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## Genetic Transformation of Forest Trees

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### 1. Introduction

Forests are complex ecosystems capable of providing a wide range of economic, social and environmental benefits. They help to regulate the water cycle, stabilize soils and moderate the climate of the Earth by absorbing and storing carbon dioxide. In addition to these environmental services, forests provide habitat to numerous species and are an important source of food, medicines and wood for humankind. In many countries, forests contribute importantly to their economic and social development through employment, the production and trade of forest products, and the protection and hosting of sites and landscapes of high cultural, spiritual or recreational value.

As a result of the increase in human population and economic activities, larger volumes of forest products, particularly wood, were required, and the natural processes were insufficient to restore the damage imposed on the forests. At present, the total forest area in the world is estimated to be nearly 4,000 million hectares, which cover about 30 percent of the global land area. Although the forest cover is still extensive, the problem of deforestation continues at an alarming rate: according to the Food and Agriculture Organization of the United Nations (FAO), in the 15 years from 1990 to 2005 the world lost 3 percent of its total forest area, representing an average decline of about 0.2 percent per year (FAO, 2007). Deforestation results from a combination of factors, including, among others, increased global demand for forest products, land-use change (e.g. conversion of forest into agriculture land) and the expansion of urban areas. A reduction in forest area can also happen through natural disasters, but deforestation is by far the most important cause of forest loss (FAO, 2007). Deforestation rates differ considerably from region to region, and figure 1 presents estimate data that illustrate the extent of losses and gains of forest land area around the world in the last two decades. It is interesting to note that deforestation is preponderant in developing countries in Latin America (with the notable exception of Cuba), Africa and Southeast Asia. The primary causes of this is the land-use change to agriculture and the production of fuelwood and charcoal through processes that are inefficient and lead to over-exploitation. Between years 2000 and 2010, however, net loss of forest area in the world decreased slightly, which is probably an indicator of the natural expansion of forests primarily due to efforts made to ensure the conservation of

biodiversity, the improvement in the establishment of forest plantations, and the genetic improvement of forest species. China, the United States and several European countries have increased their forest area mainly through the establishment of plantations, which may help reduce the harvest pressure on wild forests (FAO, 2010).

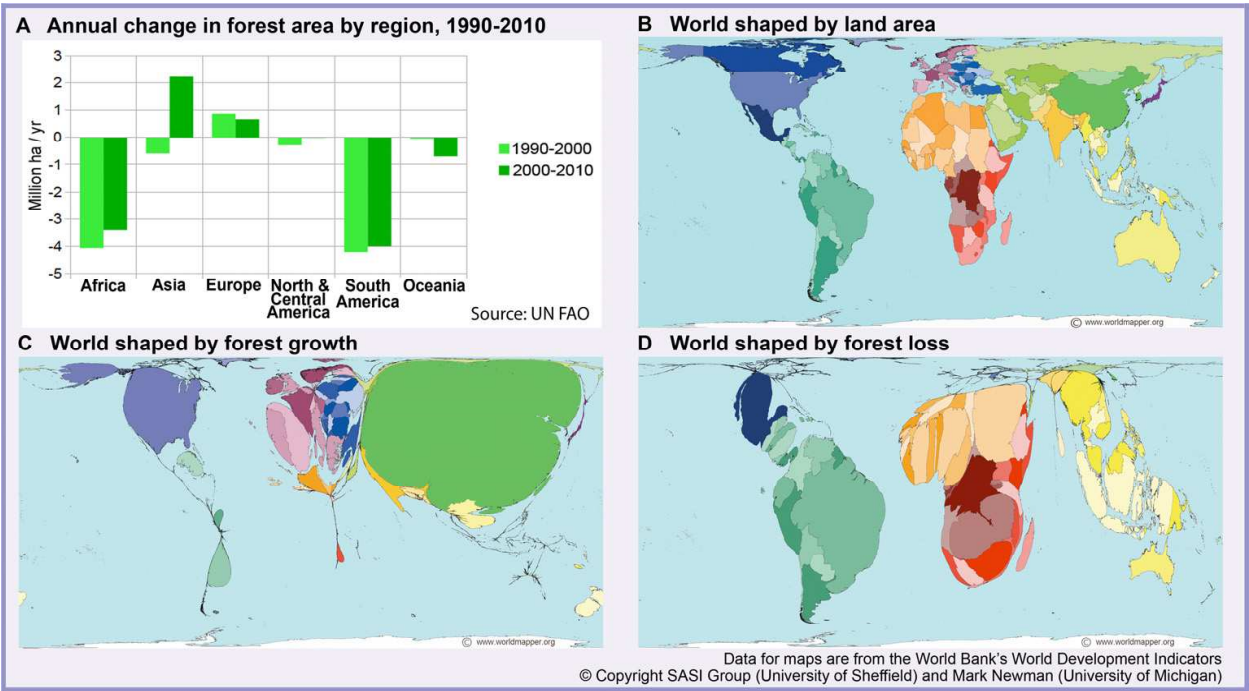


Fig. 1. Extent of forest loss and gain in the world. Territory size in C and D shows the proportion of worldwide net forest growth and loss that occurred there between 1990 and 2000.

2. Traditional tree breeding

Wood possesses physical, chemical and structural properties which have made it valuable to humanity since the earliest prehistoric times and is a renewable resource vital to the actual world economy. During the past century forestry underwent a major transition from foraging to an agricultural cropping mode on a global scale (Sedjo, 2003). The current level of demand for wood is probably exceeding what forests can supply, and this is clearly a major factor in their degradation. The establishment of plantations that can efficiently supply wood and paper pulp products and hence reduce demand for timber from virgin forests is a potential method for decreasing deforestation rates. For plantations to meet this demand, however, they must be much more productive than natural forests, thus it is essential to generate forest tree breeding programs for the selection of genetically superior individuals from large populations and their multiplication through the use of seeds or by asexual propagation (Fenning & Gershenson 2002).

Morphological and physiological traits directly related to the tree architecture, and ultimately to productivity, such as height, diameter, branch thickness or bifurcation frequency, are typical targets for genetic improvement. Breeding programs also incorporate various characteristics that enable trees to withstand a number of environmental factors, like resistance to pests, diseases, drought and other biotic and abiotic stresses (Cornelius, 1998;

Martinez-Ruiz et al., 2003). Evidently, the physical, mechanical and chemical properties, which strongly influence the suitability of wood for its direct use as timber or as a supply for the pulp and paper industry, are also relevant targets for tree improvement. The most important among these properties are wood density, lignin content, dimensional stability (shrinkage and swelling), bending and compression strength and growth stresses (Malan et al., 1996; Pryor & Willing, 1983; Raymond & Apiolaza, 2004; Turner, 2001; Zobel, 1992). More specific features, such as the content of resin and essential oils or the production of secondary metabolites are also traits of economic importance in forestry.

For the development of a forest tree breeding program, it is essential that genetic variability occurs among individuals within a population. One or more characteristics can be modified in the average population by selection and multiplication of phenotypically superior individuals (Cornelius, 1998). Since the observed variability may be due not only to the genetic background, but also to the effect of the environment, one of the major challenges in all breeding programs is to recognize and effectively separate one from the other.

A tree improvement program involves the selection of the most desirable trees from natural stands or plantations, breeding or mating of these elite trees and testing the resulting progeny. This three-step process is then continuously repeated to increase the extent to which each generation (breeding generation) exhibits the desirable traits, that is, to increase the genetic gain.

### **Selection of the raw material**

Seed stands are an important seed source for tree breeding programs. These are groups of trees identified as having superior characteristics that are formed from either natural forests or established plantations. Although seed can be obtained from natural stands, selection is more effective in plantations, since the variation due to environmental effects is less in the latter. The development of seed stands involves the selection of plantings or natural stands with an above the average phenotypic quality within the ecological zone, the improvement of the stand by removing undesirable individuals, their management to encourage early and abundant production of seed and the application of measures to reduce contamination by pollen from foreign trees, lowering the possibility of developing local breeds adapted to the site of introduction. They represent a stage prior to the formation of seed orchards and are generally not subjected to progeny testing, thus their true genetic value is not known (Lantz, 2008; Niembro, 1985; Quijada, 1980).

### **Breeding and propagation of the selected genotypes**

Replication of the phenotypically superior trees is performed in a seed orchard environment. In contrast to seed stands, seed orchards are established from the outset for the specific purpose of seed production (Zobel & Talbert, 1988). They usually consist of families of superior genetic quality which are isolated to avoid or reduce pollination from external sources. Seed orchards are managed intensively to produce abundant seeds with the highest genetic gain in a short period of time. They have helped to achieve significant improvements in aspects such as tree shape, adaptability, disease resistance, growth and wood quality (Quijada, 1980).

### **Progeny tests**

The artificial selection of trees with desirable phenotypic characteristics is assisted by progeny tests. These tests are used to estimate the genetic value of the parent trees based on the behavior of their progeny. Offspring from the selected parents are planted in

randomized, replicated tests usually established in different years and locations. Parents whose progeny perform better, on the average across all tests, are considered genetically superior. The recombinative fitness of the parents, their specific combining ability and the heritability of certain traits can be assessed through these tests (Quijada, 1980a; Roulund & Olesen, 1992; Zobel & Talbert, 1988).

These traditional practices in forestry and tree improvement remain relevant to forestry and the existing conventional programs are limited by the long reproductive cycle, long juvenile period (up to 20 years), low fertility, high levels of heterozygosity, various levels of ploidy, polyembryony, complex intraspecific incompatibility relationships, severe inbreeding depression, and the difficulty to effectively distinguish between phenotypic expression and environmental effects. Regarding the time factor, depending on the species, 5 to 20 years would be necessary for a tree to reach the reproductive maturity, 10 to 100 years to produce a marketable crop and 8 to 10 years to complete a breeding cycle (Lantz, 2008). For that reason, biotechnology plays an important role as a potential tool for the improvement of trees in much less time than was previously needed, either directly with genetic engineering, or by other procedures developed using the knowledge generated.

Biotechnology is a collection of various disciplines, including conventional methods of breeding and cultivation, which allow the management of biological systems for human benefit. Biotechnological methods are being developed worldwide to complement conventional breeding programs in commercial forests, in order to obtain continuous increases in production without increasing the land use (Burdon, 1994; Gomez-Lim & Litz 2004). Besides, the new era of biotechnology offers techniques that overcome the biological barriers that are common in the woody species. These techniques include: *in vitro* cultivation of cells and tissues, genotypic selection, genetic engineering, and molecular markers.

### 3. Genetic transformation

Innovations in the propagation methods and the introduction of fast-growing exotic species have increased industrial wood production and even reshaped regional and international patterns of generation and trade of forest products. Additionally, industrial forestry is advancing on two fronts with achievements in tree improvement as a result of traditional breeding techniques and with important research efforts oriented towards the production and commercialization of transgenic trees (Fenning & Gershenzon, 2002). 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. Although most of the productivity gains to date have been accomplished largely by traditional selection and breeding, transgenic trees are becoming increasingly common worldwide. Genetically modified (GM) trees can potentially make the breeding results observable more rapidly and reduce the development times, thus increasing productivity in plantations and reducing the exploitation pressure on natural forests (Fenning & Gershenzon, 2002; van Frankenhuyzen & Beardmore, 2004).

#### 3.1 Biotechnological tools for the genetic transformation of trees

Compared to genetic transformation of bacteria, where the transgene is integrated into a single cell and then it passes to the next generation, the genetic transformation of forest trees is not an easy task to achieve because it requires the establishment of protocols for the



regeneration of whole plants from individual cells (Figure 2). Other problematic barriers to the genetic improvement of trees, either by traditional breeding or genetic transformation, are their large size and long breeding cycles. The foreign DNA can be introduced using different approaches, and the following is a description of the techniques that have been used in different studies performed in the field of genetic transformation of forest trees (see also Table 1).

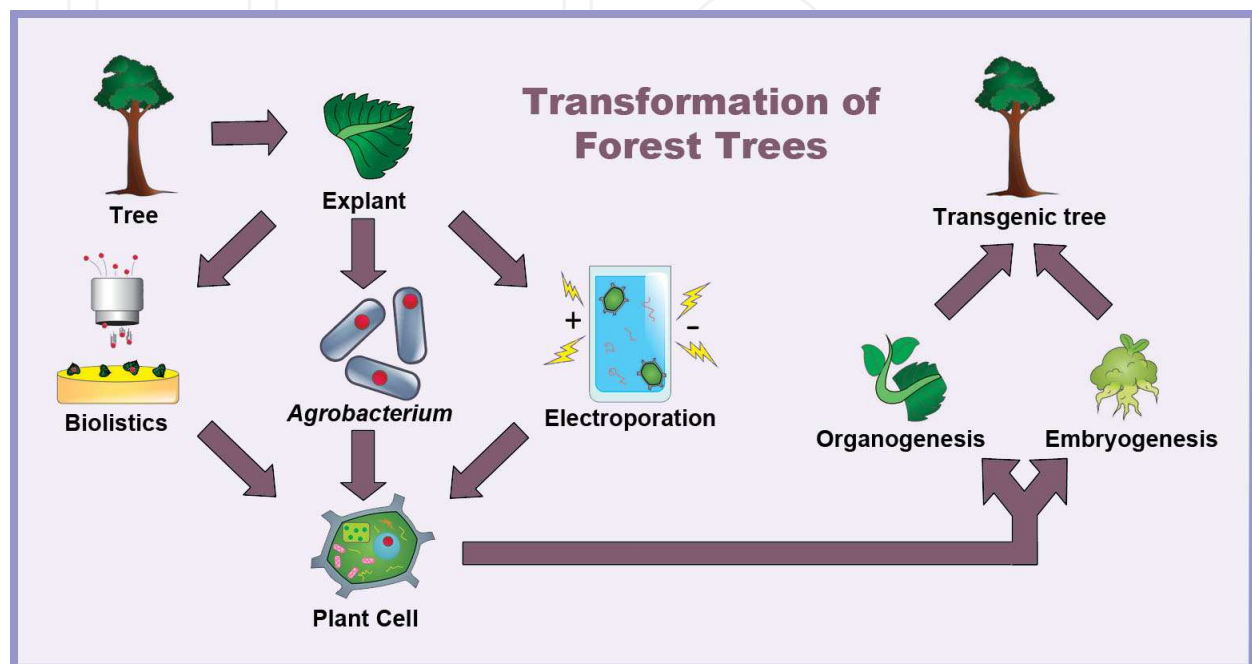


Fig. 2. Schematic diagram of the tree transformation process.

### Agrobacterium-mediated gene transfer

Among the several techniques used for transformation, *Agrobacterium* is the most widely used transformation tool, accounting for 80% of the transgenic plants produced so far (Broothaerts et al., 2005). *Agrobacteria* are plant pathogenic organisms capable of infecting a wide variety of dicotyledonous species causing tumoric diseases on infected host plants. *Agrobacterium tumefaciens* and *A. rhizogenes* harbor an extra-chromosomal genetic component, called the Ti (for tumor-inducing) or Ri (for root-inducing) plasmid. During infection, *Agrobacterium* inserts a region of this plasmid, known as the T-DNA (for transferred-DNA), into plant cells, and this DNA fragment is then integrated into the plant chromosomes. The T-DNA contains genes which expression causes aberrant growth of plant cells through the synthesis of growth hormones. These genes can be replaced by any gene(s) of interest without loss of DNA transfer and integration functions. In this way, these novel genes will be transferred to plant cells during *A. tumefaciens* infection (reviewed by Gelvin, 2003).

For the efficient production of transgenic plants the optimization of the *Agrobacterium*-plant interaction is probably the most important aspect to be considered. The use of this system is restricted by the host-range of these bacteria, since some plant species are not susceptible to the infection. Conifers are more difficult to transform with *A. tumefaciens* compared to hardwood species, and mature tissues are in general more recalcitrant to the infection. In addition, even though the DNA transfer has been demonstrated in several woody plants, in

Species	Technique	Vector	Transgen	Plant regeneration	Reference
<i>Pinus radiata</i>	Biolistic transformation	pRC 101	<i>nptII</i> and <i>uidA</i> genes	Yes (Embryogenesis)	Walter <i>et al.</i> 1998
<i>Pinus radiata</i>	Biolistic transformation	pMYC3425+pRN2 or pMYC3425+pCW132 or pMYC3425+pAW16	<i>cry1Ac</i> and <i>nptII</i> or <i>cry1Ac</i> and <i>nptII</i> or <i>cry1Ac</i> , <i>nptII</i> and <i>uidA</i> genes respectively	Yes (Embryogenesis)	Grace <i>et al.</i> 2005
<i>Quercus suber</i> L.	Cocultivation with <i>Agrobacterium tumefaciens</i>	pBINUbiGUSINT	<i>nptII</i> and <i>uidA</i> genes	Yes (Embryogenesis)	Álvarez & Ordás, 2007
<i>Picea abies</i> [L.] Karst	Biolistic transformation	pASCCR-BAR	<i>ccr</i> gen fused in antisense orientation	Yes (Embryogenesis)	Wadenbäck <i>et al.</i> , 2008
<i>Paulownia elongata</i> S.Y. Hu	Biolistic transformation	pBI121	<i>nptII</i> and <i>gus</i> genes	Yes (Organogenesis)	Castellanos-Hernández <i>et al.</i> 2009
<i>Castanea dentata</i>	Cocultivation with <i>Agrobacterium tumefaciens</i>	pCAMBIA 2301	<i>nptII</i> and <i>uidA</i> genes	Yes (Embryogenesis)	Andrade <i>et al.</i> 2009
<i>Populus tremula</i> x <i>Populus tremuloides</i> <i>Populus tremula</i> x <i>Populus alba</i>	Cocultivation with <i>Agrobacterium tumefaciens</i>	pG3KGB and pG3MKGB,	<i>nptII</i> , <i>gfp</i> and <i>bar</i> genes	Yes (Organogenesis)	Li J. <i>et al.</i> 2009
<i>Populus alba</i> x <i>Populus berolinensis</i>	Cocultivation with <i>Agrobacterium tumefaciens</i>	pROK2	<i>nptII</i> and <i>JERFs</i> genes	Yes (Organogenesis)	Li. Y. <i>et al.</i> 2009
<i>Leucaena leucocephala</i>	Cocultivation with <i>Agrobacterium tumefaciens</i>	pCAMBIA3201	<i>bar</i> and <i>uidA</i> genes	Yes (Zigotic immature embryos)	Jube & Borthakur 2009
<i>Prunus serotina</i>	Cocultivation with <i>Agrobacterium tumefaciens</i>	PsAGRNAi	<i>nptII</i> and <i>PsAG</i> genes	Yes (Organogenesis)	Liu & Pijut 2010
<i>Betula platyphylla</i> Suk.	Cocultivation with <i>Agrobacterium tumefaciens</i>	pCAMBIA-2301	<i>nptII</i> , <i>gus</i> , <i>bgt</i> genes	Yes ( <i>In vitro</i> propagation)	Zeng <i>et al.</i> 2010

Species	Technique	Vector	Transgen	Plant regeneration	Reference
<i>Quercus robur</i>	Cocultivation with <i>Agrobacterium tumefaciens</i>	pBI121 or pUbiGUSINT	<i>nptII</i> and <i>gus</i> genes or <i>nptII</i> and <i>uidA</i> genes respectively	Yes (Embryogenesis)	Vidal <i>et al.</i> 2010
<i>Hevea brasiliensis</i>	Cocultivation with <i>Agrobacterium tumefaciens</i>	pCAMBIA 2301-GFP or pCAMBIA 2300-GFP	<i>uidA</i> , <i>nptII</i> and <i>gfp</i> genes or <i>uidA</i> , <i>nptII</i> , <i>gfp</i> and <i>EcGSH1</i> genes respectively	Yes (Embryogenesis)	Leclercq <i>et al.</i> 2010
<i>Populus</i>	Cocultivation with <i>Agrobacterium tumefaciens</i>	pSKI015 or pSKI074	<i>bar</i> or <i>nptII</i> respectively	Yes	Busov <i>et al.</i> 2010

Table 1. Recent reports of transgenic forest tree species and the method of transformation used. \*See van Frankenhuyzen and Beardmore (2004), Ahuja (2009) and Harfouche *et al.* (2011) for more information of transformed and regenerated forest trees.

many cases no subsequent plant regeneration has been obtained. The first transgenic tree, a hybrid poplar (a woody angiosperm), was produced more than 20 years ago (Fillatti *et al.*, 1987) using *A. tumefaciens*. The first transgenic conifer plants were produced based on the use of *A. rhizogenes* in European larch (Huang *et al.*, 1991). Since then, this genetic transformation system has been successfully applied to other forest tree species, including aspen, cottonwood, eucalyptus, walnut, pine and spruce (Henderson & Walter, 2006).

**Biolistic-mediated transformation**

Biolistic transformation has become the method of choice for introducing genes into cell organelles. The method involves bombarding target cells with microscopic (1 µm diameter) DNA-coated tungsten or gold microprojectiles, which are accelerated mainly through compressed gases (helium, nitrogen or carbon dioxide). Exogenous DNA may integrate into the genome of the cells if they are in a competent physiological state and the physical conditions for delivery are appropriate for the species concerned (Klein *et al.*, 1987). This technique has been used to produce transgenic plants from recalcitrant coniferous or monocotyledonous species, but the transformation efficiency remains generally low and usually results in a high number of transgene inserts in the genome. For these reasons, *Agrobacterium*-mediated protocols are usually preferred over direct DNA transfer techniques.

**Protoplast transformation**

Protoplasts are produced by the enzymatic digestion of the cell walls of plant cells usually isolated from the leaf mesophyll, and are often grown in a liquid suspension culture. Protoplasts can be transformed by direct DNA uptake, following polyethylene glycol pre-treatment, by microinjection or by electroporation. Although many studies have resulted in successful transient expression of a transgene in cell-derived protoplasts (Bekkaoui *et al.*, 1995), very few have described the regeneration of transgenic trees (Chupeau *et al.*, 1994).



This is probably due to the lack of suitable methods to recover whole plants from protoplasts of most tree genotypes.

### **Regeneration of transgenic woody plants**

Plants are regenerated through one of two methods: organogenesis (direct or indirect) or somatic embryogenesis. The former involves the generation of organs, such as shoots and roots, from various plant tissues or undifferentiated cell masses (calli), whereas the latter leads to the production of embryos from somatic tissues. Regardless of the approach used, the process of *in vitro* regeneration is often genotype-dependent and its feasibility has to be assessed on a case by case basis.

Direct transformation of mature material is not easily achieved because of the low transformation competence and regeneration potential of adult tree tissues. Moreover, juvenile and adult tissues show marked differences in their responses to organogenesis and embryogenesis induction in tissue culture, with a progressive loss of competence during the transition to the mature phase. Mature to juvenile phase manipulation in the tree tissue has been a common practice for clonal propagation, either by grafting, rooting of cuttings, micropropagation or somatic embryogenesis, occasionally resulting in either rejuvenation or reinvigoration, i.e., the transient appearance of juvenile characteristics, which can be advantageous for genetic transformation (von Aderkas & Bonga, 2000). While *Agrobacterium tumefaciens*-mediated transformation is most successful with hardwood species using organogenic or embryogenic technologies, biolistic transformation can be used most successfully with embryogenic cultures of both softwoods and hardwoods. This means that the development of GM trees is highly dependent on the availability of a reliable, reproducible propagation system (Campbell et al., 2003).

### **3.2 Targets for forest-tree engineering**

Tree breeding programs are generally aimed to increase the volume of wood produced or to enhance its properties and quality for the desired end-uses. The approaches used to achieve this goal include the biochemical modification of wood characteristics and trunk structure to increase its growth rate and alter its shape. Other targets for the improvement of tree performance relate to the enhancement of the root system and canopy performance, and to all aspects of tree development and the interaction with its biotic and abiotic environment (Altman, 2003; Campbell et al., 2003). In the following sections, the use of genetic transformation to perform modifications directed at the improvement of these characteristics will be described citing some examples of its application to woody species.

#### **Modification of lignin content and composition**

Cell walls can account for up to 95% of the mass of woody plants, where the main components are cellulose, hemicelluloses and lignin. The tensile strength of wood fibers is primarily determined by cellulose and hemicelluloses, while lignin mediates adhesion between the fibers. Cellulose comprises approximately 30-60% of softwood (gymnosperm) cell walls and approximately 60-65% of hardwood (angiosperm) cell walls. Hemicelluloses comprise approximately 15-35% of both softwood and hardwood cell walls, although the percentage may exceed 40 in some hardwoods. Lignin accounts for 22-37% and 14-35% of the cell wall mass of softwoods and hardwoods, respectively (BeMiller, 2001; Mai et al., 2004). This makes lignin the second most abundant organic compound on Earth after cellulose, accounting for approximately 25% of plant biomass. Lignin confers mechanical

strength to the cell wall and due to its hydrophobicity, it waterproofs the vascular elements, playing an important role in the conduction of water and solutes. Furthermore, because of its cross-linking with other cell wall components, it minimizes the accessibility of cellulose and hemicellulose to microbial enzymes. Hence, the presence of lignin is associated with reduced digestibility of the plant biomass, providing a defensive barrier against pathogens and herbivores (Boudet & Grima-Pettenati, 1996; Campbell et al., 2003).

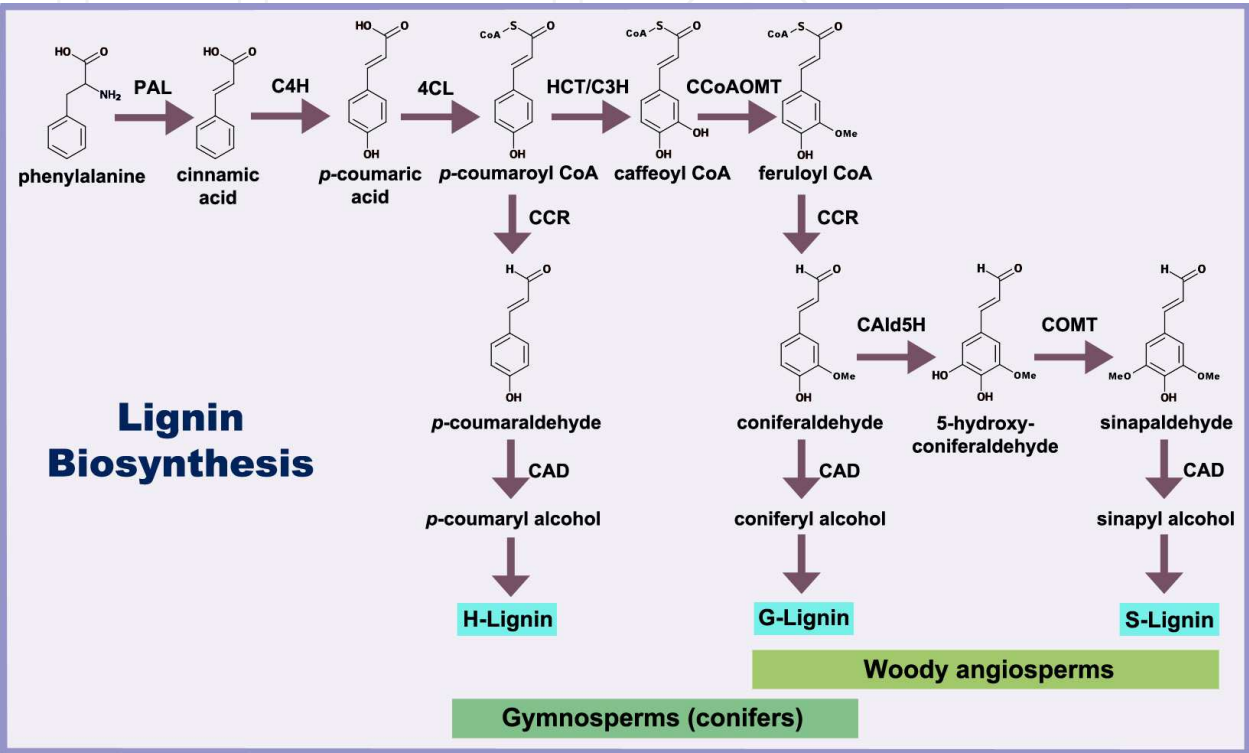


Fig. 3. Schematic diagram of the lignin biosynthetic pathway in forest trees.

Despite the fact that lignins play so important roles in plants, in the manufacture of pulp and paper, cellulose microfibrils are the component of the cell wall that is desired, and they need to be liberated from the lignin matrix through expensive and polluting processes. Additionally, residual lignin components are susceptible to oxidation and will cause a yellowing of the resultant pulp or paper if they are not thoroughly removed or bleached. Therefore, it is highly desirable to develop means by which lignin content is decreased, or make lignins more extractable, while maintaining basic structural integrity and the resistance of wood to herbivores and pathogens (Boudet & Grima-Pettenati, 1996; Campbell et al., 2003). Trees with reduced lignin would also improve the efficiency of their conversion into biofuels, especially during the pre-treatment step used in fermentation systems for the production of liquid biofuels from lignocellulosics (Hinchee et al., 2009).

Lignins result from the oxidative coupling of three monomers, namely p-coumaryl, coniferyl and sinapyl alcohols (also named monolignols), which give rise to p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units of lignin, respectively (Figure 3). Lignins exhibit a high degree of structural variability depending on the species, the tissue, the cells and the environmental conditions. Gymnosperm (softwood) lignin is predominantly lignin with G units, but also contains a smaller amount of H units. Angiosperms (hardwoods) typically possess G-S lignin. The presence of S units makes hardwoods, in general, a better choice for

paper production because the lignin is more easily extractable using chemical techniques (Peña & Séguin, 2001).

The biosynthesis of lignins proceeds from intermediate metabolism through a long sequence of reactions involving (1) the shikimate pathway which supplies phenylalanine and tyrosine, (2) the phenylpropanoid pathway from phenylalanine to the cinnamoyl CoAs which are the general precursors of different phenolic compounds and (3) the lignin specific pathway which channels the cinnamoyl CoAs towards the synthesis of monolignols and lignins (Figure 3; Boudet & Grima-Pettenati, 1996). It has become clear that lignin content and composition can be modified in genetically engineered trees by manipulation of several key enzymes, especially phenylalanine ammonia lyase (PAL), cinnamoyl alcohol dehydrogenase (CAD), O-methyltransferase (OMT), peroxidases, and laccases. However, many of the enzymes and reactions in the lignin biosynthetic pathway still need to be characterized (Altman, 2003; Boudet & Grima-Pettenati, 1996; Campbell et al., 2003).

Hu et al. (1999) were among the first to demonstrate the potential of genetic engineering for modifying lignin in trees for industrial applications. Aspen (*Populus tremuloides*) was transformed with antisense 4-coumarate:coenzyme ligase (4CL) constructs that resulted in a 45% reduction in lignin content. They also demonstrated that this genetic modification had the added advantage of indirectly increase cellulose content in about 15%. Later, the same group reported that the co-transformation of the antisense 4CL and sense coniferaldehyde 5-hydroxylase (Cald5H) into the same species produced trees with up to 52% less lignin and 30% more cellulose than the wild-type control trees, showing that the genetic modification of these genes had an additive effect (Li et al 2003). A more limited reduction in the lignin content (about 10%) was obtained from the down-regulation of 4CL in hybrid poplar (*Populus tremula* X *Populus alba*) and it led to reduced growth and serious physiological abnormalities (Voelker et al., 2010). Lignin reduction in this species was associated with a loss in wood strength and stiffness (Voelker et al., 2011). Similarly, the RNA interference suppression of coumaroyl 3'-hydroxylase (C3'H) in hybrid poplar trees (*Populus alba* X *grandidentata*) caused reduction in lignin content, and the growth characteristics of these trees were significantly impaired, resulting in smaller stems and reduced root biomass when compared to wild-type trees, as well as altered leaf morphology and architecture (Coleman et al., 2008).

The use of a transgenic approach to reduce the lignin content or alter its composition has been achieved not only in angiosperm tree species, but also gymnosperms like the Norway spruce (*Picea abies* [L.] Karst) expressing the gene encoding cinnamoyl CoA reductase (CCR) in antisense orientation, showing a reduction in lignin content of up to 8% (Wadenback et al., 2008). In another gymnosperm, the conifer *Pinus radiata*, the suppression of 4CL using a RNA interference (RNAi) construct substantially affected plant phenotype and resulted in dwarfed plants with a "bonsai tree-like" appearance. Micro-structural changes included the formation of weakly lignified tracheids that displayed signs of collapse (Wagner et al., 2009). Studies in populations of forest tree hybrids have shown a negative correlation of biomass growth and lignin content, implying that selection for improved growth rate could be accompanied by a reduction in lignin content (Novaes et al., 2010). However, it is clear from some of the above-mentioned experiences with transgenic trees, that a minimum amount of lignin is needed for anchoring the cellulose and hemicellulose components together for proper cell wall genesis and maintenance of structural integrity, and that a concentration below this tolerable limit would probably lead to cell wall disorganization and growth alterations (Koehler & Telewski, 2006).

Increasing lignin content can also be a target of improvement for those interested in using wood as fuel, since that modification would increase the thermal energy of wood. Pure cellulose has a calorific value of ~8,000 British thermal units per pound (BTU/lb), equivalent to ~18,600 kilo Joules per kilogram (kJ/kg), whereas that of pure lignin is ~11,000 BTU/lb (~25,600 kJ/kg). There is a high correlation between the heating value of wood and the lignin content. Increasing lignin content from 25% to 35% would increase the calorific value of wood by approximately 450 BTU/lb (1050 kJ/kg). This might be accomplished by achieving the opposite of the lignin reduction strategies mentioned above, namely the over-expression of a limiting enzyme in the biosynthetic pathway (Hinchee et al., 2009; White, 1987).

#### **Alteration of the tree form, performance, and growth rate**

The main objective of modifying phytohormones level in forest trees was to increase tree size, biomass production or wood quality. Induction of the GA 20-oxidase gene from *Arabidopsis* in hybrid aspen has resulted in an increase of bioactive gibberellin levels and subsequently faster growth in diameter and height, larger leaves, more numerous and longer xylem fiber and increasing biomass (Eriksson et al., 2000). This gene could be used to increase biomass production in forest trees or the use of its antisense can reduce tree size, which makes harvesting easier. In walnut, the expression of chalcone synthase decreases flavonoids synthesis and enhances the production of adventitious roots (Diouf, 2003). Expression of *iaaM* and *iaaH* auxin-biosynthetic genes from *Agrobacterium tumefaciens*, as well as *rolC* and *rolB* genes from *Agrobacterium rhizogenes*, has been shown to alter the growth patterns and development of transgenic forest trees. In addition, peroxidase genes and genes involved in directing cellulose biosynthesis have also been shown to affect stem elongation of several forest trees. It should be also noted that undesired alterations, such as reduced apical dominance and the breaking of axillary buds, could also occur (Altman, 2003).

Nitrogen availability is one of the main constraints for plant growth and limits production without fertilizer supplies. Development depends not only on the inorganic nitrogen available in the soil, but also on recycling within the plant, particularly in situations with limited nitrogen. However, many genes encoding proteins playing a key role in nitrogen fixation and assimilation have been isolated and characterized. Glutamine synthetase (GS) plays a significant role in both nitrogen uptake and recycling, as it catalyses the incorporation of ammonium into glutamine, the precursor to glutamate. Glutamine is also the precursor for all other plant N-containing compounds. In an attempt to alter tree growth, GS has been an important target for genetic engineering. One of these, the gene encoding glutamine synthetase under the control of 35S promoter was introduced in poplar. (Gallardo et al., 1999). The generated transgenic trees showed increased protein and chlorophyll content and a significantly greater net growth in height. Sulphur is an essential element found mostly in its reduced form as the amino acids cysteine and methionine. In plants, cysteine is used either in the synthesis of proteins, or can be further metabolized to methionine, glutathione (GSH) and phytochelatins. Given the biological significance of sulphur in plant development, it has also been a key target for genetic engineering in trees, particularly GSH formation (Diouf, 2003).

#### **Herbicide resistance**

Herbicide-resistant transgenic crops are considered one of the major successes of genetic engineering, being one of the major products of the first generation of agricultural



biotechnology. They are intended to reduce weed control costs, increase control flexibility, facilitate the use of low-tillage (and thus reduced erosion) cropping systems, and enable broad-spectrum, environmentally benign herbicides to be more readily employed. In herbaceous plants, there is a constant need for extensive weed control throughout the growing season. Direct competition with weeds also occurs in trees and it is especially important early in the tree's life cycle. Thus, although constitutive over-expression of herbicide-resistance genes is highly desirable throughout the life cycle of herbaceous crops, transgenic trees do not necessarily need to express resistance after they establish control of the site (Altman, 2003; Campbell et al., 2003). The first report on genetic transformation of forest trees was a herbicide-resistant poplar obtained through the introduction of the *aroA* gene, which confers resistance to glyphosate (Fillatti et al., 1987). Since then, transgenic trees with resistance to that and other herbicides, including chlorsulphuron, chloroacetanilide and phosphinothricine (glufosinate), have been generated (Campbell et al., 2003; Diouf, 2003).

### **Herbivore and pathogen resistance**

Damage to forest trees caused by both native and introduced pests and pathogens is of global importance. These biotic stresses significantly affect forest growth and productivity, with substantial economic consequences. By virtue of selecting and propagating superior individuals and families, domestication inevitably involves a narrowing of genetic diversity. One of the consequences of this is that the domesticated population will not possess all of the alleles that are present in wild progenitors that confer resistance to herbivores and pathogens. Furthermore, as the domesticated population grows larger, and the more uniform it is planted, there is selection pressure on herbivores and pathogens to overcome any resistance mechanism that the domesticated population may possess. This is particularly true for forest trees because genotypes remain in the environment for a period of time that usually encompasses many more generations of the herbivore or pathogen. Consequently, a major goal of breeding programmes is to introduce alleles that confer robust and durable herbivore and pathogen resistance. Different insects feed on different tree parts, and their damage can sometimes be a limiting factor for tree growth and survival. In practice, the use of insecticides is rather limited in forestry, due in part to the large forest areas and tree size. Thus, insecticide application is usually restricted to nurseries and young or small plantations. Genetic engineering for insect control has been achieved in several forest trees using either the Bt toxin (from *Bacillus thuringiensis*) or insect digestive-system inhibitor genes. The Bt toxin binds to the epithelial glycoproteins of the intestine of insects, especially the midgut, and causes fatal leakage of fluids between the intestine and the hemocoel. This toxin specifically affects insects belonging to the lepidopteran, dipteran and coleopteran orders of insects, which include a number of major herbivores of forest tree species (Campbell et al., 2003; Diouf, 2003). The other approach is the expression of genes involved in the proteinase inhibitor system (Altman 2003; Campbell et al., 2003). Genetic transformation using gene coding for Bt or proteinase inhibitors could lead to reduced damage and chemicals used in the environment.

### **Restriction of gene-flow and early flowering**

The long delay in the onset of flowering in forest trees, which lasts up to 30 or 40 years in some species, is an important constrain for inbreeding as a means for identifying and fixing beneficial recessive mutations and introgression/backcrossing as a means to increase the



frequency of rare alleles in breeding populations. The opposite (the ability to prevent the floral transition) is also desirable in trees for other reasons. Forest trees grown under intensive culture usually flower earlier than in the wild and produce large quantities of pollen and seed. It would be desirable that domesticated trees flower later or not at all so that additional resources for vegetative growth are available. Moreover, flowering is a major constraint to the use of genetic engineering in tree improvement. Because most forest trees have an abundance of wild or feral relatives, outcross, and display long-distance gene flow via pollen and sometimes seed, there is likely to be considerable public concern about large-scale use of genetically engineered trees and the spread of transgenes to the ecosystem. Since some forest trees are very close to their wild-type relatives, gene flow within and among genetically engineered forest trees can be rather extensive. Furthermore, gene flow from transgenic to wild-type plants may be especially problematic in forest trees because they produce large amounts of pollen and seeds which are easily dispersed over relatively long distances. Thus, current efforts are aimed at the use of developmental stage-dependent promoters, the co-engineering for reproductive sterility, and the use of naturally sterile or low-fertility tree hybrids (e.g., triploid hybrid poplars). Limiting the use of transgenic trees to nurseries, thus harvesting them before they reach their reproductive age, is another potential precaution. Advanced research efforts towards these goals include engineering for flower sterility through flower-specific expression of cytotoxic structural genes, and using sense/antisense or promoter suppression of specific homeotic reproductive development genes. In addition, induction of early flowering is beneficial in terms of reducing the tree's breeding cycle, allowing early characterization of transgene inheritance in young, small, transgenic seedling progeny (Altman, 2003). The major obstacle to engineering sterility in forest trees is simply demonstrating that a tree is reliably sterile under field conditions, and for many species, inefficient transformation, regeneration and field-testing capabilities are serious impediments. Transgenic trees with sterility constructs have been generated and some established in field tests, but the results cannot be observed until the trees reach maturity. These studies usually need to employ trees that lack nearby wild relatives, or provide other containment procedures (e.g. physical isolation or biological buffer zones), so that gene dispersal into wild populations is minimal. This points to another reason why the ability to induce early flowering is important: to speed the development and verification of sterility transgenes, preferably while in the greenhouse (Campbell et al., 2003). It has been shown that over-expression in juvenile tissues of flowering meristem-identity genes such as *LEAFY* (*LFY*), *APETALA1* (*AP1*) or *FLOWERING LOCUS T* (*FT*) from *Arabidopsis thaliana* and homologues from other plants leads to early flowering in different tree species (Campbell et al., 2003; Cervera et al., 2009).

### **Abiotic stress tolerance**

In general, most natural forest-tree species are well adapted to their environment, exhibiting high ecological competence. However, forestation with plantation-improved or imported and exotic tree species will probably reveal their sensitivity to several ecological factors. Cold, drought, salinity, and heavy metal toxicity are the main stresses specifically affecting trees, which are subjected to many annual changes during their life cycle. Genetic engineering for cold tolerance would allow the use of cold-sensitive species in northern areas, as well as providing better protection of native plants from chilling damage. Drought and salinity tolerance is particularly important for forestation in arid and semiarid areas to prevent forest losses and desertification. Drought stress is primarily osmotic stress, which causes the

disruption of homeostasis and ion distribution in the cell. Salt stress is an increasingly important issue throughout the world, and it is imposed by two factors: water deficit due to osmotic stress, and the accumulation of ions that negatively affect biochemical processes. A number of genes have been tested in attempts to increase salt tolerance in trees (El-Khatib et al, 2004; Kawazu, 2004; Tang et al., 2007). The use of plants to remove contaminants from the environment is known as phytoremediation, and this technology has recently been applied to several environmental problems, including disposal of municipal wastewater, biofiltration of farm and industrial runoff, and the remediation of soils spoiled by industrial processes. Because this technology is less costly, less invasive, more aesthetic, and often yields a usable product (e.g. biomass), it has many advantages over traditional, engineering-based methods. Several plant species have been considered for phytoremediation efforts, but trees have most recently been identified as particularly useful vehicles because they produce large amounts of biomass, have far-reaching roots and are perennial, although leaves may need to be collected for incineration (Altman, 2003; Diouf, 2003; FAO, 2010; Giri et al., 2004).

#### 4. Distribution and commercialization of GM trees

It is clear from the previous section that the production and commercialization of GM trees on a large scale offer numerous potential benefits. However, some concerns have been raised by environmental groups about their potential dangers, and GM trees have been banned in forest plantations certified by the Forest Stewardship Council (FSC) regardless of the source of genes, traits imparted, or whether for research or commercial use. Some researchers argue that this ban on research is counterproductive because it makes it difficult for certified companies to participate in the research field needed to assess the value and biosafety of GM trees. Furthermore, genetic modification could be an important tool for translating new discoveries from tree genomes into improved growth, quality, sustainability, and pest resistance (Strauss et al., 2001). The first genetically engineered tree, reported by Fillatti et al. (1987), was developed by a team of scientists from the University of Wisconsin, the Forest Service of the United States (US), and the biotechnology company Calgene (now part of Monsanto). Since then, dozens of other forest tree species have been genetically engineered for research purposes, though none have seen commercial use. The only commercialized tree in the US to date is papaya, a horticultural tree which was made virus resistant via genetic engineering and is now in widespread use in Hawaii (Gonsalves, 2006). This case has involved practically no environmental risk because papaya is an introduced species in Hawaii, thus lacking close wild relatives and because the Pacific Ocean is an effective barrier to transgene escape. A virus-resistant plum tree has already been deregulated by the US Department of Agriculture (USDA) and authorized by the US Food and Drug Administration (FDA) and is awaiting final approval by the US Environmental Protection Agency (EPA). Despite this success, no genetically engineered forest trees have yet been commercialized in the US (Sedjo, 2010). A transgenic poplar (*Populus nigra*) transformed with the Bt gene *Cry1Ac* was developed in China and used in field testing as early as 1994. In 2000, the Chinese regulatory authority allowed the establishment of about one million trees on about 300 hectares, though this release is more oriented toward forestation in parts of China where pests restrain the establishment of forests than toward the commercial production of wood (Sedjo 2005).

From a technical point of view, the use of genetic engineering would make it possible to modify forest tree species introducing the desired traits faster than through a traditional

breeding approach. However, given the regulatory restrictions associated with the commercialization of transgenic trees, the added costs and time needed to determine their long-term impact on the environment may countervail any advantages that the genetic transformation has over traditional breeding. For these reasons, an extensive commercialization of GM trees is not anticipated in the near future (Sedjo, 2010).

## 5. Ethics and biosafety

Regulatory issues related to transgenic plants concentrate on health, safety, and environmental risks. Health and safety concerns arise when humans or animals consume transgenic plants or their byproducts, which is generally not a problem for forest trees. The concerns about the environmental effects of the transgenic plants include fears that the GM plant itself might become a pest or, of greater concern, the possibility that a transferred gene might “escape” and alter the genetic composition of a wild relative, perhaps increasing the competence of the native plant and turning it into an invasive pest. In addition, an escaped gene might affect a non-transformed species and compromise its usefulness as a raw material for developing improved hybrids in the traditional way (van Frankenhuyzen & Beardmore, 2004; Walter, 2004). Although genetic containment systems have long been requested by ecologists and other scientists to reduce a number of undesired effects of genetically engineered crops (NRC, 2004; Snow et al., 2005), there has been strong pressure on companies and governments against the use of any forms of ‘Terminator-like’ containment technology (ETC, 2006). In agriculture, these concerns are primarily about control of intellectual property and the forced repurchase of seed by farmers. But in the forestry area, there has also been activism against containment technology because of a lack of confidence that it will be fully effective, concerns about loss of biodiversity associated with the modification or loss of floral tissues (Cummins & Ho, 2005), and legal uncertainties and liability risks from the dispersal of patented genes. These biological concerns occur despite the intention to use such technology mainly in plantations that, due to breeding, high planting density and short life spans, already produce few flowers and seeds compared with long-lived and open-grown trees. The powerful inverse association between forest stand density and degree of tree reproduction is widely known (Daniel et al., 1979). There is also an abundance of means to avoid and mitigate such effects at gene to landscape levels (Johnson & Kirby, 2004; Strauss & Brunner, 2004). Government regulations against the dispersal of genes from research trials also pose very substantial barriers to field research to study the efficiency of containment mechanisms (Strauss et al., 2004; Valenzuela & Strauss, 2005). The production by genetic engineering of trees that are unable to produce viable pollen or seeds, has been proposed (Strauss et al., 1995), but the genetic containment technology is, itself, difficult and highly controversial, requiring special social conditions even to carry out research. Another approach proposed to impede transgene escape is their targeting into chloroplast or mitochondrial genomes in species where these organelles are maternally inherited and therefore, the introduced traits would not be transmitted by pollen. Although most of angiosperm tree species show maternal inheritance of the chloroplast genome, most conifers exhibit a strictly paternal inheritance, thus this may be an option for preventing transgene escape only in angiosperm trees. However, this methodology is not infallible, since low levels of paternal inheritance may still occur in angiosperms (Ahuja, 2009; Ruf et al., 2007). Even though a plastid transformation system has already been developed for poplar, improvements are still needed (Okumura et al., 2006).

GM trees transformed with the purpose of providing resistance to pest and pathogens have been of particular concern. They may impose a selection pressure for the development of pests resistant to the defense mechanism introduced. The production of resistance-related components by these trees may not only suppress target organisms, but may also affect beneficial insects and plant symbionts as well as other micro-organisms involved in decomposition and nutrient cycling (Hoenicka & Fladung, 2006). Regarding the selection of resistant pests, the use of mixed populations of insect-resistant GM and susceptible non-GM trees shows resistance of the overall stand. Such strategy might help reduce the selection pressure on pests to overcome the introduced resistance mechanism in the transgenic trees and minimize the risk of mass disease outbreaks (Fenning & Gershenzon, 2002; Hu et al., 2001). It is important to note, however, that despite conventional tree breeding is a widely accepted and practiced technology, it is not completely risk-free. Traditional breeding is an intrinsically imprecise process since the new variations, created through recombination or mutations, are usually not well characterized at the genetic level. This appears to be much less controllable than genetic engineering, where only one or a few characterized genes may be either added or their expression altered (Henderson & Walter 2006). Specific studies addressing GM tree-related effects on the environment have reported that no changes in the ectomycorrhizal fungal community were found after transgenic poplars were used in the field for eight years and no unintended impacts of transgenic pine trees were observed for above-ground invertebrate communities over a period of two years (Schnitzler et al., 2010; Stefani et al., 2009). Furthermore, available information on the performance and safety of GM trees in field trials around the world reveals that none of them has reported any substantive harm to biodiversity, human health or the environment (Walter et al., 2010).

Transgenic tree germplasm is generally regulated at the country level throughout the world, as it is for other transgenic crops. Current concerns about GM trees are similar to those about agricultural crops, even though the majority of activities on genetic transformation are experimental and regulated under very strict conditions. The use of generic engineering in forestry will only succeed when the potential environmental impacts of genetically engineered trees are assessed and balanced against the costs of not pursuing this technology.

## 6. Conclusion

For the world to be supplied with the wood it needs without further degradation of forest areas, it is necessary to look for alternatives for the sustainable production of forest goods. One of these alternatives is the establishment of plantations, which productivity has increased considerably due to the development and improvement of forest management practices and to the introduction of new germplasm generated through traditional breeding. However, the generation of these new genotypes has been slow, because of the long life cycle of trees and the long time they require to reach reproductive maturity, which delay genetic crossing and the phenotypic evaluation of progeny performance.

Biotechnology, and particularly genetic engineering, is a valuable tool that can help to accelerate the process of tree improvement, especially through the introduction of traits that can be rapidly determined in juvenile plants. Forest tree biotechnology has taken advantage of the techniques developed for the genetic transformation of agricultural crops, but in spite of the great progress achieved so far, there are still important constraints that limit the generation of transgenic trees: the restricted susceptibility to *Agrobacterium* infection, the effect of the genotype in the development of efficient protocols for plant regeneration, and



the limited knowledge of tree physiology for the selection of potential target genes. In that context, transgenic trees are important not only as an alternative to increase wood production and quality, but also as tools for the study of different aspects of tree biology, which in concert with the recent developments in genomics and gene cloning techniques, will accelerate our ability to discover and introduce new value-added traits.

The research carried out in the area of molecular biology of forest trees is not comparable to the efforts implemented for similar studies in annual crops, mainly in terms of total funding resources and number of scientific groups. Nevertheless, although trees are not as easy for laboratory work as the herbaceous plants due to their size and life cycle, they are being increasingly used for research and many physiological and developmental processes specific to trees are being studied at the molecular level.

The use of transgenic trees may be assumed to raise fewer concerns to the general public than food crop plants, since the final products are not ingested and, therefore, no effect on the human health is expected. However, there is a great interest in the potential environmental effects of the GM trees, including the risks associated with the spread of transgenes to native populations. Several alternatives to restrict this potential transgene flow have been proposed, including the reproductive sterility and the transformation of plastids.

There is no doubt that the evaluation not only of the real commercial value, but also of the environmental safety of the GM trees is important, and the concerns of the public must be addressed through extensive field testing before they are planted on a commercial scale. Ironically, however, the legal framework created to regulate the release of untested GM plants into the environment makes it almost impossible to establish field trials where the potential impacts on the environment can be assessed.

Forest tree biotechnology offers a great potential and a significant progress in the field has been made to date. This area of research is expected to advance rapidly over the coming years, but this development must be accompanied by appropriate regulations and social acceptance if the transgenic trees are expected to enter the market.

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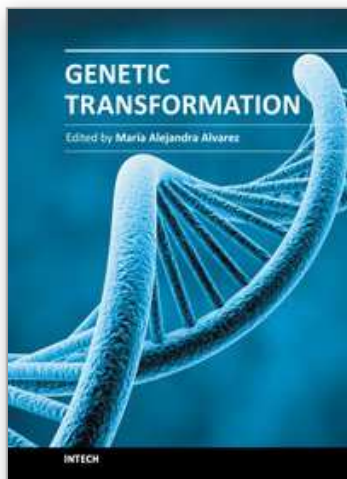


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Genetic transformation of plants has revolutionized both basic and applied plant research. Plant molecular biology and physiology benefit from this power tool, as well as biotechnology. This book is a review of some of the most significant achievements that plant transformation has brought to the fields of Agrobacterium biology, crop improvement and, flower, fruit and tree amelioration. Also, it examines their impact on molecular farming, phytoremediation and RNAi tools.

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