17. Genetic Transformation in Conifers

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Introduction

Several attempts at the genetic improvement of tree species have been made, but in comparison with crop plants the efforts as well as the results have been rather limited. The most commonly used approaches have involved selection of superior genotypes from natural outbred populations, mutations, and intra- and inter-specific hybridization under controlled conditions. While the conventional methods have proven remarkably successful in yielding improved genotypes that could be stabilized by back crossing, the techniques of cloning, marker-aided selection, and genetic engineering when integrated with the conventional breeding programs, will dramatically improve genetic gains. Conventional methods of genetic improvement involve a recombination of pre-existing gene pools within a limited range of sexually compatible taxa. The process of backcrossing and selection takes several generations before a desired set of genes can be transferred to a selected species. Genetic manipulation through recombinant DNA permits us to cross the barriers of incompatibility, not only among species and genera but also among kingdoms. Genetic engineering provides new tools for mixing genetic information in plants from a vast pool of existing genes as well as genes designed by human intervention, i.e. synthetic gene sequences. Furthermore, undesirable genes in the plant genome can be selectively silenced in the target tissues by the antisense approach (Bourque, 1995; Lee and Douglas, 1997). Site-specific mutagenesis, homologous recombination, and the use of specific promoters provide a precise means of controlling specific gene expression and its manipulation to achieve optimal genetic improvements.

Genetic Transformation in Plants

Several techniques are used to introduce biologically active genes into plant cells. These include: direct uptake, microinjection, electroporation, liposome fusion, viral- and *Agrobacterium*-based vectors, and biolistic bombardment. Each one seems to have certain advantages and some major disadvantages. For a detailed description of different techniques, see Jones (1995), Gartland and Davey (1995) and Christou (1996).

Direct uptake of DNA from the surrounding medium and the expression of foreign genes by plant cells has been demonstrated in several cases (see reviews by

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Shillito and Potrykus, 1987; Lazzeri and Shewry, 1994). Further enhancements of direct DNA uptake can be achieved by adding polyethylene glycol (PEG) or co-precipitation with calcium phosphate. Delivery of DNA into plant protoplasts by microinjection improves the efficiency of direct transformation, however, the technique is very cumbersome and inefficient in handling large numbers of cells.

Electroporation, or the facilitation of DNA uptake under the influence of an electric field can dramatically improve the efficiency of transformation (for review see Nickoloff 1995; Lurquin, 1997). The availability of an affordable electroporation apparatus also makes this method a viable option for most laboratories. In the liposome fusion method, nucleic acids are encapsulated in synthetic phospholipid vesicles and under appropriate incubation conditions are allowed to fuse with plant protoplasts. Advantages of the liposome-mediated gene transfer include protection of nucleic acids from digestion by nucleases in the culture medium and the high efficiency of nucleic acid delivery into protoplasts. Potentially several kb long nucleic acids can be delivered by this method. Recent availability of a variety of commercial products (e.g. Transfectam – Promega, Madison, WI, Lipofectin and LipofectAMINE, Life Science Technol., Grand Island, NY, etc.) that aid in DNA transfer has made this a widely used method for transfection in animal cells. For all of the above techniques, the preferred target cells are protoplasts, which makes these techniques suitable only in cases where plant regeneration from protoplasts is possible. While protoplast isolation has been demonstrated from a variety of coniferous tissues, regeneration of whole plants from protoplasts is not commonly observed (Bekkaoui *et al.*, 1995).

A number of viruses, particularly the Caulimoviruses and Geminiviruses, have been suggested as useful vector systems for the transfer of selected cloned genes into plant cells (Mushegian and Shepherd, 1995; Palmer and Rybicki, 1997). Whereas the DNA carried by a virus is stably expressed in the transformed (infected) plant, in no case the integration of the transferred gene into the host genome has been demonstrated. Unambiguous transformation of higher plants with foreign genes has been achieved with *Agrobacterium tumefaciens* and *A. rhizogenes* (for review see Weising and Kahl, 1996). With the vast amount of information already available regarding gene maps, the role of the *vir* and the T-regions, and the border fragments, it has been possible to greatly modify the plasmid to develop efficient gene transfer vectors for a variety of plant cells, including tree species (Zambryski, 1992; Han *et al.*, 1996; Gartland and Davey, 1995; Tinland, 1996). Genes transferred via *Agrobacterium* show stable integration and Mendelian inheritance.

The technique of biolistic bombardment with DNA-coated gold or tungsten particles has yielded high frequency transformations in numerous species (for review see Christou, 1994; Chibbar and Kartha, 1994). This technique does not rely on the availability of single cells, nor is the cell wall an impediment for transfer of DNA. The technique has been successfully applied to transform cells of intact organs and tissues of plants as well as animals (Christou, 1994).

Several important factors make it impossible to critically compare and evaluate the efficiency and the effectiveness of different methods of transformation for a given tissue. In no case have different methods been tested with the same tissue,

the same gene/promoter construct, and in the same laboratory. The single most important factor governing the applicability of a particular technique for a given species is the mode of regeneration of whole plants from single cells in that species. Nevertheless, it is obvious that by using a variety of gene transfer systems, it is now possible to introduce foreign genes into almost any plant cell. The foreign DNA is often integrated randomly in the plant genome, and major problems still exist in achieving the integration of specific transgenes at specific sites in the host genome. (Flavell, 1994; Jorgensen *et al.*, 1996). Further complexities in the site of integration involve variable number of copies of the transgene, re-arrangements of DNA sequences, differential amplification of segments of the transgene, and selective silencing of the transgenes (Deroles and Gardner, 1988). These variations lead to differential and unpredictable expression of the transgene, thus affecting the behavior of transgenic tissues/plants. Another, as yet unexplained complexity comes from 'co-suppression' where expression of the native as well as the transgene are often suppressed when the two share some common sequences (Matzke and Matzke, 1995). Koziel *et al.* (1996) have discussed several strategies that can be used to optimize the expression of transgenes in plants.

A variety of promoters from *A. tumefaciens*, cauliflower mosaic virus (CaMV), and higher plants have been fused with a number of reporter genes to study their expression in transgenic plants. Among the promoter sequences used to achieve constitutive expression of the foreign genes, the CaMV-derived promoters are among the strongest ones. Numerous modifications of the CaMV 35S promoter have made it highly suitable for foreign gene expression in almost any plant. Although many of the crop plants have reached the stage of field trials and commercial plantations (Dale *et al.*, 1993; Moffat, 1998), the need to extend the basic gene transfer technology to woody plants still remains unfulfilled. This is particularly true for the conifers, which would benefit uniquely from these techniques for genetic improvement but pose certain unique problems for regeneration in tissue culture.

Transformation in Conifers

Establishment of routine cell and tissue culture techniques with herbaceous plants was a major step allowing for the rapid advancement of genetic engineering applications with these species. In contrast, while considerable effort for cell culture and regeneration of many tree species has been underway (see earlier volumes in this series, Jain *et al.*, 1995a,b,c), the technical level of conifer tissue culture is far less developed than that of the herbaceous plants. The variety of tissues from which regeneration of whole plants can be routinely obtained is rather limited. While multiple shoots can be regenerated from juvenile tissues of some species, these tissues are not quite suitable for transformation by most techniques due to the limited material available for use. The most reliable tissue in conifers that is capable of regeneration is the embryogenic cell masses obtained from the culture of zygotic embryos. Embryogenic cell masses can be initiated from juvenile

material or zygotic embryos on many conifers (Jain *et al.*, 1995a,b,c). In the case of pines, the starting material is often limited further to a narrow window during the early stages of development of the zygotic embryos. Therefore, most of the studies on transformation of conifers have employed the embryogenic cell masses.

As described above, the techniques of direct DNA uptake, microinjection, electroporation, and liposome fusion all depend upon the availability of protoplast regeneration, and thus are not well suited for transformation of conifers. Similarly, viral vectors, although quite useful for expression of foreign genes in many plants, are not reliable for integration of the foreign DNA and its expression in tissues not infected with viruses. Although *A. tumefaciens* can infect most conifers, and thus should be a suitable vector for transformation (see review by Ellis, 1995), the lack of regeneration from callus creates a major problem for its use. Only a few reports on the transformation of conifer tissues with *A. tumefaciens* have appeared in the literature, and in almost all cases the resulting transformed tissue was only a callus. Therefore, biolistic bombardment, which is capable of transferring foreign DNA to virtually any cell type, becomes the only viable approach for transformation of conifers.

Although genetic transformation in several species of conifers has been demonstrated (Table 1; see also Ellis, 1995), and the potential of such techniques in the genetic improvement of commercially important species has been discussed thoroughly, only a few reports on the production of transformed plants expressing commercially useful genes have been published to date. To the best of our knowledge, no large scale field trial of genetically engineered coniferous species expressing a commercially useful gene has been undertaken. This is in contrast to the millions of hectares of cultivation of genetically engineered corn, canola, cotton, tomato, soybean and a few other agricultural crops (Moffat, 1998). A review of literature on conifer transformation further reveals that most reports of conifer transformation only demonstrate the technique of transformation using a reporter gene and/or a selection marker gene. Only in a few instances, have the genes of potential commercial value been used. Among the reporter genes, the GUS (β-glucuronidase) expression under the control of a modified CaMV 35S promoter has been the preferred choice. In a few studies, the effectiveness of different promoter sequences has been compared either in transient assays or in stable transformations.

Current Status of Transformation of Conifers

A recent review by Ellis (1995) summarized the published literature on reports dealing with transformation of gymnosperms, mostly conifers. Without duplicating the efforts of Ellis, the following discussion provides a technique-based summary of the work with conifers, identifying achievements in each case and evaluating the current situation. Unfortunately, only a few detailed reports of the production of transgenic conifer species have been published since 1995, although personal communications with Dr. Christian Walter (New Zealand Forest Research Institute, Rotorua, New Zealand) indicate a great deal of progress in this area.

Table 1. Summary of published results on transformation in conifers. f/a to table T=Transient expression; S=Stable expression.

Species	Tissue	Gene	T/S	Product	Test	Reference
Agrobacterium						
Pseudotsuga menziesii	Seedlings	Opines	S	Callus	Opine	Morris *et al.* 1989
Pinus ponderosa	Seedlings	Opines	S	Callus	Opine	Morris *et al.* 1989
Tsuga heterophylla	Seedlings	Opines	S	Callus	Opine	Morris *et al.* 1989
Abies procera	Seedlings	Opines	S	Callus	Opine	Ellis *et al.* 1989
Picea glauca	Seedlings	Opines	S	Callus	Opines, hormones	Ellis *et al.* 1989
Picea engelmannii	Seedlings	Opines	S	Callus	Opines, hormones	Ellis *et al.* 1989
Picea sitchensis	Seedlings	Opines	S	Callus	Opines, hormones	Ellis *et al.* 1989
Pseudotsuga menziesii	Seedlings	Opines	S	Callus	Opines, hormones	Ellis *et al.* 1989
Larix decidua	Seedlings	Opines	S	Buds/plants	Opines, Southern	Huang *et al.* 1991
Larix decidua	Hypocotyl	Bt, aroA	S	Plants	Bt/glyphosate resis.	Shin *et al.* 1994
Taxus brevifolia	Shoots	Opines	S	Tumours	Opines	Han *et al.* 1994
Taxus baccata	Shoots	Opines	S	Tumours	Southern	Han *et al.* 1994
Picea abies	Seedlings	Opines	S	Roots	Opines	Magnussen *et al.* 1994
Pinus contorta	Seedlings	Opines	S	Roots	Opines	Magnussen *et al.* 1994
Pinus nigra	Seedlings	Opines	S	Rooting	Opines	Mihalijevic *et al.* 1996
Larix kaempferi × *L. decidua*	Emb. Cells	NPTII	S	Emb/plants	PCR/Southern	Levée *et al.* 1997
Picea sitchensis	Emb. Cells	GUS	T	Cells	Western/stain	Drake *et al.* 1997
Biolistic bombardment						
Pinus taeda	Cot. cells	GUS	62d	Tissue	Stain	Stomp *et al.* 1991
Picea glauca	Emb/callus seedlings	GUS	T	None	Stain	Ellis *et al.* 1991
Picea abies	SE mature	NPT/ GUS	T/S	Cells	PCR/Southern, stain	Robertson *et al.* 1992
Picea mariana	Emb. cells	GUS	T	None	Stain	Duchesne and Charest, 1991, 1992
Larix decidua	Emb. cells	GUS	T	None	Stain	Duchesne and Charest, 1992
Larix × *eurolepis*	Emb. cells	GUS	T	None	Stain	Duchesne and Charest, 1992
Larix × *leptoeuropae*	Emb. cells	GUS	5-6d	None	Stain	Duchesne *et al.* 1993
Larix leptolepis	Emb. cells	GUS	5-6d	None	Stain	Duchesne *et al.* 1993
Picea glauca	Cot. SE	GUS/ NPT	2-8 mo	Tissue	Stain/Southern	Bommineni *et al.* 1993
Picea glauca	SE	GUS/Bt	T/S	Callus, embryos	PCR, Southern, Bt	Ellis *et al.* 1993
Pinus radiata	Emb. cells	GUS	35d	Callus	Stain	Walter *et al.* 1994
Picea abies	Emb. cells	GUS	T	None	Stain	Yibrah *et al.* 1994

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Table 1. Continued

Species	Tissue	Gene	T/S	Product	Test	Reference
Pinus sylvestris	Buds/calli	GUS	T	None	Stain	Aronen *et al.* 1994
Chamaecyparis nootkatensis	Pollen	GUS/ NPT	T	None	ELISA	Hay *et al.* 1994
Tsuga heterophylla	Pollen	GUS/NPT	T	None	ELISA	Hay *et al.* 1994
Pinus banksiana	Pollen	GUS/NPT	T	None	ELISA	Hay *et al.* 1994
Picea mariana	Pollen	GUS/NPT	T	None	ELISA	Hay *et al.* 1994
Pinus sylvestris	Buds	GUS	T	None	Stain	Aronen *et al.* 1994
Pinus radiata	Cotyledon	GUS	T/ 20/d	None	Stain	Rey *et al.* 1996
Picea mariana	SE/SE tissue	GUS/NPT	S	Cells, embryos, plants	Stain, ELISA, PCR	Charest *et al.* 1996
Pinus sylvestris	Pollen	GUS	T	None	Stain/PCR	Häggman *et al.* 1997
Picea abies	Pollen	GUS	T	None	Stain/PCR	Häggman *et al.* 1997
Electroporation						
Picea glauca	Protoplast	GUS/CAT	T	Protoplasts	Stain/CAT	Bekkaoui *et al.* 1988
Picea mariana	Protoplast, Emb. cells	CAT	T	Protoplasts	^{14}C/CAT assay	Tautorus *et al.* 1989
Pinus banksiana	Protoplast Emb. cells	CAT	T	Protoplasts	^{14}C/CAT assay	Tautorus *et al.* 1989
Picea glauca	Protoplast	GUS/CAT	T	Protoplasts	Stain/CAT	Wilson *et al. 1989*
Picea glauca	SE/tissue	GUS/CAT	T	Protoplasts	Stain/CAT	Bekkaoui *et al.* 1990
Pinus banksiana	SE/tissue	EMV	T	Protoplasts	Stain/CAT	Bekkaoui *et al.* 1990
Picea mariana	SE/tissue	EMV	T	Protoplasts	Stain/CAT	Bekkaoui *et al.* 1990

The ability of *A. tumefaciens* to infect conifers was reported as early as 1935 (reviewed in DeCleene and DeLey, 1976), but it was not until the late 1980s, however, that *Agrobacterium* was used experimentally to transform conifer tissues to produce crown gall tumors (Clapham and Ekberg, 1986; Dandekar *et al.*, 1987). Morris *et al.* (1989) and Ellis *et al.* (1989) compared several strains of *A. tumefaciens* for their ability to induce crown gall tumor formation on a number of conifer taxa. Their results showed that some strains were significantly more potent than others for the induction of tumours. It was further established that the integration of T-DNA was not a problem in conifers, and that the *A. tumefaciens* promoters from nopaline synthase (NOS) and octopine synthase (OCS) genes were functional in the conifer cells. *Picea* species were generally found to be more susceptible than the pines.

Using binary vectors containing either luciferase or NPTII genes, Ellis *et al.* (1989) showed the effectiveness of the commonly used CaMV 35S promoter for

stable gene expression in a few conifers. Huang *et al.* (1991) later confirmed the ability of *A. rhizogenes* for gene transfer in wounded hypocotyl tissues of European larch (*Larix decidua*). Some other examples of transformation of conifers by *Agrobacterium*-based vectors which used either opine synthesis, hormone autonomy, or a marker gene are listed in Table 1. A detailed list of the conifer species that can be infected by *Agrobacterium* also appears in Ellis (1995) and Häggman and Aronen (1996). The first commercially useful genes (Bt for insect resistance and aroA for glyphosate resistance) were transferred to European larch using *A. rhizogenes* (Shin *et al.*, 1994). Regeneration of plants involved direct shoot–bud induction from the wounded hypocotyl region of zygotic embryos. Transformed plants were tested for the presence and expression of the foreign genes and were found to be tolerant to glyphosate. While the Bt-transgenic needles were not toxic, the insect larvae consumed significantly smaller amounts of the transgenic needles than the control needles. Magnussen *et al.* (1994) and Mihalijevic *et al.* (1996) demonstrated the use of wild-type *A. rhizogenes* to improve rooting ability of *Pinus nigra* explants. Two of the recent reports on transformation of conifers by *A. tumefaciens* include the expression of the GUS gene in Sitka spruce (Drake *et al.*, 1997) and the production of transgenic hybrid larch (*Larix kaempferi* × *L. decidua*) plants from embryogenic cell masses (Levée *et al.*, 1997). Plant regeneration via somatic embryogenesis was reported in the latter.

Wilson *et al.* (1989) demonstrated the use of PEG-mediated DNA transfer into white spruce (*Picea glauca*) protoplasts. Bekkaoui *et al.* (1988, 1990) and Tautorus *et al.* (1989) studied the effects of voltage, temperature, plasmid concentration and conformation, and different promoter sequences on transformation of black spruce (*Picea marina*) and Jack pine (*Pinus banksiana*) protoplasts by electroporation. They found that while the 35S and nopaline synthase promoters were equally effective in driving the expression of the chloramphenicol acetyltransferase (CAT) gene in transient expression assays, a tandem repeat of the 35S promoter was substantially better than a single copy. Significant differences were observed among different cell lines of the same species. No stable expression was obtained in any of these studies. Earlier, Gupta *et al.* (1988) had shown that the 35S promoter was also active in protoplasts of Douglas fir (*Pseudotsuga Menziesii*) and loblolly pine (*Pinus taeda*).

The biolistic bombardment method has been used successfully for both transient as well as stable expression of a number of genes in conifers. Ellis *et al.* (1991) conducted a detailed comparative study of the expression of several promoters in three different white spruce tissues using an electrically discharged bombardment device. While several constitutive and inducible promoters from *Arabidopsis*, soybean, and maize were active in this species, 35S promoter was the most active. The embryogenic callus cells showed the least amount of foreign gene expression in this study as compared with seedlings and zygotic embryos. Stomp *et al.* (1991) reported the expression of the GUS gene under the control of 35S promoter in loblolly pine using a particle gun (model BPG-4). Häggman *et al.* (1997) have demonstrated the feasibility of pollen transformation in *Pinus sylvestris* and *Picea abies* by biolistic bombardment. However, no transformed plants were reported

from the fertilized ovules using bombarded pollen. The first case of stable transformation of a conifer tissue (Norway spruce) by this method was reported by Robertson *et al.* (1992), who used a 35S promoter attached to the GUS gene and the NPT II gene. Tissue capable of growth on kanamycin was shown to have stable integration but had lost its embryogenic potential.

Duchesne and Charest (1992) compared several different promoter sequences for expression of the GUS gene in embryogenic cell lines of *Larix decidua* × *leptolepis* and *Picea mariana*, again using biolistic bombardment. While the wheat Em gene promoter (abscisic acid-inducible) was found to be better than most others, the two cell lines did not show significant differences. The study involved only transient expression. Duchesne *et al.* (1993) later reported a detailed analysis of the response of 22 haploid and diploid cell lines of four different species of *Larix* to bombardment with the GUS gene-coated tungsten particles of four different sizes. There was little difference among the different species or cell lines.

Similar studies have been reported on comparisons of different promoter sequences and different embryogenic cell lines for Norway spruce (Robertson *et al.*, 1992; Yibrah *et al.*, 1994), white spruce, red spruce, black spruce and *Larix* × *eurolepis* (Charest *et al.*, 1993), cultured somatic embryos of radiata pine (Walter *et al.*, 1994), cultured cotyledons of radiata pine (Rey *et al.*, 1996), pollen grains of lodgepole pine (*Pinus contoria*), yellow cypress (*Chamaecyparis nootkatensis*), western hemlock (*Tsuga heterophylla*), Jack pine (*Pinus banksiana*), black spruce (Hay *et al.*, 1994), Norway spruce (Martinussen *et al.*, 1994), and buds from mature trees of Scots pine (*Pinus sylvestris*) (Aronen *et al.*, 1994, 1995). Tandem duplications of the 35S promoter were found to increase the effectiveness of the original promoter sequence in several cases. Genotypic influence has also been reported for some species. In none of these cases were any transgenic plants regenerated.

Ellis *et al.* (1993) were the first to obtain regeneration of transformed plantlets from embryogenic tissue of white spruce bombarded with DNA-coated gold particles. The embryogenic cells and the plantlets showed the expression of GUS, NPTII, and the Bt genes. All three genes were integrated into the plant genome. The callus and the seedlings both showed sublethal levels of Bt protein when tested with spruce budworm feeding experiments. Soon after, Bommineni *et al.* (1993) observed stable expression of the GUS gene in white spruce embryogenic tissue. Christian Walter's group at the New Zealand Forestry has successfully used biolistic bombardment of the embryogenic tissue to produce transformed plants of radiata pine (Walter *et al.*, 1994 and personal communications). In all of these cases, duplicated CaMV35S-derived promoters were used to drive the expression of GUS gene.

Charest *et al.* (1996) obtained transgenic plants of black spruce using a similar approach. They found no phenotypic effects of the integration and expression of the GUS and the NPTII genes on regenerated plants. Personal communications with Dr. Christian Walter at NZ Forestry indicate that embryogenic cell lines of several pine species have been transformed and the transgenic plants have been brought to the greenhouse stage.

Targets for Genetic Manipulation

Numerous areas have been the target of genetic manipulation in crop plants; the most obvious areas of success being insect resistance, virus resistance, herbicide tolerance, fruit ripening, and ornamental features. Most of these areas are equally important targets for transformation of the commercially valuable coniferous species. A brief discussion of some of the target areas relevant to conifers is given below.

Resistance to Insect Pests

Insects contribute a major threat to trees, causing severe damage to natural as well as cultivated forests. Insect control has generally been achieved by the use of insecticides, many of which have severe negative impact on the environment and often become ineffective due to the development of resistance in insect populations. Thus the development of insect resistant genotypes of commercially valuable conifer species will be of tremendous value both economically and environmentally. Considerable success has been achieved in imparting insect resistance in many crop plants through genetic engineering. Three commonly used strategies are: (1) the overexpression of δ-endotoxin proteins of the bacterium *Bacillus thuringiensis* (Bt), (2) over-expression of protease inhibitor genes found in some plants, and (3) the production of proteins in the plant cells that interfere with insect development and/or metabolism. The Bt endotoxins are naturally occurring toxins, which are highly effective against a variety of insect pests. These toxins are quite specific for different insect species and, therefore, have been used successfully to kill specific pests of a crop with no major environmental harm. Overexpression of the natural endotoxin genes or their mutated forms have been used to produce transgenic plant varieties that have shown resistance in a number of field trials. Some of these crops have been approved for commercial production (Brunke and Meeusen, 1991; Koziel *et al.*, 1993). There is no reason to believe that the same strategies cannot be employed to generate insect-resistant conifers of commercial importance. Furthermore, it has been suggested that this strategy should be quite effective in the control of nematodes, mites, and all types of insects. The expression of genes coding for neuropeptides and other enzymes that may interfere with insect development or mating/feeding behavior has also been suggested as a means to achieve insect tolerance in plants. A major concern for the use of any of these approaches is the potential for development of resistance in the insect populations against these compounds, thus rendering the whole crop vulnerable to increased losses (Raffa, 1989; McGaughey and Whalon, 1992). However, the concurrent use of more than one approach in the same genotype should minimize the risk of developing resistance by the insects.

Resistance to Viral, Bacterial and Fungal Pathogens

Among the most devastating pathogens of plants, viruses are the hardest ones to control by chemical methods. However, viral resistance has also been one of the

easiest targets for genetic improvement of plants through genetic engineering. For quite some time it had been known that virus infection in plants results in cross protection against related viruses. This led to the demonstration of virus resistance in transgenic tobacco plants expressing the coat protein of tobacco mosaic virus (Powell-Abel *et al.*, 1986). These observations were soon followed by reports on the use of either complete or partial coding sequences of coat proteins of a number of RNA and DNA viruses to impart viral resistance in several plant species. Later studies showed that in addition to transgenic expression of the viral coat protein, a number of other strategies could be equally effective in imparting resistance to viruses using transgenic techniques. These include the use of virus replicase (RNA-dependent RNA polymerase) genes, viral protease genes, mutated viral-movement protein genes, antisense strand gene expression, and satellite RNAs (for review see Kavanagh and Spillane, 1995; Pappu *et al.*, 1995). Considerable progress has also been made in understanding the host response to viral infection, leading to the use of this information to overexpress host genes involved in this response. The examples include pathogenesis-related (PR) proteins, systemic acquired resistance (SAR) proteins, and anti-viral or ribosome-inactivating (RIP) proteins (Kahl and Winter, 1995). It has also been suggested that mammalian, monoclonal antibody (mAb) producing genes and mammalian 2′–5′ oligoadenylate synthetase gene-based mechanisms may be used in the future to achieve either strain-specific or broad-based viral resistance in plants. The fact that several transgenic major crop species have been field-tested for virus resistance, and that some of these have been approved for commercial production, provides a strong basis for the use of this approach for virus resistance in conifers.

Whereas the use of transgenic approaches to impart insect and viral resistance in plants are example of success, engineering tolerance against major bacterial and fungal pathogens has not been practical thus far. In the case of bacteria and fungi, the pathogen genes are not quite suitable for transfer to the host plant. The strategies used in some cases have involved the overexpression of genes that interfere with growth and development of the pathogen, or degrade the pathogen cell wall material, or counteract the effects of the toxin produced by the pathogen. Some of these approaches for bacterial pathogens include: (1) the expression of antimicrobial cysteine-rich thionines (Bohlmann and Apel, 1991), (2) the expression and secretion of avian or T4 bacteriophage lysozyme (Trudel *et al.*, 1992; Düring *et al.*, 1993), and (3) expression of a tabtoxin-inhibiting acetylase (Anzai *et al.*, 1989) or a toxin-resistant target enzyme (De La Fuente-Martinez *et al.*, 1992). In all cases only partial reversal of pathogenesis-related symptoms was achieved. For fungal pathogens, the transgenic approaches have involved (1) increased production of phytoalexins (Hain *et al.*, 1993), (2) increased production of antifungal proteins, such as chitinases, β-1-3-glucanases (Broglie *et al.*, 1991; Neuhaus *et al.*, 1991), and (3) increased induction of hypersensitive reaction involving programmed cell death (Martini *et al.*, 1993; Strittmatter and Wegener, 1993). A recent report on the cloning of a chitinase gene from several pine species (Wu *et al.*, 1997) should open the way for overexpressing a homologous gene to achieve fungal resistance in pines and other conifers. A possible future approach for

antimicrobial resistance may involve the production of plant antibodies (also called plantibodies) directed against specific bacterial or fungal proteins. The current status of the success of genetic engineering approaches to produce pathogen resistant plants has been reviewed by Herrera-Estrella and Simpson (1995). So far, no single strategy seems to have resulted in the production of pathogen-resistant plants that have been tested at the field level.

Resistance to Herbicides

Herbicide resistance was one of the earliest targets of genetic engineering in plants and considerable progress has been achieved with many commercial crops. Genes for resistance against all major groups of herbicides have been cloned and later used in overcoming the toxic effects of different herbicides. The different strategies have been based upon the ability to increase metabolism of the herbicide (detoxification), overexpression of the alternate pathway enzymes where the herbicide interferes with the biosynthesis of an essential metabolite, and introduction of a mutated gene for the target enzyme/protein. Field trials have been completed for a number of transgenic crop plants and commercial production has been approved (Dale *et al.*, 1993). While some initial success with such genes has been demonstrated in some hardwoods (e.g. *Populus*; Han *et al.*, 1996), such research with conifers has barely been started. In fact the use of herbicides in cultivated forestry is relatively recent and the need is only now being recognized.

Tolerance to Abiotic Stresses

In addition to facing attacks from pathogens, plants are constantly being exposed to a variety of natural and man-made abiotic stress factors which have a significant negative impact on growth and development. Some of these stress factors include salinity, drought, cold, heat, chemical pollutants, and herbicidal, fungicidal and insecticidal treatments. As the stress factors are varied, so are the plant responses to them. Plants also show some systemic general responses to stress. The complex stress response must begin with stress perception, transduction of some signal, and eventually changes at the cellular and molecular level. A number of stress-induced genes and other compounds have been identified, some of which are similar in plants, algae, fungi and bacteria (Ingram and Bartels, 1996). A better understanding of this response should lead to potential genetic manipulation of the plants to minimize the impact of stress on their growth and development. For example, the halophytic bacteria and plants have mechanisms to exclude Na^+ from the cells while accumulating high levels of K^+. This is achieved by regulating antiporter activity of the plasma membrane and tonoplast. In recent years, a number of genes for ion pumps have become available thus opening the door for their genetic manipulation in plants (Jia *et al.*, 1992; Bohnert *et al.*, 1995). An alternate approach to tolerance of both high salt and osmotic stress is the accumulation of organic osmolytes and other metabolites, such as glycerol, arabitol, mannitol, sugars, proline, glycine-betaine, and probably polyamines (Rhodes and Hanson,

1993; Ober and Sharp, 1994; Chiang and Dandekar, 1995; Ye *et al.*, 1997). The biosynthetic pathways for these products have been elucidated, providing a great potential for plant improvement through genetic engineering (Bartels and Nelson, 1994; Ingram and Bartels, 1996). Although the feasibility of genetic manipulation of the cellular levels of many of these compounds has been experimentally demonstrated, and the effectiveness of this approach in achieving stress tolerance in the transgenic plants implied from laboratory data, no large scale field studies have been reported thus far (mannitol – Tarczynski *et al.*, 1993; Thomas *et al.*, 1995; fructans – Pilon-Smits *et al.*, 1995; glycine-betaine – Ishitani *et al.*, 1995, Lilius *et al.*, 1996; proline – Kavi Kishore *et al.*, 1995; trehalose – Romero *et al.*, 1997). In a similar way, genetic manipulation of the aquaporins (a group of water channel proteins that regulate water balance in plants – Chrispeels and Maurel, 1994; Kaldenhoff *et al.*, 1995) may provide a practical way to achieve drought tolerance in plants. Other commonly observed proteins that are induced in response toosmotic stress and other stresses are the LEA (late embryogenic abundant) proteins, dehydrins, osmotin, metallothionein-like proteins, calmodulin, proline-rich proteins, heat-shock proteins, etc. Coding sequences for many of these proteins have been cloned and characterized and thus can be used for their genetic manipulation. Abscisic acid appears to be a common factor for the transduction of stress signal in many cases.

For achieving heat and cold tolerance, the use of heat shock proteins and antifreeze proteins has been discussed (Hightower *et al.*, 1991; Murata *et al.*, 1992; Wallis *et al.*, 1997; Graham *et al.*, 1997).

Wood and Pulp Quality

Two major users of conifers are the pulp-paper industry and the timber industry. Both require very different wood products, and in many ways, the requirement of the two are somewhat mutually exclusive. Whereas the pulp/paper industry requires fibers that are rich in cellulose and low in lignins, particularly those lignins that are hard to remove during pulping, the timber industry uses different criteria for wood quality. As our understanding of the biosynthesis of different types of lignins increases (Boudet *et al.*, 1995; Whetten and Sederoff, 1995; Douglas, 1996; Campbell and Sederoff, 1996; MacKay *et al.*, 1997), we should be able to manipulate the amounts as well as the chemistry of lignins in wood. Lignin production is a very complex process with a tremendous amount of variation among different plant species. It is an extremely heterogeneous polymer, the biosynthesis of which is affected by environmental, nutritional and other factors that affect growth and development. Presently, it is difficult to anticipate the consequences of genetic manipulation of the biosynthetic pathways of lignin on growth, developmental and morphogenetic behavior of plants. Several attempts have been made to genetically manipulate lignin biosynthesis via the transgenic approach (Sederoff *et al.*, 1994; Atanasova *et al.*, 1995; Doorsselaere *et al.*, 1995; Kajita *et al.*, 1997). In addition to contributing to the strength of wood, lignin also plays a crucial role in the ability of wood to resist pathogen infection and rotting.

This will require alteration of lignin chemistry in ways quite different from those that are needed for wood strength or pulping quality (Yao *et al.*, 1995).

Like lignin, the deposition of cellulose in the secondary cell wall is also quite important in determining wood quality and its use. In contrast to lignin, however, cellulose chemistry is highly conserved in plants. While its chemistry is simple in that only a few steps are involved in its biosynthesis, it is only recently that we have identified the enzymes involved in cellulose biosynthesis (Kudlicka and Brown *et al.*, 1996b; Arioli *et al.*, 1998; Delmer, 1998). The most difficult and variable aspect of cellulose deposition is the underlying matrix that determines the cellulose fiber length, orientation, thickness of the secondary cell wall, the composition of other cell wall components, and other structural features of the cell wall. While increased cellulose biosynthesis in plant cells has been achieved through genetic engineering, other parameters of cellulose deposition have not been an easy target. It is expected that during the next few years, we will have a better understanding of the regulation of cellulose biosynthesis and deposition in the cell wall in plants. The production of cellulose in genetically engineered bacteria, and studies with *in vitro* cellulose biosynthesis in cotton ovules, should provide extremely useful information to increase cellulose production in appropriate plant tissues and also its deposition in the cell wall. This is an area where conifers can be the prime target for genetic manipulation. Pulping and timber quality probably will dictate different approaches to manipulation of cellulose deposition in a given conifer species.

Reproductive Physiology

A major concern for the field plantations of genetically engineered plants is the risk of spreading the transgene or the transgenic plants and its impact on natural plant populations. This becomes even more critical for the conifers which have large populations of natural forests and spread their pollen grains through wind pollination at great distances. While the spread of the seeds of transgenic plants may be controllable to some extent, cross fertilization of the wild populations via wind-spread transgenic pollen cannot be controlled. The only viable options to limit the spread of the transgenic plants by seeds or the transgene by pollen are to impact complete male sterility, if not a complete lack of fertility, in the transgenic trees. The question then is, will the advantage of limiting the spread of transgene in this way outweigh the advantages of breeding of commercially useful genotypes with the transgenic material? In other words, limiting the fertility of transgenic trees will force us to depend almost entirely on their propagation by cloning. This will also limit our ability to amplify the gains of genetic engineering for a given trait by incorporating it into a broad genetic background of the species, which can be done only by conventional breeding. The best hope to achieve a practical solution to this problem will be to use fertility controlling genes under the regulation of a promoter that can be experimentally induced or suppressed so that infertility can be reversed when desired for breeding purposes. While a number of potential mechanisms for controlling male sterility have been discussed (some

have also been shown to be quite effective) and some progress in controlling the flowering phenomenon has also been made (see Strauss *et al.*, 1995 for review), suitable promoters to achieve the reversal of infertility have yet to be identified and tested for transgenic expression.

Another commercially useful target will be the genetic manipulation of early maturation and cone production in conifers for use in fast breeding cycles to incorporate the technology of genetic engineering into traditional breeding and seed production technologies. Our understanding of the process of flowering has tremendously increased over the past few years. A number of genes controlling the timing of flower production, flower morphology, and the development of flowers have been identified in several plants and some have been tested under transgenic conditions (both sense or antisense expression) in heterologous hosts. These genes should help us produce transgenic conifer trees which show early flowering, thus enhancing our capability of reducing the breeding cycle and, in turn, increasing the breeding potential. Similar genes need to be identified and characterized in important conifer species. This will aid in increasing the genetic background of material used in agroforestry and allow us to manipulate complex characteristics of growth, yield and morphology of the trees.

Biomass and Other Characteristics

Total biomass of a tree is determined by overall growth rates and organic matter accumulation in various tissues of the tree. These are highly complex phenomena which are dependent upon the interplay of plant hormones, primary productivity, partitioning, transport and cellular metabolism. In most cases, a single gene may not contribute significantly to the overall biomass yield of a plant due to the complex interplay of homoeostatic controls of plant metabolism. However, the results of Robson *et al.* (1996) on the genetic engineering of harvest index (total leaf biomass) in tobacco by overexpression of a phytochrome gene indicate the possibility of increasing some aspects of productivity of plants by a single gene transfer.

Genetic improvement of the embryogenic potential of cell cultures may be another area of great significance for the transgenic approach. The demonstration of improved and prolonged somatic embryogenic potential of carrot cell lines through genetic manipulation of polyamine metabolism (Bastola and Minocha, 1995) may provide the basis for targeting certain metabolic pathways that play an important role in development. This approach would require a better understanding of the molecular and biochemical events associated with somatic embryogenesis.

Some of the other areas that can be targeted for genetic improvement of conifers include: juvenility and maturity, fast growth of ornamental trees, shape and branching pattern of ornamental trees, aging and senescence, better utilization of nutrients, mycorrhizal interactions, heavy metal tolerance, fragrance, etc. For many of these characteristics, genes have already been identified and cloned, and thus should become available for transgenic expression.

Future Perspectives

Recent advances in transfer of multigene constructs with polycistronic DNAs (Lough *et al.*, 1997), the transfer of large (several hundred kb) DNA molecules (Hamilton *et al.*, 1996), targeting of the transgene to specific sites in the genome, manipulation of the 5′ and 3′ untranslated regions of the gene to optimize gene expression (Koziel *et al.*, 1996), site directed mutagenesis, the use of scaffold attachment region (SARs) genes (Allen *et al.*, 1993), gene disruption and replacement through homologous recombination (Merlon and Hooykaas, 1995; Miao and Lam, 1995; Kempin *et al.*, 1997), and a multitude of other approaches to regulate the transfer and expression of foreign genes in the recipient cells/plants, should provide exciting new ways to produce highly desirable plants, including conifers. An obvious need in this regard is a concerted effort at testing the technology with economically important tree species using genes of commercial value, some of which are already available and many more are being cloned and characterized. Ultimately, the selection of the gene(s) and the plant will be determined by a balance of economic gains and potential risks of environmental damage or purturbance in the biodiversity of natural populations.

The long-term (25–50 years) rotation cycle for production of useful tree products is a problem for planning of specific targets for genetic engineering of trees. The changing technology for the usage of timber, production of pulp and paper, and the usage of tree biomass for energy production (all requiring different types of plantations), raise the most fundamental question 'What trees should be planted today to meet the needs of mid 21st century'? Pathogen evolution and development of resistance over the years could make the plantations highly prone to damage after decades of growth in the field.

The testing of genetically improved stocks typically requires at least 5–10 years for fast growing conifers, e.g. *Pinus radiata*, and even more time for most other species. Quantitative trait markers associated with desirable characteristics are currently not available to aid in early selection and evaluation of the improved genotypes. Thus, a broad spectrum of genetically improved stocks (cell lines) must be maintained in the storage banks for rapid cloning and plantation at a later date. Although cryopreservation can help in this situation, this technology has not been optimized for most conifer species (Day and McLellan, 1995).

Plantation forests during their maturation cycle often serve other human needs such as recreation, wildlife feeding and hunting grounds, and a multitude of urban and rural tourism-associated activities. We must develop policies and strategies to treat densely-planted agroforestry areas at par with agricultural land to potentially limit its usage for other needs.

Entire populations of genetically engineered stocks must be clonally propagated because the normal reproductive cycles of most conifers are too long to take advantage of heterosis. Thus the transferred gene will often be in a heterozygous form in most of the tree plantations.

Ultimately, the success of genetic engineering of conifers will depend upon economic gains to the forester which, due to their long harvesting periods, requires

long-term investment into controlled research, development and evaluation of this technology.

At present, the best that can be said is that we have started to think about and plan for using the gene transfer technology for genetic improvement of trees. There have also been sporadic demonstrations of success in the use of presently available techniques of transformation. Soon, we must demonstrate phenotypic improvement of the product at the field level and demonstrate its ultimate gain to the forester. This is likely to come first with the hardwood species, such as *Populus* and *Eucalyptus*, which are relatively faster growing than the conifers but share a number of commercially useful traits with them (Han *et al.*, 1994).

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