

## HARDWOOD TREE BIOTECHNOLOGY

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### SUMMARY

Due to the commercial importance of some conifer species, advances in conifer biotechnology often appear to overshadow equally significant advances in the biotechnology of angiosperm forest trees. However, progress with some hardwood forest trees has been just as promising as that made with conifers, and in some areas, has surpassed conifer biotechnology, particularly in the past few years. Until relatively recently, progress with *in vitro* propagation and gene transfer in hardwood forest trees was confined primarily to the genus *Populus*. Similarly, compared to other hardwood species, the greatest progress has been made both in the areas of genomics and modification of wood quality traits in this genus. However, the advances in *in vitro* propagation, in general, and somatic embryogenesis, in particular, have brought mass clonal propagation of other top commercial hardwood trees, in particular *Eucalyptus*, closer to reality and gene transfer systems have been reported for a number of them. While by far the most extensive application of genomic tools, including genomic sequencing, expressed sequence tags, transcript profiling and molecular markers, has also been made with *Populus*, these tools are now being applied to wider range of hardwood species. Just as with conifers, potential biotechnology applications for hardwood forest species include development of trees with faster growth, altered wood quality, and insect and disease resistance. In addition, some hardwood species are being manipulated for such non-traditional uses as phytoremediation. Given these advances and the worldwide importance of the products derived from them, it is likely that *in vitro* propagated and transgenic hardwood forest trees will have just as great an impact on commercial forestry and our environment as the top coniferous species.

*Key words:* expressed sequence tags; gene transfer; *in vitro* propagation; molecular markers; somatic embryogenesis; transcript profiling.

### INTRODUCTION

Compared to agronomic crops, forest trees have undergone relatively little domestication (Merkle and Dean, 2000). Consequently, advances in forest biotechnology have received relatively little attention compared to those reported for the top crop species, even though biotechnology could potentially have a greater impact on forestry and forest products than it has had on agronomic crops. Similarly, over the past four decades, the focus of both conventional tree breeding programs and, more recently, biotechnology research, often has seemed to be limited to a relatively small group of coniferous forest species (pines, spruces), primarily due to their substantial economic importance, particularly in developed countries, for construction timber as well as for pulp and paper. For example, in the United States, loblolly pine (*Pinus taeda*) is more widely planted than any other tree species, with about one billion seedlings planted annually (Whetten et al., 2001). Approximately 45% of the commercial forest area in the southern United States is loblolly pine and almost one-half billion seedlings are planted annually on other continents (Schultz, 1999). However,

in several parts of the world, hardwood forest trees, rather than conifers, are the major sources of forest products, and in some cases, the only source of high-value products such as veneer for high-quality furniture, cabinetry and flooring. With regard to their silviculture, hardwoods also have a distinct advantage over most pines (with the notable exception of radiata pine) and spruces in another respect – their potential for vegetative reproduction via coppicing. Finally, with regard to the application of genomics, some angiosperm trees have a striking advantage over conifers in that the sizes of their genomes are in some cases orders of magnitude smaller than those of conifers (e.g. the size of the *Populus* genome is only about 550 Mbp, compared to that of loblolly pine, which is 15480 Mbp). Thus, the application of biotechnology to hardwood forest trees as a group is worthy of evaluating with regard to the progress made to date and the potential impact it may have in different regions of the world. Here, we will highlight the recent progress with *in vitro* propagation and genetic transformation of commercially important hardwood species and genomics and molecular breeding, and potential commercial, or other applications of this technology with hardwoods.

As is widely known among the forest biotechnology community, due to a number of factors, the vast majority of reports on transformation, genomics, and other biotechnology applications to

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hardwood trees have been concentrated in the genus *Populus*. Poplars were the first forest trees to be regenerated *in vitro* via adventitious shoots (Winton, 1968; Wolter, 1968), the first to be genetically transformed (Fillatti et al., 1987) and the first to have a sequenced genome (Brunner et al., 2004; Tuskan et al., 2004a), so it is to be expected that work with members of this genus would continue to dominate the hardwood biotechnology literature. Since a number of excellent reviews are available on various aspects of *Populus* biotechnology, in some cases we will refer the reader to these, rather than try to recapitulate information that is already summarized elsewhere. It is also true that the topic of hardwood tree biotechnology is so broad that it is impossible to generate a truly comprehensive review. In particular, it would be unrealistic to try to include information on every hardwood timber and pulp species from throughout the world, let alone those of horticultural value such as coffee, rubber, and cacao. So, this review will not contain long tables attempting to cite every report on a hardwood tree that has been propagated *in vitro*, genetically transformed or to which molecular tools have been applied. Our primary intent with this review is to present a snapshot of the current status of biotechnology research with hardwood forest trees by summarizing the information on *in vitro* propagation, gene transfer, and genomics for a sample of hardwood timber and pulp species of significant economic and/or ecological importance in different parts of the world and offer comments on the implications of this research.

#### IN VITRO PROPAGATION

Hardwood trees were among the first plants cultured *in vitro* (Gautheret, 1940), although the first reports of production of intact, free-living angiosperm trees (*Populus tremuloides*) did not appear until several years later (Winton, 1968; Wolter, 1968). The primary applied goal of *in vitro* culture of hardwood forest trees has traditionally been mass clonal propagation of desirable genotypes, although more recently *in vitro* hardwood tree cultures have assumed another role as target material for gene transfer. While the first plantlets from cultures of both hardwood and coniferous trees were produced via adventitious shoots, in the case of conifers, the forest industry has focused its recent *in vitro* propagation research efforts on somatic embryogenesis (Gupta et al., 1993; Handley et al., 1995). Matters are not nearly so clear-cut with regard to mass *in vitro* propagation of hardwoods. While somatic embryogenesis has a number of often cited advantages over other *in vitro* propagation systems for mass propagation of hardwoods (Merkle and Trigiano, 1993) and embryogenic cultures have been generated for many important hardwoods throughout the world (note the six volume set on somatic embryogenesis in woody plants edited by Jain et al., 1996), some groups such as poplars and *Eucalyptus* clearly remain more amenable to propagation via axillary shoot multiplication or adventitious shoots. In addition, as is generally the case for conifers, even the best of embryogenic systems reported for hardwoods to date continue to lack commercial viability for two reasons: (1) low frequency of regeneration for many of the most desirable clones; and (2) unproven genotypes, since most starting material for the cultures is derived from seeds or seedlings. However, as we illustrate in the next section, the past decade has seen encouraging progress with regard to scaling up of somatic embryo and somatic seedling production and with propagation of proven genotypes via somatic

embryogenesis from tissues of mature trees of some important species.

#### North American Hardwoods

As indicated earlier, work on *in vitro* propagation of *Populus* species, including North American aspens, cottonwoods and hybrids in both groups, has been widely reported, beginning in the 1960s. Excellent reviews of poplar regeneration via shoot culture (McCown, 1997), organogenesis (Kang and Chun, 1997), and somatic embryogenesis (Park and Son, 1997), as well as other reviews on poplar *in vitro* manipulation can be found in Klopfenstein et al. (1997) and a more recent review covering all aspects of *in vitro* manipulation of poplars was provided by Confalonieri et al. (2003).

Probably the highest value North American hardwoods for which *in vitro* propagation systems have been reported are black walnut (*Juglans nigra*) and black cherry (*Prunus serotina*), both of which are used for high quality veneer for furniture and cabinetry. Black cherry has been propagated via axillary shoot multiplication (Tricoli et al., 1985; Maynard, 1994) and more recently, via adventitious shoots regenerated from leaf explants (Hammatt and Grant, 1998). As Reidiboym-Talleux et al. (1998) have described a promising embryogenic system for the related horticultural species, sweet cherry (*Prunus avium*), native to Asia Minor, such a system may some day be adapted for black cherry propagation. Black walnut has been regenerated via axillary shoot multiplication (Stefan, 1989) and adventitious shoots (Long et al., 1995). Given the successful development of an embryogenic regeneration system for the related horticultural species, English walnut (*Juglans regia*; Tuleke and McGranahan, 1985) and its application to produce transgenic plants (see section on gene transfer), it would appear that a similar productive system could be developed for other highly desirable *Juglans* species, but transfer of the protocol has been problematic. While somatic embryogenesis has been reported for the species (Neuman et al., 1993; Long et al., 1995), it is unclear if somatic seedlings have yet been produced. Similarly, reports of somatic embryogenesis in the related, valuable butternut (*Juglans cinerea*) resulted in very few complete plantlets (Pijut, 1993, 1999).

Among the US southern hardwoods of commercial importance for pulp and timber, highly productive organogenic and/or embryogenic regeneration systems have been reported for black locust (*Robinia pseudoacacia*), yellow-poplar (*Liriodendron tulipifera*) and sweetgum (*Liquidambar styraciflua*). Due to its durability and toughness, the leguminous tree black locust was at one time grown in plantations to be used for railroad ties. Although its commercial importance has virtually disappeared in the US, the tree has become a top timber producer in such eastern European nations as Hungary (Keresztesi, 1983). Black locust has been propagated by axillary shoot multiplication (Chalupa, 1983; Barghchi, 1987; Davis and Keathley, 1987), adventitious shoots (Han and Keathley, 1989; Arrillaga and Merkle, 1993), and somatic embryogenesis (Merkle and Wiecko, 1989). Arrillaga et al. (1994) demonstrated that black locust somatic embryos could be employed to produce synthetic seeds. Yellow-poplar grows to the largest size (60m) of any hardwood tree in the eastern US and is used for various forest products. It was one of the first North American hardwoods for which a high-frequency embryogenic regeneration system was developed using suspension cultures that were synchronized using

size fractionation (Merkle and Sommer, 1986; Merkle et al., 1990). Several regeneration systems have been reported for sweetgum, a fast-growing southern bottomland hardwood that is increasingly being used by the southern pulp and paper industry, including axillary shoot multiplication (Sutter and Barker, 1985), adventitious shoot production (Sommer, 1981; Brand and Lineberger, 1988, 1991; Kim et al., 1997), and somatic embryogenesis (Sommer and Brown, 1980). More recently, Merkle et al. (1998) and Merkle and Battle (2000) reported that embryogenic cultures could be initiated from mature sweetgum trees using either staminate or pistillate inflorescence explants collected from dormant buds. Vendrame et al. (2001) produced embryogenic cultures from *L. styraciflua* × *L. formosana* hybrid seeds, resulting in some clones with heterotic growth rates and Dai et al. (2004) recently demonstrated that somatic embryo production from the hybrid sweetgum cultures (as well as *L. tulipifera* × *L. chinense* hybrid embryogenic cultures) could be scaled up by synchronizing embryogenic suspensions via size fractionation.

Two genera in the *Fagaceae* for which *in vitro* propagation systems would be highly desirable are *Quercus* (oaks) and *Castanea* (chestnuts and chinkapins). In addition to their critical ecological role as providers of mast for wildlife, oaks are major sources of timber for flooring, furniture and cabinetry in the US. Micro-propagation of North American oaks via axillary shoot multiplication has been described as being very difficult (McCown and McCown, 1987). Unfortunately, reports of somatic embryogenesis in North American oaks are rare, and while the phenomenon has been reported for *Quercus rubra* (Gingas and Lineberger, 1989; Gingas, 1991; Rancillac et al., 1996), *Quercus alba* (Gingas and Lineberger, 1989) and *Quercus bicolor* (Gingas, 1991), none of these studies resulted more than a few regenerated plants. This situation is in stark contrast to that with European oak species, where considerable progress has been reported (see below).

American chestnut (*Castanea dentata*) was once a major timber and nut-producing tree of the Appalachians, but was devastated by the chestnut blight fungus (*Cryphonectria parasitica*) during the first half of the twentieth century. As a part of the effort to restore the tree, several labs have worked on *in vitro* propagation, as recently reviewed by Vieitez and Merkle (2004). Despite some progress, propagation of American chestnut via axillary multiplication has not been very efficient (Read and Szendrak, 1995; Xing et al., 1997). However, several years of work developing an embryogenic regeneration system for the tree (Merkle et al., 1990; Carraway and Merkle, 1997; Xing et al., 1999; Robichaud et al., 2004) are finally beginning to payoff and some embryogenic lines can now be manipulated in suspension culture to produce hundreds of somatic seedlings (Andrade and Merkle, 2005).

#### European Hardwoods

As with North American species, *in vitro* propagation of European poplar species and hybrids has a long history. In fact, several hybrids between North American and European aspens and cottonwoods have been propagated via organogenesis from a variety of explants (Kang and Chun, 1997; Confalonieri et al., 2003). Extensive research on *in vitro* propagation of a wide variety of European hardwoods, including *Acer*, *Betula*, *Quercus*, *Fagus*, *Prunus*, *Tilia*, and *Ulmus* has been conducted over several years by Chalupa (1987). Among European hardwoods of high commercial

value, the most significant recent progress with *in vitro* propagation systems has been in the development of embryogenic regeneration systems for *Castanea*, *Ulmus*, and *Quercus*. As was the case with American chestnut in the US, European chestnut (*Castanea sativa*) was a major timber and nut-producing species in many European countries until it was devastated by the chestnut blight fungus in the 1940s. Although the appearance of hypovirulent strains of the chestnut blight fungus have ameliorated the impact of the blight to some extent, ink disease caused by *Phytophthora* remains a major problem, and restoration of the tree has become a priority of the European Economic Community (Heiniger, 2001). Decades of research on *in vitro* propagation of chestnut have been performed by the Vieitez lab in Spain, and the most recent summary of this work, as well as other chestnut research can be found in Vieitez and Merkle (2004). Vieitez (1995) successfully regenerated several plantlets of *C. sativa* × *C. crenata* hybrids via somatic embryogenesis using zygotic embryos as explants. Similarly, Saur and Wilhelm (2005) regenerated some plantlets from embryogenic cultures of pure *C. sativa*, initiated from ovaries, ovules and immature zygotic embryos. A promising report by Corredoira et al. (2003a) indicated that embryogenic cultures could be initiated from seedling leaf explants of *C. sativa*, and that proliferation of new embryos continued via direct repetitive embryogenesis or via callus derived from somatic embryo cotyledons. If the leaves from the mature trees can also be used to initiate embryogenic cultures, this would allow elite European chestnut genotypes to be propagated via this route.

Another important hardwood genus devastated by a fungal disease in both North America and Europe during the twentieth century was *Ulmus*. The fungus *Ophiostoma ulmi* caused Dutch elm disease in most North American and European elms, and devastated elm populations on both continents during the 1920s–1940s. More recently, new strains of the fungus have emerged with sufficiently different cultural and molecular characters to warrant their designation as a new species, *Ophiostoma novo-ulmi*. Elms were among the first trees from which adventitious shoots were produced (Gautheret, 1940) and propagation of elms via axillary shoot multiplication has long been practiced (Biondi et al., 1984; Chalupa, 1987; McCown and McCown, 1987; Cheng and Shi, 1995). However, it was not until relatively recently that significant progress has been made with systems capable of regenerating plantlets via adventitious shoots or somatic embryos. As with chestnut, these routes for *in vitro* propagation of elms have been pursued as part of restoration efforts and good progress has been made, particularly in Europe. Reports indicating the ability to regenerate adventitious shoots from elm leaf explants (Bolyard et al., 1991; Fenning et al., 1993; Bolyard, 1994; George and Tripepi, 1994; Kapaun and Cheng, 1997) were promising, since this route offered the possibility of engineering fungal resistance genes into elm species via leaf-disk transformation. More recently, another potentially useful route for elm transformation has opened, with the publication of reports of somatic embryogenesis from immature zygotic embryos of *Ulmus minor* and *Ulmus glabra* (Corredoira et al., 2002, 2003b), and from leaves of mature *U. minor* trees cultured on a medium with kinetin (Conde et al., 2004).

Of all commercially important European hardwoods, arguably the most significant progress has been made with some species of *Quercus*. As in North America, European oaks such as the pedunculate oak (*Quercus robur*) and sessile oak (*Quercus petraea*) are of substantial ecological and commercial value, being important

providers of high-quality timber for construction, furniture, flooring casks and poles. The outer bark of cork oak (*Quercus suber*), primarily grown in Portugal and Spain, is the source of the world's supply of cork for wine bottle corks and many other applications. In contrast to the situation with North American oaks, great progress has been made in the development of embryogenic regeneration systems for European oaks. Somatic embryogenesis in the genus was last summarized in a review by Wilhelm (2000), but even since that date, new progress has been reported in multiple European oaks. As with many hardwood species, some early reports of somatic embryogenesis in European oaks employed zygotic embryos as explants (e.g. Chalupa, 1990). However, in other reports, leaf or stem segments from seedlings of *Q. suber* (El Maataoui et al., 1990; Fernandez-Guijarro et al., 1995) and *Q. robur* (Cuenca et al., 1999) and leaves from mature *Quercus ilex* trees (Feraud-Keller and Espagnac, 1989) could be induced to initiate embryogenic cultures. More recently, robust embryogenic systems for propagation of *Q. suber* and *Q. robur* have been established from leaf explants from epicormic shoots collected from mature trees, some of which were over 100 yr old (Hernandez et al., 2003a, b). The fact that these cultures could be maintained by repetitive embryogenesis and that an average of 40% of *Q. suber* somatic embryos germinated (Hernandez et al., 2003b) are indications that this approach may become a very powerful one for clonal propagation in a valuable genus that has long been recalcitrant to vegetative propagation.

#### Tropical Hardwoods

The loss of the world's tropical forests has been accelerating at an alarming rate. While much of this deforestation is due to conversion of rain forest to farm and pasture land, the harvesting of tropical hardwoods for forest products, most strikingly for fuelwood, also contributes substantially to these losses. For example, according to recent statistics from the Food and Agriculture Organization (FAO) of the United Nations, over 545 million m<sup>3</sup> of wood is consumed for fuel annually in Africa, which is over six times the amount of wood consumed for all other purposes (roundwood, sawnwood, pulp, etc.) combined (FAO, 2005). One of the promises of forest biotechnology that is often put forth is that the increased efficiency of fiber production on intensively managed plantations, including those on which clonally propagated and perhaps engineered trees will be deployed, will translate to decreased pressure on natural forests, especially tropical rainforests, as sources of fiber (Sedjo, 1999; Campbell et al., 2003). Of course, this potential will remain unrealized unless progress with *in vitro* propagation is extended to the high-value tropical species that are extensively harvested, such as teak (*Tectona grandis*), rosewood (*Dalbergia sissoo* and *Dalbergia latifolia*) and mahogany (*Swietenia macrophylla*). However, given the huge removals of tropical forests associated with fuelwood, it could also be argued that specialized plantations of exotic species, such as fast-growing *Eucalyptus* and pines, could be deployed specifically for fuelwood production, to lessen the pressure on native tropical hardwoods.

Of the valuable tropical hardwoods listed above, the most progress with *in vitro* propagation has been reported with rosewood, the timber which is valued for veneer for furniture, cabinetry and plywood. Somatic embryogenesis in rosewood has been reported by Muralidhar Rao and Lakshmi Sita (1996), Das et al. (1997) and Singh and Chand (2003). Rosewood has also been regenerated via

adventitious shoots (Lakshmi Sita et al., 1986; Pradhan et al., 1998b; Pattnaik et al., 2000; Singh et al., 2002) and via axillary multiplication (Pradhan et al., 1998a), including the use of encapsulated nodal segments as artificial seeds (Chand and Singh, 2004).

Unfortunately, comparatively little progress has been reported on *in vitro* propagation of some of the other tropical hardwood species that are under the most pressure. The strong, durable timber from teak, native to the tropical forests of Southeast Asia, is valued for furniture and construction. *In vitro* propagation of the species has been restricted to axillary shoot multiplication (Mascarenhas et al., 1993; Bonal and Monteuiis, 1997; Tiwari et al., 2002). RAPD markers indicated that plantlets derived via this route had a high clonal fidelity (Gangopadhyay et al., 2003). A report of somatic embryogenesis in the species did not result in plantlet regeneration (Kushalkar and Sharon, 1996). Reports of efficient *in vitro* propagation protocols for mahogany are also rare. The three species composing the genus *Swietenia* produce what many regard as the most beautiful wood in the world, but almost all mahogany wood in the commercial market is provided by big-leaf mahogany (*S. macrophylla*), native to Mexico, Central America and countries in the northern half of South America. It is also only this species of mahogany in which *in vitro* propagation work has been reported. The tree has been propagated via axillary multiplication (Venketeswaran et al., 1988; Murayama and Ishii, 1999), but the only report of adventitious bud production, from mahogany seedling epicotyl explants, apparently did not result in plantlet production (Cerdas et al., 1998). Maruyama and Ishii (1999) initiated repetitively embryogenic mahogany cultures from shoot tips of *in vitro* shoot cultures on a zeatin medium. Although the embryos germinated, conversion to plantlets was below 5%.

The ability to establish new plantations is especially critical in the dry tropics, where areas have been virtually deforested due to continual over-harvesting, particularly for use as fuelwood. While, as indicated earlier, there may be potential for substituting non-native *Eucalyptus* and pines for fuelwood production in these regions, some leguminous trees may offer a more promising route for reforestation due to their drought resistance and ability to fix nitrogen, providing timber and forage in these stressful environments. Besides rosewood, one genus of tropical leguminous tree in which *in vitro* propagation has been extensively reported is *Acacia*. *Acacia* is one of the most widespread and widely used leguminous genera in the world and includes some 1200 species distributed in Africa, Australia, Asia and the Americas (Vengadesan et al., 2002a). Uses include timber, fuel wood, charcoal, pulp, gum, numerous chemicals and wasteland reclamation. *In vitro* propagation of *Acacia* was thoroughly reviewed by Vengadesan et al. (2002a). Several *Acacia* species have been propagated *in vitro*, but most of these reports have described axillary shoot multiplication. Although one report described regeneration of plants from cotyledon-derived callus via organogenesis (Vengadesan et al., 2003), members of the genus have been characterized by a relatively low frequency of plantlet regeneration via this route (Vengadesan et al., 2002a). While somatic embryogenesis has been reported in numerous *Acacia* species, including *A. arabica* (Nanda and Rout, 2003), *A. sinuata* (Vengadesan et al., 2002b), *A. mangium* (Xie and Hong, 2001), *A. nilotica* (Garg et al., 1996), *A. senegal* (Shahana and Gupta, 2000), *A. catechu* (Rout et al., 1995), and the Mexican species *A. farnesiana* and *A. schaffneri* (Ortiz et al., 2000),

only a few of these could be described as producing sufficient numbers of plants to be called propagation systems. Notable among these reports was that by Garg et al. (1996), in which triploid somatic embryos were obtained from endosperm explants of *A. nilotica*.

Curiously, a tree widely recommended for reforestation in tropical regions and that has been described as one of the best timber producers of the tropics, *Gmelina arborea*, a member of the *Verbenaceae*, is represented by almost no reports of *in vitro* propagation. To date, the only reports of *in vitro* propagation of the species have employed axillary shoot multiplication from seedling explants (Kannan and Jasrai, 1996; Naik et al., 2003; Valverde-Cerdas et al., 2004).

### *Eucalyptus*

The tremendous potential of *Eucalyptus* species to produce timber and fiber for pulp and paper along with the rising commercial dominance of members of this genus on the world market make it an obvious target for *in vitro* propagation and genetic manipulation. As *Eucalyptus* species have gained in economic importance, an increasing number of reports have appeared on *in vitro* propagation of the members of this genus. As is the case with *Populus*, it is simply beyond the scope of this review to adequately summarize all of the progress in the genus, much of which has been made in the past 5 yr. Regeneration via adventitious shoot production from seedling explants or shoot cultures derived from them has been reported for *E. grandis* (Laskshmi Sita and Rani, 1985), *E. urophylla* (Tibok et al., 1995), *E. nitens* (Bandyopadhyay et al., 1999), *E. globulus* (Azmi et al., 1997; Bandyopadhyay et al., 1999; Nugent et al., 2001), *E. tereticornis* (Subbaiah and Minocha, 1990), and *E. grandis* × *E. urophylla* hybrids (Cid et al., 1999). As a prelude to their transformation work, Mullins et al. (1997) tested 24 *E. camaldulensis* genotypes and 12 other *Eucalyptus* species and hybrids for their ability to produce adventitious shoots from leaf explants of *in vitro*-grown seedlings. They reported successful adventitious bud induction for 13 of the *E. camaldulensis* clones and some success with four other species, but at generally lower frequencies. In a unique report, *E. botryoides* was regenerated via adventitious shoots derived from nodule cultures grown in a vertically rotated incubator (Ito et al., 1996). An early report on adventitious shoot production from *E. grandis* indicated that shoots could be obtained from nodal segments of 5-yr-old trees (Lakshmi Sita and Rani, 1985). Propagation of a proven *Eucalyptus* clones via organogenesis from explants from micropropagated plants has also been reported for *E. gunnii* (Herve et al., 2001), *E. camaldulensis* (Muralidharan and Mascarenhas, 1987), and *E. grandis* (Laine and David, 1994).

While the number of reports of somatic embryogenesis in the genus have been increasing and include work with *E. citriodora* (Muralidharan and Mascarenhas, 1987; Muralidharan et al., 1989), *E. grandis* (Watt et al., 1991), *E. globulus* (Bandyopadhyay et al., 1999; Nugent et al., 2001; Pinto et al., 2002), *E. nitens* (Bandyopadhyay et al., 1999), and *E. dunnii* (Termignoni et al., 1996), actual production of somatic seedlings has been restricted to *E. citriodora* (Muralidharan et al., 1989), *E. globulus* (Pinto et al., 2002), and *E. grandis* (Watt et al., 1991). Pinto et al. (2004) compared the DNA levels of somatic embryos, zygotic embryos, and leaves from mother trees using flow cytometry and concluded that

the somatic embryos did not differ significantly from the mother tree in DNA content, although the zygotic embryos did. Overall, despite promising progress with *Eucalyptus* regeneration via embryogenesis, organogenesis remains the *in vitro* propagation system with most potential for *in vitro* manipulation, particularly with regard to gene transfer applications (see below).

### GENE TRANSFER TECHNOLOGY AND ITS APPLICATION

The first report of a stably transformed hardwood forest tree (Fillatti et al., 1987) is almost two decades old, yet real progress in transformation of hardwood trees has remained concentrated in a few genera. We summarize the research with some of the more important hardwood forest trees below.

### *Populus*

As is the case with *in vitro* propagation, by far the most progress with transformation has been accomplished with poplars, and a number of excellent reviews summarizing transformation research with members of this genus and hybrids are available (e.g. Charest et al., 1997; Kim et al., 1997; Confalonieri et al., 2003). In addition to these reviews, reports describing the development of transformation protocols tested for a wide range of poplar species and hybrids, including both aspens (Tzfira et al., 1997) and cottonwoods (Han et al., 2000) are available. Although most poplar transformation has been accomplished with *Agrobacterium*-mediated transformation, poplars have also been transformed via biolistics (McCown et al., 1991) and electroporation of protoplasts (Chupeau et al., 1994). Since it was in *Populus* species that gene transfer was first accomplished, it is no surprise that the largest number of genes of potential commercial importance have been tested in members of this genus. In fact, a gene of commercial interest, *aroA*, conferring resistance to the herbicide glyphosate, was the first gene engineered into a forest tree (Fillatti et al., 1987). Other genes of commercial interest that have been transformed into poplar species include those conferring resistance to additional herbicides (De Block, 1990; Confalonieri et al., 2000; Gullner et al., 2001), insect resistance (Klopfenstein et al., 1991; Leple et al., 1995; Delledonne et al., 2001; Genissel et al., 2003; Wang and Constabel, 2004), pathogen resistance (Liang et al., 2001), flowering control (Weigel and Nilsson, 1995; Rottmann et al., 2000), and wood quality, in particular, lignin quality (Van Doorselaere et al., 1995; Tsai et al., 1998; Franke et al., 2000; Pilate et al., 2002), and quantity (Hu et al., 1999; Zhong et al., 2000). Recently, Li et al. (2003) reported that aspen trees co-transformed with antisense 4-coumarate-CoA ligase, and sense conifer aldehyde 5-hydroxylase genes had up to 52% less lignin, 64% higher syringyl/guaiacyl ratio and 30% more cellulose than the wild-type control trees. Thus, the report demonstrated not only that aspens could be co-transformed by inoculating explants with multiple *Agrobacterium* cultures carrying different vectors, but that aspen trees could be engineered for changes in both lignin quantity and quality at the same time, as well as cellulose content.

One relatively new area for which transgenic poplars are being generated is phytoremediation, the use of plants to stabilize, reduce or detoxify pollutants. Eastern cottonwood plantlets engineered with a modified bacterial mercuric ion reductase gene (*merA*) showed increased resistance to mercuric ion *in vitro* over wild-type trees,

presumably due to their ability to reduce the mercuric ion to less toxic elemental mercury (Che et al., 2003). Other trees are currently being engineered with genes with the goal of using them for phytoremediation of organic or heavy metal pollutants. A mammalian cytochrome P450 gene that showed striking enhancement in metabolism of trichloroethylene (TCE) when engineered into tobacco plants (Doty et al., 2000), has been engineered into hybrid poplar and the trees are currently being tested for their ability to handle TCE and other organic pollutants (personal communication, Dr Sharon Doty, University of Washington).

The relative ease of generating transgenic poplars has also resulted in their use as vehicles for the study of basic tree physiology, as thoroughly reviewed in Herschbach and Kopriva (2002).

#### Other Temperate Forest Hardwoods

While in most cases not advanced as the work with poplars, interesting and useful progress has been made with a number of other temperate forest hardwood trees, and potential deployment of some of these trees may ultimately have greater commercial and ecological impacts than the deployment of transgenic poplars. Two North American hardwoods that have already been transformed with genes of potential commercial importance are sweetgum and yellow-poplar. Sweetgum has been transformed via *Agrobacterium* inoculation of leaves, followed by regeneration of shoots from the leaves (Sullivan and Lagrimini, 1993) or nodule cultures (Chen and Stomp, 1992), and by microprojectile bombardment of nodule cultures (Kim et al., 1999). A tobacco anionic peroxidase gene conferred some resistance to feeding by caterpillars and beetles on the leaves of the transgenic sweetgum trees, compared to leaves from wild-type trees (Dowd et al., 1998). Yellow-poplar was transformed via microprojectile bombardment of embryogenic cultures (Wilde et al., 1992). Later, this system was used to introduce the *merA* gene into yellow-poplar, and similar to the work with eastern cottonwood, the regenerated plantlets showed increased resistance to mercuric ion *in vitro* over wild-type trees (Rugh et al., 1998).

Black locust was once a major contributor of strong, durable wood for use as railroad ties in the United States, but was devastated by locust stem borer (*Megacyllene robiniae*). Thus, one long-term goal might be to engineer the tree with genes conferring insect resistance. Given its successful deployment on mine spoils, the tree may also make a suitable target for engineering with phytoremediation genes. Han et al. (1993) produced transgenic black locust plantlets following co-cultivation of seedling hypocotyl segments with *Agrobacterium rhizogenes*, although the leaves of the transgenic plants had an abnormal morphology. Igasaki et al. (2000) co-cultivated black locust leaf and stem explants with *Agrobacterium tumefaciens* to produce hygromycin-resistant calluses, over 90% of which regenerated transgenic plants. Zaragosa et al. (2004) used sonication-assisted *Agrobacterium* transformation (SAAT) with the *bar* gene, which confers resistance to phosphinothricin, to produce transgenic black locust from cotyledon explants.

Some of the most advanced work with engineering genes of potential economic importance into hardwood forest trees has been accomplished with birches, which are among the most important hardwoods in boreal regions. *Agrobacterium*-mediated transformation of birches was first reported for *Betula platyphylla* var.

*japonica* (Mohri et al., 1997) and *Betula pendula* (Keinonen-Mettälä et al., 1998). Transgenic *B. pendula* trees engineered with the sugar beet chitinase 4 gene, and that had high levels of mRNA for the gene, showed a high degree of resistance to *Pyrenopeziza betulicola*, which causes leaf spot disease in birch, in greenhouse tests (Pappinen et al., 2002). While birch trees transformed with this gene were not significantly more resistant to leaf spot disease than wild-type controls in the field after 3 yr, they were more resistant to birch rust (*Melampsorium betulinum*) than controls (Pasonen et al., 2004). Finnish scientists also regenerated *B. pendula* trees engineered with constructs designed to induce sterility, with the long-term goal of preventing the spread of transgenes from genetically engineered trees to wild relatives. Trees of an early flowering *B. pendula* clone engineered with a *BpMADS1::BARNASE* construct either formed no male inflorescences, or if these did form, they aborted early or contained no stamens (Lemmettyinen et al., 2004). Similarly, the development of both male and female inflorescences was prevented in *B. pendula* engineered with a *BpFULL1::BARNASE* construct (Lännpää et al., 2005).

Much of the effort that has gone into developing transformation of hardwood trees has been with the long-term goal of engineering them for resistance to devastating diseases or insect pests. However, outside of work in poplars and birch, examples of trees successfully engineered with either insect or disease-resistant genes are very few. As detailed earlier in the section on *in vitro* propagation, two of the most famous examples of trees devastated by fungal diseases are the chestnuts and the elms, both of which have been attacked by fungal pathogens in Europe and North America. The English elm (*Ulmus procera*) was transformed by infecting the proliferating shoot cultures with a wild-type strain of *Agrobacterium*, resulting in tumors from which transformed shoots were regenerated (Fenning et al., 1996), and by Ri-plasmid mediated transformation of internodal segments, after which dwarf shoots were regenerated from hairy roots (Gartland et al. 2001). Gartland et al. (2000) regenerated phenotypically normal transgenic English elm plants following co-cultivation of internodal stem segments from shoot tip cultures with *Agrobacterium*. In an upcoming review of the application of biotechnology to deal with Dutch elm disease, Gartland et al. (2005) indicate that English elm plantlets transformed with antifungal genes have been produced, and that these are currently being tested for their ability to resist the Dutch elm disease fungus. As mentioned earlier, *in vitro* propagation research with American elm has lagged, but recently, details were published on a leaf piece transformation system for the tree (Newhouse et al., 2005). The authors indicated that one gene that has already been transformed into American elm with their system produces an antimicrobial peptide that may enhance resistance to Dutch elm disease. The relatively recent availability of embryogenic regeneration systems for some elms may help speed transformation efforts for these trees, since these cultures often make useful target material for gene transfer.

A similarly encouraging story is also emerging for the development of transformation systems for chestnuts, so that antifungal genes can begin to be tested for their ability to confer chestnut blight resistance to these trees. The development of embryogenic regeneration systems for chestnut species, summarized earlier in the review, has provided suitable target material for transformation experiments. Carraway et al. (1994) used biolistics to

transform embryogenic cultures of American chestnut, but were unable to regenerate transgenic plants. Corredoira et al. (2004) achieved a transformation frequency of 25% and regenerated stably transformed European chestnut trees by co-cultivation of leaf-derived embryogenic cultures with *Agrobacterium*. The first American chestnut trees engineered with a potential antifungal gene were produced when an oxalate oxidase (*OxO*) gene was transferred via *Agrobacterium*-mediated transformation of embryogenic cultures (Polin et al., 2005). The *OxO* gene, which is from wheat, was previously shown to confer resistance to the poplar pathogen, *Septoria musiva*, when engineered into *Populus × euroamericana* (Liang et al., 2001). The oxalate oxidase enzyme encoded by the gene was shown to break down oxalic acid, and since, as is the case with *S. musiva*, *C. parasitica* infection involves the killing of tissue with oxalic acid, the overexpression of this gene in chestnut may confer resistance to the blight fungus. The availability of a robust embryogenic regeneration system has also recently allowed the genetic transformation of cork oak, via a similar approach used with chestnut embryogenic cultures (Alvarez et al., 2004).

The development of an efficient gene transfer system could be a great help to the improvement of black walnut, and even more critically butternut, which is danger of being lost to the butternut canker disease (*Sirococcus clavigignenti-juglandacearum*). The availability of an embryogenic regeneration system for English walnut resulted in the development of an *Agrobacterium*-mediated gene transfer system (McGranahan et al., 1988, 1990) that has been used to test a number of genes in this species of *Juglans* (Dandekar et al., 1998; Escobar et al., 2002). The development of a similar system for butternut may allow the testing of antifungal genes that may be effective against butternut canker.

### *Eucalyptus*

As indicated in the section on *in vitro* propagation, great effort has been put into the development of organogenic and embryogenic regeneration systems for *Eucalyptus* species, and in many cases the goal was to provide suitable target material for gene transfer. Despite the application of multiple approaches to the members of the genus, including biolistics (Sartoretto et al., 2002) and SAAT (Gonzalez et al., 2002), very few reports have appeared on stable transformation and regeneration of *Eucalyptus* species. The first reports demonstrated that *E. camaldulensis* could be transformed via co-cultivation of leaf explants from *in vitro*-grown plants (Mullins et al., 1997) or seedling hypocotyls (Ho et al., 1998) with *A. tumefaciens*. More recently, in the only report of *Eucalyptus* transformation involving a gene of potential commercial importance to date, Tournier et al. (2003) used *Agrobacterium* co-cultivation to transform leaf explants from *in vitro* shoot cultures of *E. grandis* × *E. urophylla* hybrids with antisense cinnamyl alcohol dehydrogenase (CAD) gene from *E. gunnii*. Some regenerated plants showed up to 78% down-regulation of CAD activity.

### Commercial Deployment of Transgenic Hardwood Forest Trees

In the 20 years that have passed since the first report of transgenic tree production, there is no doubt that substantial advances have been made in the ability to produce hardwood transgenic trees carrying and expressing genes of potential

commercial value. Yet transgenic forest trees have only been deployed on a commercial scale in one country, and even the exact extent of the deployment remains unclear. *Populus nigra* engineered with the *CryIAc* gene from *Bacillus thuringiensis* was first approved for environmental release in China in 1998 and commercialization was approved in 2002 (Su et al., 2003). The latest available reports, while unofficial, indicate that at least one million transgenic *P. nigra* trees have been established in plantations in China to date (Su et al., 2003).

### HARDWOOD GENOMICS

Genomic technologies offer enormous potential for relatively rapid advances in tree improvement and domestication, including those that can be made through breeding programs and direct gene transfer. The application of genomic technologies to the elucidation of fundamental molecular and cellular processes that control growth and development in hardwood forest tree species, a prerequisite for the development of many tree improvement strategies, is in the early stages. A number of genomic resources have recently become available for hardwoods with many others under development and poised to expand the resources that are available for tree improvement and domestication programs. These include genomic sequencing, EST sequencing, transcript profiling, gene transfer, molecular markers for mapping and breeding, as well as other genetic selection strategies. The massive data sets that are generated by these methods and the need to manage and analyze these data necessitate the integration of bioinformatics into virtually every aspect of genomic research.

In this section, our objective is to provide a summary of the current status and near-term prospects for genomic resources that are available for research in hardwood forest tree biotechnology. *Populus* genomic resources have been the subject of several excellent reviews and readers will be directed to these and references contained therein for detailed information on specific topics.

### Transgenic Approaches for Functional Genomics

Extensive mutant collections and the ability to produce populations of tagged mutants is a powerful tool for the *Arabidopsis* model system. Although there are constraints on this approach in a woody perennial, such as plant size and generation time, these and other alternative approaches are feasible for the hardwood model *Populus* (Strauss et al., 2004). High throughput methods for generating lines by RNA interference are under development and T-DNA based approaches for transposon tagging are being investigated (Wullschleger et al., 2002a). A high-throughput system has been developed for activation tagging in *Populus*. Identification of a transgenic line over-expressing a *GA 2-oxidase*, created by the insertion of a transcriptional enhancer in the gene promoter, and subsequent characterization of the dwarfed mutant provided insight into the role of gibberellin in tree growth and development and may also have near-term applications for plantation forestry (Busov et al., 2003).

### Genome Sequencing

As with many taxonomic groups, model systems play a central role in fundamental research of tree genomics and biotechnology.

The availability of numerous genomic resources, including a complete genome sequence, extensive EST collections, a facile transformation system, short generation times, and mutant collections, have placed *Arabidopsis* at the forefront of research into fundamental biological processes of plants.

Tracheary element differentiation can be studied in cell culture systems such as *Zinnia*, *Pinus*, and *Arabidopsis* (Fukuda and Komamine, 1980; Möller et al., 2003; Oda et al., 2005). In addition, *Arabidopsis* has the potential to be exploited as a model species for research into some areas of growth and development of secondary vascular tissues (Chaffey, 2002; Chaffey et al., 2002). Under appropriate conditions, *Arabidopsis* will undergo secondary growth with the secondary xylem comparable to that of hardwood tree species. However, *Arabidopsis* has limitations for extrapolation of information to woody perennials (Wullschleger et al., 2002b). Features of anatomy and physiology that are specific to woody perennials, such as variation in earlywood and latewood, mature wood and juvenile wood, formation of heartwood (HW), juvenility, and cambial dormancy, will require studies in perennial woody species.

*Populus* has become a well-established model system for forest trees (Bradshaw et al., 2000; Wullschleger et al., 2002a). In addition to the utility of the genus in fundamental research, many poplar species are economically important for timber, pulp and paper, and as a source of raw materials for the production of biofuels (Taylor 2002). *Populus* species are also a dominant species in many ecosystems, providing habitat for other plant, animal, and microbial species. In addition to providing bioresources, *Populus* species provide a wide range of ecosystem services including carbon sequestration, water purification, and oxygen production (Brunner et al., 2004).

*Populus* is the first tree species for which the complete sequence of the genome is available (Brunner et al., 2004; Tuskan et al., 2004a). *Populus* as a model system complements the use of *Arabidopsis* in fundamental plant research by providing a tractable system for research into molecular and cellular processes that are unique to tree species. Genomic sequences are necessary to obtain information on regulatory elements, which cannot be derived from EST sequences. Differences in many aspects of anatomy and physiology between herbaceous annuals and woody perennials are likely to be controlled by regulatory variation rather than differences in structural genes (Strauss and Martin, 2004). Comparative genomics can be used to examine orthologous genes and their functions, syntenic relationships between taxa, regulatory sequences, and conservation of signal transduction pathways (Brunner et al., 2004). An initial comparison of *c.* 300 kb from *Populus* BAC clones with the sequence of the *Arabidopsis* genome revealed substantial regions of microcollinearity between the genomes and conservation of synteny (Stirling et al., 2003).

#### Expressed Sequence Tag Collections

EST databases facilitate examination of the protein coding portion of the genome and allow comparative analysis of expression between different tissues, developmental stages, and environmental conditions. The development of EST resources for *Populus* is more advanced than for any other hardwood species. The EST database for *Populus* currently contains more than 121 000 sequences comprising a unigene set of 25 000 sequences from 19 tissues

(Bhalerao et al., 2003b; Sterky et al., 2004; Moreau et al., 2005). EST resources include over 4000 full-length cDNA sequences assembled from the EST collection (Sterky et al., 2004) and 4500 non-redundant clones from a full-length enriched cDNA library constructed from stress-treated leaves, of which approximately 62% represent full-length cDNA clones (Nanjo et al., 2004).

*Eucalyptus* has a genome size similar to that of *Populus* (Paux et al., 2004). Development of EST resources for *Eucalyptus* has progressed significantly advanced in the past few years. The Genolyptus Project, a consortium of universities, companies, and government has generated approximately 120 000 EST sequences from this species (Grattapaglia, 2004). Another group has generated approximately 2500 EST sequences from a *Eucalyptus* xylem subtractive library (Paux et al., 2004).

Black locust (*Robinia pseudoacacia* L.) is being utilized for the study of HW formation (Yang et al., 2003). Approximately 3000 EST sequences representing over 2500 unigenes have been generated from cDNA libraries of *Robinia* bark/cambial zone, sapwood region and the sapwood–heartwood transition zone (Yang et al., 2004). In addition to the utility of EST sequences from this species in dissecting the regulation of sapwood and HW formation, *Robinia*'s phylogenetic position will permit comparative genomic analysis of wood formation between the groups that contain woody perennial and herbaceous taxa.

#### Transcript Profiling

EST sequences form the basis of many approaches utilizing functional genomics to examine the molecular and cellular mechanisms that control plant morphology and physiology. EST sequences derived from various tissues, developmental stages, and environmental conditions can be used to identify the genes that are involved in growth and development of different plant tissues, including wood formation in trees. The representation of EST sequences in a given tissue or under specific conditions reflects the abundance of a gene transcript in that tissue (Sterky et al., 2004). Comparison of EST sequences from different taxa can also be used to infer homology of genes present in different genomes and to assign genes to functional groups. EST sequences are also used as templates to create microarrays, which can contain thousands of genetic elements printed on a platform the size of a microscope slide. The resulting arrays can be hybridized to probes generated from different tissues, developmental zones, or environmental conditions.

The first studies in hardwood species utilizing EST sequences focused on identification of genes involved in wood formation and characterization of the corresponding expression profiles. These were subsequently expanded to investigate genes and gene regulation involved in distinct developmental stages of wood formation. Sterky et al. (1998) analyzed EST sequences from cambial region and developing xylem. Comparative sequence analysis was used to classify known sequences into functional categories. EST sequences representing genes involved in protein synthesis were elevated in the cambial region relative to the developing xylem whereas expression of genes involved in cell wall formation were elevated in developing xylem. Hertzberg et al. (2001) prepared sections from each of several developmental zones from the stem of *Populus* and performed a comparative analysis of gene expression across the developmental gradient. Microarrays



prepared using EST sequences were hybridized to probes generated for various developmental regions of the stem. Many of the EST sequences from *Populus* had homologs in *Arabidopsis* that were linked to specific cellular processes such as cell cycle, regulation of cell identity, cell expansion, and secondary cell wall biosynthesis. Overall, *Populus* homologs for these genes showed expression profiles in developmental regions that were consistent with their putative functions.

The work of Sterky et al. (2004) expanded upon earlier EST studies in *Populus*, both in the number of EST sequences and the range of tissues from which they were derived. The analysis included the cambial zone, different developmental regions of the wood-forming zone, and tension wood (TW). EST sequences were assigned functional classifications where possible and expression levels were compared among the various tissues using microarrays. EST sequences from wood-forming tissues clustered together, and elevated expression of genes within specific functional classes in certain tissue types generally reflected the physiological state of the tissue. This included elevated transcript levels for a number of histone and ribosomal proteins in the shoot meristem as well as up-regulation of genes encoding photosynthetic proteins in young leaves. This analysis also revealed high conservation in primary DNA sequence from different *Populus* species and suggested that microarrays can be used to examine gene expression in different *Populus* species and species within the related genus *Salix*.

One of the first genomic studies of wood formation in *Eucalyptus* used a subtractive library to identify EST sequences preferentially expressed in developing xylem (Paux et al., 2004). Colony arrays and real-time RT-PCR were used to confirm transcripts that were differentially expressed in xylem and leaves. Sequences that were preferentially expressed in xylem included components of the auxin signaling pathway, specific members of gene families involved in cell wall biosynthesis, and genes encoding enzymes involved in lignification.

Comparative analysis of the coding regions of the *Populus* and *Arabidopsis* genomes revealed a high degree of similarity, which is consistent with the suggestion that many differences between perennial and annual species are controlled by variation in gene regulation (Sterky et al., 2004). Karpinska et al. (2004) identified a number of transcription factors from *Populus* EST sequences that belong to the MYB gene family. Differential expression of specific members of this multi-gene family in various tissues, including the developmental zone where secondary cell wall formation occurs, supports a role for regulatory control of secondary vascular tissue development. Over 98% of genes represented by full-length sequences had putative homologs in *Arabidopsis* (Sterky et al., 2004) and a number of putative homologs were identified in xylem EST sequences from *Eucalyptus* and *Populus* (Paux et al., 2004). The presence of both herbaceous and woody species within different taxonomic groups is also consistent with the hypothesis that the major changes in the coding content of the genome are not required for transition between the herbaceous and woody habit.

The process of wood formation can be divided into five major stages, cell division, expansion, secondary cell wall biosynthesis, programmed cell death (PCD), and HW formation (Plomion et al., 2001). The size and organization of the vascular meristem and secondary xylem in trees permits the dissection and characterization of gene expression at different developmental stages of wood formation.

*Cell division and expansion.* Plant meristems represent a population of undifferentiated stem cells. The cambial initials divide to produce mother cells, which proceed through subsequent cell divisions followed by expansion and differentiation. In hardwood tree species, a primary meristem, the procambium, gives rise to the vascular cambium, a cylindrical arrangement of undifferentiated stem cells from which secondary vascular tissues are derived. Schrader et al. (2004b) used a high-resolution transcript profiling approach to investigate the molecular basis underlying maintenance of the vascular cambium in *Populus*. Their analysis suggested that conceptual subdivision of the cambial zone into anatomically indistinguishable cambial initials and mother cells is supported by differences in the transcriptomes of cells within the cambial zone. Comparative analysis of data generated from this study with data from the study of primary meristems suggested conservation of mechanisms for maintenance of meristematic regions.

*Secondary cell wall biosynthesis.* Understanding the molecular basis of variation in wood properties is one of the primary foci of hardwood biotechnology. The major components of the secondary cell walls of vascular tissues are cellulose, hemicellulose, and lignin. There has been significant progress in unraveling the mechanisms of cellulose synthesis in recent years. However, knowledge of the process remains incomplete. Identification of the first genes involved in the synthesis of hemicellulosic polysaccharides was reported within the past two years and these are the members of the cellulose synthase-like gene family (Dhugga et al., 2004; Liepman et al., 2005). A recent study identified 34 genes representing glycosyltransferases and glycosidases that are differentially expressed during xylem development in *Populus* (Aspeborg et al., 2005). The biosynthesis of lignin is better understood than that of cell wall polysaccharides. However, the regulation and variation of lignin biosynthesis remains an active area in plant research. Furthermore, compensatory mechanisms have been suggested for the regulation of secondary cell wall composition. A cellulose-deficient mutant in *Arabidopsis* had increased lignin content including ectopic lignification (Boudet et al., 2003). In *Populus*, down-regulation of a lignin biosynthetic enzyme reduced lignin and increased cellulose, but a mutant of *Arabidopsis* with reduced lignin had no increase in cellulose (Boerjan et al., 2003).

Cellulose is the major component in the secondary cell wall of the vascular tissues that make up wood and wood fiber, and cellulose synthesis in the primary cell wall is critical in cell morphogenesis. The catalytic subunits of the cellulose synthesis complex are encoded by the *CesA* multi-gene family, which is part of a larger gene superfamily that includes the cellulose synthase-like (*Csl*) genes (Richmond and Somerville, 2000). A specific membrane-bound cellulase, korrgan, and sucrose synthase are also involved in cellulose synthesis of the plant cell wall (Lane et al., 2001; Salnikov et al., 2001). Microtubules of the plant cytoskeleton have been implicated in the deposition of cellulose (Baskin, 2001); however, details of the relationship between microtubules and cellulose deposition remain unclear.

Studies of *Arabidopsis* mutants led to the identification of three specific members of the *CesA* gene family that were functionally non-redundant and required for normal cellulose synthesis in the secondary cell walls of vascular tissue (Taylor et al., 2003). Subsequent studies in rice (*Oryza sativa*), *Populus*, and loblolly pine

(*P. taeda* L.) support the evolutionary and functional conservation of the secondary cell wall *CesA* genes in seed plant growth and development (Joshi, 2003; Tanaka et al., 2003; Djerbi et al., 2004; Joshi et al., 2004; Nairn and Haselkorn, 2005). Consequently, the secondary cell wall *CesA* genes in hardwood species are attractive targets for the modification of wood properties.

Lignins are the second most abundant plant biopolymers, comprising a range of complex heteropolymers (Boerjan et al., 2003). Lignins are integral structural components of cell walls in specific types of plant cells, such as those of vascular tissue, and are important for strength, water transport, and disease resistance (Rogers and Campbell, 2004). *Populus* has been extensively utilized in the study of lignin and the effects of modification through mutation and transgenic approaches. These transgenic approaches included the modification of multiple genes involved in lignification (Li et al., 2003). In addition to the importance of understanding lignin biosynthesis and deposition in the context of fundamental plant biology, variation in quantity and composition of lignin in plant cell walls is important to the industries that use hardwoods as raw materials. Altered properties in pulping for papermaking have been demonstrated for transgenic plants with modified lignin properties (Baucher et al., 2003; Boerjan et al., 2003). The ability to manipulate lignin as part of cell wall modification is an important area of focus as society increasingly looks towards the utilization of plant biomass as a renewable resource for sustainable development (Boudet et al., 2003).

Transcriptional control of lignification is supported by experimental evidence and post-transcriptional regulatory mechanisms are likely to play a role in lignin synthesis, transport, and integration into the cell wall matrix. Reduced expression of several different enzymes involved in lignin biosynthesis in transgenic plants causes reduction in the quantity of lignin to varying degrees as well as altered ratios of guaiacyl (G) and syringyl (S) units (Boerjan et al., 2003).

The number of enzymes involved, the tissue specificity, and the developmental control of lignification suggest the possibility of common regulatory mechanisms for various components of the process. Circadian control of lignification has also been demonstrated (Rogers and Campbell, 2004). Common regulatory elements, AC elements, were identified in the promoters of various genes involved in the lignin biosynthetic pathway. Promoter analysis of a *Eucalyptus* hydroxycinnamoyl-CoA reductase (CCR) and a hydroxy cinnamyl alcohol dehydrogenase (*EgCAD2*) demonstrated that AC elements were required and sufficient for directing expression in xylem and differentiating vascular tissue, respectively. The *EgCAD2* promoter also directed wound-induced expression. Expression patterns of transgene constructs in different species suggest conserved regulatory mechanisms in woody perennial and herbaceous annual plants.

The involvement of members of at least four different families of transcription factors in control of lignification has been demonstrated (Rogers and Campbell, 2004). Specific members of the MYB and LIM families of transcription factors recognize AC promoter elements and alter the expression of lignin biosynthetic genes. Members of the KNOX and MADS families of transcription factors also affect lignification. However, mechanisms of regulation are less clear and could result from direct interaction with gene promoters or through regulation of developmental processes to which the control of lignification is intimately tied.

*Programmed cell death and heartwood formation.* The final developmental stages in wood formation involve PCD and HW formation. Moreau et al. (2005) used comparative analysis of *Populus* EST sequences and microarray experiments to examine the changes in gene expression in the region where xylem fibers undergo cell death. Genes that were differentially expressed in the region undergoing cell death included specific members of the ACP, GRP, and extension-like protein families that are components of the cell wall. Transcripts that encode products involved in amino acid metabolism, proteolysis, signaling, and vacuolar regulation were also among those differentially regulated during PCD. This study highlights the potential of functional genomics to elucidate not only the genetic control of PCD in wood formation but, also changes in cell walls that may be part of the process.

HW is formed in the stems of most hardwood species and is associated with cell death and accumulation of extractives, the most abundant being phenolic compounds (Yang et al., 2003). Approximately 3000 EST sequences were generated from black locust (*R. pseudoacacia* L.) cambial, sapwood, and transition zones cDNA libraries. Transcript profiling, using microarrays and probes generated from distinct developmental zones, was used to investigate the molecular basis of HW formation, including seasonal variation in gene expression (Yang et al., 2004). Functional classification of genes with specific or elevated expression in distinct zones was generally consistent with the physiological processes associated with those zones. EST sequences from sapwood included genes involved or implicated in cell wall biosynthesis and sugar transport. Genes involved in secondary metabolism, including those for flavonoid synthesis, were up-regulated in the sapwood–heartwood transition zone. Of 1873 genes expressed in the transition zone, 569 genes showed differential expression between summer and fall. Data from these studies were also consistent with a proposed role for ethylene as a regulator of HW formation and the hypothesis that carbohydrates are imported to the transition zone and utilized in the synthesis of extractive phenolic compounds.

TW is formed in hardwood species on the upper side of the leaning stem in response to gravitropic stimuli. The altered morphology and cell wall composition of TW, including reduced lignin content, higher cellulose content, presence of an inner cell wall G-layer, and lower microfibril angle (MFA), makes this an attractive model for genomic investigations into the control of wood formation (Plomion et al., 2001; Pilate et al., 2004). EST sequences from TW were included in transcriptome analysis of wood formation in *Populus* (Sterky et al., 2004). Paux et al. (2005) applied transcript profiling to examine differential gene expression during development of TW and normal wood (NW) in *Eucalyptus*. Of 231 genes preferentially or specifically expressed in xylem, 196 were differentially expressed in TW (Paux et al., 2004). Plomion et al. (2003) applied proteomic methods to the study of TW formation in *Eucalyptus* and identified proteins that were differentially expressed in TW. Genes encoding components of the cellulose synthesis complex and the lignin biosynthetic pathway were among those differentially expressed in TW and NW (Paux et al., 2005). Interestingly, an *Aux/IAA* gene identified in the *Eucalyptus* study is a putative ortholog of an *Aux/IAA* gene implicated in TW formation of *Populus*, suggesting conserved mechanisms of TW formation in hardwood species.

*Other hardwood tree traits.* *Populus* provides a model system for processes that are unique to woody perennials, such as dormancy,

that cannot be effectively studied in *Arabidopsis*. Dormancy involves complex changes in the physiology of cambial tissues including those relating to cell proliferation, cold hardiness, desiccation, and accumulation of storage compounds. Regulation of dormancy in trees is an area of major interest for potential modification of growth and development in tree improvement strategies. Schrader et al. (2004a) used a functional genomics approach to examining transcriptional changes in cambial tissues during dormancy. EST sequences were obtained from active and dormant cambium and used for *in silico* analysis as well as microarray experiments. They observed an overall reduction in transcriptome complexity of dormant cambium. Expression of genes involved in cell cycle processes was maintained and down-regulation of *PIN* homologs was consistent with the reduction of basipetal polar auxin transport during dormancy. Other changes included the up-regulation of ubiquitin ligases involved in auxin signaling and a gene that encodes a repressor of gibberellin responses. These observations suggest possible changes in both auxin and gibberellin signaling during dormancy of cambial meristems.

Autumn senescence in *Populus* was examined by EST sequencing and microarray analysis (Bhalerao et al., 2003a; Andersson et al., 2004). Similarities were observed between this process in the woody perennial and senescence in annual plants such as the induction of proteases. Changes in gene expression reflected a transition in energy generation from photosynthetic activity to respiration, oxidation, and nutrient mobilization. Increased transcriptional activity preceded the onset of visible senescence, reflecting a shift in the physiological status of leaves.

Transcriptional profiling also has been applied to study changes in the transcriptome in response to environmental stress, wounding, and pathogenic infection. Comparative analysis of EST sequences from *Populus* that had been subjected to dehydration, salinity, chilling, heat, abscisic acid, and H<sub>2</sub>O<sub>2</sub> revealed that most of the *Populus* genes identified by similarity to *Arabidopsis* stress-related sequences were up-regulated by stress treatment in *Populus* (Nanjo et al., 2004). Homologs of the ERF/AP2-domain transcription factor family, which function in environmental stress responses, were among those identified and individual members of the family exhibited differences in expression patterns. Systemically induced wound responses have also been examined in *Populus* (Christopher et al., 2004). A significant percentage of the EST sequences generated in this study represented genes potentially involved in defense and secondary metabolism, including novel members of the Kunitz trypsin inhibitor-like genes and the chitinase-like genes. Changes in gene expression induced by mechanical wounding and infection by a viral pathogen were examined by transcript profiling in *Populus* (Smith et al., 2004). Significant representation of genes involved in PCD and cell wall reinforcement was observed in the wound-response EST sequences. Genes for metallothionein-like proteins, and genes involved in cell wall remodeling were observed in the EST sequences derived from viral infection of *Populus* leaves. These studies highlight the potential for transcript profiling in aspects of hardwood biology other than those addressing wood formation.

#### Molecular Markers and Mapping

Molecular markers such as AFLP, RAPD, and microsatellite (SSR) markers, provide powerful tools for the construction of genetic

and physical maps. They are also utilized in marker assisted breeding programs, including those for hardwood species. Genetic maps are useful in determining the relationship between genes and QTLs (Taylor, 2002). In addition, markers have applications for assessment of genetic diversity in populations and in paternity analysis. Markers have been utilized in hardwood tree species and are useful for comparative mapping of related taxa. Bradshaw et al. (1994) constructed the first linkage map for a hardwood species with an estimated 50% coverage of the *Populus* genome. Recently, Yin et al. (2004) reported the most complete map to date for *Populus* with genome coverage exceeding 99.9%. Development of SSR markers has been accelerated in *Populus* by the use of genomic sequencing (Tuskan et al., 2004b). Due in part to the relatively low cost of development, molecular markers are currently available for more hardwood species than any other genomic resource.

Grattapaglia et al. (2004) have utilized microsatellite markers in breeding programs for *Eucalyptus*. They have reported an alternative approach to conventional progeny trials by paternity testing of superior individuals from a hybrid seed orchard. Microsatellite markers have also been used to fingerprint unrelated individuals in an elite breeding population of *Eucalyptus* (Kirst et al., 2005). Moran et al. (2002) generated a genetic linkage map containing RFLP and microsatellite loci as well as ESTs, candidate genes and isozymes. The maps include putative locations of a number of QTLs, including those for traits such as density, cellulose, pulp yield, fiber length, and microfibril angle.

Microsatellite markers have been developed for the genus *Quercus* and used for a variety of applications, including an investigation of the genetic structure of *Q. petraea* in Ireland (Muir et al., 2004). The results indicated that despite reduction of the species to small, fragmented populations due to deforestation, high levels of genetic diversity and outcrossing as well as low differentiation were present in the remnant populations. Markers have also been used to investigate the genetic structure of sugar maple (*Acer saccharum*) populations and demonstrated that a large portion of variation exists within populations and differences between regions represented less than 2% of the variation (Gunter et al., 2000). Microsatellite markers from *Quercus* and *Castanea* have also been used for comparative mapping, and a high percentage was transferable between taxa (Barreneche et al., 2004). Microsatellite markers developed for two *Quercus* species have been used for fingerprinting *C. sativa* cultivar: (Bocacci et al., 2004).

As part of the effort to restore American chestnut following its devastation by the chestnut blight fungus, Kubisiak et al. (1997) mapped several putative resistance loci in American chestnut × Chinese chestnut (*Castanea mollissima* Blume) hybrids using molecular markers. Markers have also been used to identify species and hybrids within the genus *Castanea* (Kubisiak, 1999). Dodd et al. (2005) used AFLP markers to investigate the inter- and intra-population variation in susceptibility to *Phytophthora ramorum* infection *Quercus agrifolia*. The results suggest that individual genotypes vary in their susceptibility to infection, that several loci are involved in resistance, and that the genetic variation exists within populations.

Microsatellite markers have recently been developed and cross-species transfer evaluated for the genus *Ulmus* (Whiteley et al., 2003). Watanabe et al. (2004) reported the development of RAPD markers for fingerprinting and clone management of teak (*Tectona grandis*). These and other studies highlight the utility of

molecular markers for the study of a wide variety of biological questions and applications for genetic mapping in hardwood taxa. Continuing development of these resources for an increasing number of taxa has great potential for fundamental and applied research in hardwood tree species.

### CONCLUSIONS

Hardwood forest trees are a world-wide resource that mankind has depended upon for fiber, fuel, and food for millennia. As demand for timber and paper continues to grow around the world, this resource will come under greater pressure, requiring more and more efficient means of growing and managing this resource. Overall, the application of such biotechnological tools as *in vitro* propagation, gene transfer, and molecular breeding to forest trees, including hardwoods, is still in its infancy compared to agronomic crops. It is also true that progress applying these tools to commercially important hardwoods has been very uneven, and as illustrated in this review, extremely species dependent. However, there is also no doubt that these tools are already having an impact on how some hardwood trees are being bred, propagated, deployed, and managed. All can make contributions to raising the efficiency with which we produce and use our hardwood forest tree resource and thereby help make sure this resource is available for future generations.

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