the mature developmental phase of most tree species for adventitious root initiation. Cutting-based tree breeding programs are expensive and time-consuming because juvenile phase rooting-competent cuttings must be planted and allowed to mature before most desirable phenotypes can be evaluated. Presently, clonal propagation by cuttings of select genotypes at maturity is not feasible for most economically important forest tree species.

Past research on plant developmental anatomy and endogenous growth substances has not provided conclusive evidence for the basis for the competence for adventitious root initiation in cuttings. We developed an *in vitro* experimental system with tobacco in order to examine molecular mechanisms underlying the competence to initiate adventitious roots from cuttings. Our system consists of adventitious rooting-competent wild type tobacco and adventitious rooting-incompetent, monogenic auxin-resistant mutant individuals that were isolated from the wild type via UV mutagenesis by M. Caboche and colleagues. Phloem parenchyma or inner cortical parenchyma cells in mutant stem cuttings callus in response to exogenous IBA treatment but do not undergo adventitious root morphogenesis as do analogous cell types in IBA-treated wild type cuttings. The callus response to auxin but lack of root morphogenesis in mutant tissue is similar to the auxin response in mature developmental phase cuttings of many tree species. In this poster, we demonstrate how RNA blot and histochemical analyses can be used with our system to identify genes whose auxin-induced expression may be correlated with competence for adventitious root initiation.

A LINKAGE MAP FOR SUGI (*Cryptomeria japonica*) BASED ON RFLP, RAPD AND ISOZYME LOCI

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We have constructed a linkage map for sugi (*Cryptomeria japonica* D.Don) based on restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and isozyme loci using three-generation pedigree prepared for genetic analysis of the heartwood color. One hundred and twenty-eight RFLP (123 cDNA and five genomic probes), 33 RAPD, two isozyme and one morphological (dwarf) loci were segregated in 73 progenies. Of the 165 segregating loci, 147 loci were distributed into 20 linkage groups. Ninety-one of them with confirmed map position were assigned to 13 linkage groups, covering a total of 887.3 cM. Clustering of the markers with distorted segregation were observed in six linkage groups. While distortions with the reducing number of homozygotes from female parent were found in three clusters, the reducing number of homozygotes from male parent was found only in one cluster.

As a first step for the assignment of linkage groups to specific chromosomes, a panel of the trisomics DNAs were hybridized with the RFLP markers specific for each of the 13 assigned linkage groups and the relative intensities of the hybridization signals were compared. Three linkage groups were assigned to the extra chromosomes of the trisomics.

HISTOLOGY OF CONIFER EXPLANTS RESPONSIVE TO EMBRYOGENESIS *IN-VITRO*

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Primary explant types, which produce somatic embryos in conifers, are restricted mostly to juvenile tissues such as precotyledonary zygotic embryos, and rarely to cotyledonary embryos and seedling explants (from 7-30 day old seedlings). Explants from mature trees, when cultured, have not demonstrated embryogenic potential. Whether embryogenic potential exists in meristematic tissue types in the mature tree, such as cambium, vegetative and reproductive buds, young female cones (at the time of meiosis of the megaspore mother cell) and prophylls besides apical meristem, remains to be investigated.

In the present study, we have histologically characterized different tissue types in conifer explants that produced somatic embryos *in vitro*. Both whole mounts and microtome sections (10 μ thin) of the responsive explants were stained with nuclear and cytoplasmic specific stains and examined under confocal phase contrast microscope. A comparison of these tissue types with those from mature explants was made to characterize anatomical similarity between juvenile and mature tissues.

CHROMOSOME ANALYSIS AND DNA HOMOLOGY IN GENUS *LARIX* USING EMBRYOGENIC CULTURES.

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The effects of different concentrations of hydroxyurea (HU) and amphidicolin (APH) on mitotic index (MI) were compared in embryogenic suspensions of *Larix decidua, L. leptolepis* and *Larix* X *eurolepis*. The highest increase of MI was obtained with HU at 1.25 mM and 0.6% colchicine. A detailed karyotype analysis was made on the somatic complement of *L. decidua, L. leptolepis* and their hybrid. The karyotypes are generally asymmetrical with the smaller chromosomes being more submedian than the larger ones. The two species have three pairs of chromosomes with secondary constrictions. The topography of chromosome 7 of *L. decidua* and chromosome 9 of *L. leptolepis* was found to be the most significant cytotaxonomic characteristic. The hybrid larch that we analyzed was cytologically stable. Genomic *L. decidua* probe hybridized strongly to dots of DNA from *L. leptolepis* indicating that there is a high sequence homology between these two species.

HEAT MEDIATED BRANCHING IN *IN VITRO*-GROWN *HANCORNIA SPECIOSA*

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Hancornia speciosa fruit is processed in small scale by juice and ice cream industries in Brazil. The fruit also has potential to be used as a natural flavonoid source for the tobacco industry.

Seeds of *H. speciosa* rapidly lose viability after harvest and attempts to induce rooting in cuttings have not been successful. These characteristics limit the propagule supply and consequently the crop acreage and fruit availability. A micropropagation technique for *H. speciosa* has been developed (Pereira-Netto, 1991), however multiplication rates (MRs) are not adequate for commercial purposes.

Axillary branch proliferation has been shown to be a powerful tool for MR enhancement in many species. Nevertheless, *H. speciosa* has strong apical dominance and does not respond to cytokinins for axillary bud stimulation, even in the presence of 10µM TDZ. However, during growth temperature curve analysis, it was observed that an increase in temperature from 25 to 31°C induced a 6 times increase in MR, while temperatures between 33 and 35°C induced a 6 times increase in branching (number of lateral branches measuring 1 cm long or more). Significant (11 times) drop in MR was found when the temperature changed from 35 to 37°C. At the temperature of 39°C, both, MR and branching were zero. Callus production was stimulated by an increase in temperature from 25 to 33°C, while a further increase in temperature induced a steep decline on callus production. Growth of the heat-released axillary buds may be a promising way to effectively enhance the *H. speciosa* multiplication rate *in vitro*.

TRANSGENIC POPLARS EXPRESSING A CYSTEINE PROTEINASE INHIBITOR ARE TOXIC TO COLEOPTEROUS

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In France, two Coleopterous, *Chrysomela populi* and *Chrysomela tremulae* that feed on poplar leaves, are responsible for severe damages in poplar plantation. Analysis of proteolytic activity in the gut of *Chrysomela tremulae* larvae show that cysteine proteinases are the major digestive enzymes. In in vitro test, this proteolytic activity is inhibited by the addition of the rice proteinase inbitor OCI. Therefore, we introduced the OCI coding sequence fused to a strong constitutive promoter into a hybrid poplar clone *Populus tremula* x *P. tremuloides* very sensitive to *Chrysomela* attack. Transgenic poplars were assessed for their ability to express the active form of OCI and for their toxicity against *C. tremulae*. OCI protein was detected in transgenic poplars by Western blot analysis. Three of them, exhibiting high level of OCi protein, were used as feeding material for in vitro insect assays. When ingested, foliage from these three poplars slows down larvae growth and development and increases the percentage of mortality when compared to what happens with foliage from control trees: the average mortality rises from 4,5% with control tree up to 40% for transgenic foliage. These results give good evidence for the use of cysteine proteinase inhibitor to limit insect attacks in poplar plantation.

A NEW METHOD FOR HANDLING EMBRYOGENIC TISSUE OF WHITE SPRUCE

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Optimization of embryo production techniques is required for commercialization of somatic embryogenesis in white spruce. The following technique is an easy method for handling of embryogenic tissue on solid media. Embryogenic tissue is placed on pieces of sterilized Nitex mesh and squashed into the mesh using the back of a spoon. This produces a flat mat of tissue with uniform contact with the media. Transfer of the tissue to subsequent media can easily be done by transferring the whole mesh to the new media. Flattening the tissue by this method does not appear to have a detrimental effect and in some clones may even be beneficial.

This technique has potential applications for mass production of embryos. Increasing the size of the initial inoculum creates a large surface area (whole petri dish or greater) capable of growing hundreds of somatic embryos. Since the mesh is easily transferred, a short regrowth period on maintenance media is readily arranged. The mature embryos are produced in a uniform mat making them easier to pick. We have also found that we can use stainless steel to replace Nitex to make a reusable carrier, thus saving costs.

PLANTING STOCK QUALITY: LOBLOLLY PINE SOMATIC EMBRYO PLANTS AND SEEDLINGS

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Somatic embryos plants and seedlings from three controlled-cross loblolly pine families were raised for a fall planted field trial comparing growth traits. Somatic embryos were germinated in mid May on solid germination medium. Plantlets were then transferred to Ray-Leach tubes in late June and maintained under greenhouse mist treatments for four weeks. Following the mist treatments, plants were gradually acclimated to full-sun conditions. Survival during the greenhouse acclimation phase averaged 75 percent across all lines.

Two seedling populations, large and small, were raised to bracket somatic embryo plant size. Stratified seeds were sown in late July (large seedlings) and mid August (small seedlings). Seedlings were grown in the greenhouse until they exhibited two-to-three cm of epicotyl elongation, when they were transferred to full-sun conditions.

In late October 120 trees of each plant type (large seedlings, somatic embryo plants, small seedlings) were destructively harvested. Measurements of shoot length as well as shoot and root dry weights were taken. Somatic embryo plants had well structured root systems with shoot/root dry matter ratios similar to the seedlings: 2.7 for small seedlings, 2.4 for somatic embryo plants, and 2.1 for large seedlings. Somatic embryo plants could be characterized as short and heavy. In height they were similar to the small seedlings while their total dry weights were similar to the large seedlings. All somatic embryo plants were smaller than optimum indicating that a more appropriate time to germinate somatic embryos for a fall field planting would be mid April instead of mid May.

OPTIMIZATION OF *IN VITRO* ROOTING OF MATURE *Q. ROBUR*: EVALUATION OF THE ROOT SYSTEM BY IMAGE ANALYSIS*

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The efficiency to micropropagate recalcitrant forest tree species, such as *Q. robur*, is limited by the loss of *in vitro* morphogenetic competence, such as rooting ability of the mature material. The goal of this research was to improve the *in vitro* rooting efficiency of shoots derived from mature oak trees. Using video image analysis techniques, we measured the area and perimeter of the root system, giving us a better quantification of the root system development.

Six *Q. robur* clones were previously established *in vitro* from shoots originated by forced flushing of branch segments collected from the crown of 70-300 year-old trees. Shoot multiplication stage was achieved by culturing decapitated shoots placed horizontally on the multiplication medium (Plant, Cell, Organ and Tissue Culture,1994 in press). Preliminary rooting experiments showed that an initial five-day dark period improved the rootability of IBA (3 mgl^{-1},7 days) treated shoots. However, darkness produced shoot necrosis, negatively affecting the survival of the regenerate plantlets. Alternatively, shoots were treated with IBA containing medium (5-50 mgl^{-1}) for 24h and then they were transferred to an auxin free medium with 1% activated charcoal (AC). The root systems developed in medium with or without AC were evaluated by image analysis. A basal darkness treatment was also performed in order to test whether the effect of AC on rooting was either due only to darkness or to absorbing inhibitory components. Better results were achieved in AC containing medium. With this method, shoot quality was preserved, and an acceptable rooting percentage with promotion of the root system was obtained.

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EFFECT OF ELEVATED CO2 ON COLD HARDENING AND DEHARDENING OF RADIATA PINE, PONDEROSA PINE, AND DOUGLAS-FIR.

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Timely development of adequate cold hardiness in autumn and avoidance of its premature loss in late winter is important to survival of woody perennials. Few studies have explored the impact of elevated atmospheric CO2 on this process, but indications are that cold hardening might be retarded. We grew radiata pine, ponderosa pine, and Douglas-fir to useable size in a greenhouse, then gave them simulated autumn, winter, and spring conditions in controlled environments under ambient (ca. 350 ppm) or elevated (700 ppm) CO2. Cold hardiness was measured weekly by freeze induced electrolyte leakage from foliage and at less regular intervals by a whole plant freeze test. The two tests gave comparable answers. First order results show little or no difference in cold hardiness between CO2 levels. However, all three species showed periods of one to two weeks when there were significant differences. The greatest difference was during cold hardening of ponderosa pine under an 8 hour day at 10°C and 3°C night, when it appeared to harden faster under 700 ppm CO2.

USE OF FORCING SOLUTION AND *IN VITRO* TECHNIQUES TO RESCUE CHESTNUT (*CASTANEA* SPECIES)

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Chestnut is difficult to propagate *in vitro*. The timing for harvesting of explant materials is critical, although many factors need to be considered for successful micropropagation. Previous research with spirea and 5-leaf aralia demonstrated that the forcing solution techniques extended the availability of high quality explant material, thus expediting micropropagation. However, preliminary research illustrated that chestnut is very difficult to force and the new forced softwood growth is very short-lived. This made micropropagation even more difficult. It was found that at about 7 days from bud break, the forced chestnut softwood growth (about 2 cm long) served as the best explant material. If longer than this timing window, the new growth would die off. If shorter, the explants had a high contamination rate and phenolic exudation, and explants would not regenerate. Shoot proliferation and callus regeneration were achieved by culturing good quality explants on woody plant medium supplemented with 0.1 mg BA per liter. The new shoots grew vigorously *in vitro* with apparent normal morphology.

IN VITRO SHOOT MULTIPLICATION AND REGENERATION FROM REJUVENATED PROPAGULES OF MATURE PECAN

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Superior pecan (Carya illinoensis) trees are generally identified at the mature stage of development. In some cases trees are very old, which makes vegetative propagation difficult. Effective methods are needed to multiply selected individual trees, which are old but showing desired characteristics. Micropropagation of mature pecan was investigated using rejuvenated materials. Rejuvenation techniques were applied to adult (10-year-old or older) trees by cross-cutting at the trunk or wounding the trunk by a shallow cut. Juvenile sprouts developed at the stump or the base of the trunk within the following growth season. Stump sprouts were collected and used as micropropagules for the *in vitro* experiment. After surface-sterilization with 15% bleach, nodal cuttings were placed on a shoot regeneration medium modified from woody plant medium. Shoots initially developed from axillary buds within 2-4 weeks of *in vitro* culture. Multiple shoots then regenerated from single-node explants during subcultures. Roots were induced from elongated shoots when cultured on a medium containing indolebutyric acid (10 mg/l). The average of multiplication rate and performance of the *in vitro*-derived regenerants from the rejuvenated sprouts were the same as those developed from young seedling-derived material. Therefore, this improved system can serve as an alternative method of pecan regeneration and is specially useful for clonal multiplication of elite trees once they are identified.

INVITED SPEAKERS

MANUSCRIPTS

THE ROLE OF CARBOHYDRATE METABOLISM IN LIGNINOLYSIS

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Introduction

Extracellular enzymes of the lignin-degrading system of *Phanerochaete chrysosporium* include lignin peroxidase (LiP), manganese peroxidase (MnP) and glyoxal oxidase (GLOX). Peroxide generated by GLOX is an oxidant required in the peroxidase catalytic cycles. Subsequent to the discovery of GLOX, the metabolites glyoxal and methylglyoxal were found to be produced by cultures of *Phanerochaete* during secondary metabolism, thus the name glyoxal oxidase. The derivation of these metabolites from the carbohydrate growth substrate is not known and can not be readily explained from established pathways of intracellular metabolism. Our recent efforts to elucidate the metabolic pathway for the conversion of wood-derived carbohydrates have identified additional substrates for GLOX. Importantly, further metabolism of these substrates may directly involve catalysis by the extracellular peroxidases. Literature on the chemistry of these compounds shows them to be reactive, susceptible to autoxidation with the generation of active oxygen species, and in some cases, to support oxidative reactions with peroxidases without exogenous peroxide. This suggests that the peroxidases of *P. chrysosporium*, studied almost exclusively in the context of lignin degradation, may also have an important role in the breakdown of carbohydrate-derived metabolites.

Peroxide Production

The discovery of GLOX began with the assumption that if glucose or xylose were not the substrates for the extracellular reduction of O_2 to H_2O_2, then intermediary metabolites derived from the carbohydrates might be the substrates (7). A wide assortment of compounds were tested and a number of aldehydes and alpha-hydroxycarbonyl compounds were found to be substrates (Figure 1). The non-specificity of the oxidase was unexpected and put into question which of the

known substrates were physiologically important, if any. In the search for a physiological substrate, analytical techniques were developed to test for substrates in culture (7). Time-course studies indicated that glyoxal and methylglyoxal were present during secondary metabolism of *Phanerochaete*. However, because of the non-specificity of the enzyme, the possibility of a series of physiological substrates could not be excluded.

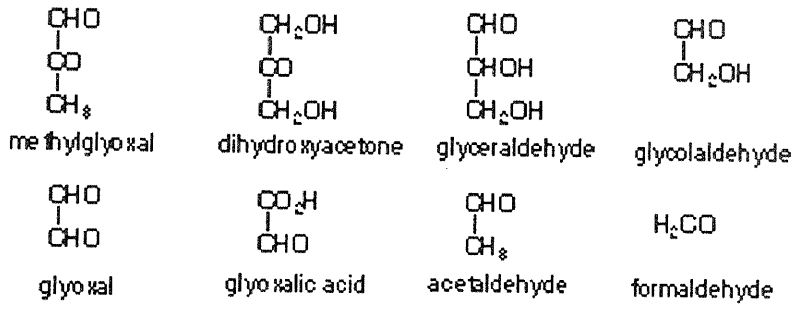

Figure 1. Glyoxal oxidase substrates (6, 7).

Kinetic analysis of GLOX was used to determine if a substrate was likely to play a physiological role (6). Accordingly, the reaction conditions were designed to mimic ligninolytic cultures; GLOX activity was coupled to the oxidation of veratryl alcohol with lignin peroxidase at pH 4.5. Of the substrates tested, the best is the extracellular metabolite methylglyoxal with a K_m of 0.64 mM and an apparent k_{cat} of 198 s^{-1} under air-saturated conditions. However, there is little variation in the k_{cat} values for the various substrates and the efficiency of catalysis appears to be principly governed by the K_m values. Because the concentration of detected metabolites in culture (glyoxal and methylglyoxal) is considerably lower that the corresponding K_m values, the rates of oxidation would be roughly proportional to the concentration levels in culture. Using published kinetic parameters for GLOX and concentration values of metabolites in culture, analysis does not clearly favor methylglyoxal over glyoxal as being a dominant source of peroxide. This is because considerably more glyoxal is found in the experimental cultures.

Two-carbon Fragment Metabolism

Another approach to identify physiologically important metabolites is to establish the metabolic pathway in a series of enzymatically-catalyzed reactions. Accordingly, we have studied the oxidation of glycolaldehyde in GLOX-LiP coupled reactions (3). Glycolaldehyde is also of interest because it is a proposed product of ligninolysis (14). This suggests that once the oxidase system is "primed" with substrates derived from carbohydrates, the system can in part be perpetuated by the action of lignin peroxidase on lignin itself. To test the possibility of sequential oxidations of glycolaldehyde, we needed high levels of active GLOX for stoichiometric determinations which was possible using recombinant GLOX. Results indicated that three equivalents of peroxide were obtained with glycolaldehyde, two equivalents with glyoxal, and one equivalent with glyoxylic acid. These results are explained by the sequence of reactions in Figure 2, and would indicate a great economy in extracellular peroxide production.

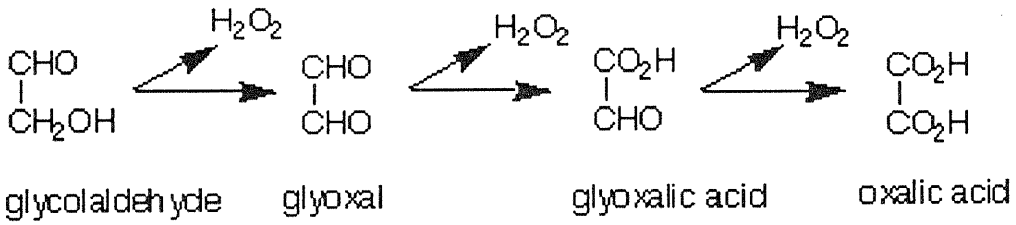

Figure 2. Sequential oxidation of glycolaldehyde yielding three peroxide equivalents.

Three-carbon Fragment Metabolism

We have also followed sequential oxidations catalyzed by GLOX with substrates that are three-carbons long as a possible means of identifying additional physiologically important metabolites. When GLOX is coupled to lignin peroxidase, for each mole of methylglyoxal, veratryl alcohol (a lignin peroxidase substrate) and oxygen consumed, one mole each of pyruvate and veratraldehyde is produced (6). The pyruvate formed is not a substrate for GLOX or LiP and therefore the analysis in this case was relatively straightforward. The scenario

with dihydroxyacetone, another substrate for GLOX (Figure 1), appears to be considerably more complicated. This is because multiple oxidations are likely that involve intermediates that are not commercially available, that are labile, and for which analytical techniques for their detection have not been established. For example, both Figures 3 and 4 present plausible pathways for the oxidation of dihydroxyacetone given the reactivities of GLOX with methylglyoxal (6) and glycolaldehyde (Figure 2). Notably, our preliminary results show that dihydroxyacetone is oxidized by rGLOX in coupled peroxidase reactions to give triose reductone, propane-trione and hydroxypyruvic acid (Green, Mozuch, Kersten, unpublished). The lack of standards of known concentration has impeded stoichiometric determinations.

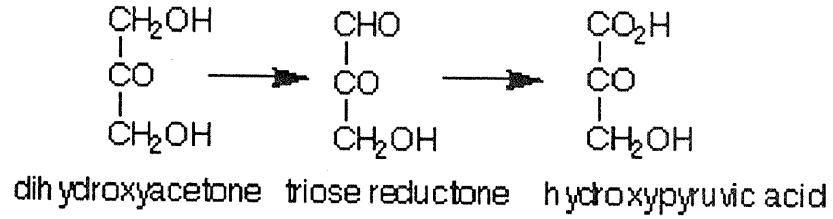

Figure 3. Sequential oxidations of dihydroxyacetone at C_1.

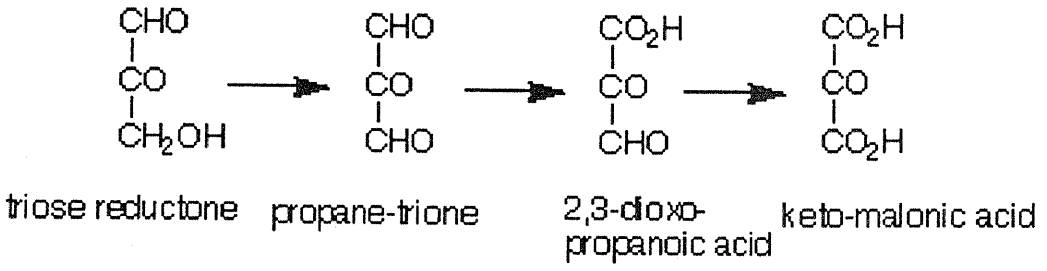

Figure 4. A plausible sequence of oxidations for dihydroxyacetone at C_1 and C_3 is shown. Side reactions would be expected because of the reactivity of the intermediates.

Implications beyond Peroxide Genesis

The possibility of metabolites as described above present further considerations worth exploring for their potential role in the decay of wood. There are numerous examples in the literature for which a peroxidase catalyzes unexpected reactions in the presence of a specific redogenic substrate, and without addition of exogenous peroxide. Examples include, the hydroxylation of phenol to hydroquinone and catechol by horseradish peroxidase (HRP) in the presence of dihydroxyfumaric acid (2), oxidation of malonaldehyde by HRP in the presence of Mn^{2+} (10), aerobic oxidation of triose reductone by crystalline turnip peroxidase (16), oxidations with indoleacetic acid, oxalate, oxalacetate, and ketomalonate by various plant peroxidases ((17) and references therein), oxidation of hydroxypyruvate by HRP, Mn^{2+} and dichlorophenol (5), and the oxidation of linear aldehydes (4, 8). The examples with triose reductone, hydroxypyruvate, ketomalonate, and oxalate are of particular interest in light of two- and three-carbon fragment metabolism discussed above.

Examples of peroxidase-catalyzed reactions without the use of exogenous peroxide have already been described with enzymes from *Phanerochaete*. Early reports of MnP indicated that the enzyme has 'oxidase-type' activity with specific substrates such as NADPH and GSH (11, 12). Detailed studies of reactions with GSH have been provided (15). However, the physiological significance of reactions with NADPH or GSH has not been demonstrated. More recently, it has been shown that oxalate supports the oxidation of Phenol Red by MnP without the addition of peroxide, and that oxalate can be found in cultures of *P. chrysoporium* (9). The oxidation of oxalate by LiP in the presence of veratryl alcohol and Mn^{2+} is also of interest as an example of an oxidase activity of LiP (1, 13). Studies using labelled carbohydrate precursors should bring to light the significance of additional redogenic substrates in ligninolysis.

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HOW DO WHITE-ROT FUNGI REALLY DEGRADE LIGNIN?

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Lignin peroxidases (LiPs) are likely catalysts of ligninolysis in many white-rot fungi, because they have the unusual ability to depolymerize the major, recalcitrant, nonphenolic structures of lignin (1). However, direct evidence for such a role has been lacking, in part because these enzymes could not be demonstrated to depolymerize lignin in vitro. To address this problem, we developed a new method for the treatment of lignins with LiP in vitro, and used this approach to characterize enzyme-mediated changes in ^{14}C- and ^{13}C-labeled synthetic lignins (2).

The lignins were dispersed in aqueous buffer/organic solvent mixtures, and were then oxidized with H_2O_2 and a purified isozyme of *Phanerochaete chrysosporium* LiP, which were added slowly from separate containers. This procedure was designed to maximize access of the enzyme to the water-insoluble lignin, and to minimize bimolecular coupling of enzyme-generated lignin free radicals.

Gel permeation chromatography of the treated, radiolabeled polymers showed that LiP catalyzed their cleavage to give soluble lower molecular weight products. To a lesser extent, the enzyme also polymerized the lignins, which is attributable to the fact that purified LiP, unlike the intact fungus, provides no mechanism for the removal of lignin fragments that are susceptible to repolymerization. However, the net result of the treatment was depolymerization. A lignin with a number-average molecular weight of 4200 was depolymerized under our reaction conditions to give products with a number-average molecular weight of 850.

^{13}C NMR experiments with a-^{13}C-labeled synthetic lignin showed that LiP generated new benzylic aldehyde groups in the polymer, as shown by the appearance of NMR signals between 190 and 192 ppm vs. tetramethylsilane. This

result can only be attributed to cleavage of the polymer between C_a and C_b of its propyl sidechain, which is the result predicted from our knowledge of the LiP reaction mechanism (1). LiP activity towards lignin in vitro therefore accounts adequately for the initial steps of ligninolysis by *P. chrysosporium* in vivo.

However, these results do not mean that LiPs provide the only route for fungal ligninolysis. Some white-rot fungi have been reported to degrade lignin without expressing LiP when they are grown on defined media (3,4). One possibility is that these fungi use weaker oxidants, such as manganese peroxidases or laccases, to attack relatively labile phenolic structures in lignin. These structures are, however, infrequent in the natural polymer. Alternatively, these organisms may in fact produce LiP, but only on their natural substrate, wood. Previous work has shown that some so-called LiP-negative fungi contain LiP-like genes, which may not be expressed on defined growth media (5). A third possiblility is that these fungi can attack the more recalcitrant nonphenolic structures in lignin, but that they use some oxidant other than LiP for this purpose.

To address this problem, we compared the abilities of the known LiP producer *P. chrysosporium* with those of a reported nonproducer, *Ceriporiopsis subvermispora*, to degrade a synthetic lignin with normal phenolic content, a lignin with all phenolic units blocked, and a dimer, 1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol, that represents the major nonphenolic structure in lignin. *P. chrysosporium* mineralized all three models rapidly in defined medium, whereas *C. subvermispora* showed appreciable activity only towards the more labile phenolic compound under these conditions. However, in its natural environment, wood, *C. subvermispora* mineralized all of the models as rapidly as *P. chrysosporium* did. Defined media therefore fail to elicit a key component of the ligninolytic system in *C. subvermispora*. An isotope trapping experiment with the dimeric model showed that a LiP-dependent pathway was responsible for about half of dimer mineralization in wood by *P. chrysosporium*, but was responsible for no more than

6-7% of mineralization by *C. subvermispora* in wood. Therefore, *C. subvermispora* has mechanisms for the degradation of nonphenolic lignin that are as efficient as those in *P. chrysosporium*, but which do not depend on LiP.

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REMEDIATION OF PCP- AND CREOSOTE-CONTAMINATED SOILS USING WOOD-DEGRADING FUNGI

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Introduction

Until very recently, the literature concerning biotreatment of hazardous organic compounds (xenobiotics) dealt almost exclusively with bacteria. It is now becoming apparent that fungi, especially wood-degrading fungi, also play an important role in xenobiotic degradtion, and that they have potential for remediating contaminated soils and waters. The higher basidiomycetous fungi probably play the major role in recycling the carbon in lignocellulosics, the most abundant renewable organic materials on earth. Thousands of species of these fungi degrade the complex structural component lignin, thereby gaining access to the cellulose and hemicelluloses, which with lignin, make up lignocellulosics. During recent years, it has become clear that the lignin-degrading enzyme system of these fungi is quite nonspecific and can degrade a wide variety of man-made as well as natural non-lignin compounds. The objective of our research is the deliberate harnessing of the xenobiotic-degrading abilities of lignin-degrading fungi for remediating soils contaminated with xenobiotics. This work has demonstrated that fungal bioaugmentation, where the fungi are added to the soil via a solid substrate inoculum, is useful for remediation of pentachlorophenol (PCP)-contaminated soils and may also be useful for creosote-contaminated soils. In this paper we describe: the fungi and their xenobiotic-degrading abilities; our experiences on the application of fungal bioaugmentation to remediation of PCP- and creosote-contaminated soils and; aspects of fungal bioaugmentation that will require further developments before commercialization of the technology, will be described.

Studies of PCP degradation in soil by lignin-degrading fungi

Bioaugmentation of PCP-contaminated soils with selected lignin-degrading fungi has been shown to cause a rapid and extensive decrease in PCP concentrations in a variety of soils under ideal laboratory conditions (Lamar et al. 1990a, Lamar et al. 1990b). For example, PCP decreases in 3 soils, that were spiked with 50 mg PCP kg^{-1} soil and inoculated with the lignin-degrading fungus *P. chrysosporium*, averaged 98% after 8 wks (Lamar et al. 1990a). Although this fungus has been shown to mineralize PCP in aqueous culture (Lamar et al. 1990b, Mileski et al. 1988), decreases of PCP in the three soils were primarily due to conversion to non-volatile products. The nature of these products, whether they were soil-bound or extractable was greatly influenced by soil type. The extractable and primary transformation product in PCP-spiked Marshan soil cultures of both *P. chrysosporium* and another lignin-degrading fungus, *P. sordida* was pentachloroanisole (PCA) (Lamar et al. 1990b). In *P. chrysosporium* cultures, 46% of the overall 96% PCP decrease was due to conversion to and accumulation of PCA. However, both fungi were able to degrade some of the PCA. For example, after peak PCA accumulation (1 wk) where 64% of the PCP was methylated, there was an 18% decrease in the anisole concentration during the following 7 wk in *P. chrysosporium* cultures. Decreases of PCA in *P. chrysosporium* and *P. sordida* soil cultures was not suprising because the anisole was mineralized in liquid cultures of both organisms to similar extents as was PCP (Lamar et al. 1990b).

In Batavia and Zurich soil microcosms inoculated with *P. chrysosporium* conversion to nonextractable products was the predominant fate of PCP (Lamar et al. 1990a). The nature of the nonextractable compounds has not been elucidated. However, chlorophenols including PCP have been coupled to humic materials via copolymerization reactions catalyzed by fungal phenoloxidases such as laccases and peroxidases in *in vitro* reactions (Dec and Bollag 1990, Bollag et al. 1988, Sakar et al. 1988) and in soil microcosms (Cheng et al. 1983). The resulting

xenobiotic-humic acid hybrid polymers are non-toxic (Lyr 1962, Bollag et al. 1988) and relatively resistant to microbial degradation in soils (Dec and Bollag 1988). Direct and indirect evidence that copolymerization of xenobiotic and humic materials occurs in soils as a result of the activity of fungal phenol-oxidizing enzymes includes the immobilization of 2, 4-dichlorophenol by a laccase from *Geotrichum candidum* in soil columns (Shannon and Bartha 1988) and the humification of benzo(a)pyrene by *P. chrysosporium* in soil microcosms (Qiu and MacFarland 1991), respectively.

We have reported significant but less extensive PCP decreases (i.e. 88% to 91%) in two small-scale field studies (e.g. total weight of soil for all treatments ≤ 24 tonnes). In these studies the ability of lignin-degrading fungi to decrease concentrations of PCP as the sole soil contaminant (Lamar and Dietrich 1990) and in mixtures with creosote (Lamar et al. 1993, Davis et al. 1993) was evaluated. In the first study, inoculation of a sandy gravel soil pH 9.6 contaminated with a commerical PCP-containing wood preservative (250-400 mg PCP kg^{-1} soil) with either *P. sordida* or *P. chrysosporium* resulted in an overall decrease of 88% to 91% of the PCP in the soil in 6.5 weeks (Lamar and Dietrich 1990). The second study was a field treatability study that was conducted at the former Brookhaven Wood Preserving pole-treatment facility in Brookhaven, MS. The objectives of the treatability study were to evaluate the ability of several lignin-degrading fungi to decrease the concentrations of PCP and PNA components of creosote in a clay soil pH 3.8 contaminated with K001 sludge and to select the best fungal treatment for use in a large-scale demonstration study that was conducted at the same site. K001 sludge is described as bottom sediment sludge from the treatment of waste waters from wood preserving processes that use creosote and/or PCP (CFR 1990). Intial soil concentrations of PCP and total PNA analytes averaged 700 mg kg^{-1} and 2000 mg kg^{-1}, respectively. The best fungal treatment was inoculation of the soil with *P. sordida*. This resulted in an 89% removal of PCP after 8 wks (Lamar et al. 1993).

In the first field study, a small percentage (8 to 13%) of the PCP decrease caused by *P. chrysosporium* or *P. sordida* was due to methylation and accumulation of PCA (Lamar and Dietrich 1990). In this case, decreases in PCA concentrations after peak accumulations of the anisole, which occured 3 wks after inoculation, were insignificant. However, the physiological activity of the fungi during the period after peak PCA accumulation may have been severly restricted by low soil temperatures that averaged 10°C. Decreases in the PCP concentrations during this period were small relative to those that occurred during the first 3 wks. However, a slower rate of PCP decrease during the last 3 wks of the study may have been caused by a limited residual supply of readily available PCP, and/or by restricted physiological activity caused by low soil temperatures. In the treatability study, approximately 11% (80 mg kg^{-1}) of the PCP was transformed to PCA after 4 wks (Lamar et al. 1993). However, after 8 wks only 3% of the overall PCP decrease was caused by formation of PCA. Thus, *P. sordida* caused a substantial decrease in the PCA concentration. This decrease occurred concurrently with significant PCP decreases. The results of these field studies (Lamar and Dietrich 1990, Lamar et al. 1993) and the soil microcosm study (Lamar et al. 1990a) indicated that both *P. chrysosporium* and *P. sordida* can degrade PCA once it is produced. In the treatability study there was also a 70% removal of PNAs in soil inoculated with *P. sordida* after 8 wks (Davis et al. 1993). Toxicity analyses using seed germination and root elongation tests demonstrated significant detoxification of a creosote-contaminated soil after treatment with *P. chrysosporium* (Baud-Grasset et al. 1993). Extent of detoxification was highly correlated to decreases in the total concentration of 3- and 4-ring PNAs. The ability of *P. chrysosporium* , *P. sordida* and other fungi (Loske et al. 1990) to cause substantial decreases in PNA concentrations suggests that, with improvements in the extent of PNA removal, they might also be useful for remediation of creosote-contaminated soils

The feasibility of large-scale fungal bioaugmentation was evaluated by assessing the ability of the lignin-degrading fungus *P. sordida* to decrease the soil

concentrations of pentachlorophenol (PCP) and 13 priority pollutant PNA creosote components in a field-demonstration study. This study was also performed at the Brookhaven, MS site. Inoculation of the contaminated soil with the fungus (fungal treatment) at a rate of 10% (dry wt inoculum dry wt $soil^{-1}$) was compared to amending the soil (amended control) with sterile inoculum substrate also at a 10% rate (dry wt substrate dry wt $soil^{-1}$) and no treatment (nonamended control), for their effects on PCP and PNA concentrations over a 20 week period. The fungal treatment and controls were chosen on the basis of results obtained from the above-mentioned field treatability study that was conducted during the previous year (Lamar et al. 1993, Davis et al. 1993). The initial soil PCP and total measured PNA concentrations for the three treatments averaged 1058 mg kg^{-1} and 1210 mg kg^{-1}, respectively. The concentrations of PCP and PNAs varied greatly throughout the contaminated soil volume. Thus initial concentrations also varied between the treatability and demonstration studies. Despite very low initial levels of fungal biomass, determined from ergosterol levels in the inoculum and inoculated soil, there was a 64% PCP decrease to a residual concentration of 362 mg kg^{-1} in the fungal treated soil after 20 weeks of treatment. This residual concentration was statistically less than the 715 mg kg^{-1}(30% decrease) and the 985 mg kg^{-1} (19% decrease) PCP concentrations observed in the amended control and the non-amended control soils, respectively. There were significant decreases in the concentrations of 3- and 4-ring PNAs in all treatments. With the exception of acenapthene, decreases in the concentrations of 3-ring PNAs were significantly greater in the nonamended control soil than in either the amended control or fungal treated soils. Conversely, decreases in the concentration of 4-ring PNAs tended to be greater in the fungal treated and amended control soils than in the nonamended control soils. The concentrations of 5- and 6-ring PNAs were not decreased significantly by any of the treatments.

Commercialization of Fungal Bioaugmentation

The results of these field investigations demonstrate that (1) lignin-degrading fungi can be successfully inoculated into and cultivated in soils contaminated with relatively high concentrations of PCP (up to 1,000 mg kg^{-1}) alone or in combination with creosote (4,000 mg kg^{1} total measured PNAs) and (2) concentrations of PCP and low-molecular-weight PNAs are greatly reduced as a result of the activity of these fungi. The abilities of these organisms to decrease the concentration of PCP in two soils with very diverse chemical and physical characteristics lends support for the continued development of a fungal-based treatment technology to remediate soils contaminated with organic pollutants. Several important components of the fungal technology require additional development before this technology can become commercially viable. First, fungal inocula that are easy to apply must be able to be produced economically. Next, continued investigation of contaminant fate in fungal inoculated soils and fungal strain selection is required. Finally, the extent of contaminant degradation must be improved.

The development of an inexpensive and effective fungal inoculum is crucial to successful implementation of fungal-based soil remediation. Currently, the production and delivery of the inoculum is the most expensive component (currently $1.32/kg) of the technology. In the small-scale field trials, only modest amounts of fungal inoculum (≤ 500 kg per fungus) were applied. Scale-up of fungal bioaugmentation, as it is currently practiced, will require the production and delivery of much greater quantities of inoculum. For example, at an inoculation rate of 10% (dry weight basis), inoculation of 100,000 tonnes of soil would require 25,000 tonnes, wet weight (60% moisture content) of inoculum. Inoculum costs can be reduced by developing more economical production methods and by producing inocula with greater inoculum potential (i.e. weight of fungal biomass produced per weight of inoculum). The latter will allow inoculation rates to be reduced, thus lowering the costs of inoculum.

Mechanized techniques for inoculum application will also be necessary for large-scale operations. In the demonstration study, use of a large screw auger grain mixer to mix the inoculum into the contaminated soil resulted in severe dimunition of inoculum potential . Therefore, in addition to low costs and ease of application, inocula that maintain inoculum potential through the mixing process need to be developed. Wood chips and a nutrient-fortified mixture of sawdust and grains have served well as substrates for fungal inocula in these field studies. These materials provide a niche in which the lignin-degrading fungi can outcompete indigenous microbes and survive the toxic environment of the contaminated soil. For large-scale applications, the delivery and application of the necessary quantities of high-quality inocula made with these materials would be difficult to economize in their present form. Novel production and inoculum formulations involving lignocellulosic or other substrates are expected to result in economical inocula that more effectively take advantage of the niche (i.e. the substrate(s)) and growth habits of these organisms.

The effectiveness of remediation treatments is currently assessed by measuring, through direct chemical analysis, the extent of contaminant(s) removal and/or by determining if contaminants are transformed to innocuous products. Very little is known about the fate of contaminants in soil due to the activity of lignin-degrading fungi. We have found that *P. chrysosporium* removes PCP *per se* from soils primarily by converting it to soil-bound products (PCP-humic material copolymers) (Lamar et al. 1990a). Similarly, *P. chrysosporium* enhanced the polymerization of the creosote component benzo[a]pyrene into soil humic materials (Qiu & McFarland 1991). For certain contaminants the fungal technology works, apparently by transforming pollutants by decreasing their bioavailability through stabilizing them by polymerization with soil organic matter. This type of transformation may not be the fate for all the hazardous compounds that these organisms are able to degrade. The nature and stability of the such pollutant-humic copolymers or soil-bound residues is not known. Work with chemically synthesized humic acid-xenobiotic copolymers suggests that

xenobiotics bound to humic materials through enzymatic polymerization reactions are relatively stable (Bollag & Liu 1985, Haider & Martin 1989). The nature, stability, and toxicity of the soil-bound transformation products, under a variety of conditions, require continued evaluation.

More than 1,500 species of lignin-degrading fungi have been estimated to exist in North America alone. Almost nothing is know about the pollutant-degrading capabilities of other wood decay fungi, including brown rot or soft rot fungi, or other common soil fungi. Most of the research on the pollutant-degrading abilities of these organisms has been focused on relatively few species and on *P. chrysosporium* in particular. Fungal strains with superior biotransformation abilities are expected to be identified through simple screening procedures.

Although the most effective fungal treatments in the Oshkosh and Brookhaven studies decreased PCP concentrations by approximately 90%, a significant amount of PCP remained in the soil at the end of each study. Insufficient removal of contaminants is a common drawback of bioremediation treatments in general. In both field studies, the ultimate extent of PCP removal probably was restricted by the extremely low soil temperatures encountered toward the end of the investigations because of the negative effect of these temperatures on fungal growth and activity, and therefore the length of the treatment period. However, the residual PCP simply may have been unavailable to the fungi. In addition to providing optimum environmental conditions for fungal growth and activity, techniques to enhance the availability of contaminants to fungal or other microbial degradation systems may facilitate more complete contaminant removal.

In summary, the development of a technology that employs lignin-degrading fungi to remediate contaminated soils is promising. Actual implementation of the technology on a commercial scale will require further developments in inoculum production and in techniques to improve the effectiveness of the treatment.

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THE ROLE OF SECONDARY METABOLITES IN THE FUNCTIONING OF MN AND LIGNIN PEROXIDASES FROM *PHANEROCHAETE CHRYSOSPORIUM*

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Phanerochaete chrysosporium is a wood decay fungus of the "white-rot" type that has been extensively characterized for its lignin-degrading activity. Under conditions that trigger the synthesis of the ligninolytic system, it secretes a large number of hemeprotein peroxidases which catalyze the depolymerization of lignin. These peroxidases can be divided into two isozymes families based on the catalytic activities, the lignin peroxidases and the Mn peroxidases.

Lignin and Mn peroxidases possess a catalytic mechanism similar to other peroxidases. The native ferric enzyme reacts with H_2O_2 to form the two-electron oxidized intermedtiate, compound I. Compound I oxidizes one substrate molecule via one-electron to form the one-electron-oxidized enzyme intermediate, compound II. Compound II then returns to resting enzyme by oxidizing a second substrate molecule by one electron. The distinguishing differences between the various peroxidases is the nature of the reducing substrate. Mn peroxidases are unique in their catalytic dependence on Mn ions. Both compounds I and II are capable of oxidizing Mn^{2+} to Mn^{3+}, which in turn can oxidize phenolic substrates.

While the lignin and Mn peroxidases found in the extracellular fluid of ligninolytic fungal cultures have received the bulk of the attention devoted to understanding lignin depolymerization, there are other components present which are proving to be critical to the proper functioning of these enzymes. *P. chrysosporium* also secretes a H_2O_2-generating system under ligninolytic conditions. This system is composed of glyoxal and an enzyme, glyoxal oxidase, which oxidizes glyoxal by two electrons to generate H_2O_2. Additionally, veratryl alcohol is produced as a secondary metabolite.

The rate of catalysis of the Mn peroxidases is dependent on the presence of Mn chelators. We investigated whether *P. chrysosporium* produces chelators which would stimulate Mn peroxidase. We found that oxalate is produced by *P. chrysosporium* and that the appearance of oxalate in the extracellular fluid of *P. chrysosporium* correlated with the production of Mn peroxidase and lignin peroxidase. This correlation suggested a role for oxalate in the oxidative reactions catalyzed by these lignin-degrading enzymes, so we investigated the effect of oxalate on Mn peroxidase activity. Oxalate stimulated Mn peroxidase-catalyzed phenol red oxidation in a concentration-dependent manner up to 500 μM. This is within the range of oxalate concentrations found in the extracellular fluid of *P. chrysosporium*. Aside from stimulating the rate of phenol red oxidation, oxalate was also able to increase the net amount of phenol red oxidized. This result suggests that oxalate might stabilize Mn^{3+} from spontaneous decomposition.

The reaction of Mn peroxidase compound II with Mn^{2+} is much slower than that of compound I. The rate is also highly dependent on the chelation state of the Mn^{2+}. In the presence of succinate the reduction of compound II is relatively slow. Succinate is not a strong Mn^{2+} chelator and at the concentration of 20 mM, Mn^{2+} exist predominantly as free Mn^{2+}. The rate accelerates when oxalate is used to complex the Mn^{2+}. Oxalate has a much higher formation constants than succinate such that, at the concentrations employed, Mn^{2+} is predominantly complexed. This suggests that compound II does not react with free Mn^{2+}.

To determine the accessibility of the Mn^{2+} binding site, experiments were also performed with a polymeric complexing agent, poly-L-glutamate. Although a low amount of stimulation is observed, the results are not different from those obtained with succinate alone. The average molecular weight of this complex is 14, 000, much larger than that of the organic acids tested, which suggests that the organic acid/Mn^{2+} complexes require direct access to the active site of Mn peroxidase in order to stimulate the reactivity of Mn^{2+} with Mn peroxidase.

The reactivity of the various oxalate/Mn^{2+} complexes (i.e. 1/1 versus the 2/1 chelator to Mn^{2+} ratio) was studied by varying the oxalate concentration at constant Mn^{2+}. The formation constants are known for many of these complexes; thus the relative fraction of each Mn^{2+} complex can be calculated. Only the 1:1 complex of oxalate/Mn^{2+} reacts with compound II. Interestingly, at concentrations of oxalate which favor the 1:1 oxalate/Mn^{2+} complex, the 2:1 oxalate/Mn^{3+} complex is favored. This suggest that once formed, Mn^{3+} is released from the enzyme as the 2:1 complex.

Surprisingly, we also discovered that oxalate is capable of supporting Mn peroxidase activity without added H_2O_2. In incubations containing Mn peroxidase, Mn^{2+} and oxalate, phenol red was oxidized after a long lag period. If any one of the components omitted, no phenol red oxidation occurred. The lag period could be eliminated by the addition of low levels of H_2O_2. No oxidized phenol red was formed when the reactions were carried out under anaerobic conditions, indicating that oxygen is required for this oxalate-supported oxidative activity.

Glyoxal and glyoxal oxidase are also secreted into the extracellular fluid of *P. chrysosporium*. One of their oxidation products, glyoxalate, can also support Mn peroxidase activity in the absence of added H_2O_2 . As superoxide scavengers superoxide dismutase and tetranitromethane can inhibit this activity, it appears that superoxide formation is involved in the process.

In the case of both oxalate- and glyoxalate-supported Mn peroxidase activity, H_2O_2 is likely produced from the Mn^{3+}-dependent oxidation of the organic acid. The newly generated H_2O_2 can then be utilized by Mn peroxidase for generation of more Mn^{3+}, resulting in further amplification of H_2O_2 production. It is unlikely that this, rather than the glyoxal oxidase system, is the primary means of providing H_2O_2 to the ligninolytic peroxidases of *P. chrysosporium*; however, the ligninolytic system of *P. chrysosporium* is induced under a variety of nutrient

limitations and there are a multitude of ligninolytic enzymes. Therefore, it would not be surprising if there are multiple mechanisms for H_2O_2 generation in this fungus.

Veratryl alcohol is a secondary metabolite also produced by ligninolytic cultures of *P. chrysosporium*. It has been proposed that veratryl alcohol acts as a redox mediator between lignin peroxidase and lignin. In this scheme, veratryl alcohol is first oxidized by a single electron to form the cation radical. This radical then diffuses for an unknown distances and oxidizes other substrates such as anisyl alcohol, which is not oxidized efficiently by lignin peroxidase unless veratryl alcohol is included in the incubation mixture.

Another proposed role for veratryl alcohol is to prevent the H_2O_2-dependent inactivation of the enzyme. With poor substrates, such as anisyl alcohol, the enzyme undergoes more rapid H_2O_2-dependent inactivation, so the role of veratryl alcohol might be to prevent these inactivations.

We found that neither hypothesis explains all the data for anisyl alcohol oxidation. Our results provide an explanation for the enhancement of anisyl alcohol oxidation by veratryl alcohol. We found that protection of the enzyme by veratryl alcohol from inactivation is only a secondary consequence. The basis for the enhancement phenomenon is explained by the inertness of anisyl alcohol to compound II but not compound I. Inclusion of veratryl alcohol allows the enzyme to return to the ferric state and complete the catalytic cycle.

Anisyl alcohol does not readily support steady-state turnover of lignin peroxidase. Incubation of the enzyme with 4 mM anisyl alcohol and 0.3 mM H_2O_2 resulted in low levels of anisaldehyde formation . The stoichiometry indicate that the enzyme went through 3.3 turnovers. Lowering the H_2O_2 concentration or increasing the anisyl alcohol concentration resulted in a slight increase in the amount of anisaldehyde formed. At the highest concentration of 80 mM, we were able to

obtain approximately 140 turnovers. All of these yields are much lower than the amount of anisaldehyde formed in the presence of 0.1 mM veratryl alcohol.

When the H_2O_2 concentration was lowered to equal one equivalent of enzyme -- to allow for formation of compound I without any excess H_2O_2 remaining -- reaction of anisyl alcohol with this compound I preparation resulted in slightly less than one half of the expected amount of anisaldehyde. This suggests that only compound I and not compound II reacts with anisyl alcohol. In contrast, oxidation of veratryl alcohol by compound I resulted in near stoichiometric formation of veratraldehyde .

The reactivity of anisyl, veratryl and 3,4,5-trimethoxybenzyl alcohol with compound I was studied by presteady state techniques. The plot of k_{obs} *versus* anisyl alcohol concentration is linear. A rate constant of $2.3 \times 10^3\ M^{-1}s^{-1}$ was calculated from the slope. A rate constant of $1.5 \times 10^5\ M^{-1}s^{-1}$ was calculated for veratryl alcohol and $2.4 \times 10^4\ M^{-1}s^{-1}$ for 3,4,5-trimethoxybenzyl alcohol.

On the other hand, very low reactivity is observed for anisyl alcohol with compound II. Increasing the anisyl alcohol concentration up to 80 mM did result in an increase in k_{obs}. Fitting the data revealed a Kd of 27 mM for anisyl alcohol. However, these results are most likely due to a 1% contaminant in anisyl alcohol which can convert compound II to ferric enzyme. If so, then the Kd for this unknown contaminant would be 0.27 mM. This Kd is in line with the Kd values obtained for other substrates of lignin peroxidase. The presence of this contaminant would explain the low number of turnovers still seen under steady-state conditions .

In light of the finding that anisyl alcohol does not react with compound II, an explanation is possible for the phenomenon of veratryl alcohol enhancement of anisyl alcohol oxidation. The stimulation can be explained by the necessity of a

second substrate to convert compound II to resting enzyme. This would complete the catalytic cycle and allow for continued turnover of the enzyme.

In summary, the extracellular fluid of *P. chrysosporium* contains, in addition to the Mn and lignin peroxidases thought to carry out the depolymerization of lignin, secondary metabolites necessary for the proper functioning of those peroxidases. These include oxalate, and veratryl alcohol. Oxalate, at the levels secreted by the fungus, stimulates Mn peroxidase activity by chelating Mn^{2+} in a 1:1 complex. It may also be involved in H_2O_2 generation, supplementing that produced by the glyoxal oxidase system secreted by the fungus. Veratryl alcohol may have a role in returning lignin peroxidases to their catalytically competent native state when oxidizing substrates that react readily with the compound I form of the enzyme, but not with the compound II form.

THE MOLECULAR CLONING AND EXPRESSION OF LACCASE GENES FROM THE WHITE-ROT BASIDIOMYCETE *POLYPORUS PINSITU*

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Lignin is a structurally complex aromatic biopolymer which is resistant to degradation. The degradation of lignin is an important step in the pulp and paper making process. It has been demonstrated that the extracellular enzymes of some white-rot fungi are able to degrade lignin to some extent (Kirk and Farrell, 1987). These extracellular enzymes include peroxidases, lignin and manganese peroxidases, and laccases. Laccase are multi-copper enzymes which catalyzes the oxidation of phenolic compounds. Laccases are found in both plants and fungi (Mayer, 1987). In plants, laccases are involved in lignification (O' Malley et al., 1993). In fungi, besides a role in delignification, laccases appear to be involved in sporulation (Leatham and Stahmann, 1981), pigment production (Clutterbuck, 1972; Smith et al., 1989), and plant pathogenesis (Geiger et al., 1986; Marbach et al., 1985). Laccase genes from the non-lignolytic fungi *Neurospora crassa* (Germann et al., 1988) and *Aspergillus nidulans* (Aramayo and Timberlake, 1990) have been cloned. Laccase genes from the white-rot lignolytic fungi *Coriolus hirsutus* (Kojima et al., 1990), *Phlebia radiata* (Saloheimo et al., 1991), *Coriolus versicolor* (Iimura et al., 1992) and a newly isolated lignolytic basidiomycete PM1 (Coll et al., 1993) have been cloned. In addition to the industrial application of laccases in delignification there are also other potential applications such as in the polymerization of phenolics in liquids, the oxidation of dyes and dye precursors , and polymerization of lignin and lignosulfates.

Polyporus pinsitus is a white-rot fungus which has lignolytic activity (Bottcher et al., 1988). As seen in several other fungi, extracellular laccase production in *P. pinsitus* is induced approximately 15 fold by the addition of 2,5-xylidine (Bollag

and Leonowicz, 1984) We report here the purification of laccases from an induced culture of *P. pinsitus* , the cloning of the genes coding for laccase, and the heterologous expression of a *P. pinsitus* laccase gene in *Aspergillus oryzae.*

Laccase activity was purified from a concentrated culture broth of an induced *P. pinsitus* culture. The purification involved anion exchange chromatography and gel filtration. Three peaks of laccase activity were found after anion exchange chromatography. These three peaks were further purified by gel filtration. All three laccase fractions have an apparent molecular weight of ~130 kDa as determined by gel filtration. After gel filtration all three fractions were electrophoretically pure. From SDS-PAGE analysis, all three laccase fractions have an apparent subunit molecular weight of ~65 kDa suggesting the active laccases are dimers. Glycosylation accounts for about 5-10 kDa of the total mass. The isoelectric points of the three forms are different, the isoelectric points are 3.5, 5-6, and 6-6.5. The N-terminal sequences of the three laccase fractions were determined; two of the fractions have identical N-terminal sequences suggesting they are perhaps posttranslational variants. The third form has unique N-terminal sequence. Work is in progress to determine the biochemical differences between the purified laccases.

Based on the N-terminal sequence of one of the purified laccases, degenerate oligonucleotides were used to isolate a partial cDNA. The partial cDNA was used as a probe to screen a cDNA library and genomic banks of *P. pinsitus.* Five genomic clones have been isolated whose predicted proteins have homology to other basidiomycete laccases. The five laccases share from 60 -80% identity at the amino acid level. All five laccase genes encode typical signal sequences suggesting that all are secreted proteins. The structural organization of the five genes differ with the number of introns ranging from 8 to 12. The position of some of the introns are conserved while others are not strictly conserved. Two of the genes are appear to code for the laccases purified from the 2,5-xylidine induced culture (above). We are currently working to determine the levels of

expression of the five laccase genes in both induced and uninduced cultures. A karyotype of *P. pinsitus* has been determined, and localization of the genes to individual chromosomes will be presented.

The use of laccase in industrial applications will only be feasible if large quantities of the enzyme can be made at a reasonable cost. Even under inducing conditions the levels of laccase produced by *P. pinsitus* are low. We have chosen to produce laccase in a heterologous system. Expression constructs have been made and used to transform *Aspergillus oryzae*. The protein produced by *Aspergillus oryzae* transformants is secreted and as determined by a number of biochemical methods appears to be identical to the laccase produced by *P. pinsitus*.

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APPLICATION OF GENETIC DIAGNOSTICS TO PLANT GENOME ANALYSIS: COMPARISON OF MARKER SYSTEMS IN SOYBEAN

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A variety of marker systems has been developed over the years to facilitate the analysis of complex plant and animal genomes. Classical genetic phenotypes and isozyme markers, the first genetic markers, pose severe limitations for detailed genome investigation; their use is complicated by problems with phenotypic penetrance or heritability, and by the difficulty involved in constructing multiply marked lines. In addition, these markers are not numerous enough in most species to permit high resolution mapping. More recently, several different DNA-based markers systems have been developed which circumvent many of these problems. Based solely on nucleotide polymorphism, DNA markers are potentially limitless in number and their identification and use are far less affected by phenotype.

The utility of restriction fragment length polymorphisms (RFLPs) as codominant markers for plant genome analysis has been quickly realized, and the identification and use of RFLPs is now being applied to a wide range of species. The effective use of RFLP markers, however, has been hampered by the relatively large amount of genetic material required and the labor involved in performing the analysis. Alternatively, methods based on the polymerase chain reaction (PCR) are technically less demanding and offer greater experimental simplicity. The random amplified polymorphic DNA (RAPD) assay (William, et al., 1990, 1993), for example, can identify an extremely large number of genomic polymorphisms, requires minimal amounts of input DNA and is technically simple and straightforward compared to RFLP (Carlson, et al., 1991; Chapparo, et al., 1992; Hong, et al., 1993). The dominance of RAPD markers, however, poses some limitations for genetic mapping, and the development of codominant, PCR-based

markers and maps involving simple sequence repeat (SSR), or microsatellite, sequences is currently underway for many plant and animal species (Lynn Senior and Huen, 1993; Akkaya, et al., 1992 ; Morgante, and Olivieri, 1993; Morgante, et al., 1994: Lagercrantz, et al., 1993; Hazan, et al., 1992). Shown to be abundant, well dispersed, and highly polymorphic in many plant genomes, SSRs are predicted to be extremely useful for genetic mapping in plants. Finally, multiplexed genome assay methods, such as amplified fragment length polymorphism (AFLP) and inter-repeat amplification can reveal several polymorphisms simultaneously (Zabeau and Voss 1993; Zietkiewicz, et al., 1994). Although these multiplexed methods are becoming increasingly useful for marker discovery, fingerprinting and mapping, their routine use may be somewhat limited because they are technically demanding than either RAPD or SSR assays.

The choice of marker system to use for a particular application will depend to some degree upon the simplicity and ease of use of the assay and on the technical expertise of the investigator; however, the most important consideration for marker choice is the type of genomic information required from the analysis. Each assay method makes an estimate of genomic polymorphism and identifies polymorphic markers in a distinct way and each has different inherent biases (Rafalski, et al., 1994). The various marker systems differ in their capacity to detect polymorphisms in a given population (infomativeness) and in the number of loci that can be simultaneously detected per experiment (multiplex ratio). Therefore, it is useful and highly instructive to compare directly the ability of these various systems to identify polymorphisms and generate useful genetic markers that can be applied to multiple populations. Although all of these methods have been applied separately towards analysis of individual genomes, we know of no case whereby all have been directly compared on a common set of individuals. Below we describe the results of a direct comparison of these assay methods on a set of 12 soybean genotypes, from which we can draw conclusions about their relative efficiency for estimating polymorphism levels and genetic similarities.

An initial RFLP survey of soybean germplasm, together with co-ancestry (pedigree) information, allowed the identification of 12 soybean genotypes that collectively account for 92% of the allelic variation in the gene pool of North American soybean cultivars (Delanney, et al., 1983). Genetic material from these 12 genotypes, which include 10 Glycine max cultivars and 2 Glycine soja accessions, was used as template for four different genome assays. A previous RFLP analysis (Rafalski and Tingey, 1993) had identified 110 RFLP loci that were polymorphic between a Glycine max cultivar, Bonus, and PI81762, an accession of Glycine soja. Using conventional Southern blotting and probe hybridization, these RFLP loci then were assayed for polymorphism in the entire set of 12 lines. Similarly, 66 random 10mer primers were used for a RAPD analysis of the 12 cultivars, and the results scored on agarose gels stained with ethidium bromide. The same set of templates was examined by PCR with primer pairs representing 36 soybean SSR loci (AG, AC and AT repeat sequences all are represented), and the polymorphisms visualized and scored using high resolution polyacrylamide gels. Finally, DNA from all 12 lines was used as template for a multiplexed AFLP analysis with two different restriction enzyme combinations. A total of 337 products derived from just 7 amplification reactions could be well resolved on polyacrylamide gels and scored for polymorphism.

For any of these four assays, a marker (i.e., locus, probe, or band on a gel) was scored as polymorphic if at least two alleles could be identified among the set of the 12 cultivars. For these purposes, both dominant and codominant polymorphisms were scored similarly; if at least one cultivar in the set had a different allele from the others, the marker was scored as polymorphic. Because of the RFLP probe preselection conditions (previously identified polymorphism between Bonus and PI81762), all 110 RFLP loci were classified as polymorphic; however, previous data had revealed that only ~25% of arbitrarily chosen RFLP probes identify a polymorphism in the soybean genome. Only 37 of the 66 RAPD primers (56%) revealed a polymorphism in at least one cultivar, in contrast to the

finding that all 36 SSR loci (100%) were polymorphic among the 12 lines. Of the 337 bands amplified by AFLP, only 136 (40%) were polymorphic.

These raw data were then be used to estimate the expected heterozygosity and effective multiplex ratio for each marker system. The practical utility of a marker system depends on both of these values; that is, a marker's utility is essentially a balance of the polymorphism revealed by a single reaction with the degree to which a single reaction or experiment can detect multiple polymorphisms. A reasonable estimate of marker utility, therefore, is essentially a product of information content and mutliplex ratio. A summary of the results for this marker comparison in soybean is shown below:

marker system	Effective multiplex ratio[a]	Expected Heterozygosity[b]	Marker Index (marker utility)[c]
RFLP	0.25	0.41	0.10
RAPD	0.56	0.41	0.23
SSR	1.0	0.60	0.60
AFLP	19.0	0.32	6.08

[a]The number of products from a single amplification reaction multiplied by the fraction of the products that are polymorphic.

[b]A measure of a marker's information content. Expected heterozygosity, H, equals $1-\sum p_i^2$, where p_i is the allele frequency for the i-th allele. For this calculation, only the polymorphic bands from each reaction were considered.

[c]A product of effective multiplex ration and exptected heterozygosity.

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CUSTOMIZING LIGNIN THROUGH BIOTECHNOLOGY

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The properties of wood are dependent on several different aspects of cell biology and biochemistry, including tracheid and fibre cell length, wall thickness, lumen volume, lignin content and lignin composition. In woody species, all of these attributes exhibit substantial variability [Zobel and van Buijtenen 1989], and they are directly or indirectly controlled by the genetic programming in the cell's nucleus. With the advent of recombinant DNA and plant transformation technologies, it has become possible to consider modifying that programming to improve specific attributes. The current state of genetic engineering only realistically allows us to deal with single gene traits, where the gene product is usually a specific enzyme (structural genes). However, the identity of the genes (and enzymes) responsible for most aspects of cell wall formation has yet to be established, with the notable exception of lignin.

This complex phenylpropanoid polymer provides the hydrophobic matrix which surrounds and permeates the polysaccharide network laid down during cell wall synthesis. On one hand, lignin is essential for cell wall rigidity and the structural integrity of wood, but on the other, it must be stripped from the wall in the production of high quality wood pulp. As a result, a substantial part of the production cost and environmental impact of industrial paper production results from the need to remove lignin from wood. Customizing lignin content, either through reduction of total lignin levels, or by generating a shift in polymer composition toward a more easily removed "high syringyl" lignin, has therefore become a desirable goal for forest biotechnology.

The basic aim of plant genetic engineering is, in effect, to create "mutants", but to do so in a directed fashion, rather than by the classical random mutagenesis techniques. In this regard, it is interesting that very few "lignin mutants" have

been identified from natural populations. A recent attempt to identify lignin mutants within the small *Arabidopsis* genome after EMS mutagenesis was also unsuccessful [Dharmwardhana, Ellis and Carlson, unpublished observations]. One implication of this apparent low natural mutability of the lignin trait might be that proper functioning of the biosynthetic pathway is so crucial to plant survival that loss of function at any point results in a lethal phenotype. It is also possible that each step in the biosynthetic process is functionally redundant; i.e. the products of more than one gene are able to catalyze each reaction. If the former scenario is correct, it will be essential to create finely tuned genetic changes that impact only lignin formation, and only within the desired spatial and temporal context. If the pathway proves to possess high redundancy, it could make the use of suppression constructs more challenging than usual. The studies underway in several laboratories are beginning to provide some insight into these aspects of lignin genetics.

It is generally accepted that lignin biosynthesis begins with the deamination of L-phenylalanine by the enzyme phenylalanine ammonia-lyase (PAL) to yield cinnamic acid, and ends with the oxidative polymerization of a mixture of hydroxycinnamyl alcohols to yield a three-dimensionally cross-linked lignin. Between these events exists a matrix of reactions, most of which have the potential to also participate in other biosynthetic sequences in addition to lignin formation. The canonical lignin biosynthetic sequence involves sequential ring hydroxylations and O-methylations, as well as activation, and then reduction, of the carboxyl function [Davin *et al* 1992]. Both peroxidase [Ferrer *et al* 1990] and laccase [Bao *et al* 1993] have been proposed to be responsible for the final polymerization step.

Ideally, attempts to modify the overall flux of carbon to lignin would target unique steps for which no metabolic by-pass is known; candidate reactions would be those catalyzed by PAL and by cinnamate-4-hydroxylase (C4H), which are essential for the generation of the 4-hydroxyphenylpropene skeleton from which

all lignin monomer structures are derived. When PAL activity in transgenic tobacco was partially suppressed by introduction of a heterologous PAL gene driven by the CaMV 35S promoter (which generally permits gene expression in a wide range of tissues), the lignin content of the transgenic plants was indeed reduced. However, other phenotypic changes were also obtained, including localized necrosis, distorted leaf morphology and reduced fertility [Elkind *et al*]. It seems unlikely that these pleiotropic effects are the direct result of reduced lignification; instead, they probably reflect the disruption of other cellular processes that depend on specific phenylpropanoid metabolites for proper function. Unless there exists a form (or population) of PAL in woody plants that is solely committed to lignin biosynthesis, interfering genetically at this early step may well be impractical. Isoforms of PAL have been detected in several plant species, and most plants examined appear to possess a family of PAL genes, but whether specialized lignin-specific forms of PAL exist remains to be determined. The same concerns would apply to the subsequent C4H step, although there is still relatively little information concerning this enzyme or its corresponding gene.

The next step, activation of 4-coumaric acid by the enzyme 4-coumarate:CoA ligase (4CL), is a crucial junction point in the lignin biosynthesis pathway. The product of the 4CL reaction, 4-coumaryl CoA, can be acted upon by several different enzymes that direct it into various branches of the phenylpropanoid pathway. These branches can lead to formation of hydroxycinnamoyl esters (both soluble and insoluble), flavonoids, stilbenes or benzoic acids, as well as lignin. How this traffic is organized at the subcellular level remains uncertain, but it seems likely that the array of potential reactions is segregated within plant tissues, and perhaps even within a given cell. Isoforms of 4CL with different substrate specificities could conceivably play a role in directing 4-coumarate into specific metabolic options. For instance, isoforms of 4CL in poplar have been reported to differ in their ability to activate sinapic acid [Grand *et al* 1983]. Expression of different patterns of 4CL isoforms in different cells, or at different times, in poplar tissues could result in a bias toward, or against, synthesis of syringyl-type

monomers in the biochemical feedstock for lignification. We have been exploring the possibility of exploiting such differential isoform function to create a "high syringyl" lignin in transgenic poplar. Analysis of the 4CL mRNA populations in hybrid poplar (*P. trichocarpa* x *deltoides*) by Douglas and colleagues [Allina and Douglas, unpublished results] has revealed at least two closely related classes of 4CL gene products, although it is always possible that other 4CL genes exist that display low homology to these two. On the other hand, at least three isoforms of 4CL activity can be resolved in protein extracts from developing xylem of poplar [Pri-Hadash and Ellis, unpublished results]. These all use ferulic acid efficiently as a substrate but differ in their affinity for sinapic acid; the pattern of substrate preference among the isoforms differs, however, from that reported earlier for 4CL preparations from *Populus x euramericana* [Grand *et al* 1983]. In order to establish the relationship between the 4CL genes and enzymes, we are characterizing recombinant poplar 4CL proteins both immunologically and kinetically. Experiments are also underway to modulate 4CL activity in transgenic plants (tobacco, poplar) through over-expression and antisense suppression of each poplar 4CL cDNA in turn.

Creation of guaiacyl-type lignin monomers requires a second hydroxylation of the ring, at position 3, followed by an O-methylation of the 3-hydroxyl. The enzyme responsible for the latter reaction, catechol O-methyltransferase, has been purified, and the cDNA has also been cloned from a number of sources, including alfalfa and poplar. It appears that the substrate specificity of angiosperm COMT enzyme enables it to convert both caffeic acid to ferulic acid, and 5-hydroxyferulic acid to sinapic acid, although COMT from gymnosperms is reported to act much less efficiently on 5-hydroxyferulic acid [Kuroda *et al* 1975]. If this difference (rather than inefficient 5-hydroxylation of ferulic acid) is responsible for the prominence of the guaiacyl substitution pattern in gymnosperm lignin, it might be possible to insert a bi-functional angiosperm COMT into transgenic conifers, once that technology is ready. However, suppression of COMT expression also offers the potential to restrict the overall flux through the pathway, and this possibility is

being investigated by Chiang and colleagues at Michigan Technological University [T. Higuchi, personal communication].

The step in the phenylpropanoid pathway that establishes the potential for part of the product stream to bear a syringyl substitution pattern, as opposed to the ubiqiutous guaiacyl pattern, is that catalyzed by the enzyme ferulate 5-hydroxylase. Relatively little is known about this enzyme from any source, but a recent study of *Arabidopsis* mutants affected in phenylpropanoid metabolism has resulted in the identification of a genetic locus that controls introduction of the 5-hydroxy function [Chapple *et al* 1992]. Tagging of the same locus with a T-DNA insertion has also allowed recovery of the wild-type structural gene, which shows strong homology to other cytochrome P-450 hydroxylases [C. Chapple, personal communication]. If this proves to be the structural gene encoding ferulate 5-hydroxylase, it should provide an excellent entrypoint for genetically engineering major changes in lignin monomer composition.

Reduction of the Coenzyme A derivatives of the hydroxycinnamic acids probably commits the product stream largely to lignin synthesis. The resulting aldehydes are further reduced to the primary alcohols by the enzyme cinnamyl alcohol dehydrogenase (CAD), which has been purified and cloned from several sources. There is some evidence for the existence of CAD species that differ in their affinity for the three main hydroxycinnamyl alcohols, which again offers an opportunity to alter lignin composition by manipulation of this step. The dedication of the CAD reaction to lignin biosynthesis, however, makes it an attractive target for suppression of the overall metabolic flux to lignin. Early results from attempts to reduce CAD activity in transgenic tobacco have shown that it is possible to significantly suppress lignin accumulation in this fashion [Schuch 1993].

The reactions that convert the newly synthesized hydroxycinnamyl alcohols to a lignin polymer have yet to be clearly defined. In the canonical model, the alcohols

are converted to 4-O-glucosides by one or more glucosyltransferases for storage and/or transport, then hydrolyzed by *beta*-glucosidase activity, transported from the cytoplasm to the cell wall space, and polymerized *in situ* by peroxidase and/or laccase. Most of the evidence for this sequence derives from correlations between developmental lignification patterns and distribution of enzyme activities and/or metabolite pools. The attractiveness of these distal reactions as sites for genetic engineering a block in lignin synthesis, however, has recently focussed more attention on this portion of the pathway. We have undertaken the characterization of *beta*-glucosidases from cambial tissue of *Pinus contorta*, and have been able to detect and purify a coniferin-specific glucosidase [Dharmawardhana, Ellis and Carlson, unpublished results]. Isolation of the corresponding cDNA is currently underway, and with this in hand, we hope to be able to suppress the activity of this enzyme in lignifying tissue. The results should help clarify the role of the glucosylation-hydrolysis cycle in lignin biosynthesis, and may provide another potential regulation site.

The nature of the final polymerization reaction is still unclear. Correlative evidence in different systems has implicated both peroxidase and laccase as the catalytic agents, but the final answer is almost certainly going to prove to be more complex than identification of a discrete enzyme. The polymerization process generates spatially and temporally differentiated types of lignin, but whether these patterns are established through nucleation patterns, through precursor supply, or by participation of different species of polymerizing enzyme, is unknown. The availability of cDNA clones for both peroxidases and laccase has recently made it possible to design experiments aimed at answering these questions. Initial attempts to modulate peroxidase activity in transgenic tobacco and tomato plants have yielded mixed results, however; lignin content was profoundly affected in one case [Lagrimini *et al* 1990], but not in another [Sherf *et al* 1993]. The multiplicity of peroxidase isoforms observed in plant tissues may ultimately make it difficult to block peroxidase activity efficiently.

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REGULATORY EVENTS IN SOMATIC EMBRYOGENESIS - TRANSCRIPTIONAL AND TRANSLATIONAL

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We have been using somatic embryos of alfalfa (*Medicago sativa* L.) as a model system to study the deposition of storage reserves, particularly proteins, during their development. Somatic and zygotic embryos of many dicot species frequently exhibit gross morphological similarities during their development (Gray and Purohit, 1991), e.g. they pass through the globular, heart and torpedo stages. Such is the situation for both types of alfalfa embryogenesis (Xu and Bewley, 1992). But striking differences in development can occur, not least of which is the failure of somatic embryos to form a suspensor, and the absence of two well-formed cotyledons in the mature embryo (Steward et al, 1958; Xu and Bewley, 1992). Our interest has been in the metabolic consequences of this incomplete development, and to establish the differences between somatic and zygotic embryos at the subcellular level of organization.

An appropriate measure of the integrity of embryogenesis *in vitro* is the synthesis of polymeric reserve materials such as proteins, carbohydrates and triacylglycerols, for this event is characteristic of zygotic embryo development (Bewley and Black, 1994). Moreover, with regard to the nature of the subunit or polypeptide composition of some storage proteins is usually cultivar specific, as has been shown for alfalfa (Krochko et al, 1990). There has been an extensive characterization of the storage proteins within zygotic seeds of alfalfa, where they are present mainly within the cotyledons, but also in the axis (Krochko and Bewley 1988, 1990; Coulter and Bewley, 1990). There are two low-salt-soluble proteins: a low molecular weight (LMW) 2S, disulphide-bonded, sulphur-rich protein and a heterogenic 7S oligomeric protein (alfin) containing polypeptides ranging in molecular mass from 14-50 kDa. A larger 11S oligomeric protein (medicagin) is made up of acidic (39-49 Kda) and basic (20-24 Kda) polypeptides and is extractable only in high-salt buffers. Somatic embryos contain the same

types of proteins, but they are both quantitatively and qualitatively different. The amount of protein synthesized in somatic embryos, on a dry weight basis is only about 20% of that in zygotic embryos, and there is also a marked difference in the proportion of the three major storage proteins in the mature embryos. Whereas the 11S and 2S proteins predominate in the zygotic embryos (in ratios 11S:7S:2S of 3:1:2), in the somatic embryos there is proportionately more 7S protein (11S:7S:2S ratio is 1:4:2). Also, even the ratios of the component subunits of the 11S protein are different in the somatics, with at least one of the acidic subunits being absent.

During zygotic embryo development the 7S storage protein is synthesized one stage (approx. 2-3 days) earlier than either the 11S or 2S, which commence synthesis at the same time (Krochko et al, 1992, 1994). The 7S protein is also the first to appear during somatic embryo development, and while the 11S protein appears within the next two days, the appearance of the 2S protein is considerably delayed. Thus both the composition, and the coordinate regulation of the appearance of the three major storage protein types in alfalfa seeds is disrupted during somatic embryogenesis.

Northern blot analysis of the messenger RNAs for the storage proteins, using appropriate cDNAs, during the cotyledonary stages of development of somatic embryos reveals that there is a correlation between the amounts of message present and protein synthesized (Pramanik et al, 1992). Thus, as is normally the case during zygotic embryogenesis, there is transcriptional control of storage protein synthesis. Likewise, during the equivalent (cotyledonary) stages of somatic embryo development, the mRNAs for the 2S, 7S and 11S proteins are abundantly present; again, this is indicative of transcriptional control. It is possible that for the 2S storage protein, some post-translational control is also in operation. For this protein accumulates only in low amounts in somatic embryos, and may not be targeted correctly to the protein bodies after its synthesis, thus making it susceptible to proteolytic enzymes present within the cytoplasm (Bewley

et al, 1993). In contrast to these observations, northern blot analysis of the storage protein mRNA population during the early stages of somatic embryogenesis (globular, heart and torpedo stages) reveals the presence of 2S, 7S and 11S messages, even though none of the storage proteins is being synthesized at this time (Pramanik et al, 1992). This means that storage protein messages are present, but there is some post-transcriptional block which prevents them being translated during the early stages of embryogenesis.

The occurrence in eukaryotic cells of untranslated mRNAs has been reported, especially in animal cells undergoing embryogenic and morphological changes (Rosenthal et al, 1980). These mRNAs are associated with proteins in the cytoplasm of the cell, as messenger-ribonucleoprotein complexes (mRNPs), also known as the repressed fraction. Messages can be recruited from this fraction (which at times during animal embryogenesis can contain up to 80% of the total mRNA complement of a cell) into he active polysome fraction where they are used for the synthesis of proteins. Does a similar mechanism of mRNA repression occur in alfalfa somatic embryos during the globular to torpedo stages of development? It appears so, for a greater proportion of the population of mRNAs for all three storage proteins is present in the mRNP fraction (Pramanik et al, 1992). This is consistent with there being no storage protein accumulation in the seeds at this time. Later in development, during the cotyledon stages of somatic embryogenesis, this distribution is reversed, with more of the messages being present in the active polysomal fraction. This, again, is consistent with the increase in storage protein accumulation, especially the 7S and 11S, during the late stages of embryogenesis. During the cotyledonary stages of zygotic embryo development, when storage protein accumulation is maximal, their mRNAs are found largely within the polysomal fraction.

The storage protein messages present within the mRNP fraction during early somatic embryogenesis are potentially active, for when they are extracted from the cells, deproteinized, and placed in an *in vitro* protein synthesizing system, the

storage proteins are translated (Pramanik et al, 1992). Thus it is possible that inactivation of mRNAs within the mRNP complex is due to their association with repressor proteins. Using uv-cross-linking experiments (Bewley et al, 1993, and unpublished data) we have been able to identify several proteins that are intimately associated with mRNAs in both the polysomal and cytoplasmic mRNA (repressed) fractions. Many of the proteins (ranging in molecular mass from 15-150 kDa) associated with both fractions are qualitatively similar throughout embryogenesis, although their concentrations in the two fractions vary. However, some developmentally stage-specific proteins were found to be associated with the repressed mRNA fraction during the early stages (pre-cotyledonary) of somatic embryo development. These proteins inhibited protein synthesis when added to an *in vitro* protein-synthesizing system; proteins from the later stages (cotyledonary) did not. In contrast, proteins from the polysomal fractions of cotyledonary zygotic embryos enhanced *in vitro* protein synthesis. Regulation of protein synthesis at the translational level during early somatic embryogenesis, therefore, could be the result of sequestering of mRNAs with specific repressor proteins. Either the cellular complement of these proteins changes during development, with them not being synthesized or present at stages when storage protein synthesis is occurring, or else the storage protein mRNA itself changes in its ability to bind repressor proteins (perhaps because of interactions with other proteins).

At the practical level, there are many problems to be faced when sowing somatic embryos under field conditions, to insure their germination and successful conversion to seedlings. Establishment of seedlings after germination of zygotic embryos, i.e. following emergence from the seed, requires the mobilization of the major storage reserves, the catabolites from which are used as metabolic building-blocks for further growth. Since many somatic embryos are poor in reserve content compared with their zygotic counterparts, this deficiency has to be accommodated if seedling establishment is to occur. One approach is to encapsulate the somatic embryos in a medium which provides sufficient nutrients for early growth, although there are logistical problems with relation to this. If

polymeric compounds are used, then enzyme degradation of them will be necessary, and if low molecular weight compounds are used, osmotic problems could result upon imbibition, thus preventing or slowing germination and growth. It would be more satisfactory to increase the endogenous storage reserve content of the embryos themselves. This might be achieved through manipulations of the media on which the somatic embryos are developing, e.g. increasing available N content (e.g. glutamine) to improve protein content, or maintaining the embryo developmental mode longer by adding abscisic acid (ABA) to the medium. While both may increase reserve synthesis, it has not been to the level found in zygotic embryos. Transfer of genes, for example for storage proteins, into tissues from which somatic embryos are formed may enhance the reserve content if they are expressed during embryogenesis. But our work points to the fact that defects in storage protein content of somatic embryos may not reside simply in underexpression of storage protein genes. Post-transcriptional events, such as mRNA repression and mis-targeting, so that the translated products are hydrolyzed by cytosolic enzymes, may play a role too. It will be much more difficult to overcome these latter deficiencies.

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CONIFER SYNTHETIC SEEDS.

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Synthetic seed technology based on the use of somatic embryos, is expected to revolutionize propagation of conifers. Proliferating embryogenic cultures from conifers consist of immature embryos, which undergo synchronous maturation in the presence of abscisic acid and elevated osmoticum. One normal aspect of zygotic embryo development is maturation drying, which allows seeds to be stored and promotes normal germination. Conditions of culture have been obtained that yield mature conifer somatic embryos that possess normal storage proteins and fatty acids and which survive drying to low moisture contents. This enables large numbers of quiescent somatic embryos to be produced throughout the year and stored for germination in the spring, which simplifies production and provides plants of uniform size. This paper focuses on the development of conifer somatic embryos, desiccation tolerance, germination and plant formation.

Water stress during embryo development

Water stress and concomitant moisture loss during development, followed by late stage drying occurs naturally during zygotic embryo development. Embryo drying enables seeds to be stored for prolonged periods and drying enhances germination and growth by altering protein synthesis from a developmental to a germinative mode (Bewley and Black, 1985; Kermode, 1990). Research concerning somatic embryogenesis has recently been aimed at causing somatic embryos to function as artificial seed with regard to storage and handling qualities (Gray and Purohit, 1991). Restricting water uptake using osmotica and ABA has been studied for its ability to retain plant embryos in a developmental mode while suppressing germination (Kermode, 1990; Attree and Fowke 1993). Permeating osmotica (e.g., sucrose) are frequently used to generate a water stress and thereby promote embryo development during *in vitro* culture of angiosperm and conifer somatic and zygotic embryos. During prolonged culture, however, such osmoticum will be

taken up by plant cells leading to osmotic recovery and no reduction in water content (WC), which may be detrimental to embryo development. In contrast, non-permeating osmotica restrict water uptake, so provide a natural drought stress during embryo development (Attree et al., 1991; Attree and Fowke, 1993).

Studies of white spruce somatic embryos have shown that suitable osmotica for promoting the development of white spruce somatic embryos should have a molecular size greater than about 30 Å. Thus, polyethylene glycols (PEGs) and dextrans of molecular weights greater than about 1000 and 6000, respectively, provided a non-permeating form of water stress which promoted somatic embryo development; cultures showed a three to seven fold increase in maturation frequencies and recovered embryos had higher accumulations of storage lipids (Attree et al. 1992) and proteins (Misra et al. 1993), in comparison to somatic embryos cultured in control medium containing low osmoticum (3 % sucrose). High levels of permeating osmotica such as low molecular weight PEGs, mannitol, or sucrose, resulted in culture death, presumably due to uptake of the solutes following prolonged contact.

Maturation drying

Drying of conifer somatic embryos to moisture contents below those observed for mature zygotic embryos excised from dry seed was first described for white spruce (Attree et al. 1991, 1992). During culture with ABA and PEG 4000, somatic embryo development in white spruce was marked by well defined phases. An initial phase occurred during the first two weeks on development medium in which there was little gain in fresh weight (FW) or decline in moisture content (MC) and corresponded to a period of rapid cell division. This was followed by a maturation phase (weeks 3-8) during which storage reserves were deposited. The maturation phase consisted of two parts; a desiccation intolerant (predesiccation) period (weeks 3-5) during which a rapid decline in MC and rapid increase in dry weight (DW) was observed, and somatic embryos only survived slow drying. This was followed by a desiccation tolerant stage (weeks 5-8) when the somatic

embryos survived rapid drying once the MC had declined below about 55 % and when DW approached or exceeded WC values. The final phase after the 8th week, was the artificially imposed drying phase in which MC reached as low as 5 % following rapid drying, resulting in quiescent somatic embryos. Drying was essential for subsequent normal growth of white spruce somatic embryos following development with PEG (Attree et al., 1991).

Slow drying of white spruce somatic embryos in controlled environments of 81-43 % RH yielded conversion frequencies of 78-84 % for dried somatic embryos following one year of frozen storage at -20 °C, similar to the 77 % recorded for control somatic embryos germinated without freezing or storage. This demonstrates that drying treatments may be used effectively for long-term storage of mature somatic embryos during large scale embryo production. Plantlets grown from desiccated somatic embryos have undergone continued growth and establishment in soil.

Scaling up production of conifer somatic embryos in a bioreactor

The mature somatic embryos in the above studies contained high levels of storage reserves, but were less vigorous than their zygotic counterparts. The cultures were maintained in Petri dishes containing agar solidified media and frequent transfer of developing embryos to fresh media was required. As a consequence, methods for large scale micropropagation are labour intensive and costly.

Somatic embryos of white spruce, black spruce, and Norway spruce have been cultured in simple continuous flow solid support bioreactors. Following maturation in a bioreactor white spruce somatic embryos exceeded the growth extension recorded for *in vitro* grown excised zygotic embryos by 20 % (Attree et al. 1994). The somatic embryos turned green and commenced growth at 99 % frequency following desiccation to 8 % moisture, showing that they were extremely tolerant to late stage maturation drying. Somatic embryos converted to plantlets at a frequency of 92 %, compared to just 80 % following development on agar

medium then desiccation. Maturation, desiccation tolerance, and postgerminative growth were all improved following culture in bioreactors compared to culture on agar medium in Petri dishes, but somatic embryos produced by either method possessed similar lipid levels, which were over three times the level of zygotic embryos. Both culture methods also produced somatic embryos with fatty acid compositions which were comparable to the fatty acid compositions of zygotic embryos. This suggests that the better growth from the bioreactor grown cultures was due to an enhanced supply of nutrients or the efficient removal of waste products, resulting from the medium flow. Additionally, the large air space in the culture chamber may have provided a more suitable gaseous environment encouraging normal development. The continuous-flow culture chamber type bioreactor is less sophisticated than a mechanically-stirred submerged liquid bioreactor, so is considerably cheaper. Running 25 units seven times per year could yield over one million mature somatic embryos. The high vigour of the somatic embryos described here suggests that the increased level of storage lipid within somatic embryos compared to zygotic embryos (Attree et al. 1992) has in part compensated for the absence of a nutritive megagametophyte. This should be of value in creating synthetic seeds of conifers capable of germinating *ex vitro*.

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HARDWOOD PROPAGATION AND GENE TRANSFER VIA SOMATIC EMBRYOGENESIS

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Embryogenic cultures of hardwood forest trees promise to be highly useful for propagation and gene transfer applications, provided that some basic limitations characterizing these systems can be overcome. Currently, the major limitation to propagating hardwood tree species by somatic embryogenesis is the fact that most systems employ genetically unproven seed or seedling explants. A secondary problem is the low efficiencies of plantlet production reported for most systems. Progress in solving these problems is being made. Reports of embryogenesis from mature (or at least non-seed) tissues of hardwood trees are appearing in the literature with increasing frequency (e.g. Gingas 1991, Jorgensen 1989, Michler and Bauer 1991). Furthermore, some applications of embryogenic hardwood cultures can be realized now, in spite of the current limitations. Over the past 10 years, we have initiated embryogenic cultures of a number of hardwood forest trees. These cultures were all initiated from immature zygotic embryos and thus the cultures derived from them are simply the products of embryo cloning. Even though these cultures originated from genetically unproven material, they have proven valuable for a number of applications, including propagation of superior full-sib families, rare hybrids and rare species, protoplast culture, artificial seeds and microprojectile-mediated gene transfer. Here, each of these applications will be illustrated using embryogenic systems we have developed in our laboratory.

Mass propagation of full-sib material (*Liriodendron tulipifera*)

Somatic embryogenesis in yellow-poplar (*Liriodendron tulipifera*) tissue cultures was first reported by Merkle and Sommer (1986), and the protocol for culture initiation has remained basically unchanged since being optimized by Sotak et al. (1991). The optimal developmental stage of zygotic embryo explant for initiation of embryogenic cultures was found to be the globular stage, 0.3 mm or less in diameter, which appears 7-9 weeks following pollination (Sotak et al. 1991).

Proembryogenic masses (PEMs) began to appear from the explants within 2 months of culture on a Blaydes' (Witham et al. 1971) induction medium containing 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.25 mg/l benzylaminopurine (BAP), 1 g/l casein hydrolysate (CH) and 40 g/l sucrose. Proliferation of PEMs was maintained by monthly passage to fresh induction medium. To initiate development of somatic embryos, PEMs were transferred to semisolid basal medium (same as induction medium, but lacking growth regulators). Over the past five years, however, we have developed a protocol for mass production of synchronous populations of embryos from suspension cultures of PEMs, which were initiated from cultures on semisolid medium. Embryogenic suspension cultures were size fractionated on stainless steel screens and the fraction of PEMs between 38 µm and 140 µm was collected on filter paper using a Buchner funnel. The filter with PEMs was placed on semisolid basal medium and incubated under fluorescent light (16 hr/day) at 30° C. Using this method, a highly synchronous population of embryos was produced within two weeks following plating of PEMs (Merkle et al. 1990). Mature embryos with well-developed cotyledons were selected and transferred to Petri plates containing basal medium lacking CH for germination. We found that CH inhibited somatic embryo germination, probably by raising the osmotic potential of the medium. Eliminating it from the basal medium promoted vigorous germination and cotyledon greening within one week (Merkle et al. 1990). Following transfer to Magenta GA7 vessels containing 100 ml of a modified Risser and White's (1964) plantlet development medium, an average of 32% of these somatic embryos converted to plantlets (Merkle et al. 1990). This protocol was applied to 9 embryogenic lines to produce over 5500 somatic embryo-derived plantlets for field testing (Merkle et al. 1991). Recently, we found that skipping the step on plantlet development medium actually resulted in more vigorous plantlets (see section on *Magnolia* spp. for experimental results). Newly-germinated somatic embryos with greening cotyledons are now routinely transferred directly from Petri dishes to potting mix and acclimatized in a humidifying chamber by gradually lowering the relative humidity from 100% to ambient conditions over 6-8 weeks. During this

time plantlets are fertilized weekly with 1 ml of a modified Hoagland's solution.

Mass propagation of rare hybrids (*Liriodendron tulipifera* x *Liriodendron chinense*)

Initiation of hybrid *Liriodendron* embryogenic cultures followed the same protocol as for yellow-poplar culture initiation, except that the explant material was immature hybrid zygotic embryos. To generate hybrid seeds, flowers were collected from a Chinese tuliptree (*Liriodendron chinense*) and the anthers used for controlled pollinations of yellow-poplar mother trees. Aggregates of samaras resulting from the pollinations were harvested 8 weeks post-pollination and embryos and endosperm excised to initiate cultures on the yellow-poplar induction medium described above. In contrast to yellow-poplar embryogenic cultures, hybrid *Liriodendron* cultures tended not to maintain growth as PEMs while exposed to 2,4-D. Instead, they proliferated via repetitive embryogenesis as globular- or even later-stage embryos. Thus, they were not as amenable to suspension culture as yellow-poplar embryogenic cultures and efforts to use the fractionation/plating protocol described above were unsuccessful. However, clusters of immature hybrid somatic embryos transferred to basal medium developed into mature embryos after 4-6 weeks. Mature embryos germinated vigorously following transfer to basal medium lacking CH. We found that newly germinated embryos can be immediately planted in potting mix and acclimatized as described above for yellow-poplar. Hybrid *Liriodendron* plantlets have shown evidence of hybrid vigor by their rapid growth rates and leaf sizes that are 2-3 times the size of parental leaf sizes. We have verified the hybrid nature of all of these embryogenic lines and plants derived from them by Southern hybridization analysis with a species-specific DNA marker (Merkle et al. 1993).

Mass propagation of rare species (*Magnolia* spp.)

Using our experience with yellow-poplar as a basis, we initiated experiments to establish embryogenic cultures of number of magnolia species native to the southeastern United States: Sweetbay magnolia (*Magnolia virginiana*), fraser magnolia (*M. fraseri*), yellow cucumbertree (*M. cordata*) (Merkle and Wiecko

1990), bigleaf magnolia (*M. macrophylla*; Merkle and Watson-Pauley 1993), pyramid magnolia (*M. pyramidata*; Merkle and Watson-Pauley, in press), and southern magnolia (*M. grandiflora*; unpublished). Some of these species, such as yellow cucumbertree and pyramid magnolia, are quite rare, and even genetically unproven clones of these trees are of considerable horticultural value. Culture initiation protocols and media were the same as for yellow-poplar, with one main distinction: The optimal developmental stage of zygotic embryo to explant for initiation of embryogenic cultures was reached much sooner following pollination in magnolias than in yellow-poplar. We found that 3-5 weeks post-pollination magnolia seeds had the highest embryogenic potential (Merkle and Wiecko 1990).

Each magnolia species responded somewhat differently to culture on yellow-poplar induction medium. Some species proliferated as PEMs and could be maintained on the 2,4-D-supplemented medium, while others declined with long-term exposure to 2,4-D, but maintained repetitive embryogenesis on basal medium following an initial pulse with the auxin. As with yellow-poplar, germination of mature somatic embryos of all magnolia species was accomplished by transferring them to basal medium lacking CH and incubating in the light at 30° C. Germinants transferred to yellow-poplar plantlet development medium in test tubes or GA7 vessels continued to grow, but both root and shoot development were slow. Because of this lack of plantlet vigor in vitro, we conducted an experiment to compare development of newly germinated pyramid magnolia somatic embryos in vitro versus that ex vitro. We found that frequency of plantlet production (conversion) from germinants was higher and the resulting plantlets were more vigorous when germinants were transferred directly to potting mix and grown in the humidifying chamber instead of being maintained in vitro on plantlet development medium. Ex vitro-converted pyramid magnolia somatic embryos had, on the average, leaves that were 3 times larger and root systems that were 9 times longer than in vitro-converted somatic embryos of the same age (Merkle and Watson-Pauley, in press). Based on these results, our standard protocol for newly germinated somatic embryos of all of our magnolia and *Liriodendron* cultures is to

transfer them directly to potting mix for conversion in the humidifying chamber.

Although no organized field testing of magnolia somatic embryo-derived plantlets has been conducted, those trees which have been planted out resemble seedling-derived trees in form, growth rate and bud phenology. Surprisingly, somatic embryo-derived trees of both sweetbay magnolia and yellow cucumbertree have produced flowers as early as 2 seasons following outplanting (unpublished).

Artificial seeds (*Robinia pseudoacacia*)

Embryogenic black locust (*Robinia pseudoacacia*) cultures were initiated at a low frequency (average 12%) by culturing developing seeds collected only 2-3 weeks post-anthesis on Finer and Nagasawa's (1988) 10A40N medium, which was originally developed for embryogenic soybean cultures (Arrillaga et al. 1994). Cultures were maintained as suspensions of PEMs in liquid 10A40N medium with 3 mg/l 2,4-D, and produced populations of singularized embryos approximately 1 month following transfer to either semisolid or liquid basal 10A40N medium. We tested alginate-encapsulated and naked black locust somatic embryos for their potential to be employed as artificial seeds by sowing them either on half-strength basal medium or on potting mix in GA7 vessels. On half-strength medium, 71% of naked embryos and 41% of encapsulated embryos converted to plantlets. Fifteen days of cold treatment increased conversion rates up to 95% for naked embryos and 80% for encapsulated embryos. Conversion rates of naked and encapsulated embryos sown directly on potting mix were much lower (18% and 7% respectively), but demonstrated the potential for somatic embryos of this species to produce plantlets directly under ex vitro conditions (Arrillaga et al. 1994).

Protoplast culture (*Liriodendron tulipifera, Magnolia cordata*)

Prior to the advent of microprojectile-mediated gene transfer, we developed a system for isolation and culture of protoplasts from embryogenic suspensions with the goal of using them for gene transfer via electroporation or PEG-mediated

DNA uptake. Embryogenic yellow-poplar suspensions proved to be sources of highly regenerative protoplasts (Merkle and Sommer 1987, Wilde et al. 1989). Highest protoplast yields (1.7×10^7 protoplasts/g) were obtained from 20 day-old suspensions, which were digested using a mixture of Cellulysin and Macerase for 24 hours. Following isolation, protoplasts were plated in beads of regeneration medium solidified with low melting point agarose, and cultured in plates flooded with liquid regeneration medium. Cultured yellow-poplar protoplasts regenerated cell walls within 3 days and the first cell divisions generally occurred within 4 days. Cell colonies of 8 or more cells were observed within 2 weeks and calli were visible without a microscope within 3 weeks. Plating efficiency (percent of protoplasts forming cell colonies) ranged as high as 30%. Suspension cultures could easily be established from the protoplast-derived calli by placing the agarose droplets containing the calli into liquid induction medium in flasks on a gyratory shaker. Within a few weeks, calli expanded out of the droplets and PEMs proliferated rapidly in suspension. PEMs from suspensions regenerated from the protoplast-derived calli produced somatic embryos following transfer to basal medium, and plantlets regenerated from these embryos appeared normal.

Species from the two subgenera of *Magnolia* are sexually incompatible, making it impossible to use sexual hybridization to breed the flower coloration characterizing some Asian species into our white-flowered North American species. Protoplast fusion to generate somatic hybrids between species in different subgenera could potentially could be used to overcome this barrier. In preliminary experiments, we have used techniques similar to those used with yellow-poplar to isolate and culture protoplasts from an embryogenic suspension of yellow cucumbertree. To date, however, embryogenic cultures have not been regenerated from the isolated protoplasts (unpublished).

Microprojectile-mediated gene transfer (*Liriodendron tulipifera*, *Castanea dentata*)
One application for which we have found embryogenic suspension cultures to be highly amenable is gene transfer via microprojectile bombardment (Klein et al.

1988). Using a DuPont Biolistics PDS-1000 particle gun, we (Wilde et al. 1992) stably transformed embryogenic cells of yellow-poplar, from which transgenic plantlets were regenerated via somatic embryogenesis. Briefly, our transformation protocol was as follows: Plasmid DNA (pBI121.1) containing genes encoding the reporter β-glucuronidase (GUS; Jefferson et al. 1987) and the selectable marker neomycin phosphotransferase (NPTII) was precipitated onto 1.1 μm tungsten or gold particles. The particles were used to bombard individual cells and small cell clusters that had been isolated by size fractionation of an embryogenic yellow-poplar suspension and collected on a filter paper disk. After 2 days of incubation on semisolid induction medium, filters were transferred to induction medium containing 100 μg/ml kanamycin. After 5-6 weeks, kanamycin-resistant PEMs, approximately 1 mm in diameter, were transferred individually to fresh antibiotic-containing plates. Suspension cultures were initiated from the resulting lines by transferring PEMs to liquid induction medium containing 50 μg/ml kanamycin, which was sufficient to inhibit growth of nontransformed yellow-poplar suspensions.

The ability to grow these cultures as suspensions was particularly desirable as it allowed stringent selection pressure to be applied, thereby reducing the likelihood of regenerating chimeras. Southern analysis indicated that independently transformed embryogenic sublines carried from 3 to 30 full-length copies of the GUS gene. A histochemical assay for GUS activity employing the substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) revealed a heterogeneous staining pattern in PEMs, which seemed to indicate that they were chimeric for the GUS gene. However, extracts from cell clusters reacting positively (blue) or negatively (white) with this substrate both demonstrated GUS activity when a fluorometric assay was performed using the substrate 4-methylumbelliferyl-β-D-glucuronide (MUG). Furthermore, somatic embryos regenerated from transformed sublines were uniformly GUS positive, using the histochemical assay. Plantlets derived from transformed somatic embryos and grown in the greenhouse continue to express GUS and NPT II in leaves and roots

over 3 years following transformation (unpublished). Currently, we are employing microprojectile-mediated transformation of embryogenic yellow-poplar cells to test genes with the potential to confer resistance to heavy metal ions or to modulate lignin biosynthesis.

Based on our results with microprojectile-mediated transformation of yellow-poplar, we tested microprojectile bombardment of embryogenic American chestnut (*Castanea dentata*) suspensions as a potential vehicle for generating transgenic American chestnut trees carrying genes conferring resistance to the chestnut blight fungus, *Cryphonectria parasitica*. Embryogenic American chestnut cultures were initiated from immature seeds collected from surviving trees throughout the range of the species (Merkle et al. 1991, Carraway et al. 1994). A low percentage of the immature seeds continuously cultured on Woody Plant Medium (Lloyd and McCown 1980) supplemented with 3 mg/l 2,4-D and 0.25 mg/l BAP produced repetitively embryogenic cultures. Somatic embryos were produced by transferring clusters of PEMs to medium lacking 2,4-D (basal medium). American chestnut PEMs were grown in suspension culture, size fractionated and bombarded with microprojectiles carrying the same plasmid as was used with yellow-poplar. While 16 transgenic cell lines were obtained using this method and have continued to express the foreign DNA for almost 2 years, no somatic embryos or plantlets have been obtained from these lines to date (Carraway et al. 1994).

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ENGINEERING RESISTANCE TO THE INSECT PEST CODLING MOTH IN APPLE AND WALNUT

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Over the millennia the study of horticulture and forestry has had a significant impact on the domestication of a wide variety of tree crops, creating important industries and a demand for their products. The 21st century poses a unique set of challenges to the production, storage and preservation of these products that can be met using novel germplasm and production practices that will sustain yield and quality while at the same time be gentle on the environment and the food chains vital to human health. The technology to produce transgenic tree crops will have an important and powerful impact on some of the immediate problems such as disease and pests. However, commercial success will depend upon their integration with the conventional technologies such as plant breeding, plant propagation, production physiology, integrated pest management and post harvest technology.

Gene engineering, theoretically allows the introduction of any gene from any source, greatly enhancing the pool of germplasm/genes that can be used, for a particular tree species. The major limitations, apart from ones imagination, are the stark reality of gene availability and the more mundane but essential development of transformation and/or gene transfer methodologies for the production of transgenic tree crops and ultimately for the regulation of expression of the introduced genes.

Codling moth (CM), the focus of our research, is a major pest of apple, pear and walnut; it also attacks prune, quince, hawthorne and certain varieties of plum. The major economic damage is to the fruit. Each spring, the first generation of adult

codling moths mate and lay eggs on the tiny developing fruit, at the base of leaf clusters or on leaf surfaces near the fruit. In apples, newly hatched CM larvae tunnel their way to the core of the fruit where they feed on the developing seeds. In California each year there are at least 3 generations of CM per growing season. Since fruit are the primary target and the larvae eat a very small portion of the surface, timing of insecticide sprays should coincide with egg hatch. CM is currently controlled mainly by chemical insecticides like "Guthion" (azinphosmethyl), "Lorsban" (chlorpyrifos), "Supracide" (methidathion) and "Imidan" (phosmet), with Guthion being the insecticide of choice on walnut, apple and pear. Because of its feeding behavior CM is difficult to control with spray applications. Secondary insect pest problems are caused by the application of chemical pesticides which kill CM, as well as impact predators of mites and aphids, leading to increased populations. Alternatives to chemical insecticides have had limited success and, it is difficult to grow apples or walnuts organically, especially in regions that have several generations of CM in a growing season. Additionally, there is the cost of materials and application that has to be considered for any management practice to be economically feasible. There is a general feeling that future availability of many chemical pesticides is limited in view of the growing sensitivity to environmental and food safety issues. Research and development costs of new and safer chemical pesticides and the cost of registration are rapidly escalating. We are exploring the alternative of expressing genes in tree crops to battle insect pest problems.

The approach used in this study involves the insecticidal activity of proteins made by the common soil bacterium *Bacillus thuringiensis* (Bt). The insecticidal properties of crystal/spore suspensions of *Bacillus thuringiensis* have been exploited commercially for many years in products such as `Dipel' (Abbot Laboratories), `Javelin' (Sandoz) and `Thuricide' (Sandoz). Products like these have been in use for the past thirty years. These products have been shown to be safe to humans, birds, bees and other mammals and even other nontarget insects. We have tested purified preparations of the insecticidal crystal proteins (ICPs)

encoded by the *cry*IA(b) and *cry*IA(c) genes and found them to be very toxic to CM (Vail *et al.*, 1991). Transgenic plants expressing an ICP of Bt have been obtained in tobacco (Vaeck *et al.*, 1987), tomato (Fischoff *et al.* 1987), and cotton (Perlak *et al.*, 1991b). These transgenic plants expressed sufficient quantities of ICP to protect the plants from damage caused by the feeding larvae of tobacco or tomato hornworm, and tobacco budworm.

The *cry*IA(c) gene was introduced using the binary plasmid pWB139 into both walnut and apple. The plasmid pWB139 was constructed by Dr. W.M. Barnes, Washington University School of Medicine, St. Louis, MO., and has a wound inducible gene that encodes for a fusion protein containing the insecticidal activity of the *cry*IA(c) gene from the HD-73 strain of *Bacillus thuringiensis* with the kanamycin resistance gene product APH(3')II. The fusion gene is regulated by the 35S promoter of the cauliflower mosaic virus and wound inducibility is regulated by the presence of a fragment of the tomato protease inhibitor I gene and contains the sequences of exon 3 and part of the 3' region (Dandekar *et. al.*, 1994a). Therefore, successful expression of this chimeric fusion gene would give a product that gives resistance to the antibiotic kanamycin, allowing selection of transformed plants as well as is toxic to insects by virtue of the presence of the *cry*IA(a) sequences.

Analysis of 10 embryo lines and 13 shoot lines regenerated from the transformed walnut embryos (Dandekar *et. al.*, 1994a) and 27 transgenic apple shoot lines (Dandekar *et. al.*, 1994b) revealed low levels of expression as judged from the biochemical and insect bioassay data (Dandekar *et. al.*, 1994ab).

Since we had previously documented the sensitivity of this insect to pure preparations of the *cry*IA(c) gene product (Vail *et al.*, 1991), the problem could be at the transcriptional or post transcriptional levels. We believe the difficulty is due to the wild type gene sequences of Bt that results in several different problems; a) a strong codon bias preferring A/T in the third position when the preference in

plants is for codons XXC/G rather than XXA/T , b) presence of the unusual codons that have CG or AT in the second and third position, these are absent in most eukaryotes and lastly, c) we could identify at least 28 instances throughout the sequence where undesirable sequences were present. These undesirable sequences could cause instability in mRNA and thus affect protein accumulation (Dandekar *et. al.*, 1994a). Interestingly, transgenic plants expressing wild type ICP were obtained in tobacco (Vaeck *et al.*, 1987) and tomato (Fischoff *et al.* 1987) and although the level of expression was low and variable (Vaeck *et al.*, 1987) sufficient quantities of ICP was present to protect the plants from damage by the larvae of tobacco hornworm, tomato hornworm, and tobacco budworm.

Recently a synthetic gene containing an altered codon bias and removal of undesired sequences was introduced into cotton that demonstrated a much higher level of expression resulting in the mortality of both tobacco budworm and cotton bollworm, *Helicoverpa zea*(Boddie) (Perlak *et. al.*, 1991ab). Recently we have introduced into walnut synthetic *cry*IA(c) genes supplied to us by Dr. D. Fischoff from Monsanto Co. and Dr. Kevin McBride at Calgene Inc. in order to investigate the above mentioned possibilities. We now have several embryo lines expressing these synthetic genes which result in high levels of mortality when fed to CM, indicating a high level of expression of *cry*IA(c).

The next step is to regenerate the transgenic tissues to obtain plants for further testing. It will be important to identify those plants that show damage below 5%, which seems to be the threshold that is commercially acceptable and to measure yield and quality protection with and without the use of chemical pesticides.

Other potential concerns are regulatory and public acceptance issues. These concerns will almost certainly have been addressed by the time the transgenic apple or walnuts come into the marketplace because tomato and cotton will be introduced much earlier.

Another concern is the possibility of development of resistance to the transgenic plant material by CM. This is a controversial issue and will be crucial for the long term deployment of these transgenic plants. First and foremost the risk should be assessed by monitoring susceptibility of different populations of CM in the western USA. This would determine how susceptible the local populations of CM are to these proteins and to detect any genetic variability that could infer the possibility of resistance development.

A variety of resistance management strategies will also have to be explored to diminish the possibility of resistance development. There are several strategies that have been proposed, including integration with other compatible biological control methods like the use of insect predators or mating disruption techniques. Less chemical pesticides usage will allow the build up of predator populations. Mating disruption maybe more efficient because of the lower population levels of CM in the transgenic orchards.

Providing 'refugia' (safe sanctuary) has been proposed as a way to delay or avoid resistance development. Experiments with various orchard planting patterns containing both transgenic and wild type trees would have to be tested. The wild type trees will provide a refugia for susceptible insects thus maintaining a viable population to breed out resistance should it develop and be a recessive trait.

Another approach to avoid resistance development is to transform with multiple transgenes that are lethal towards CM but that do not share the same target site in the insect. This would mean identifying new gene products that are lethal to CM.

One of the areas that we will investigate is temporal and tissue specific expression of the introduced transgene. This may be very important because in our case the insect feeds on fruit, so we can avoid some of the other secondary pests by concentrating expression specifically in the fruit in a tissue specific manner. In addition expression can be regulated temporally in a specific tissue (e.g. fruit)

such that the gene is expressed in young but not mature ripe fruit, affecting earlier generations but not later generations of CM during the growing season. This approach could be another powerful resistance management strategy.

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USE OF SYNTHETIC HYPOVIRUS TRANSCRIPTS TO ESTABLISH TRANSMISSIBLE HYPOVIRULENCE IN PATHOGENIC FUNGI

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Strains of the chestnut blight fungus, *Cryphonectria parasitica* , that are infected with members of the genus hypovirus of the newely designated virus family *hypoviridae* often exhibit reduced virulence (Day *et al.*1977). These hypovirulent strains can serve as biological control agents by virtue of their ability to convert virulent strains to hypovirulence as a result of virus transmission during anastomosis (fusion of hyphae) (Anagnostakis and Day, 1979; Grente and Berthelay-Sauret, 1978; Van Alfen *et al.*1975). Natural and artificial application of hypovirulent *C. parasitica* strains have been quite effective in controlling chestnut blight in orchard settings throughout many regions of Southern Europe (Mittempergher, 1978; Turchetti, 1982). European hypovirulent *C. parasitica* strains were imported into the United States in 1972 and were found in glasshouse studies to be effective in controlling chestnut blight caused by North American *C. parasitica* strains on American chestnut trees (Van Alfen *et al.*1975; Anagnostakis and Jaynes, 1973). Subsequent release of these strains into North American forest ecosystems resulted in demonstrable but limited disease control (Anagnostakis, 1990; Fulbright *et al.*1983; Fulbright *et al.*1988; Garrod *et al.*1985). A major contributing factor to the reduced level of biocontrol efficacy observed in North America appears to be a high degree of vegetative incompatibility deversity in the resident virulent *C. parasitica* populations that restricts anastomosis and accompanying virus transmission.

The development of an infectious full-length cDNA copy of a hypovirus RNA was recently reported (Choi and Nuss, 1992). Strains transformed with these infectious cDNA clones (engineered hypovirulent strains) contain both chromosomally integrated viral cDNA and cytoplasmically replicating, cDNA-derived viral RNA (Choi and Nuss, 1992). Thus, engineered hypovirulent *C. parasitica* strains are able to transmit hypovirus genetic information through mating, a mode of

transmission not available to natural hypovirulent strains. This property circumvents barriers to cytoplasmic virus transmission imposed by the fungal vegetative incompatibility system (Chen *et al.*1993). By deleting a specific portion of hypovirus genetic information in the context of the infecious cDNA clone, it has also been possible to increase the level of conidiation by an engineered hypovirulent *C. parasitica* strain without affecting the level of hypovirulence (Craven *et al.*1993). It is anticipated that these properties will result in enhanced biological control, especially for fungal populations that have perviously been refractory because of extensive vegetative incompatibility diversity (Nuss, 1992).

We recently showed that the cDNA-derived hypovirus dsRNA present in engineered hypovirulent strains originates as a large nuclear transcript that subsequently undergoes pre-mRNA splicing and trimming of nonviral nucleotides (Chen *et al.*1994b). These observations suggested that it should be possible to estabilish a hypovirus infection by introduction of a synthetic copy of a hypovirus dsRNA coding strand into fungal spheroplasts. This prediction was confirmed by introducing synthetic hypovirus transcripts into fungal spheroplasts by electroporation (Chen *et al.*1994a). Transcripts corresponding to the viral RNA-coding strand were synthesized from a full-length cDNA copy of the prototypic hypovirus CHV1-713 L-dsRNA in plasmid pLDST. Electroporation of spheroplasts derived from virus-free virulent *C. parasitica* strains consistently yielded mycelia containing a dsRNA species that comigrated on agarose gels with CHV1-713 L-dsRNA. In contrast, no infections occurred when spheroplasts were electorporated with either the linearized template plasmid or RNase treated cell-free transcription reaction products, indicating that the synthetic transcripts are the source of L-dsRNA present in the transfected colonies. This was confirmed by transfecting with a modified synthetic viral transcript containing a unique *Not* I site at L-dsRNA map position 12,038 and showing by RT-PCR that the dsRNA present in the resulting transfectants contained the corresponding molecular tag.

These results provided the first demonstration that a synthetic transcript corresponding to the coding strand of a mycovirus dsRNA can initiate an infection when introduced into fungal spheroplasts.

Although the genus *Cryphonectria* (family Valsaceae) is widely distributed geographically and includes several plant pathogens (Barr, 1978; Micales and Stipes, 1986; Roane *et al.*1986), *C. parasitica* is the only member of the genus for which hypoviruses have been reported. It was, therefore, of interest to determine whether other taxonomically related fungal species possessed the capacity to support hypovirus replication if transfected with the full-length hypovirus transcript. Species selected for this analysis included three members of the same genus and one member of the genus *Endothia* (family Gnomoniaceae) within the same order (Diaporthales). These were *Cryphonectria cubensis* (Bruner) Hodges, the causal agent of a canker disease of *Eucalyptus* spp. in many tropical areas of the world (Hodges, 1980); *Cryphonectria havanensis* (Bruner) Barr, a less well characterized pathogen of *Eucalyptus* (Roane *et al.*1986), a nonpathogenic saprophytic species, *Cryphonectria radicalis* (Schw. ex Fries) Barr (Roane *et al.*1986) and *Endothia gyrosa* (Schw. ex Fries) Fries, a serious canker pathogen of *Quercus* spp., mainly pin oak (Appel and Stipes, 1984). To our knowledge, these species have not been shown previously to harbor hypoviruses and hypovirus dsRNA is not transmissible from *C. parasitica* to these species via anastomosis (our unpublished observations). However, all four of these fungal species were capable of supporting hypovirus replication following transfection with the infectious single-stranded CHV1-713 L-dsRNA transcript (Chen *et al.*1994a). These results demonstrated that fungal species other than *C. parasitica*, including a representative of a separate taxanomic family (*E. gyrosa*), have the capacity to support hypovirus replication.

Significantly, virulence of *E. gyrosa* on both pin oak and red oak was severly reduced following hypovirus infection. The low level of virulence exhibited by the virus-infected strains, coupled with the observation that hypovirus RNA is

transmissible from a transfected *E. gyrosa* strain to virus-free isolates via anastomosis, suggests the potential use of hypovirus-infected *E. gyrosa* strains for field control of pin oak blight. The expansion of hypovirus host range to include other tree pathogens suggests that virus-mediated hypovirulence will find broader applications in addition to the biological control of chestnut blight.

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THE USE OF TRANSGENIC BACULOVIRUSES FOR CONTROL OF FOREST PEST INSECTS

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Introduction. The *Baculoviridae* are a diverse family of viral pathogens characterized by a complex replication cycle that culminates in the occlusion of virions in a crystalline protein matrix (Blissard & Rohrmann, 1990). Baculovirus virions contain a single molecule of circular, supercoiled, double stranded DNA that ranges in size from 90 to over 165 kb in different species of the virus. The genome is packaged in enveloped, rod-shaped nucleocapsids, 30-60 nm in diameter and 250-300 nm in length. The two genera of baculoviruses (nuclear polyhedrosis viruses [NPVs] and granulosis viruses [GVs]) are distinguished by the size and morphology of the occlusion body. NPVs replicate in cell nuclei and are characterized by the occlusion of numerous virions in large (1-15uM) polyhedron-shaped occlusion bodies comprised predominantly of a single protein called polyhedrin. The occlusion body is surrounded by a structure called the polyhedron envelope or polyhedron calyx. In contrast, GVs are occluded in small (0.15-0.5uM) ellipsoidal-shaped occlusion bodies called granules and there is normally a single virion per granule and replication occurs either in the cytoplasm or in an undefined region present following the breakdown of the nuclear membrane.

Host distribution. Infection by baculoviruses is limited exclusively to arthropods, predominantly holometabolous insects (Adams & McClintock, 1991; Martignoni & Iwai, 1986). NPVs have been reported to infect about 300 species of Lepidoptera, 20 species each of Diptera and Hymenoptera, and a single species of Trichoptera. In addition, NPVs are infectious for several species of the crustacean order, Decapoda (shrimp), and are a major problem in the shrimp farming industry in Asia. Individual viruses have a very limited host range and normally only infect closely related insects within a single order. In contrast to NPVs, GVs are limited to Lepidoptera and about 150 species have been reported to be infected.

Ecology. Baculoviruses play a major role in regulating the level of insect populations in the environment. Many insect populations are characterized by cycles of expansion and subsequent collapse. As the number of insects increase, occlusion bodies that contaminate soil or vegetation from past baculovirus epidemics are ingested by susceptible insects and initiate an infectious cycle. When the insects die they disintegrate and contaminate surrounding vegetation. This leads to the virus being disseminated by wind, birds and insects. Thus the virus can spread over vast areas and can significantly contribute to the eventual collapse of an insect population. For example, in forest ecosystems, insect infestations can encompass several million hectares and baculoviruses often play a prominent role in the eventual collapse of these large populations.

Baculovirus insecticides. NPVs are highly virulent for insect pests and have been shown to be effective in insect control programs. Currently an NPV pathogenic for the velvetbean caterpillar is used to treat approximately one million hectares of soybeans per year in Brazil (Moscardi, 1989). Despite such instances, the use of NPVs for pest control is not widespread. This can be attributed to both the narrow host range of individual NPVs, and the fact that insects may survive for several weeks after infection. During this time they continue feeding and therefore can inflict extensive crop damage prior to death. This limits their commercial potential when contrasted to chemical insecticides that are often rapidly toxic to a large number of different insect pests. However, because broad spectrum chemical insecticides often destroy beneficial insects, and can be acutely toxic to vertebrates, alternative means of controlling insects have become increasingly attractive.

Genetic engineering to improve baculovirus insecticides. The advent of genetic

engineering led to a renewed interest in baculovirus insecticides because of the potential to alter baculovirus genomes to accelerate the infection. This could result in more rapid kill of target insects and make baculoviruses more attractive for use in insect control programs. The approaches being investigated include the following:

a) expression of genes that alter insect development. A diuretic hormone that regulates insect water balance has been cloned into *Bombyx mori* NPV and used to infect *B. mori* larvae (Maeda, 1989). This infection resulted in a major reduction in haemolymph volume and a modest reduction in the time to kill larvae. Juvenile hormone esterase (JHE) causes a reduction in JH titers which is associated with a cessation in feeding and initiation of metamorphosis. *Autographa californica* multinucleocapsid NPV (AcMNPV) expressing JHE was constructed and it was found that only insects infected at the first instar showed a reduction in feeding (Hammock et al, 1990). It was thought that the lack of effect on later instars could be due to an inability of the JHE to overcome insect JH synthesis, the fact that JHE is unstable, or that the production by virus of ecdysteroid UDP-glucosyl transferase (see below) could reduce the impact of JHE.

b) expression of insect-specific toxins. *Bacillus thuringiensis* delta endotoxin has been expressed in baculoviruses (Martens et al, 1990; Merryweather et al, 1990). The initial studies showed no effect. This was thought to be due to the fact that the toxin remained intracellular and therefore is unable to interact with cell membranes. In other studies insect-specific neurotoxins have been expressed in baculoviruses. In one instance a mite neurotoxin (Tomalski & Miller, 1991) and in another a scorpion neurotoxin gene (Stewart et al, 1991) was expressed by a recombinant baculoviruses. In both cases, an improvement in the effectiveness of the virus was observed by a reduction in the time it took for the virus to kill infected larvae.

c) deletion of a baculovirus gene that prolongs juvenile stages. Ecdysteroids are involved in molting between instars and between instars and pupae. The AcMNPV ecdysteroid UDP-glucosyl transferase (EGT) gene encodes an enzyme that inactivates ecdysteroids thereby prolonging juvenile instars (O'Reilly & Miller, 1989). While this is advantageous for the virus, because it prolongs the time of the larval instars and consequently the time for virus production, it is not an advantage for the insecticidal action of the virus because it prolongs the feeding stage. Therefore this gene has been deleted from AcMNPV in order to accelerate the infection.

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IN SITU HYBRIDIZATION AND GENOME ORGANIZATION IN FOREST TREES

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In situ hybridization has made significant contributions to the understanding of how the various classes of DNA sequences are organized within the plant genome. This has had important applications among angiosperms ranging from chromosome identification in barley (Leitch and Heslop-Harrison 1993) and the integration of physical and genetic maps of rice (Gustafson and Dille 1992) to assessing the three dimensional organization of DNA sequences at interphase (Rawlins et al. 1990). The technology of i*n situ* hybridization is also making important contributions to the understanding of comparative plant genome organization (Korenberg and Rykowski 1988; Ganal et al. 1988), evolution (Wienberg et al. 1992), genome mapping (Lichter et al. 1990; Ried et al. 1992), and the analysis of hybrids (Anamthawat-Jonsson et al. 1990). *In situ* hybridization permits specific DNA sequences to be physically mapped along metaphase chromosomes or interphase chromatin. Chromosomal target sequences may range from short (1 kb) unique sequences (Bhatt et al. 1988) to long tandemly arrayed repetitive elements (Rayburn and Gill 1985; Bedbrook et al. 1980), whole chromosomes (Wienberg et al. 1990) or entire genomes (Schwarzacher et al. 1992).

Gymnosperms, for which so little is known about genome organization, are particularly promising candidates for this technology, especially in the conifer species for which there are established tree genetics programs and for which genetic linkage maps are being constructed (Tulsieram et al. 1992). With the exception of chromosome numbers (Khoshoo 1960) and data from reassociation kinetics (Rake et al. 1980; Dhillon 1987), little is known of the nuclear genome in gymnosperms. Conifer genomes are large (white spruce, has one of the smaller conifer genomes with 2C = 17 pg. or 1.7×10^{10} base pairs; Miksche and Dhillon

1981) and consist of as much as 75% noncoding DNA. *In situ* hybridization has potential applications in genome mapping in conifers for piecing together linkage groups separated by areas of low or no recombination, for the quick assessment of the degree of similarity between genetic maps from different genotypes or related species, for sorting out multilocus probes in RFLP mapping, and for ordering contiguous, large clones such as YAC and cosmid inserts for map-based cloning of genes. These applications require a sensitive and accurate *in situ* hybridization protocol. There are few reported applications of *in situ* hybridization with gymnosperms, however. We will discuss our work on an *in situ* hybridization protocol for white spruce (*Picea glauca* (Moench) Voss.) and its related species and it's use in molecular karyotyping. Since the chromosomes of white spruce and most conifer species since are predominantly metacentric and of similar size (Khoshoo 1960), chromosome identification is difficult. Classical banding methods have also met with only limited success (MacPherson and Filion 1981). The *in situ* hybridization of repetitive DNA probes such as 5S ribosomal RNA genes, the nucleolar organizer regions (18S/26S rRNA genes), rDNA intergenic spacer sequences, centromeric repeats, telomere sequences, and total genomic DNA, is proving to be an effective alternate means of karyotyping in spruce.

Since the resolution and sensitivity of nonisotopic *in situ* hybridization procedures employing fluorescent detection systems and sophisticated imaging instruments now rival those of autoradiographic techniques (Lawrence et al. 1990; Lemieux et al. 1992),we chose to develop a fluorescence *in situ* hybridization (FISH) procedure for white spruce (2n = 24). Biotin labeled probes are hybridized to metaphase chromosomes preparations and hybridization sites are detected by fluorescein-labeled anti-biotin antibodies. We developed the technique using an 18S/26S ribosomal DNA probe from soybean, which is an ideal target sequence since rDNA loci consist of one to hundreds of tandemly arrayed repeats and are generally found at chromosomal sites that can be morphologically confirmed (the nucleolus organizers).

Conifer fluorescence *in situ* hybridization protocol

Preparing suitable metaphase chromosome spreads is the most critical and difficult aspect of FISH experiments in white spruce. Although time consuming, slide pretreatments prior to squashing minimize the loss of cellular material during the procedure. The fact that spruce root tips are very rigid and the white spruce chromosomes quite long pose problems in obtaining metaphase preparations with well separated chromosomes. The complete digestion of cell walls with cellulase and macerase or pectinase is essential prior to squashing the colchicine treated root tips. Prior to hybridization, slides are stained with 0.2 ug/ml DAPI, metaphases photographed, then destained in 3:1 methanol:acetic acid. Chromosome preparations are also treated with RNAase and then pepsin to reduce cytoplasmic background. For other details of our FISH protocol for conifers, refer to Brown et al. (1993). Image acquisition and processing are performed using a confocal laser scanning microscope. The confocal laser scanning microscope's ability to digitally record the FITC signal independently from that of the propidium iodide counterstain signal and to image via photon counting provides a high level of sensitivity in detecting weak hybridization signals. This can also be accomplished with a cooled CCD camera on an epifluorescent microscope, with appropriate software.

Mapping 18S/26S rRNA genes in spruce

We have localized the major ribosomal DNA (rDNA) loci on metaphase chromosomes and in interphase nuclei of white spruce by FISH with a rDNA probe from soybean. White spruce was found to have fourteen rDNA sites, one site present on each of 7 separate chromosome pairs. This is one of the highest numbers of rDNA loci yet reported among plant species. As a general rule, conifers may contain more rDNA loci than angiosperms. Typically, angiosperms carry 2-10 rDNA loci per cell (Appels and Honeycutt 1986). Of the few conifers for which data is available, radiata pine (*Pinus radiata* D. Don) has at least 10 sites (Cullis et al. 1988), white spruce has 14 sites (Brown et al., 1993), and Scots pine (*P. sylvestris* L.) has at least 16 sites (Karvonen, personal communication).

The position of the rDNA loci together with morphological features permits, in the best chromosome preparations, all homologous pairs of chromosomes to be distinguished.

Mapping 5S rRNA genes in spruce

We amplified the 5S rRNA genes from white spruce by the polymerase chain reaction. An amplification product of approximately 220 bp was observed and cloned into ddT-tailed pBluescript. One clone was sequenced and verified to be a 5S rRNA gene by comparison of the coding region to the published sequences for *Pinus radiata* and several angiosperm 5S rRNA genes (Cullis et al. 1988). Southern hybridization of this clone to *Bam*HI partial digests of genomic DNA revealed a "ladder" of hybridizing fragments confirming the tandemly arrayed organization of these genes in the white spruce genome. The chromosomal location of 5S rRNA in white spruce and Sitka spruce was determined by FISH. The 5S rRNA genes in both species are located on a single pair of homologous chromosomes at a position adjacent to a secondary constriction in both species. The 5S rRNA probe provides an additional chromosome marker to distinguish this pair of chromosomes from others with secondary constrictions situated a midway along the chromosome arm.

Mapping a centromeric tandem array in spruce

A *Sau*3A clone from white spruce, designated S31, was identified as a repetitive DNA by screening a partial library with genomic DNA. Southern hybridizations indicated that this clone was also tandemly organized in the white spruce genome. Further analysis showed that approximately 10,000 copies of the 142 bp monomer are present per haploid genome. We have mapped S31 by FISH to the centromeric region of 4 chromosome pairs in white spruce and 5 pairs in Sitka spruce. Variations in signal intensity were reproducible among all metaphases examined and likely represent differences in copy number at each site. S31 is one of the first centromeric DNA sequences isolated in plants

Using the morphological features provided by DAPI staining, it can be shown that only one S31 site is shared between the Sitka and white spruce. This is somewhat surprising considering the close evolutionary relationship and the ease of interspecific hybridization between the two species.

Mapping telomeres in spruce

When we use the telomeric sequence from *Arabidopsis* in FISH with spruce chromosomes, we observe only very weak signals and only on a subset of the 24 chromosomes. This indicates that either the conifer telomeric repeat sequence is not very similar to angiosperms, or that there is a diversity of and fewer than expected telomeric repeats in spruce.

Genomic *In Situ* hybridization

In British Columbia, interspecific hybridization between white spruce, Engelmann spruce (*P. engelmanni* (Parry)) and Sitka spruce (*P. sitchensis* (Bong.)) is a common occurrence where natural ranges overlap (Roche 1969; Fowler 1987). Genomic *in situ* hybridization (Schwarzacher et al. 1989) is a means of detecting introgression of foreign DNA sequence and does not require a cloned species-specific DNA sequence. This approach could be used to determine the presence and extent of introgression within natural spruce populations and may help to identify potentially elite genotypes of hybrid origin in spruce breeding programs.

Chromosome Evolution

It is expected that regions of the spruce genome essential to chromosome viability and transmission (e.g., telomeres, centromeres and origins of replication), will share structural and possibly sequence similarities to those characterized in other organisms. FISH will provide one means of confirming this. The localization of additional repetitive DNAs may reveal both interesting and valuable insight into the conifer genome. Mapping repetitive DNAs by FISH will yield a broad sketch of a large fraction of the conifer genome.

It has been postulated that the uniformity of karyotypes observed within conifer genera reflects a lower rate of chromosome evolution, in comparison to angiosperms (Khoshoo 1960). Repetitive DNAs are abundant and free to evolve without the evolutionary constraints imposed on coding sequences. Therefore they can be mapped by FISH in related species allowing comparisons of the mode and tempo of chromosome evolution, as well as aiding in verifying phylogenetic relationships among spruce species. Molecular cytogenetics using FISH is uncovering striking differences in genome organization of repetitive DNAs in conifers compared to angiosperms. Such information from molecular cytogenetics may generate renewed interest in chromosome evolution.

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PLANT RESPONSE TO BIOTIC AND ABIOTIC STRESS

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Plants synthesize discrete (and partially overlapping) suites of "defense" compounds in response to pathogens, elicitors, and mechanical wounding. Understanding the steps involved in plant perception and response to pathogen- and mechanical injury-induced stresses will advance our knowledge of how plants interact with biotic and abiotic factors in the environment. This knowledge is also a critical step toward modifying plant responses via genetic engineering.

In general, plant "defense" compounds collectively repair and compartmentalize the cellular damage imposed by the stress factor. Using elicitation as an example, the salient features of how this response is coordinated will be briefly described.

The perturbation must first be detected in some manner by the plant cell, presumably at the cell surface. Localized defense gene expression in response to pathogens and elicitors is thought to be mediated, at least in part, by an "oxidative burst" that results in the rapid production of active oxygen species at the cell surface (reviewed in Mehdy 1994). These active oxygen species may directly promote fortification of the cell wall via crosslinking of extracellular proteins. Active oxygen species also appear to activate a signal transduction pathway involving calcium influx and protein kinase activity in both spruce and tobacco cells. The intermediates that transduce the signal to the nucleus have not been defined, but synthesis of jasmonic acid (a hypothesized wound signaling intermediary) may be involved (Mehdy 1994; Farmer and Ryan 1992). The signaling occurs rapidly, suggesting the signal transduction apparatus is in place and ready for activation prior to the stress.

Certain components of the defense response are inducible; numerous genes are newly transcribed and translated in response to the intracellular signals generated

by elicitors. *Cis*- and *trans*-acting factors in the nucleus that appear to mediate elicitor-induced gene expression (Korfhage et al. 1994 and references therein) have been successfully identified in angiosperms. An important tool in these studies has been the availability of elicitable suspension cultures. Suspension-cultured pine cells (which are elicitable by chitosan; Lesney 1990) may provide a tool by which homologous *cis*- and *trans*-acting factors can be identified in gymnosperms.

Although many questions can be addressed using suspension cultures, there are certain experiments that require a whole-plant approach. Systemic signaling is one example. Pathogen interactions and cellular injury are known to promote long-distance dissemination of signals to undamaged sites elsewhere in the plant. Systemic signaling apparently evolved as an "alarm" system. Pathogen-induced systemic signaling (i.e., systemic acquired resistance) and wound-induced systemic signaling are clearly mediated by distinct mechanisms in angiosperms, and would be expected to differ as well in gymnosperms.

The well-characterized defense responses of higher plants may provide an opportunity to identify similarities and differences between angiosperms and gymnosperms with respect to transcriptional control mechanisms and signal transduction pathways. The null hypothesis would be that the factors which regulate gene expression are highly conserved among vascular plants and do not differ between angiosperms and gymnosperms. This hypothesis can be addressed by using a transgenic approach in which gymnosperm genes are tested for function in angiosperms, and vice versa. Experiments designed to test gymnosperm gene function in angiosperms can currently be performed and should be informative. Ultimately, transformation protocols developed for pines will permit direct tests of pine gene function in homologous systems.

From the perspective of molecular biology and its application to forest trees, the rationale for studying the molecular basis for responses to biotic and abiotic stress

factors is no different than for non-woody species. If forest tree breeding efforts are to be augmented by genetic engineering in the future, then a detailed understanding of how gene expression is controlled will be necessary so that introduced genes will be predictably regulated and have the desired effects. Identifying the nuclear and cytosolic factors that orchestrate defense responses, bearing in mind potential similarities and differences between gynmosperms and angiosperms, is an important step toward understanding the mechanisms by which trees interact with the environment, and manipulating expression of defense genes in transgenic trees.

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TOWARDS COMMERCIALISATION OF CONIFER EMBRYOGENESIS

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Abstract

Progress in conifer embryogenesis over the past 5 years has been promising with the establishment of large field trials of somatic seedlings, particularly with spruce. Spruce embryogenesis is somewhat of a "model system" for conifer embryogenesis and it is expected that application on a commercial scale will occur within 5 years. Pine embryogenesis has been more problematical, but is being tested on a pre-commercial scale by a number of forest companies worldwide. Carter Holt Harvey Forests Ltd. is one of the Southern hemisphere's largest plantation forest owners with 327,000 hectares of pine forest in New Zealand. It's Forest Biotechnology Centre is currently applying and scaling-up the techniques developed at the New Zealand Forest Research Institute with the aim of developing a commercially viable process. Radiata pine (*Pinus radiata* D. Don) somatic seedlings have been produced from the 1992/93 cell lines and over 2,000 embryogenic cell lines have recently been established from identical control-pollinated crosses from different seed orchards and from open-pollinated families. The best mature-embryo forming clones will be cryopreserved and will be established in clonal trials in 1996.

Constraints to the successful introduction of commercial embrogenesis are perceived by Carter Holt Harvey Forests to be: application of new techniques in a new laboratory; improving embryo quality, efficiency and the number of responding genotypes; selection of the best delivery system from "lab to forest"; and development of a cost effective system. Through all the stages of embryogenesis, physiological and biochemical profiles, molecular biology and total management information systems are used to monitor media, growth and progress in the lab and the forest. Continued advances in cryopreservation, automation, liquid

culture, encapsulation, rejuvenation, genetic fingerprinting and gene transformation are expected to further enhance the process. Application of somatic embryogenesis techniques to a range of conifers on a commercial scale is now a real possibility.

1. Introduction

Significant progress has been made in conifer embryogenesis in recent years with some methods now being advanced towards a commercial scale of production, particularly with spruces and pines. Some of the key highlights include: the field planting of more than 80,000 spruce somatic seedlings (Cervelli et al 1994, these proceedings); the development of a machine to transplant somatic seedlings from gelled media to a soil mix (Roberts et al 1994); a better understanding of the genetic influences on initiation and development of embryogenic tissue (Becwar et al 1991; Becwar and Chesick 1994; Park and Bonga 1994); bioreactors and liquid culture for scale-up (Smith et al 1991; Tautorus et al 1992); improved protocols for dessication of somatic embryos (Attree and Fowke 1993, 1994); and artificial seeds for encapsulation of conifer somatic embryos (Gupta et al 1993). The benefits of embryogenesis to the forest industry, in general, are outlined by Cervalli et al (1994, these proceedings).

In New Zealand a similar programme on embryogenesis of radiata pine has been underway at the New Zealand Forest Research Institute (NZFRI), where to date, over 4,000 somatic seedlings are in the greenhouse, nursery or forest (Smith et al 1994). Somatic embryogenesis has become more important commercially, now that there is a shortage of control-pollinated seed from trees with improved growth rates and form, disease resistance, and high or low wood density (Shelbourne et al 1989, Carson et al 1990, Burdon 1994) and that molecular biology methods offer new exciting opportunities. Vegetative multiplication by embryogenesis can make better use of the latest high-quality seed produced from advanced generation radiata pine breeding programmes. In 1993, Carter Holt Harvey Forests Limited (CHHF), New Zealand's largest plantation forest owner with 327,000 hectares of

forest (mostly radaita pine), opened a facility to develop a commercially viable embryogenesis process for radiata pine and to apply other new forest biotechnologies as appropriate. CHHF has been jointly funding the development of embryogenesis with NZFRI since 1989.

2. Development of Pre-commercial System for Radiata Pine Embryogenesis

The embryogenesis process of radiata pine has a number of stages from the initiation of embryogenic tissue, to final planting in the field. These were developed at the NZFRI and have been deployed at CHHF Forest Biotechnology Centre for the past year. All in vitro stages were carried out on gelled media. Progress to date is briefly summarised.

2.1. Initiation of embryogenic tissue

Megagametophytes from immature seeds from green cones were dissected and placed onto media in petri dishes using methods developed by Smith et al (1994, umpublished). This process was carried out during December and early January. During the 1993/94 capture season, a total of 58,084 megagametophytes were dissected and 2,299 embryogenic cell lines were captured from 8 control and 2 open-pollinated crosses (Table 1). Preliminary data shows that the seed orchard site affects the number of embryogenic cell lines that were initiated. Detailed analysis of the equivalent stages of zygotic embryo development from each seed orchard and the optimum window for each cross has been obtained but the data is not shown here. Overall, the percentage of embryogenic cell lines (clones) captured per cross varied between 1.4 and 8.4 %, based on the total initial number of megagametophytes dissected. The best controlled cross in the programme was selected because it was one of the best crosses in the previous year. In a comparison, it was found that more explants of the same controlled cross were dissected from cones from the Amberley seed orchard than from cones from the Matakana seed orchard. Three out of the four crosses yeilded a higher percentage of embryogenic cell lines from the Amberley seed orchard. The number of cones

from each orchard was approximately the same, but cones were noticeably smaller from the Matakana seed orchard.

Table 1. 1993/94 Capture - Number of megagametophytes dissected and embryogenic clones captured.

Crosses	Locations	No. of embryogenic clones	No. of mega-gametophytes	% of embryogenic clones
1x2	M*	32	1672	1.9
	A**	105	4732	2.2
3x4	M	92	3409	2.7
	A	286	7896	3.6
5x6	M	252	5056	5.0
	A	188	6450	2.9
2x1	M	186	4923	3.8
	A	367	6224	5.9
7x4	M	105	2590	4.1
7xOP	M	118	2887	4.1
5xOP	A	231	5310	4.4
2x8	A	168	1996	8.4
5x8	A	41	2832	1.4
4x9	A	128	2107	6.1
Total:	-	**2299**	**58084**	-

M* = Matakana seed orchard (located in North Island, 37.5°S, 177.5°E)

A** = Amberley seed orchard (located in South Island, 43.5°S, 177.5°E)

Crosses - Numbers 1-9 are codes for the parents, with the first number being the female parent and the second number being the male parent.

OP = open pollinated.

In a comparison between control- and open-pollinated families there was no difference in the percentage of embryogenic cell lines initiated in cones from Matakana, but in those from Amberley the number of cell lines initiated from open-pollinated cones was higher than the number from control-pollinated cones.

This comparison was only carried out with one open-pollinated and one control-pollinated cross from each seed orchard, therefore the results may not be applicable to other crosses.

2.2. Maintenance of embryogenic tissue

Embryogenic tissue was transferred to maintenance media developed by Smith et al (1993, unpublished) which contains no phytohormones. Cell lines from the 1992/93 capture season were initiated at NZFRI and subsequently transferred to the CHHF laboratory, where they were maintained for approximately one year. Selected cell lines were also cryopreserved according to methods developed by Hargreaves and Smith (1994). Cell lines from the 1993/94 capture season are being maintained, screened for mature embryo-forming potential and cryopreserved. The clones selected will form the basis of the pre-commercial production of mature somatic embryos for 1994/95.

2.3. Maturation of somatic embryos

Mature somatic embryos developed on ABA-containing media as described by Smith et al (1993, unpublished). Mature somatic embryos were harvested in 1993/94 from 62 clones initiated in 1992/93. Embryos did not develop synchronously and this facilitated repeated harvesting of high quality embryos from the same petri dish. Preliminary results have shown that maturation of embryos was found to be the most costly stage in the embryogenesis process. This was thought to be caused by the low number of embryos produced per unit of tissue or per petri dish. This differs from the results obtained by Cervelli and Senaratna (1994) where the dessication and germination stages were the most costly. Whereas interior spruce embryogenic tissue may routinely produce 200-300 mature somatic embryos per dish (Roberts, pers. comm.), radiata pine produced 20 per dish, on average.

2.4. Dessication of somatic embryos

Harvested somatic embryos were placed on a pre-germination medium prior to

germination on gelled media as desribed by Smith et al (1993, unpublished). Germination varied from 17 % -54 % and some embryos remained white and did not germinate. Using a modified high relative humidity treatment, as originally developed by Roberts et al (1990), 95-100% of all clones germinated subsequently and embryos that previously stayed white also germinated.

2.5. Germination and growing-on

Mature embryos germinated successfully on gelled media and were transplanted successfully to soil in the greenhouse to become somatic seedlings using NZFRI protocols (Smith et al 1993, unpublished) with some minor modifications. From the 1992/93 captured cell lines approximately 2,500 germinated somatic seedlings were produced from the CHHF laboratory. Small trial plantings of somatic seedlings were also established in the forest by the NZFRI research team (CHHF/ NZFRI joint venture) in 1991, 1992 and 1994. Combining the numbers from the NZFRI and from the CHHF laboratories there are approximately 6,000 radiata pine somatic seedlings in either the greenhouse, nursery or forest. Now, with the pre-commercial laboratory, an expanded capability for mass propagation and delivery of somatic seedlings for larger field trials and clonal tests has been established.

3. Constraints for Radiata Pine Embryogenesis and the Future

Although all control-pollinated crosses have been able to produce mature radiata pine somatic embryos that germinate, the number of genotypes within a cross that are able to do this needs to be increased. The transfer of embryo-forming proteins into non-embryo forming cell lines in *Picea abies* (Norway spruce) has recently been demonstrated (on Arnold et al 1994) and may be a useful approach for radiata pine.

In order to increase efficiencies in the process at all stages, new techniques have been adopted. Ongoing research in support of the pre-commercial programme will focus on process optimisation, particularly at the maturation stage, but also at all

other stages. The use of liquid culture, as described by Gupta et al (1993) needs to be investigated for cell lines at the maintenance and maturation stages respectively. The effects of liquid media on the quality of tissues can be both negetive and positive (Smith and Spomer 1994), and must be carefully monitored.

The transfer of techniques developed in the NZFRI laboratory to the CHHF laboratory has been successful overall. However, inherent differences in the environment (culture room) and microenvironment (petri dish) have affected some stages.

The development of software to manage production of somatic embryos at all stages and to "track" clones during their period in the laboratory through to planting in the forest is essential, and is underway. Much of this is carried out both manually and by computer and is labour intensive. To date, a Media Management Software system has been installed in the Forest Biotechnology Centre's computer system to assist with preparation of large batches of media and provide an inventory system for consumables, for example chemicals and containers. Software management systems in the laboratory need to link closely with forest nursery production systems and with the geographic information systems (GIS) being used to map and manage forest operations.

In addition to tracking clones by computer, it must be verified that clones are true-to-type or genetically identical at all stages in the laboratory, greenhouse, nursery and forest as part of quality control. Genetic fingerprinting or other molecular biology techniques will be essential to monitor cells, tissues and somatic seedlings.

There are many stages to the embryogenesis protocols. To reduce the number of stages in vitro, encapsulation of mature somatic embryos in an artificial seed and sowing directly into the nusery bed to produce bare-rooted planting stock, as is standard seedling production practice in New Zealand nurseries, would be an

advantage. The alternative, of placing an artifical seed into a greenhouse first and then tranplanting into the nursey bed may not be as cost effective under New Zealand conditions.

4. Conclusions

Somatic embryogenesis is now a demonstrated process for the vegetative propagation of radiata pine and many other conifers including spruces, larches, other pines and firs. Increased efficiency and quality of tissues must be achieved at all stages in order to reduce costs and to improve the quality of somatic seedlings. The embryogenesis method also has good scope for automation at selected stages. More importantly, large numbers of clonal propagules must be planted in clonal tests and embryogenic tissue of identical genotypes cryopreserved to capture clonal benefits in future. In the case of radiata pine, this could be as early as 8-10 years. The development of commercially viable methods for conifer embryogenesis will enable forest companies and research organisations to capitalise on new technologies that require embryogenesis as a delivery system for their planting stock, for example transformed clones with new or improved traits or rejuvenated tissues from mature trees.

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PILOT PRODUCTION AND SCALE-UP DELIVERY OF SPRUCE SOMATIC SEEDLINGS

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Introduction

Silvagen Inc. was incorporated in 1990, for the purpose of commercializing conifer somatic embryogenesis (SE) and related technologies. The company consists of a partnership between DRM Equities Limited, Halifax, BC Research Inc., Vancouver and Nova Scotia Research Foundation Corp., Dartmouth. The company has developed appropriate relationships with government and private players in specific areas such as tree breeding, nursery culture and market development.

Silvagen's primary focus is on spruces (*Picea*) for both forestry and ornamental landscape applications. The forestry applications are being developed primarily at BC Research Inc. for interior spruce (*Picea glauca/engelmannii* complex) and Sitka spruce (*Picea stichensis*). In order to capture and deliver elite germplasm through SE, this work is being performed in close collaboration with the British Columbia Ministry of Forests tree breeding program under the direction of Dr. Alvin Yanchuk. The SE scale-up effort has currently resulted in the development of a pilot production system capable of delivering quantities of 50,000 to 250,000 somatic seedlings into the greenhouse as a single crop.

Part One: SE as a Viable Tool for Forest Improvement

Somatic embryogenesis is a extremely potent tool for rapid vegetative propagation, and can produce high volumes of cloned embryos from a single seed. This new biotechnology may be a means for quickly and economically delivering the best of tree breeding programs to the forest on an operational scale. The impact of this technology on the viability of the forest industry may be substantial.

Clonal SE lines can be preserved indefinitely by storage in liquid nitrogen and bulked up for production at any time (Cyr et al. 1994). This unique ability to store clonal lines in this manner is significant because germplasm banks of superior material can be established and maintained at low cost while field trials are being performed. Production and delivery of somatic seedlings from the germplasm bank to the nursery can be accomplished within 6-8 months.

SE has been evaluated throughout the tissue culture, nursery production and field establishment phases. Studies of the biochemistry and patterns of gene expression during development of somatic and zygotic embryos (Flinn et al. 1991) and during germination (Cyr et al. 1991) revealed that they are similar with respect to the accumulation and utilization of storage proteins and lipids. Assessment of genetic stability during SE, using isozyme analysis (Eastman et al. 1991), and before and after cryopreservation (Cyr et al. 1994) using RFLP analysis, indicate that somaclonal variation is very infrequent compared to other micropropagation methods (Scowcroft 1984). Finally, intensive morphological and physiological assessments during nursery and field growth show that somatic seedlings are normal with respect to their performance and phenological patterns (Grossnickle et al. 1992; Grossnickle et al. 1994; Grossnickle and Major 1994a & 1994b). At present there are approximately 80,000 somatic seedlings in nursery and field trials in British Columbia and elsewhere, the oldest of which were field planted in 1990.

Somatic embryogenesis represents a technology which enables, for the first time, a small number of selected seeds - e.g. seeds from "plus" trees, seed from second-generation controlled crosses, or seed with known resistance to disease or insects - to be bulked up to produce large numbers of planting stock in a very short period of time. At present the delivery of genetically improved material is through seed orchards, and in some instances through vegetative propagation of elite seed by cuttings. While seed orchards are relatively effective, they may require 7-10 years

to become productive. Thus, the principal benefit of SE is rapid delivery of improved material (sometimes referred to as gain per unit time).

It is well known that clonal selection provides greater degrees of genetic gain. The principal barrier to clonal forestry for conifers is the inability to maintain juvenile material capable of sustained propagation during the testing and production phases for each clone. Achieving this in the long term using various cuttings and hedging strategies is limited and expensive. Because of the availability of long term germplasm banking, and the ability to produce large numbers of embryos from each clone at will, SE is ideally suited to clonal forestry.

Implementing SE enables the use of intensive selection strategies and a high degree of flexibility in the delivery of improved material. The opportunity to utilize the genetic resource available in a large breeding program is greatly enhanced. Using progeny data, selected crosses can be made for specific traits and delivered to operational reforestation within one year. Following clonal testing, the optimal clonal mix can be selected and used to match specific site requirements (Sutton et al. 1993).

Silvagen is also pursuing opportunities in applying SE to deliver valuable stock to the ornamental landscape trade. Grafting of named cultivars is expensive and results in poor form which requires years of pruning and shaping. Somatic seedlings have the trueness to type of grafts, but also demonstrate the growth and form of true seedlings.

Part Two: The Silvagen Conifer SE System

In order to meet the demand to deliver large quantities of spruce to the BC forest industry, Silvagen has developed a pilot scale Conifer SE Production System for spruce using high-volume tissue culture techniques and semi-automation (Roberts et al. 1994). The strategy employed in developing this system was to: 1. reduce

labour costs required for per-unit handling of plant material in the laboratory, 2. provide uniform, high rates of greenhouse crop establishment, and 3. provide high system though-put so that significant quantities can be delivered to the nursery in a short time window.

In this production system, suspension culture and other bulk handling techniques developed for SE bulk-up, maturation, desiccation and germination have significantly reduced production costs, since no per-unit handling of embryos or germinants is required (Cervelli and Senaratna, 1994). Quality assurance is performed at the time of ex vitro planting of 4-6 week old germinants through the use of a patent pending, semi-automated sorting and planting machine (Edmonds and Cervelli, 1993). The production system provides greater than 95% uniform crop establishment in the greenhouse in commercial volumes.

The Fast-Plant™ semi-automated planting machine was developed by the biomachinery engineers at the NS Research Foundation Corp. The machine's design was chosen to blend human intelligence, ergonomics and automation in such a way as to improve human efficiency and product uniformity, while avoiding the costs of robotics or machine vision. The original prototype has proven the unique concept of 'sandwiching' the roots of SE germinants within an open book of moistened potting mix, thereby avoiding bent or damaged roots and minimizing transplant shock.

The Silvagen Conifer SE System has proven to provide low production costs, high through put, little or no transplant shock, 95%+ greenhouse crop establishment and excellent quality and uniformity of grow out. Silvagen is currently scaling up this pilot system to full commercial production.

Conclusion

Silvagen Inc. has a primary focus on the development of SE for forestry and ornamental landscape applications. SE is a potent tool for rapid vegetative propagation, and can produce high volumes of cloned embryos from a single seed. Somatic seedlings have been proven to be physiologically identical to true seedlings from a number of tests. SE represents a technology which enables, for the first time, a small number of selected seeds to be bulked up to produce large numbers of planting stock in a very short period of time. Because of the availability of long term germplasm banking, and the ability to produce large numbers of embryos from each clone at will, SE is ideally suited to clonal forestry. Implementing SE enables the use of intensive selection strategies and a high degree of flexibility in the delivery of improved material.

The Silvagen Conifer SE System has proven to provide low production costs, high through put, little or no transplant shock, 95%+ greenhouse crop establishment and excellent quality and uniformity of grow out. Silvagen is currently scaling up this pilot system to full commercial production, and exploring applications to forestry crops worldwide.

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TAXOL - THE PRODUCTION OF AN ANTI-CANCER DRUG FROM TREES

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The dynamics of taxol production, a valuable anti-cancer drug, have changed rapidly within the past five years. In the late 1980's and early 1990's a lack of taxol supply was a major limiting factor not only for the chemotherapeutic use of taxol for the treatment of patients with ovarian cancer, but also for the testing of taxol in clinical trials against other forms of cancer. In these phase I and phase II clinical trials, taxol consistently showed activity against a wide range of cancer cells and tumors types (Rowinsky et al. 1990). Currently, the use of cultivated plants has contributed to the supply of taxol, such that supply is no longer limiting either chemotherapeutic use or clinical trials. The dynamics of taxol supply could rapidly change however with the approval of taxol for use in other forms of cancer. Taxol is currently approved as a drug for the treatment of ovarian cancer and by the end of the year should also be approved for treatment of breast cancer. One of the most optimistic results from the use of taxol for chemotherapy has been a greater than 30% response rate in patients.

Taxol is a mitotic block, disrupting and stopping cell division. It is therefore effective against any rapidly dividing cell, hence its activity against cancer cells. Of particular interest is the unique mode of action in disrupting cell division. Other well known, plant derived chemotherapeutic drugs which also act as mitotic blocks, such as the vinca alkaloids and colchicine, disrupt cell division by **destabilizing** microtubules. In contrast, taxol blocks cell division by **stabilizing** microtubules. Taxol is not specific to cancer cells and is toxic to any dividing cell, including plants cells. This toxicity may be important in the design of systems for taxol production using *Taxus* cells. However, relatively little is known about taxol synthesis within the plant, or how the plant avoids the toxic effects of taxol.

The preferred source of taxol by the pharmaceutical industry from an economic and supply standpoint would be by chemical synthesis. This would allow the drug to be manufactured in the lab without the need of the plant. Although chemical synthesis of taxol is now possible (Holton et al. 1994), considerable work is still needed prior to total synthesis becoming a commercial option. Therefore, in the foreseeable future the sole source of taxol will continue to be the plant. The biosynthetic regulation of taxol, a highly functionalized diterpene amide, within the plant is complex and poorly understood. Recent results however have aided in our understanding of the biochemistry of taxol. Numerous putative precursors to taxol have been shown to be incorporated into taxol using shoot cultures and cambium sections. These studies have shown for example that the three benzoyl moieties in taxol (two on the side chain and one on the taxane ring) can be derived from phenylalanine. These taxane precursors have also been used in tissue culture to increase taxol production (Fett-Neto et al. 1993).

The site of taxol synthesis in the plant is still unknown. The localization of taxol in the plant has been very difficult as taxol is not very soluble in aqueous solutions and is not fixed to other cellular components during routine fixation for cytology. As a consequence of this, taxol is mobilized within the tissue during dehydration and embedding. This led early on to the identity of vacuolar inclusions within phloem parenchyma cells as a site of taxol within the cell (Ellis et al. 1993). Since this report however, we have confirmed that the high level of taxol within the tannin bodies was due to the embedding process and that taxol is actually present in the cell walls. This site of taxol deposition in the plant might account for the ability of rapidly growing tissues to contain relatively high levels of taxol.

Although the primary commercial source of taxol has been the bark of *Taxus brevifolia* (Pacific yew), all *Taxus* spp. and all parts of the plants thus far investigated contain taxol, including the needles, stem and roots. This widespread production of taxol within the genus has provided an opportunity for the

production of taxol within the genus has provided an opportunity for the renewable production of taxol from faster growing horticultural *Taxus* spp., as well as the manipulation of tissue and cell cultures for the production of taxol and related taxanes. Taxol and taxane concentrations in these different species and horticultural cultivars can vary widely, for example *T. x media* shoots contain a relatively high taxol concentration while *T. baccata* shoots contain a relatively low taxol concentration and a relatively high level of the precursor taxane, 10-D baccatin. The differences in taxane profiles in field grown material do not always correlate with the levels produced in tissue culture however. For example, a cell suspension culture from *T. baccata* produced a high level of taxol (Gibson et al. 1993) in comparison with shoots from field-grown plants, and tissue cultured *T. cuspidata* showed an inverse correlation between taxol and 7-epi 10-D taxol compared to field material.

These discrepancies may highlight one reason why the commercial scale production of plant secondary metabolites in tissue culture has only been demonstrated for a few compounds. Although there is no doubt that there is a genetic component for the production of plant secondary metabolites such as taxol, environmental, physiological, and developmental components also play a role. An important tool for the study of these biochemical processes would be the identification of a particular cell or tissue type which is either directly or indirectly related to the production of the secondary compound. In an attempt to identify such a marker for taxol and to characterize the developmental regulation of taxol in the shoot, we investigated the production of taxanes in four *Taxus* varieties from bud swelling and flushing in the spring through dormancy in the winter. The shoots showed relatively little developmental regulation of taxol, as even dormant buds contained taxol. The production of taxol in shoots of 3 year-old field grown *Taxus* plants varied only 3-5 fold from bud break in the spring until bud set in the fall. These results suggest that actively growing *Taxus* tissues could tolerate the presence of this potentially toxic compound. Further, since taxol was found at all developmental stages and levels change only moderately

during development, no cytological or morphological markers correlating with taxol production were detected.

An aeroponics system, where *Taxus* plants were grown in five gallon buckets with periodic misting of the roots with a nutrient solution, was used to study the production of taxol from roots. In contrast to the shoots, taxol concentration in the roots is developmentally regulated. Older roots with a sloughing cortex contained up to 7X higher levels of taxol compared to the developmentally younger and actively elongating root tips. Because taxol levels in roots from the aeroponic system were comparable to the levels detected in the needles, root growth rates under different root harvesting regimes were determined. Although no attempt at optimization of root growth was made, between 30 - 40 gms f.w. of roots could be harvested from each plant every six weeks.

The use of tissue culture for the production of taxanes is an alternative to extraction from whole plants. In tissue culture, taxol has been produced in suspension, callus, seedling, shoot, nodule, hairy root, transformed tumor, and embryogenic cultures. Although much of the work being done is proprietary, the most advanced systems appear to be based on suspension cultures. While much optimism has surrounded the use of these cultures for the production of taxol and related taxanes, relatively little information is available on the level of production, the stability of production over time, nor the economics of production. With the economics of taxol supply rapidly changing, the role of cell cultures in the production of taxol will depend on all three of the above mentioned factors.

One of the largest changes in the past few years in taxol production strategies has been the use of taxane precursors for the semi-synthesis of taxol and taxol analogues. The most frequently cited precursor is 10-D baccatin, which is present in *T. baccata* at 5-10X the level of taxol in *T. brevifolia* bark. The conversion of 10-D baccatin to taxol can be done on a production scale and will probably be a

major source of taxol in the next few years. Extensive tests are currently underway with a taxol analogue derived in France, Taxotere, which has similar anticancer properties as taxol.

To offset the wide demand of taxol, the use of tissue culture and cultivated plants will certainly play a role in the supply of taxol and the precursors needed for the semi-synthesis of taxol. In addition, the development of active taxol analogues, such as Taxotere, and the use of peptides which mimic the action of taxol will also play a role in the future use and demand of taxol.

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Our job at the North Central Forest Experiment Station is discovering and creating new knowledge and technology in the field of natural resources and conveying this information to the people who can use it. As a new generation of forests emerges in our region, managers are confronted with two unique challenges: (1) Dealing with the great diversity in composition, quality, and ownership of the forests, and (2) Reconciling the conflicting demands of the people who use them. Helping the forest manager meet these challenges while protecting the environment is what research at North Central is all about.