

REVIEW / SYNTHÈSE

Current status and environmental impact of transgenic forest trees

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Abstract: Advances in technology for in vitro propagation and genetic transformation have accelerated the development of genetically engineered trees during the past 15 years. At least 33 species of transformed forest trees have been successfully regenerated to date. Targeted traits include herbicide tolerance, pest resistance, abiotic stress tolerance, modified fiber quality and quantity, and altered growth and reproductive development. Commercial potential has been demonstrated in the field for a few traits, in particular herbicide tolerance, insect resistance, and altered lignin content. Now that commercial implementation is feasible, at least for the few genotypes that can be efficiently transformed and propagated, environmental concerns have become the main obstacle to public acceptance and regulatory approval. Ecological risks associated with commercial release range from transgene escape and introgression into wild gene pools to the impact of transgene products on other organisms and ecosystem processes. Evaluation of those risks is confounded by the long life span of trees, and by limitations of extrapolating results from small-scale studies to larger-scale plantations. Issues that are central to safe deployment can only be addressed by permitting medium- to large-scale release of transgenic trees over a full rotation. Current regulations restricting field releases of all transgenes in both time and space need to be replaced with regulations that recognize different levels of risk (as determined by the origin of the transgene, its impact on reproductive fitness, and nontarget impacts) and assign a commensurate level of confinement. The next step in determining acceptability of transgene technology for forest tree improvement is the unconfined release of constructs that pose little risk in terms of gene escape and nontarget impacts, such as lignin-altered poplar or pine, to permit evaluation of ecological risks and environmental or agronomic benefits at relevant scales.

Résumé : Depuis 15 ans, les développements technologiques liés à la multiplication in vitro et la transformation génétique ont permis d'accélérer la mise au point d'arbres génétiquement modifiés. À ce jour, les scientifiques ont pu produire des arbres forestiers modifiés avec au moins 33 d'espèces. Les caractères cibles comprennent la tolérance aux herbicides, la résistance aux ravageurs, la tolérance aux stress abiotiques, la modification de la quantité et de la qualité des fibres, l'altération de la croissance et le développement des fonctions reproductrices. Le potentiel commercial a été démontré au champ pour quelques-uns de ces caractères, en particulier la résistance aux herbicides, la résistance aux insectes et l'altération du contenu en lignine. Alors que la commercialisation apparaît réalisable, du moins pour les quelques génotypes qui peuvent être modifiés et multipliés de façon efficace, les inquiétudes pour l'environnement sont devenues l'obstacle principal à l'approbation régulatrice et à l'acceptation populaire. Les risques écologiques associés au déploiement commercial vont de la dispersion du transgène et son introgression au sein des bassins géniques naturels à l'impact des produits transgéniques sur les autres organismes et processus écologiques. L'évaluation de ces risques est compliquée en raison de la longévité des arbres et de la difficulté à extrapoler les résultats d'études à petite échelle aux plantations à plus grande échelle. Les principaux enjeux liés au déploiement sécuritaire peuvent être abordés uniquement en permettant le déploiement à moyenne et à grande échelle d'arbres transgéniques pendant une révolution complète. Les règlements actuels qui restreignent dans le temps et dans l'espace le déploiement au champ de tous les transgènes doivent être remplacés par une réglementation qui reconnaît les différents niveaux de risque (tels que déterminés par l'origine du transgène, son impact sur le succès en reproduction ainsi que les impacts non spécifiques) et qui attribue un niveau correspondant de confinement. L'étape suivante dans la démarche visant à déterminer l'acceptabilité de la technologie transgénique en amélioration génétique des arbres forestiers vise le déploiement sans confinement de constructions qui posent peu de risque en termes de dispersion des gènes et des impacts non

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spécifiques, comme l'utilisation de pins ou de peupliers dont la lignine a été modifiée, afin de permettre l'évaluation des risques écologiques et des bénéfices environnementaux ou agronomiques à des échelles pertinentes.

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Introduction

The recent worldwide commercialization of transgenic agricultural crops has intensified public debate on risks and benefits of genetic engineering. The debate is characterized by a global trend of declining optimism, as the use of recombinant DNA technology is becoming more and more controversial. The general public remains supportive of medical and environmental applications, but there is increasing opposition to other uses, in particular genetic engineering of food crops (Gaskell et al. 2000; Priest 2000).

Although transgenic trees do not evoke immediate health concerns, as do transgenic food crops, the prospect of their commercialization is raising concerns regarding potential ecological impacts (James et al. 1998) and is fueling public opposition. Tree engineering is coming under fire from environmental groups as it has moved out of the laboratory into the field-testing stage (Kaiser 2001). The development of transgenic trees has arrived at a critical juncture: now that scientific progress has brought their use in commercial plantations within reach (Mann and Plummer 2002), environmental concerns are becoming the main obstacle to public acceptance and regulatory approval. Opponents are calling for a moratorium on commercial release until long-term environmental impacts are better understood and a stronger research-based regulatory framework is in place (Campbell and Asante-Owusu 2001). Proponents agree that more research is needed to assess risks as well as benefits (Bradshaw and Strauss 2001).

With public and scientific scrutiny becoming more intense, now is an opportune time to review the current status of transgenic forest trees in terms of their development and testing, and in anticipation of further commercial development, to review the research that has been done to address the multitude of ecological issues that are associated with their deployment. The purpose of this paper is to provide a framework for further discussion and to direct the research and regulatory effort that is needed to move transgenic trees further along the path of commercialization while ensuring ecologically responsible deployment.

The context of tree engineering

As the global need for wood increases, intensively managed plantations of fast-growing species have become an attractive option for producing industrial wood. Economic advantages of planted forests have led to large-scale plantations around the world, often utilizing species that can be propagated by cloning. Managed plantations are already supplying about 35% of the current industrial wood supply (Sedjo 1999). That share is expected to increase with growing pressures to conserve natural forests, because plantations can produce fiber on a much smaller land base.

During the last decade, biotechnology has emerged as a potential force shaping the future of plantation forestry. Several companies are combining traditional breeding methods

with marker-assisted selection, and are using "artificial seed technology" or other propagation methods to mass-produce elite planting stock for clonal forestry programs (Mann and Plummer 2002). The integration of genetic transformation — the controlled expression of foreign genes or modified endogenous genes — to further improve high-yield genotypes is a possible next step within this framework of intensifying commercial investment.

Why transgenic trees?

Improvement of trees by conventional breeding is constrained by their long reproductive cycles and complex reproductive characteristics. Those include low selfing ability, the difficulty of identifying suitable parents and controlling their mating, and the challenge of maintaining genetic gains in the face of high heterozygosity (Cheliak and Rogers 1990). Genetic engineering offers an attractive addition to conventional breeding because it permits the transfer of valuable traits into selected genotypes without compromising their desirable genetic background, while avoiding the problem of a long juvenile phase.

Genetic engineering is expected to have major benefits, primarily within the context of intensively managed short-rotation plantations with clonally propagated species. In contrast to conventional breeding, which is limited to sexually accessible variation in complex traits that typically depend on a large number of interacting genes, recombinant-DNA technology provides tree breeders with an unlimited gene pool for desirable traits, as long as those traits are controlled by one or a few genes. Endogenous genes already present in the tree genome can be modified to improve certain traits, such as fiber quality and quantity, while exogenous genes can be transferred from unrelated organisms to confer entirely novel traits, such as resistance to herbicides, diseases or pests. Traits that are currently being targeted (see "Target traits for forest tree engineering") are expected to have a positive impact on the economics of plantation forests (improved growth, reduced rotation, improved wood quality, reduced cost of pest control), or confer distinct environmental benefits during wood production (reduced pesticide use) or processing (improved pulping, reduced inputs of hazardous chemicals and energy). Proponents argue that intensification of clonal forestry, with genetic engineering providing continued gains in yield and quality, will reduce harvesting pressures on natural forest, and is therefore a necessary element of future forest conservation strategies (Strauss et al. 1999). Genetic engineering of trees could also play a key role in conservation or restoration of biodiversity, for example by engineering resistance against exotic threats in ecologically important tree species (Adams et al. 2002).

Recent progress and current status

In vitro micro propagation

The success of genetic engineering hinges on the ability

to cultivate plant tissues *in vitro*, both in the initial step of rendering the recipient competent for integrative transformation, and in the final step of regenerating whole plants from transformed cells. Techniques for the *in vitro* micro-propagation of forest trees include organogenesis (the regeneration of plants by organ formation on explants, or from cell masses or protoplasts) and somatic embryogenesis (the formation of embryos from cells other than gametes), which have been reviewed elsewhere (Chun et al. 1988; Kirby et al. 1989; Tautorius et al. 1991). Because many economically important tree species are recalcitrant to regeneration through organogenesis, in particular gymnosperms, somatic embryogenesis has become the most prominent technique. Somatic embryos can be produced for a wide range of tree species (Park 2002), but often lack commercial viability owing to unproven genotype (starting material derived from seeds or seedlings), and low frequency of whole-plant regeneration for the most desirable clones (Merkle and Dean 2000). Although mass propagation of elite clones was initially the primary goal of developing somatic embryogenesis systems for trees, the use of somatic embryos and embryogenic tissues as source material for genetic transformation has greatly facilitated progress in the development of transgenic trees.

Genetic transformation

The two most common transformation methods are *Agrobacterium*-mediated DNA transfer, and bombardment with DNA-coated micro-projectiles, so-called "biolistic" transformation (reviewed in Walden and Wingender 1995). Both methods have been used to transform tissues from a broad range of tree species (Jain and Minocha 2000), but recovery of transformed plantlets has been successful for only a limited number of species and an even smaller subset of forest-tree species (Table 1). Within that subset, optimized and efficient transformation and recovery procedures exist for only a handful of desirable genotypes, primarily angiosperms in the genera *Populus*, *Eucalyptus*, and *Liquidambar*, and more recently some gymnosperms, such as *Pinus*, *Picea*, and *Larix* (Llewellyn 2000; Mann and Plummer 2002).

The first report of genetic transformation and recovery of transformed plants of a forest tree involved the insertion of a herbicide-tolerance gene into hybrid poplar, using co-cultivation of leaf explants with *Agrobacterium tumefaciens* (Fillatti et al. 1987). Other transformation systems were developed (e.g., Leplé et al. 1992; Tsai et al. 1994; Tzfira et al. 1996; Tzfira et al. 1997), but progress was mostly confined to a few poplar hybrids that were selected for ease of transformation. Today, routine transformation procedures utilizing *A. tumefaciens* or *A. rhizogenes* exist for most poplar species and hybrids (Leplé et al. 2000), even for recalcitrant genotypes such as cottonwoods (Han et al. 1997, 2000). There has been limited but significant progress in the engineering of other deciduous genera. Transgenic plantlets have been regenerated from stem or leaf segments after co-cultivation with *Agrobacterium* in the case of American sweetgum (*Liquidambar styraciflua*) (Sullivan and Lagrimini 1993), black locust (*Robinia pseudoacacia*) (Han et al. 1993; Igasaki et al. 2000), birch (*Betula platyphylla*) (Mohri et al. 1997), English elm (*Ulmus procera*) (Gartland et al. 2000) and various *Eucalyptus* species and hybrids (Mullins

et al. 1997; Ho et al. 1998). Somatic embryogenesis-based transformation systems were used to obtain transgenic English walnut (*Juglans regia*) (Escobar et al. 2000; McGranahan et al. 1988), yellow poplar (*Liriodendron tulipifera*) (Rugh et al. 1998; Wilde et al. 1992), *Allo-casuarina verticillata* (Franche et al. 2000a), and *Casuarina glauca* (Franche et al. 2000b).

Production of the first transgenic conifer was reported a decade ago (Ellis et al. 1993). Because gymnosperms are less amenable to infection by *Agrobacterium*, initial transformation successes relied mostly on micro-projectile bombardment. Biolistic transformation of mature somatic embryos or embryogenic tissue resulted in regeneration of transgenic white spruce (*Picea glauca*) (Ellis et al. 1993), black spruce (*Picea mariana*) (Charest et al. 1996), tamarack (*Larix laricina*) (Klimaszewska et al. 1997), radiata pine (*Pinus radiata*) (Walter et al. 1998), loblolly pine (*Pinus taeda*) (Tang and Tian 2003), and Norway spruce (*Picea abies*) (Walter et al. 1999; Clapham et al. 2000). Larch was the first conifer to be stably transformed with *Agrobacterium*, initially by infecting seedlings with wild-type *A. rhizogenes* (Huang et al. 1991; Shin et al. 1994), and later by co-cultivation of embryogenic tissue with disarmed strains of *A. tumefaciens* (Levéé et al. 1997). The latter approach has proved successful for other conifer species as well, including white pine (*Pinus strobus*) (Levéé et al. 1999), loblolly pine (*Pinus taeda*) (Tang et al. 2001; Tang 2002), and Norway spruce, white spruce, and black spruce (Klimaszewska et al. 2001).

Target traits for forest tree engineering

Progress in micro-propagation and transformation of trees has gone hand-in-glove with the efforts to engineer novel traits (by transfer of exogenous genes) or to alter existing traits (either by modifying endogenous genes or by transfer of homologous genes from related species), sometimes with the specific goal of improving commercial qualities, but more often to produce model systems for basic research.

Herbicide tolerance

Engineering tolerance to herbicides has been a major target for tree engineering as a commercially attractive trait because it is expected to reduce herbicide use during the establishment phase of plantations and favour the use of environmentally benign products (Strauss et al. 1997). Poplar was the first hardwood transformed with a herbicide-tolerance gene, a mutant *aroA* gene from *Salmonella typhimurium* (Fillatti et al. 1987). Herbicide tolerance of the transformants was minimal, which was attributed to low-level expression of the *aroA* gene and localization of the mutant enzyme in the cytoplasm (Riemenschneider and Haissig 1991). When the same poplar clone was transformed with *aroA* under a stronger promoter and a gene coding for a transit peptide to direct transport of the enzyme into chloroplasts, glyphosate tolerance increased markedly in whole-plant spray tests, but was still too low to be of silvicultural interest (Donahue et al. 1994). Higher and commercially relevant glyphosate tolerance in poplars was achieved by using the *CP4* gene (an *aroA* equivalent cloned from *Agrobacterium* strain CP4) with a chloroplast transit peptide sequence (Meilan et al. 2002b), the same combina-

Table 1. Species of transgenic forest trees that have been regenerated.

Species	Reference	Transformation
<i>Allocasuarina verticillata</i>	Franche et al. 2000a	<i>A. tumefaciens</i>
<i>Casuarina glauca</i>	Franche et al. 2000b	<i>A. tumefaciens</i>
<i>Betula platyphylla</i>	Mohri et al. 1997	<i>A. tumefaciens</i>
<i>Betula pendula</i>	OECD 2003	Microprojectile
<i>Castanea sativa</i>	OECD 2003	?
<i>Eucalyptus grandis</i>	Macrae and Van Staden 1993; and others ^a	<i>A. rhizogenes</i>
<i>Eucalyptus dunnii</i>	MacRae and van Staden 1993	<i>A. rhizogenes</i>
<i>Eucalyptus nitens</i>	MacRae and van Staden 1993	<i>A. rhizogenes</i>
<i>Eucalyptus camaldulensis</i>	Mullins et al. 1997; Ho et al. 1998	<i>A. tumefaciens</i>
<i>Eucalyptus globulus</i>	MacRae and van Staden 2000	<i>A. tumefaciens</i>
<i>Eucalyptus saligna</i>	MacRae and van Staden 2000	<i>A. tumefaciens</i>
<i>Eucalyptus urophylla</i>	MacRae and van Staden 2000	<i>A. tumefaciens</i>
<i>Eucalyptus hybrids</i> ^a	MacRae and van Staden 2000	<i>A. tumefaciens</i>
<i>Juglans regia</i>	McGranahan et al. 1988; Escobar et al. 2000	<i>A. tumefaciens</i>
<i>Juglans hindsii</i> × <i>regia</i>	Vahdati et al. 2002	<i>A. tumefaciens</i>
<i>Larix decidua</i>	Huang et al. 1991; Shin et al. 1994	<i>A. rhizogenes</i>
<i>Larix laricina</i>	Klimaszewska et al. 1997	Microprojectile
<i>Larix kaempferi</i> × <i>decidua</i>	Levee et al. 1997	<i>A. tumefaciens</i>
<i>Liquidambar styraciflua</i>	Sullivan and Lagrimini 1993	<i>A. tumefaciens</i>
<i>Liriodendron tulipifera</i>	Wilde et al. 1992; Rugh et al. 1998	Microprojectile
<i>Picea abies</i>	Klimaszewska et al. 2001	<i>A. tumefaciens</i>
	Walter et al. 1999; Clapham et al. 2000	Microprojectile
<i>Picea glauca</i>	Klimaszewska et al. 2001	<i>A. tumefaciens</i>
	Ellis et al. 1993	Microprojectile
<i>Picea mariana</i>	Klimaszewska et al. 2001	<i>A. tumefaciens</i>
	Charest et al. 1996	Microprojectile
<i>Pinus radiata</i>	Walter et al. 1998	Microprojectile
<i>Pinus strobes</i>	Levéé et al. 1999	<i>A. tumefaciens</i>
<i>Pinus taeda</i>	Tang et al. 2001; Tang 2002	<i>A. tumefaciens</i>
	Tang and Tian 2003	Microprojectile
<i>Pinus sylvestris</i>	OECD 2003	?
<i>Populus deltoides</i>	Han et al. 1997	<i>A. rhizogenes</i>
	Han et al. 2000	<i>A. tumefaciens</i>
<i>Populus nigra</i>	Wang et al. 1996; and others ^b	<i>A. tumefaciens</i>
<i>Populus tremuloides</i>	Tsai et al. 1994	<i>A. tumefaciens</i>
<i>Populus tremula</i>	Tzfira et al. 1996	<i>A. rhizogenes</i>
	Tzfira et al. 1997; and others ^b	<i>A. tumefaciens</i>
<i>Populus tricarpa</i>	Han et al. 1997	<i>A. rhizogenes</i>
<i>Populus hybrids</i> ^b	Fillatti et al. 1987; and others ^b	<i>A. tumefaciens</i>
	Han et al. 1997; and others ^b	<i>A. rhizogenes</i>
	Chuveau et al. 1994	Electroporation
<i>Robinia pseudoacacia</i>	Han et al. 1993	<i>A. rhizogenes</i>
	Igasaki et al. 2000	<i>A. tumefaciens</i>
<i>Ulmus procera</i>	Gartland et al. 2000	<i>A. tumefaciens</i>

Note: *A. tumefaciens*, *Agrobacterium tumefaciens*; *A. rhizogenes*, *Agrobacterium rhizogenes*.

^aSee MacRae and van Staden (2000) for complete listing of transformed and regenerated *Eucalyptus* species and hybrids.

^bSee Leplé et al. (2000) for complete listing of transformed and regenerated *Populus* species and hybrids.

tion that is used in Monsanto's Roundup Ready® crops. Glyphosate tolerance has been obtained in some other forest tree species as well, in particular *Eucalyptus grandis* (Llewellyn 2000) and European larch (*Larix decidua*) (Shin et al. 1994), but the tolerance of the latter in greenhouse tests was below normal field application rates. Tolerance to another class of herbicides has been achieved by using a mutant acetolactate synthetase gene from *Arabidopsis*, which

conferred a high level of resistance to chlorsulfuron in tests with potted hybrid poplars at dose levels exceeding field application rates (Brasileiro et al. 1992). Research on the *bar* gene from *Streptomyces hygroscopicus* as a selectable marker for plant transformation has enabled engineering of tolerance to a third class of herbicides. Glufosinate tolerance was first achieved in poplar in the late 1980s (de Block 1990; Chuveau et al. 1994), and more recently tolerance to

operational dosages was obtained in radiata pine, Norway spruce and *Eucalyptus camaldulensis* (Bishop-Hurley et al. 2001; Harcourt et al. 2000).

The commercial potential of herbicide-tolerant trees has been demonstrated in various field trials during the past decade. Trials during the early 1990s in Europe demonstrated maintenance of effective glufosinate tolerance over 2 years in hybrid poplar with no deleterious effects on tree growth (Paques et al. 1995). Similar results were reported from multiple trials in the northwestern USA during the mid- and late-1990s with glyphosate-tolerant hybrid poplars (Meilan et al. 2000, 2002b). Those trials were the first to demonstrate tolerance to operational glyphosate dosages under field conditions. Glyphosate tolerance was also demonstrated in field tests with eucalypts in England in 1995 and later in Chile and Uruguay (Llewellyn 2000). In the USA, field trials have been conducted with sweetgum engineered for tolerance to 2,4-D (Information Systems for Biotechnology 1993), but little information is available on the results of those trials (Llewellyn 2000). Trials with glufosinate-tolerant radiata pine and Norway spruce were initiated in New Zealand at the end of 2002 (C. Walter, New Zealand Forest Research Institute, personal communication).

Insect resistance

Genetic engineering of insect resistance in trees has focused primarily on the use of delta-endotoxin genes (*cry*) from *Bacillus thuringiensis* (*Bt*). The first generation of transgenic trees expressing wild-type *cry* genes often exhibited low levels of pest resistance, as was the case in poplar, walnut, white spruce, and European larch (Dandekar et al. 1994; Ellis et al. 1993; Shin et al. 1994). An exception is the report of effective protection against lepidopteran defoliators on black poplar (*Populus nigra*) transformed with a truncated but otherwise unmodified *cryIA* gene (Wang et al. 1996). The use of synthetic *cry* genes with altered codon bias to optimize expression in plants has permitted the engineering of effective resistance. High levels of mortality (up to 100%) have been reported under laboratory conditions for lepidopteran pests on transgenic poplar (McCown et al. 1991), white spruce (Peña and Séguin 2001) and loblolly pine (Tang and Tian 2003), and for leaf beetles on eucalypt (Harcourt et al. 2000) and hybrid poplar (Génissel et al. 2003a).

Operationally effective resistance to natural insect infestations was demonstrated in the field, most notably with transgenic poplar in the USA and China. Meilan et al. (2000) reported minimal feeding damage and improved growth of hybrid cottonwood expressing the *cry3A* gene under leaf-beetle infestations in Oregon during 1998. Similar results were reported for tests with *cryIAc*-black poplar (Wang et al. 1996) infested with lepidopteran defoliators in China during 1994–1997 (Hu et al. 2001). The largest field evaluation of lepidoptera-resistant poplar took place in China: 20 000 clonally propagated transgenic trees representing three independent transformants were planted out over six provinces in 1995 (Wang et al. 1996), but no results have been reported to date. Other field evaluations of insect-resistant trees that are in progress include tests of transgenic (*cryIAb*) white spruce in Canada (A. Séguin, Canadian Forest Service, personal communication).

In view of the ability of insect pests to develop resistance to *Bt* toxins (Tabashnik et al. 2003), new sources of genetic insect resistance are being investigated. Effectiveness of several approaches has been demonstrated in the laboratory but has not yet been tested under field conditions. Significant levels of resistance to the poplar leaf beetle (*Chrysomela tremulae*) were achieved in hybrid poplar by using a cysteine proteinase inhibitor from rice (Leplé et al. 1995). Transformation of white poplar (*Populus alba*) with a cysteine proteinase inhibitor from *Arabidopsis* conferred even higher levels of beetle resistance, causing up to 100% mortality of *C. populi* (Delledonne et al. 2001). Another approach is to manipulate plant metabolites that are known to interact with insect feeding. Expression of a tobacco anionic peroxidase gene in sweetgum (Sullivan and Lagrimini 1993) provided partial resistance to some leaf-feeding caterpillars but not to others (Dowd et al. 1998). Accumulation of tryptamine in poplar transformed with the enzyme tryptophan decarboxylase reduced feeding and growth of the forest tent caterpillar (*Malacasoma disstria*) (Gill et al. 2003).

Disease resistance

Compared to tolerance to herbicides and insect feeding, engineering of disease resistance in forest-tree species is still in its infancy (Séguin 1999). Recent progress with fruit trees has demonstrated that it is feasible to engineer disease resistance in woody perennials (reviewed in Peña and Séguin 2001). One example is the development of papaya (*Carica papaya*) resistant to papaya ring spot virus (Lius et al. 1997), which constitutes the first (1997) and only commercial release of a transgenic tree. Work with forest trees is in progress to test various approaches that have resulted in disease resistance in fruit trees and agricultural crops. Examples include expression of viral coat protein genes to obtain disease resistance in walnut (Information Systems for Biotechnology 1993); the use of antimicrobial peptides in poplar and white pine and endochitinases from fungal origin against root rot in black spruce (Séguin 1999); and the insertion of defensin-like genes to obtain fungal resistance in Norway spruce (Walter and Smith 2000). Some progress in engineering disease resistance has been reported for poplar. Expression of a wheat oxalate oxidase gene delayed the onset of *Septoria* disease in leaf-disk assays (Liang et al. 2001), but was not tested in whole plants or in the field. In the only field test of engineered disease resistance reported to date, overexpression of the bacterio-opsin gene failed to elevate resistance of hybrid poplar to *Venturia* (Mohamed et al. 2001).

Abiotic stress

Worldwide problems of climate change, desertification, salinization and accumulation of toxic substances in soils are stimulating research to increase plant tolerance to abiotic stress. The complex plant response to abiotic stress makes genetic modification for increased tolerance difficult to achieve, although modification of a single trait has improved stress tolerance in several crops (see Wang et al. 2003a). Transgenic forest trees are being developed to shed light on stress tolerance mechanisms, using loblolly pine and poplar as the main models. Tang (2002) demonstrated a modest increase in salt tolerance of transgenic loblolly pine calli and

plantlets transformed with genes coding for mannitol-1-phosphate and glucitol-6-phosphate dehydrogenase, enzymes that result in the accumulation of solutes that help maintain osmotic balance. Wang et al. (1999, 2003b) used transgenic aspen to study the importance of a water-stress responsive protein (BspA) and a salinity-stress protein (SP1), and demonstrated some improvement in tolerance to water-deficit and salt stress in potted plants expressing a high level of either protein. Transgenic poplar is also used as a model to study the role of antioxidative systems in plant stress response, in particular enzymes that are involved in oxygen scavenging (Arisi et al. 1998) or the regeneration of antioxidants (Noctor et al. 1998). Overexpression of enzymes mediating glutathione biosynthesis enhanced resistance to high-light and low-temperature stress (Foyer et al. 1995) but did not improve protection of photosynthesis against acute ozone stress (Strohm et al. 2002).

The most likely application of trees with improved stress tolerance in the near future is for phytoremediation of contaminated soils. Rugh et al. (1998) used the bacterial *merA* gene with plant-optimized codon usage to express mercuric ion reductase in yellow poplar (*Liriodendron tulipifera*). Transgenic plantlets were resistant to normally toxic levels of ionic mercury in the culture medium and transpired high levels of elemental mercury. The same gene was inserted into eastern cottonwood (*P. deltoides*) with similar results and transgenic plants were able to grow in mercury-contaminated soils at an ionic mercury concentration that was lethal to wild-type plants (Che et al. 2003). A field test was initiated in 2003 to assess their performance on a mercury-contaminated site (S. Merkle, University of Georgia, personal communication).

Wood quality and quantity

Traits related to wood quality are key amongst the targets for genetic modification of trees. Because removal of lignins is a costly and energy-consuming component of the pulp and paper production process that requires the use of polluting chemicals, reducing lignin content and modifying its composition for increased pulping efficiency have become important objectives of tree engineering as an alternative to adjusting bleaching and delignification processing technologies (Christensen et al. 2000). Substantial progress has been made, aided by a growing understanding of lignin biosynthesis and its genetic control (reviewed in Tzfira et al. 1998; Grima-Pettenati and Goffner 1999), with poplar serving as the main model (Dinus et al. 2001). The most notable achievement to date is a 45% reduction in structurally normal lignin resulting from antisense-suppression of 4-coumarate:CoA ligase (4CL) (Hu et al. 1999). Leaf, stem and root growth were substantially enhanced in transgenic lines. Structural integrity was maintained at both cellular and whole-plant level in 10-month old trees, but it is not yet known how the lignin-reduced trees perform in the longer term or under field conditions. Suppression of cinnamoyl-CoA reductase has also shown promise for reducing lignin content (Boerjan et al. 1999). Down-regulation of other enzymes, such as caffeate O-methyltransferase or cinnamyl alcohol dehydrogenase (CAD), did not reduce lignin content but did result in modified lignin structure (Baucher et al. 1996; van Doorsselaere et al. 1995; Tsai et al. 1998; Lapierre et al. 1999; Jouanin et al. 2000). Depressed CAD

activity increased the incorporation of aldehydes and free phenolic groups and facilitated lignin solubilization during kraft pulping (Lapierre et al. 1999; Pilate et al. 2002). In contrast, reduced caffeate O-methyltransferase activity caused a higher resistance to kraft delignification by increasing the frequency of less extractable guaiacyl-lignin and condensed bonds (Lapierre et al. 1999). Franke et al. (2000) reported an increase in more easily extractable syringyl-lignin in transgenic poplar overexpressing a rate-limiting enzyme.

Field trials have demonstrated the commercial feasibility of using lignin-modified trees for improved pulping efficiency. Tests to evaluate pulping performance and ecological impacts of hybrid poplar expressing CAD or O-methyltransferase antisense constructs over an 8-year period were initiated in 1995 in France and England (Pilate et al. 2002). The trial was ended prematurely in 1999 when the experimental plots in England were destroyed by eco-activists. Nevertheless, the 4 years of testing showed that changes in lignin content and structure previously observed in greenhouse-grown trees (Lapierre et al. 1999) were maintained in the field. CAD-antisense poplars had characteristics that allowed easier delignification and higher pulp yield, producing 6% savings in chemical use and 2–3% increase in pulp yield. Those trials constitute the first duplicated field demonstration that pulping performance can be enhanced through genetic engineering, although greater improvements may be needed to be of commercial interest.

In contrast, engineered genotypes with improved form and growth are still far removed from any commercial application. Basic research on molecular factors controlling plant growth and differentiation has shown that auxin (*iaa*), cytokinin (*ipt*), and root-inducing (*rol*) genes from *Agrobacterium* can be used to alter tree growth characteristics and wood properties, but not necessarily in a beneficial way. The use of auxin- or cytokinin biosynthetic genes reduced growth rate, leaf size and stem diameter in transgenic aspen (Schwartzberg et al. 1994; Tuominen et al. 1995). Expression of the *rolC* gene under control of the constitutive 35S promoter in transgenic aspen resulted in stunted growth, breaking of apical dominance, reduced leaf size and stem growth, and decreased internode length (Nilsson et al. 1996; Fladung et al. 1997; Grünwald et al. 2001). Under the control of its native promoter, the same gene enhanced growth and increased stem production index during 2 years of growth in the greenhouse (Tzfira et al. 1999). Improvements in commercially important traits have been achieved by modification of plant hormone levels. Ectopic overexpression of the gibberellin (GA) 20-oxidase gene from *Arabidopsis* in transgenic poplar resulted in faster growth in height and diameter, larger leaves, more numerous and longer xylem fibers, and increased biomass in 3-month-old plants grown under greenhouse conditions (Eriksson et al. 2000). No data were provided on performance of the transgenic lines over a longer growth period or in the field.

Reproductive development

The application of tree engineering is limited to the few commercial genotypes that can be efficiently transformed and regenerated, because long generation time makes it impractical to introgress a transgene from primary transformants into commercial lines through hybridization and

backcrosses, as is done with agricultural crops. Manipulation of genes that control floral development has shown some promise in reducing generation times by inducing precocious flowering (Nilsson and Weigel 1997). Constitutive expression of the *LEAFY* (*LFY*) or *APETALA1* (*API*) homeotic genes from *Arabidopsis* in citrus resulted in trees with a generation time of 1 year from seed to seed (Peña et al. 2001). Efforts to induce normal flowering early in the juvenile phase of *Populus* have not yet met with the same success. Expression of the *LFY* gene induced flowering after 8 months instead of 8–20 years of vegetative growth, but the flowers were abnormal and did not shed pollen (Weigel et al. 1995). Response to *LFY* overexpression was highly variable among poplar genotypes, with the few genotypes that flowered precociously producing abnormal flowers and displaying altered vegetative development (Rottmann et al. 2000). Similar results were reported for ectopic expression of *PTLF*, a poplar homolog of the *Arabidopsis LFY* gene. More research is required before control over the flowering process is refined enough that it can be used as a practical approach for reducing generation times of transgenic trees.

Another objective of manipulating homeotic genes is to engineer sterility to confine potential transgene spread (see “Invasive escape and vertical gene flow”). In herbaceous plants, sterility has been achieved through sense–antisense or promoter-based suppression of floral development genes (“gene-suppression” methods), but application of this approach to trees is still at the research stage (Skinner et al. 2000). Flower-specific expression of cytotoxic genes to ablate floral organs (so-called “genetic ablation” methods, as reviewed in Strauss et al. 1995) has been applied to trees with limited success. Expression of *dtA* (Diphtheria toxin A) or *barnase* (ribonuclease) genes in poplar and radiata pine ablated floral structures but also decreased vegetative growth owing to leaky expression in nontarget tissues (Mouradov et al. 1998; Meilan et al. 2001). Transgenic poplar with cytotoxic genes under the control of a tapetum-specific promoter and poplar with sense–antisense suppressed *LFY* homeotic gene were field-tested in Oregon (Strauss et al. 1996), but results have not yet been reported.

Field releases

The current status of genetic tree engineering is reflected in the number of planned field trials. Permit applications for field releases are listed by various regulatory agencies and other institutions on the internet (see legend of Fig. 1). Although not all permits result in actual field tests, major trends can be gleaned from the electronic databases. The first field release in 1988 of glufosinate-resistant poplar in Belgium was followed by a modest growth in the number of planned field releases during the early 1990s. The number increased in the latter half of the 1990s with a broadening diversity of target traits (Fig. 1A). Of the 205 permit applications that were listed by the end of 2003, 73.5% originated in the USA, 23% in other OECD (Organization of Economic Co-operation and Development) member nations (in particular Belgium, Canada, France, Finland, New Zealand, Norway, Portugal, Spain, and Sweden), and 3.5% elsewhere (Brazil, China, Chile, South Africa, and Uruguay). Testing of four key target traits accounted for 80% of the permit applications: in particular, herbicide tolerance (32%), marker

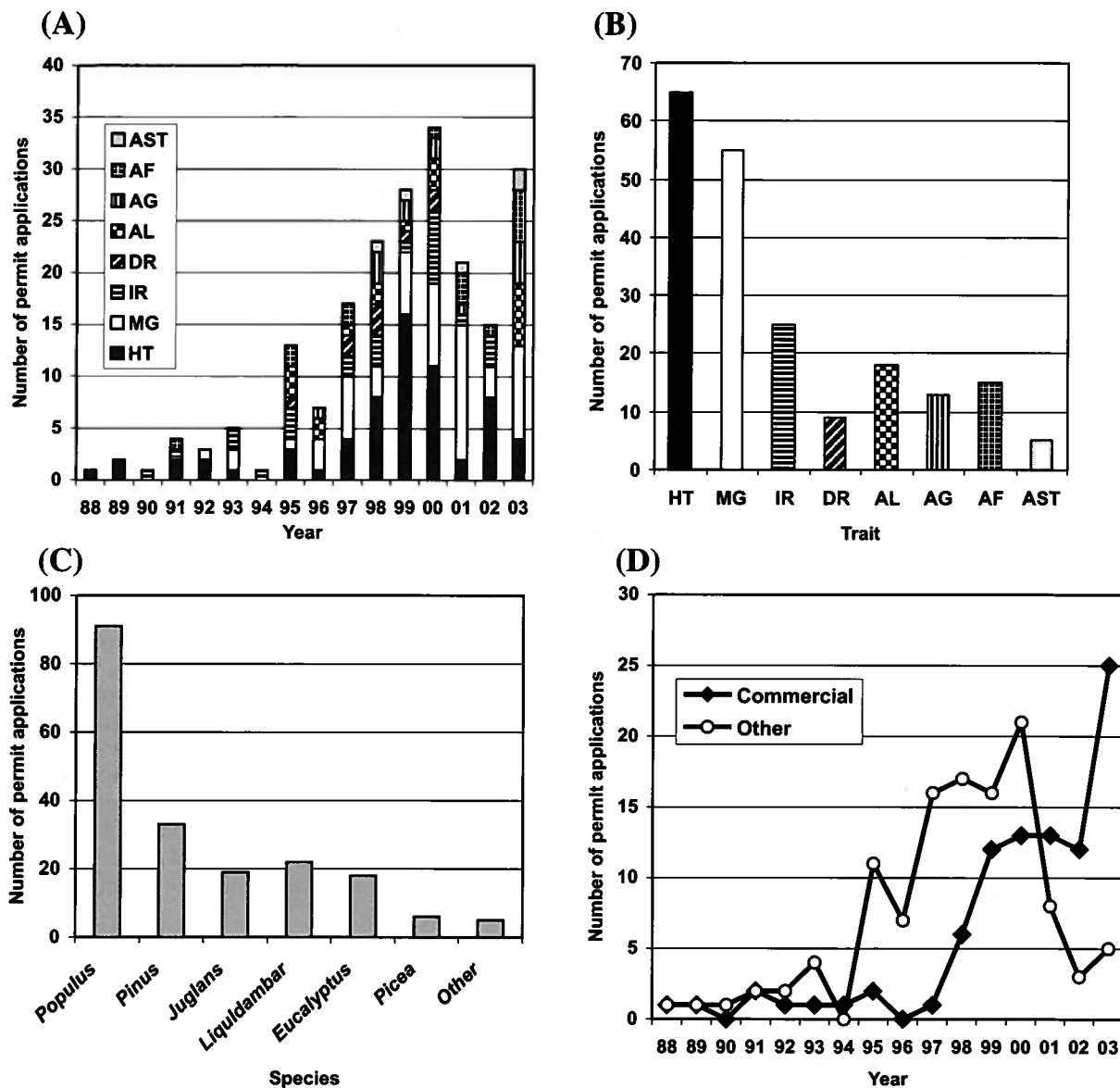
genes (27%), insect resistance (12%), and lignin modifications (9%) (Fig. 1B). About 85% of the applications pertained to field tests with transgenic *Populus*, *Pinus*, *Liquidambar*, and *Eucalyptus* (Fig. 1C), genera for which commercially feasible transformation, regeneration and propagation protocols are available.

Industrial interest in transgenic trees remained remarkably low for the first 10 years of their development (Fig. 1D). Academic and government researchers submitted most of the field release permits, mostly for the purpose of basic research, or to establish proof of concept. Some of the most promising constructs were taken to the next level by demonstrating commercial feasibility in field trials, although limited in both scale and duration (e.g., Meilan et al. 2000, 2002a, 2000b; Hu et al. 2001; Pilate et al. 2002). Having established proof of concept for key target traits, the number of noncommercial permits has dropped sharply in recent years. In contrast, the number of field releases planned by industry has been increasing steadily since the late 1990s. By the end of 2003, industry permits accounted for 46% of the permits submitted. Since 1988, companies like Plant Genetic Systems, ArborGen, Westvaco, International Paper, Union Camp, Shell, Monsanto, and Weyerhaeuser received permits for field trials, mostly with transgenic poplar, and fewer with radiata pine, sweetgum, and eucalypts. Nevertheless, commercial pursuit of transgenic trees can best be characterized as hesitant and exploratory: only a few companies (ArborGen, Westvaco, International Paper) have played an active role in the past 3 years, and their involvement appears to be aimed primarily at testing the murky waters of regulatory and public approval (30% of the permits requested in 2003 involved marker genes). The dramatic spike in company permits in 2003 is not necessarily evidence of increased commercial pursuit: it was entirely attributable to applications submitted by ArborGen, a joint venture that is in its last year of venture capital funding (Duncan 1999). To date, there has not been a concerted push for forestry commercialization of any transgenic tree genotype anywhere in the world.

Environmental impacts and research needs

Nevertheless, the prospect of commercial release is turning transgenic forest trees into the next battleground for public acceptance of biotechnology. There was little public interest in the development of transgenic trees until 1999, when a forest biotechnology conference was picketed and a plantation with lignin-modified poplar was destroyed in England (Strauss et al. 1999). The destruction of several transgenic poplar field trials in Oregon and the firebombing of offices at the University of Washington that followed attracted even more public attention (Kaiser 2001). Opposition is not limited to fringe activists but is also being voiced by environmental groups with much broader public support, such as the World Wildlife Fund (Campbell and Asante-Owusu 2001), and even by some forest accreditation bodies. For example, public ambiguity precipitated the policy decision by the Forest Stewardship Council to not certify plantations as being sustainably managed if they contain even research trials with transgenic trees, because “the use of genetically modified trees in a plantation context crosses the

Fig. 1. Number of permit applications for confined field releases of transgenic forest trees by (A) traits and year of application, (B) target trait, (C) tree species, and (D) type of permit originator. MG, marker or reporter gene; HT, herbicide tolerance; IR, insect resistance; DR, disease resistance; AG, altered growth; AL, altered lignin; AF, altered flowering; AST, abiotic stress tolerance. The following sources were used to compile this figure: Information Systems for Biotechnology 1993; Biosafety Information Network and Advisory Science 2001; Canadian Food Inspection Agency 2003; European Commission 2003; Organization for Economic Cooperation and Development 2003.



line for acceptability on grounds of social and economic equity as well as environmental risk" (Cauley 2001).

Ecological risks focus on three interrelated issues, which are equally relevant to transgenic crops. The first key concern is the possibility that the transgene will spread, either because of increased invasiveness of the transformed species itself, or transfer of the transgene to wild relatives through hybridization ("vertical" gene flow) or to unrelated organisms by nonsexual means ("horizontal" gene flow). The second concern is that new traits imparted to transgenic trees will cause unexpected perturbations to nontarget organisms or ecosystem processes. The third centers around the notion that genetic transformation is imprecise, causing instability in transgene expression over time and possible unforeseen negative effects on tree fitness ("pleiotropic effects").

Concern 1: Transgene spread

Invasive escape and vertical gene flow

The introgression of an escaped transgene into a wild population involves two phases: gene flow by dispersal of seed or vegetative propagules ("escape") or by pollination ("hybridization"), followed by establishment of the transgene in the wild gene pool (Van Raamsdonk and Schouten 1997). Of greatest concern is that the transgenic species, its offspring, or one of its wild relatives will become a weed because the transgene has increased invasiveness of its carrier, for example by conferring resistance to herbivores (Stewart et al. 1997) or because the new trait limits the ability to control escapees (e.g., herbicide tolerance, see Warwick et al. 1999). Invasive escape of the transgene can occur at three levels.

First, the transformed species itself can escape cultivation and become a weed. That concern is rooted in the experience with exotic tree species in plantation forestry. In the southern hemisphere, for example, at least 19 species of pines have escaped cultivation and invaded other habitats during the past 3–4 decades, at great economic and ecological costs (Richardson 1998). Second, the transgene can be transferred to nontransgenic individuals of the same species through pollination. Recent examples of sexual gene transfer from transgenic to nontransgenic crops (Reiger et al. 2003) or wild-type species (Quist and Chapela 2001) underscore that concern. Third, the transgene can be transferred to a wild relative through out-breeding. Transfer of transgenes from transgenic crops to weedy relatives has been demonstrated experimentally (e.g., Chèvre et al. 1997; Snow et al. 1999), but there are no known cases (yet) of transgenes escaping into weed populations from commercial scale plantings (Royal Society of Canada 2001). The risk of hybridization with wild relatives is considered to be much greater for transgenic forest trees because (i) most species are undomesticated out-breeders that will readily interbreed with related species, (ii) gene flow is often mediated by copious production of wind-borne pollen that may travel great distances (Di-Giovanni and Kevan 1991), and (iii) transgenes are likely to be used in close proximity to interfertile populations of natural or feral origin.

Because the probability of transgene escape into gene pools of wild-type or related tree species is considered high, containment of reproduction is expected to facilitate commercial release in many cases. Besides using sterile hybrids or harvesting before reproduction occurs, co-engineering for sterility is pursued around the world as the most promising approach (Skinner et al. 2000). However, the two key approaches for engineering sterility, genetic ablation and gene suppression, are expected to lead to occasional reversions to fertility (Denis et al. 1993; Flavell 1994), and it is questionable whether gene escape can be completely avoided once transgenics are deployed over large acreages and full rotations (see “Transgene stability”).

Gene flow and the ability to predict its ecological and evolutionary impacts have therefore become central issues for public acceptance of transgenic plants, whether crops or trees (Snow 2002). Short-term and historical gene flow have been studied among natural tree populations, seed orchards and plantations, but there are few studies on the effects of gene flow from forest plantations on wild populations (reviewed in Slavov et al. 2002). A major challenge is the extrapolation of short-term gene flow observations to spatial and temporal scales that are ecologically relevant (Levin 1992). The use of spatial simulation models that integrate gene flow data with landscape-scale analyses of ecosystem dynamics is an emerging solution (e.g., Higgins et al. 2000; and see below).

Predicting the fate of transgenes in a wild plant population is considerably more difficult than determining whether gene flow will occur (Royal Society of Canada 2001). The ecological consequences of gene flow depend on the impact of the transgene on reproductive fitness (Hancock 2003). Genes with detrimental effects will be lost from the population through natural selection or genetic drift. Genes that confer no selective advantage might spread but may not have long-

term ecological consequences. For example, the transfer of herbicide tolerance from a transgenic plantation to a wild-type species is unlikely to provide a competitive advantage to hybrids in the absence of herbicide applications, as was demonstrated by Crawley et al. (1993) for herbicide-tolerant canola. The first generation of transgenic trees is likely to deploy genes that do have the potential to increase fitness of its carriers, for example by enhancing survival or reproductive success of hybrid offspring under insect infestations, disease epidemics, or harsh environmental conditions. Prediction of transgene fate requires empirical data on fitness costs and selective advantage of those traits collected within the broad ecological context in which they will be deployed (Royal Society of Canada 2001). Such data are lacking, even for transgenic crops that have already been released commercially, with the exception of a few studies involving transgenic canola (Crawley et al. 1993; Fredshavn et al. 1995; Stewart et al. 1997; Snow et al. 1999). Multi-generational and multi-site field experiments, as conducted by Crawley et al. (1993), are needed to evaluate ecological performance and invasiveness of various transgene–species combinations. Such studies will be difficult, if not impossible, to do for long-lived transgenic trees, with slow growth causing delayed fitness differentials and a long juvenile period making detection of ecological impacts likely only after many years. The only realistic option is to use simulation models to extrapolate results from short-term empirical studies.

To date, only one model specifically addresses transgene flow and fate in a forestry context. Simulation of transgene effects in a variable environment (or STEVE) provides a spatially explicit representation of gene flow from hybrid poplar plantations to wild cottonwood populations in the northwestern United States as a tool to study consequences of gene flow under various transgene deployment scenarios over a 50–100 year time frame (DiFazio 2002). That model considers such factors as mating success, long distance pollen movement, seed and vegetative propagule establishment, and the competitiveness of hybrid seedlings in the wild. Modeling a variety of scenarios for fertile transgenic trees resulted in the predictions that (i) only low levels of gene flow (1–2% after 25 years) will occur when the transgene has a neutral impact on reproductive fitness, (ii) insect resistant transgenes, which confer a selective advantage, will become fully introgressed into wild-type trees, and (iii) herbicide resistant transgenes have a minor effect on transgene flow, unless there was a nearby habitat with extensive herbicide use (Difazio 2002). Models such as STEVE can establish threshold levels of acceptance (e.g., 0% or 5% transgenic gene flow from pollen) and assess the effect of a range of variables on this threshold level. More biological data on gene flow and environmental interactions are needed to improve confidence in predictions of transgene flow and fate, and similar models need to be developed for other transgenic forest trees targeted for commercialization.

Horizontal gene flow

Concern has also been expressed about the transfer of transgenes to unrelated organisms by nonsexual means. Horizontal gene transfer is a normal process for gene exchange most commonly seen among bacteria (Lorenz and Wackernagel 1994) and evolutionary arguments have been made

that it occurs between unrelated organisms, for example between plants and bacteria, and animals and bacteria (Syvanen 1994). The possibility of transgenes moving from a genetically engineered plant to plant-associated or soil-inhabiting microorganisms has raised public and regulatory concerns, particularly in view of the use of bacterial antibiotic resistance genes as selectable markers in crop transformation (Syvanen 1999). Transgenic plant DNA is readily degraded in the soil but a fraction escapes by adsorption to sand or clay particles and remains available to transform bacterial cells (as reviewed in Bertolla and Simonet 1999). That the mechanisms for horizontal gene transfer from transgenic plants to microorganisms exist was demonstrated in experiments under idealized or optimized conditions in the laboratory (Hoffman et al. 1994; DeVries and Wackernagel 1998) and in sterile soil microcosms (Nielsen et al. 2000), but such transfers are extremely rare and have not been shown to occur under natural or field conditions (Gebhard and Smalla 1999; Schlüter et al. 1995; Syvanen 1999). Horizontal gene transfer is therefore not considered a key concern. In the only study to date with transgenic trees, persistence of *nptII* (kanamycin resistance) selectable marker gene in decomposing poplar leaves was less than 4 months under natural conditions (Hay et al. 2002), as compared with 2 years in a sugar beet field (Gebhard and Smalla 1999).

Concern 2: Impacts on nontarget organisms and ecosystem processes

Even if the transgene does not "escape", it may still adversely impact other organisms, or critical ecosystem processes like decomposition, nutrient cycling, or competition. Potential negative impacts will depend on the target trait.

Pest resistance

There is a concern that engineered herbicide tolerance may lead to greater use of one class of herbicides (as dictated by the tolerance mechanism engineered in the crop species) and therefore increased selection pressure for resistant biotypes in unrelated weed species. The list of herbicide-resistant agricultural weeds has grown geometrically since the first documented occurrence of tetrazine resistance in 1968 (Warwick et al. 1999), but there are no known cases of herbicide resistance in weeds as a result of forestry applications, which occur on a much smaller fraction of total cultivation and at much lower frequency than in agriculture. Increased selection of herbicide-tolerant weeds in forest plantations as a result of transgenic herbicide tolerance in the crop species is therefore not a major concern.

In contrast, evolution of resistant pest biotypes is a key concern for any form of engineered insect resistance (Raffa 1989). In the case of insect-resistant *Bt* crops, target pest resistance is being delayed by using specific planting prescriptions (Tabashnik et al. 2003). Selective pressures will be more difficult to manage in long-lived forest trees, as they can exceed those in agricultural settings during a single rotation. In the only study with a forest insect, the initial frequency of alleles conferring resistance to *Bt* toxins was close to the highest theoretical values (Génissel et al. 2003b), which underscores the potential for resistance development. The use of tissue-specific or wound-inducible promoters (Ellis et al. 2001), multiple resistance mechanisms (Robison

et al. 1994), and refugia of untransformed trees within or near transgenic plantations (Raffa 1989) all require careful consideration within specific constraints imposed by the biology of each particular pest–transgenic host combination.

Novel selection pressures could also affect nonpest members of the insect-herbivore guild on the transformed host, which may be elevated to pest status through altered competition or indirect effects via other trophic levels (Raffa 1989). Those issues have received little or no attention within the forestry context to date. Possible long-term effects of chronic target pest suppression on competitive and trophic interactions (Schuler et al. 2001) that can shape arboreal community structure and biodiversity need to be examined before commercial release of insect-resistant transgenics.

The use of novel genes from plant, microbial, or animal origin to obtain disease or insect resistance in the foliage can result in novel routes of exposure for beneficial organisms involved in plant health, litter decomposition, and nutrient cycling. Novel proteins can enter the soil by decomposition of plant residues or via root exudates. Release from roots into the rhizosphere was first demonstrated for *Bt* toxins produced in transgenic corn (Saxena et al. 1999). The toxin protein is adsorbed to soil particles and retains biological activity well beyond expected half-lives, but with no detectable negative effects on soil-dwelling organisms (Saxena and Stotzky 2001). Reports on nontarget effects of anti-microbial proteins on saprophytic soil microflora were reviewed by Glandorf et al. (1997). Most studies indicate no obvious deleterious effects, root colonization by mycorrhizal fungi being one example that is relevant for trees, but the authors caution that studies are scarce and methods employed do not justify generalization of conclusions. Studies on the effects of insecticidal and anti-microbial proteins produced by transgenic trees in forest soils have just been initiated (A. Séguin, Canadian Forest Service, personal communication). The effect of such proteins in leaf litter from transgenic trees on organic matter decomposition and nutrient recycling in aquatic ecosystems is another issue that has not received any attention to date.

Wood quality and quantity

Reducing lignin content or altering the biochemical composition of lignin to facilitate its extraction during the pulping process may lead to various unintended side effects reducing tree fitness. For example, field studies with herbaceous perennials suggest that genes controlling lignin biosynthesis are linked with genes that influence winter survival (Casler et al. 2002). Reduced lignin content may also lead to increased population growth rates of insect defoliators, because lignin is known to reduce digestibility of plant material. Reduced lignin content can furthermore lower resistance to viral disease, as was the case in transgenic tobacco (Maury et al. 1999). Such adverse fitness effects can obviously undermine commercial potential of the transgenic. Lignin modifications may also have unintended environmental side effects: reduced lignin biomass could affect soil structure and fertility by accelerating organic matter decomposition. Higher decomposition of roots was the only significant effect observed by Pilate et al. (2002) in a 4-year study

on ecological impacts of reduced-lignin poplar in the UK and France. No other unexpected biological or ecological impacts were detected: there were no differences between transgenic and control lines in the extent of leaf damage by caterpillars, sawflies, and leaf beetles; in diversity of arboreal arthropods; in attack by *Melampsora* rust fungi; or in soil carbon, nitrogen content, microbial biomass, and respiration.

Studies have also been conducted to examine the effects of altered plant hormone biosynthesis on the establishment of symbiotic interactions between tree roots and fungi. Phytohormone balance can play a role in the formation of both ecto- and arbuscular mycorrhizas (Barker and Tagu 2000). Transgenic aspen expressing auxin-biosynthetic genes were able to form fully developed ectomycorrhizas in vitro (Hampp et al. 1996). Kaldorf et al. (2002) conducted the first field study of mycorrhization of transgenic trees in a 4-year trial of aspen transformed with the *rolC* gene from *A. rhizogenes*. Mycorrhizal colonization, diversity and community structure did not differ between transgenic and non-transgenic trees. However, one of the transgenic clones displayed depressed mycorrhization by one species, possibly because of somaclonal variation or positioning effect of the insert, indicating that genomic alteration can result in unpredictable changes in the tree's ability to form mycorrhizal associations.

Concern 3: Unstable gene expression and unexpected effects of genetic manipulation

Transgene stability

An issue that is of particular relevance for long-lived crops like trees is the stability of transgene expression over time. Expression of the transgene may be reduced or lost altogether because of "transgene silencing", a phenomenon that describes the recognition and specific inactivation of foreign DNA by the host plant (reviewed in Finnegan and McElroy 1994). The silencing of homologous endogenes (endogenous gene within the transformed genome that are homologous to the transgenes) has also been observed in a number of herbaceous transgenic plants (Wu et al. 2002; Szitty et al. 2003). Because cold-induced dormancy and other environmental conditions can trigger transgene silencing (see Maessen 1997), stability of expression is a concern, particularly in trees, which undergo numerous dormancy cycles and are exposed to a range of environmental conditions during a normal rotation. Although a few cases of transgene silencing in trees have been reported (Fladung 1999; Jouanin et al. 2000; Kumar and Fladung 2001), the cumulative experience with field and greenhouse tests suggests that transgene expression is stable, at least in primary transformants with the promoters and gene constructs that have been tested. For example, multi-year field studies with visual reporter genes in poplar (Ellis et al. 2001; Leplé et al. 2000; Meilan et al. 2002a), spruce (Ellis et al. 2001), and *Eucalyptus camaldulensis* (MacRae and van Staden 2000) have shown variation in gene expression between individuals within lines and with season, tissue type and genotype, but that gene expression was stable from year to year. In addition to stability of expression from year to year and under changing environmental conditions, the best evidence for

transgene stability is its maintenance during meiosis (Maessen 1997). The first controlled pollinations of sexually mature transgenic poplars were conducted in 1996 in France (Leplé et al. 2000), and seedlings from those crosses show the expected Mendelian segregation ratios (G. Pilate, unpublished data, cited in Ellis et al. 2001).

Field stability of transgene expression has yet to be examined over full rotations in commercial-scale plantings. That is particularly important for engineered sterility as a gene containment strategy. Total sterility cannot be guaranteed owing to spontaneous reversions and unpredictable gene silencing phenomena. Reversion and silencing frequencies need to be estimated at operationally relevant scales, possibly in conjunction with commercial release of a low-risk transgene. Current regulatory restrictions make it impossible to do so, because field tests are not permitted to continue beyond the minimum time required for sexual maturity and flowering (McLean and Charest 2000). The same restrictions preclude the testing of strategies that could be used to increase reliability of engineered sterility, such as homologues recombination to permanently delete essential floral genes, or the use of redundant (multiple) sterility mechanisms for backup (Strauss et al. 1995). This regulatory deadlock has yet to be resolved.

Somaclonal variation and pleiotropic effects

Another concern is that genetic transformation can negatively affect fitness of the transformed species (Burdon 1999). Adverse effects on fitness can arise from various aspects of the transformation process itself. During the in vitro propagation step, stable genetic changes can occur in somatic cells of the plant ("somaclonal variation"), which are often expressed as morphological abnormalities (e.g., Wang et al. 1996). The longer plant cells are maintained in unorganized culture, the greater the likelihood of genomic instability (Deverno et al. 1999). However, somaclonal variation can be avoided as a practical problem in the deployment of transgenic trees by careful selection of promoters, control over culturing conditions, minimizing the use of growth regulators to maintain or regenerate shoots, and screening out aberrant lines in greenhouse tests (Ellis et al. 2001). Strauss et al. (2001) reported morphological abnormalities in only 0.1% of 3200 independent transgenic poplar lines in 16 hybrid genotypes after years of testing in the field and greenhouse. Adverse effects on fitness of the transformed tree can also arise from disruption of existing genes or gene function by the transgene insertion. Mutations in single genes have long been known to produce multiple effects ("pleiotropic effects"). Expression of foreign genes or suppression of existing genes can be accompanied by a range of collateral changes in expression of nontarget genes, as has been reported for transgenic aspen (Eriksson et al. 2000; Kaldorf et al. 2002; Rottmann et al. 2000). Such effects may be subtle and may appear only at a particular growth stage or in response to specific environmental conditions (Royal Society of Canada 2001). Fitness differentials may not be evident until after several years of growth (Rottmann et al. 2000), and may affect long-term performance of the transformed species in terms of its tolerance to various biotic and abiotic stresses (Burdon 1999).

Moving forward

Progress during the past decade towards commercialization of transgenic trees has been nothing short of phenomenal. Several species have been engineered for specific traits, in particular herbicide tolerance, insect resistance and altered lignin, and have undergone extensive field-testing to demonstrate commercial potential. It is important to note that the lack of robust and efficient systems for transformation, regeneration, or propagation remains a key technical obstacle to broader application of recent advances beyond the few genotypes for which commercially valuable clones already exist. In addition, there are significant economic hurdles to operational implementation of scientific gains (Mann and Plummer 2002): the long time lag between costly research programs and the accrual of actual benefits (Mullin and Bertrand 1998) makes investments in tree engineering a risky business. The uncertainty over how genetically engineered trees will be received by both public and regulators adds to that economic risk. Lack of knowledge on how transgenes will interact with their aggregate environment contributes to a shaky public confidence and is a major obstacle to regulatory approval of commercial release.

The knowledge framework that is needed to ensure ecologically sound deployment of transgenic trees is clearly lagging far behind progress made during the past decade in their development and production. Public hesitation to embrace transgenic tree technology hinges on a number of ecological concerns that have yet to be addressed in a comprehensive fashion. Compared with transgenic crops (Dale et al. 2002), relatively little research has been conducted to date on ecological risks associated with the use of transgenic trees. Those risks range from short-term direct toxic impacts on nontarget organisms to community-level interactions and much more complex and longer-term impacts on evolutionary processes.

Filling in those knowledge gaps is subject to a number of severe constraints, not the least of which is the issue of testing scale. Current regulations restrict field releases of all transgenes in both time (duration) and space (size of out planting). Although small-scale and short-term testing is a necessary first step to assess benefits, as well as obvious deleterious (pleiotropic or environmental) effects, it has a high degree of uncertainty for predicting risks on a plantation scale. For example, in cases of no observable impacts, "absence of evidence cannot be equated with evidence of absence" (Royal Society of Canada 2001), as it may simply reflect inadequacies in design, scale or duration of testing. Such problems are not unique to the testing of transgenic trees (Snow and Moran Palma 1997), but they are exacerbated by their long rotation cycles. The data needed to realistically assess risks and benefits have to come from medium- to large-scale field releases over at least one full rotation, yet such tests are not permitted.

That conundrum can be resolved if regulatory agencies were to recognize that not all transgenes pose the same risk, and that there are cases where regulatory constraints can be eased to permit testing at a larger scale without undue risks. The basic tenets of regulatory systems in most industrialized countries are similar, with risk assessment being conducted on a case-by-case basis to ensure that every transgenic or-

ganism is thoroughly evaluated (OECD 1995). Elements of "familiarity" are often incorporated into the risk assessment where knowledge of the species (such as proximity of wild relatives and likely fitness impact of the transgene) can be used in conjunction with the case-by-case evaluation (OECD 1986). However, the resulting degree of regulatory control does not reflect the level of risk, because low-risk constructs are subjected to the same restrictions as high-risk ones.

Recently, James Hancock proposed a modification of the case-by-case approach and used the concept of "familiarity" to assign transgenic plants to low-, medium- or high-risk categories (Hancock 2003). The concept that risk levels can be categorized on the basis of the origin of the transgene (native or endogenous genes, homologs from related organisms, or exogenous genes from unrelated organisms) (Strauss 2003) and its impact on reproductive fitness (neutral, negative or positive) (Hancock 2003) may provide a useful framework for modifying regulations for transgenic trees, especially when adding as a third criterion the potential to cause undesirable impacts on nontarget species or ecosystem processes. Each transgenic construct can be assigned to a risk category, with each category requiring different levels of containment and testing scale. For example, the high-risk category requiring the highest level of confinement would include exogenous genes that increase reproductive fitness and can cause irreversible nontarget impacts, such as *Bt* toxin genes for insect resistance. The medium-risk category would include transgenes such as herbicide-tolerance genes, which, although selectively neutral in the natural environment and likely without severe nontarget impacts, confer a selective advantage in the presence of herbicide applications, so that the transgene could spread into wild populations and abolish a valuable silvicultural tool (e.g., herbicidal control of poplar in conifer plantations). The low-risk category would include endogenous genes that have a detrimental impact on fitness and limited nontarget impacts, such as altered-lignin and male-sterility constructs.

Field-testing regulations that recognize different levels of ecological risk and assign a commensurate level of confinement would enable research on long-term ecological risks while not discouraging commercial pursuit. Both objectives can be accommodated by moving forward with those transgenes that pose little risk in terms of gene escape and nontarget impacts. Prime candidates are altered-lignin constructs, which can be tested safely without genetic or physical confinement, for several reasons. First, lignin alterations are likely to have a detrimental impact on reproductive fitness, so that the transgenes will be selected against in the natural environment and therefore will not spread upon escape (Hancock 2003). Second, lignin biosynthetic genes are endogenous, which have already faced the sieve of natural selection so that their modification is unlikely to have unexpected (fitness) impacts (Strauss 2003). Third, transgenes mimic natural variation in lignin biosynthetic genes: CAD-antisense trees have the same phenotype as the naturally occurring "CAD-null" mutant of loblolly pine (MacKay et al. 1995, 1999), which is widely used in commercial plantations in the USA without restrictions. And fourth, altered lignin content did not have any drastic nontarget impacts during multiple years of small-plot testing (Pilate et al. 2002). The establishment of a limited number of medium to large-scale

plantations with lignin-altered pines or poplars in selected environments will permit industry to evaluate performance and economic benefits over a full rotation and across a range of environmental conditions, while permitting researchers to obtain scientific data that have to be collected at relevant temporal and spatial scales. Those data, such as estimation of gene flow from plantations to natural populations as a function of plantation size and spatial separation, can then be used to calibrate relevant models and to refine confinement requirements for higher-risk constructs.

In conclusion, much of the concern over the use of transgenic trees is due to scientific uncertainty, which produces much doubt and results in biases based on preconceived notions. To move this technology forward and address issues that are central to their safe deployment, we need relevant biological data that only medium- to large-scale release of transgenic trees over one full rotation can provide. This can be accomplished with negligible risk to the environment if low-risk transgenic trees such as lignin-altered poplar or pine are used.

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