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INVITED REVIEW

Technological advances in temperate hardwood tree improvement including breeding and molecular marker applications

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Abstract Hardwood forests and plantations are an important economic resource for the forest products industry worldwide and to the international trade of lumber and logs. Hardwood trees are also planted for ecological reasons, for example, wildlife habitat, native woodland restoration, and riparian buffers. The demand for quality hardwood from tree plantations will continue to rise as the worldwide consumption of forest products increases. Tree improvement of temperate hardwoods has lagged behind that of coniferous species and hardwoods of the genera *Populus* and *Eucalyptus*. The development of marker systems has become an almost necessary complement to the classical breeding and improvement of hardwood tree populations for superior growth, form, and timber characteristics. Molecular markers are especially valuable for determining the reproductive biology and population structure of natural forests and plantations, and the identity of genes affecting quantitative traits. Clonal reproduction of commercially important hardwood tree species provides improved planting stock for use in progeny testing and production forestry. Development of *in vitro* and conventional vegetative propagation methods allows mass production of clones of mature, elite genotypes or genetically improved genotypes. Genetic modification of hardwood tree species could potentially produce trees with herbicide tolerance, disease and pest resistance, improved wood quality, and reproductive manipulations for commercial plantations. This review concentrates on recent advances in conventional breeding and selection, molecular marker application, *in vitro* culture, and genetic transformation, and discusses the future challenges and opportunities for valuable temperate (or "fine") hardwood tree improvement.

Keywords Clonal propagation · Cryopreservation · Forest genetics · Genetic transformation · Organogenesis · Plantation forestry · Regeneration · Somatic embryogenesis

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Introduction

Hardwood forests and plantations in North America, Europe, and other parts of the world contain a wide range of temperate tree species that are an important resource for the forest products industry and to the foreign trade of lumber and logs. In addition to timber, sawlog, and veneer log production, hardwood trees are also planted for wildlife habitat, native woodland restoration, riparian buffers, erosion control, windbreaks, conservation, and watershed protection. Some of the more valuable hardwoods include alder (*Alnus* spp.), ash (*Fraxinus* spp.), basswood (*Tilia* spp.), beech (*Fagus* spp.), birch (*Betula* spp.), black locust (*Robinia pseudoacacia*), black cherry (*Prunus serotina*), chestnut (*Castanea* spp.), elm (*Ulmus* spp.), gum (*Liquidambar styraciflua*), hackberry (*Celtis occidentalis*), hard (and soft) maples (*Acer* spp.), hickory and pecan (*Carya* spp.), oak (*Quercus* spp.), sassafras (*Sassafras albidum*), sycamore (*Platanus* spp.), walnut (*Juglans* spp.), black willow (*Salix nigra*), and yellow

poplar (*Liriodendron tulipifera*). The market for these species can be very high because of the special appearance (e.g., grain, figure, texture, and color) or technical properties (e.g., strength, durability, and good machining properties) compared to lesser quality hardwoods used for fuel or pulp. These species are utilized in the manufacture of residential and commercial structures and furnishings (architectural millwork, cabinets, doors, flooring, furniture, moldings, paneling, shutters, siding, and windows) and other specialty products (barrel staves, baseball bats, billiard cues, boat interiors, butcher blocks, carvings, caskets, crates, gun stocks, hockey sticks, kitchen utensils, ladders, musical instruments, oars, skis, tool handles, toys, Venetian blinds, and woodenware).

Tree improvement of temperate hardwoods has been more limited compared to that of coniferous species and hardwoods of the genera *Populus* and *Eucalyptus* (Merkle and Nairn 2005). The demand for hardwood from tree plantations will continue to rise as the worldwide consumption of forest products increases, and the environmental, commercial, and political pressures of restricting logging of high-quality trees from natural forests also increases (for example, see references and working papers on forest plantations cited in FAO 2001). Considerable effort has been exerted over the last 40 yr in conventional tree improvement programs through breeding and selection, and strategies for breeding and tree improvement of temperate hardwoods have been developed (Burley and Kanowski 2005; Michler et al. 2005). The long generation and reproductive cycle, difficulty in conducting controlled pollinations, intermittent or scarce seed crops, and seed recalcitrance of hardwood trees are some of the limitations imposed on conventional tree breeding programs (Lantz 2007). Forest geneticists are interested in developing populations with disease resistance, superior growth, form, and timber characteristics, including straighter boles and reduced branching. Molecular marker development would be useful in determining the genetic quality and population structure of natural forests and plantations, and the quantitative genes of superior trees. Clonal reproduction of commercially important hardwood tree species is also necessary in a tree improvement program to provide improved planting stock for use in progeny testing and for production forestry. *In vitro* and conventional vegetative propagation methods will be required to produce clones of mature, elite genotypes or genetically improved genotypes. Many economically important hardwood tree species have a low genetic or physiological capacity for adventitious root formation, and are considered recalcitrant to routine, commercial-scale vegetative propagation. Genetic modification of hardwood tree species to produce trees with herbicide tolerance, disease and pest resistance, improved wood quality, and reproductive manipulations for commercial plantations is also a major aspect of a tree improvement program. Development of an effective gene transfer and efficient *in vitro* regeneration system for each hardwood species, that can be easily adapted for many genotypes, will facilitate the production of genetically improved temperate hardwood trees.

Plantation forests and the role biotechnology can play has been reviewed (Fenning and Gershenzon 2002). Other recent reviews have focused on innovative technologies that provide the basis for acceleration in forest tree improvement (Nehra et al. 2005) and *in vitro* propagation, gene transfer, and genomics for a sample of hardwood timber and pulp species (Merkle and Nairn 2005). Because *Populus* and *Eucalyptus* biotechnology has been recently reviewed, we will limit our review to the hardwoods previously mentioned. This review concentrates on conventional breeding and selection, molecular marker application, *in vitro* culture, genetic transformation, and future challenges and opportunities in valuable temperate hardwood tree improvement. A comprehensive review of the literature is impossible, but hopefully we have captured or highlighted many important species and research.

Conventional Tree Improvement

The conventional improvement of hardwoods has always lagged behind that of conifers. Deciduous hardwood species that are used for fiber or horticulture, and those with congeners used by those industries, have benefited from a research crossover effect that mostly informs biotechnological methods. Conventional breeding still relies on a mainstay of provenance trials to evaluate local adaptation, phenotypic selection to identify potentially superior parents, progeny trials to evaluate those parents, and seed orchards for the production of adapted, improved seed. A web resource that links to many of the tree improvement programs (including conifers) world-wide (http://www.genfys.slu.se/staff/dagl/Documentations/OrganisationLinks.htm) is maintained by Dag Lindgren at SLU, Umeå, Sweden. In Europe, recent activities of the British and Irish Hardwood Improvement Programme are summarized in Burley (2004), including research in ash, silver birch, wild cherry, two species of oak, sweet chestnut, sycamore (*Acer*), and walnut (two species). Recent research in Europe and elsewhere has focused at least as much on conservation of genetic resources as on the development (breeding) of resources (Eriksson 2001; Xie et al. 2002; Karagöz 2003; Hosius et al. 2006), but these two activities are closely related and interdependent. Reviews focused specifically on tree improvement in particular regions or in particular species are also available (Koski and Rousi 2005). In the US, summaries of hardwood breeding

research (Byram et al. 2000; Fralish 2002; Michler et al. 2004) reflect an intermittent pattern of State, Federal, and private investment in fine hardwood improvement that has hampered the diligent, sustained effort necessary for progress in conventional forest tree breeding.

DNA-based and Other Marker Systems

Genetic markers have become indispensable tools for understanding, managing, and improving natural and planted forest tree populations. The many marker systems and their uses, as well as the choice of optimal system for various research goals, are well reviewed (Gillet 1999; Mohler and Schwarz 2004; Ziegenhagen and Fladung 2004). For some researchers, the choice of marker system is more determined by the opportunities and constraints in their laboratory and their technical staff than by any other factors. The research infrastructure needed for different marker systems has also been reviewed (see overview link and associated content at: http://www.cgn.wur.nl/UK/CGN+Plant+Genetic+Resources/Research/Molecular+markers). Fortunately, most research questions can be investigated with any of several types of markers. An excellent decision scheme for marker choice is published by the International Plant Genetic Resources Institute (Karp et al. 1997).

Molecular Marker Applications

The discriminatory power provided by molecular markers can be used to resolve and understand hybridization and species differentiation. Examples include the infamous (to taxonomists) hybrid complexes in *Quercus* (Muir et al. 2000; Scotti-Saintagne et al. 2004b; Tovar-Sanchez and Oyama 2004; Whittemore and Schaal 1991), *Acer* (Hasebe et al. 1998; Skepner and Krane 1998; Joung et al. 2001), *Betula* (Anamthawat-Jonsson and Thorsson 2003; Palme et al. 2004), *Fraxinus* (Raquin et al. 2002), *Liriodendron* (Li and Wang 2002), *Platanus* (Vigouroux et al. 1997), *Fagus* (Ohyama et al. 1999; Gailing and von Wuelisch 2004), *Juglans* (Manos and Stone 2001; Orel et al. 2003), *Tilia* (Fineschi et al. 2003), and *Salix* (Hardig et al. 2000). Understanding the nature and origins of hybrids is important to breeders, ecologists, and taxonomists. DNA markers are the most commonly used molecular tools for identifying species and interspecific hybrids, and marker systems describing hybridization relevant to hardwood improvement have been described for *Alnus* (Prat 1988), *Betula* (Clausen 1979), *Juglans* (Potter et al. 2002), *Liriodendron* (Wang 2003), *Platanus* (Santini 2001), *Salix* (Krstinic and Kajba 1997) *Ulmus* (Pinon et al. 1999), and probably other genera as well.

The capacity of molecular markers to permit the assignment of a sample to a particular individual, provenance, stand or species within an allowable likelihood of error (Douhovnikoff and Dodd 2003) has led to a wide variety of practical applications. Conservation biologists use markers to monitor and validate the identity of accessions in *ex situ* collections (Goodall-Copestake et al. 2005); breeders and nurseries make use of the large number of alleles and high heterozygosity in most forest tree species to genotype or "fingerprint" breeding materials and to reconstruct pedigrees (Dangl et al. 2005). This technique is used more among horticultural breeders than forest tree breeders (Tobolski and Kemery 1992; Conner and Wood 2001; Boccacci et al. 2004; Pooler and Townsend 2005). Breeders have also used markers to monitor and understand levels of genetic diversity and genetic differentiation in breeding populations compared to wild relatives (Panda et al. 2003; Aradhya et al. 2004). When marker resolution and population genetic structure permit the identification of specific stands or provenances (e.g., Ferris et al. 1997; Hamann et al. 1998; Kelleher et al. 2004), then breeders can potentially make use of the untapped genetic variability located there (Ruter et al. 1999). An important use of markers related to forest management concerns the certification and characterization of seed sources (Heinze and Lexer 2000; Gregorius and von Werder 2002; Ziehe and Hattemer 2004). Novel applications of marker technology in forest genetics include forensic applications to prosecute log theft (Woeste, unpublished data) and possibly in the certification of wood products, archaeology, and paleobotany (Dumolin-Lapegue et al. 1999; Deguilloux et al. 2003, 2004).

Insight into evolutionary processes and the spatial, temporal, and demographic changes that affect them has been significantly advanced by the development of molecular markers. The conservation biology of woody plants can hardly be addressed without consideration of the types of molecular variation found in them (*see* Linhart 2000 and citations therein). With specific reference to conservation of forest genetic resources and intraspecific variation, research describing and monitoring genetic diversity has been pursued for many of the most important temperate hardwoods (reviewed in Newton et al. 1999; Eriksson 2001; see also Fjellstrom and Parfitt 1994; Machon et al. 1997; Huh 1999; Heuertz et al. 2001; Bellarosa et al. 2003; Fineschi et al. 2003; Rusanen et al. 2003; Cros 2004; Rowden et al. 2004; Tsumura et al. 2004; Goodall-Copestake et al. 2005).

Pollen, in many important hardwood species, is wind-dispersed. The lacuna in our understanding of male parentage has been colored in by studies in a variety of genera (Merzeau et al. 1989; Streiff et al. 1999; Heuertz et al. 2003). Until the development of genetic marker

systems, it was not possible to characterize the flow of pollen in disturbed sites (Sork et al. 2002; Goto et al. 2004), natural stands (Dow and Ashley 1996; Garcia et al. 2005), or breeding populations (Arbeloa et al. 2006; Grauke et al. 2006).

Genetic Maps

Genetic maps are useful or even necessary tools for whole genome selection (marker-assisted backcross breeding), quantitative trait loci (QTL) analysis, and other trait mapping procedures, gene discovery, studies related to genomics and genome evolution, and studies of species differentiation. The quality of a genetic map is determined by the number and types of markers used, and the size and types of population(s) used to analyze segregation. The existing genetic maps for most hardwoods are not as useful as those for agronomic crops because in most hardwood species large full-sib populations are difficult to generate, the number of markers is relatively small, and most maps are based on markers that are not tied to physical or transcriptional landmarks such as expressed sequence tags (ESTs), which would increase their usefulness for genomic and other types of research. There are, nevertheless, published genetic maps for a number of hardwood tree species and genera:

Species or genus	Markers used in the map	Reference
Betula pendula	SSR, AFLP	Pekkinen et al. 2005
Carya illinoinensis	RAPD, AFLP	Beedanagari et al. 2005
Castanea sativa	RAPD, ISSR, isozymes	Casasoli et al. 2001
Fagus sylvatica	RAPD, AFLP, SSR	Scalfi et al. 2004
Juglans	RAPD, RFLP	Woeste et al. 1996a
Prunus	SSR	Howad et al. 2005
Quercus robur	RAPD, SCAR, Minisatellite Isozyme, SSR, 5S rDNA	Barreneche et al. 1998
Salix	AFLP, RFLP	Tsarouhas et al. 2002
	AFLP, SSR	Hanley et al. 2002

Gene discovery, genomics, and other "-omics" methods were first developed for animal research, but later applied to model plants. These methods are now being applied in forest trees grown for fiber (Brinker et al. 2004; Kirst et al. 2004; reviewed in Plomion et al. 2005). Genomic research in fine hardwoods can be divided into gene characterization and genome-wide approaches, including microarrays. Gene characterization studies may be based on the analysis of genes shown in model systems to affect important physiological processes such as photosynthesis and senescence (Valjakka et al. 1999; Sillanpää et al. 2005). A second approach is to identify differentially expressed genes in a hardwood species of interest (Label et al. 2001; Beritognolo et al. 2002). Often, sequences for these genes are derived from EST databases generated by sequencing cDNA libraries from tissues of interest (Connors et al. 2001). Techniques such as differential display (Schafleitner and Wilhelm 2002; Gil et al. 2003) and representational difference analysis can be used to find regions of genomic differentiation between samples or species (Zoldos et al. 2001). Whole genome analysis via microarray (Yang et al. 2003; Yang et al. 2004; Quere et al. 2005) or proteome analysis using 2-D gel electrophoresis (Jorge et al. 2005) holds out promise for hardwoods that is being realized in crop plants (Dunwell et al. 2001).

DNA Markers and *In Vitro* Technologies

DNA-based molecular markers have been used to identify and verify the origins and stability of *in vitro* cultures and plants regenerated from culture. RAPD and AFLP have been used for this purpose when specific sequence data are not available (Vendrame et al. 2000; Sanchez et al. 2003; Martins et al. 2004). Microsatellites, which are hypervariable, are especially sensitive and effective markers for this type of research (Wilhelm et al. 2005; Lopes et al. 2006) and for monitoring somatic mutation in long-term storage (Ryynanen and Aronen 2005) because they are relatively easy to use once primer sequences have been identified. Markers can be used in conjunction with *in vitro* propagation to increase the efficiency of breeding by assigning paternity to zygotic embryos in culture, making possible subsequent selection on genotype (Hormaza 1999).

The efficiency of plant transformation and regeneration may be improved by a better understanding of the molecular biology of critical steps in the process. Genomics and research in non-hardwood species (Brinker et al. 2004; Lippert et al. 2005; Zamboni et al. 2005) make possible smaller-scale, candidate gene approaches to understand embryogenesis and development *in vitro*. Proteins such as the heat shock proteins (Puigderrajols et al. 2002), legumins, and dehydrins (Sunderlikova and Wilhelm 2002) are expressed by large gene families with members that may be useful as markers of the physiological condition of somatic embryos. Later stages of plant development *in vitro* may also be monitored using RNA or protein-based markers. Antisense chalcone synthase was found to enhance adventitious rooting of walnut, probably by altering the flavonoid metabolism of microshoots and, thereby, auxin flow (El Euch et al. 1998).

Marker-assisted selection (MAS) includes a class of breeding decisions based at least partly on genotypic data. One common type of genomic data used in making breeding decisions is the mapping of QTL. Quantitative trait loci are loci affecting quantitative phenotypic traits that are mapped to a specific region of the genome. Typically, QTL are identified in the progeny of crosses between two phenotypically distinct parents. Most often, QTL mapping is performed in self-fertilizing species, but methods have been developed for outcrossing species as well (Cervantez-Martinez and Brown 2004). QTL are not genes but blocks of genes in linkage disequilibrium; that is, they cosegregate because they are physically linked to one another. Genes responsible for quantitative variation have been found within QTL (Fridman et al. 2000; Paran and Zamir 2003), but the genetic structure of QTL is often complex (Ross-Ibarra 2005) and QTL usually contain large numbers of genes (almost always >10), some of which are coordinately regulated (Thomson et al. 2006). QTL mapping requires a large number of mapped, DNA-based markers evenly spaced in the genome, an accurate pedigree, and accurate phenotypes for each of the members of the pedigree. Results from QTL analysis increase in reliability as more phenotypes are scored. Some QTL in hardwoods have been shown to be stable over time (Casasoli et al. 2004) and space (Scotti-Saintagne et al. 2004a), but other QTL have proven less stable (Tsarouhas et al. 2002). QTL can be stable across environments (Shepherd and Jones 2004) or show strong genotype × environment effects (Slate 2005). The case for practical molecular mapping in forest trees has been made (Wu et al. 2000), but maps and pedigrees are scarce commodities for many hardwood species. It is possible to map QTL in unmanipulated, natural populations (Slate 2005), but the interpretation of the results is not straightforward. Once QTL are located in one member of a family, they may sometimes collocate to orthologous map loci in related species or genera (Shepherd and Jones 2004; Gailing et al. 2005; Casasoli et al. 2006), but the stability of QTL across populations, especially populations under greatly different selective pressures, cannot be assumed (Slate 2005). Whereas QTL have been used in the improvement of a large number of crops, the practical and theoretical limitations of QTL in breeding are many (Xie and Xu 1997; Bernardo and Charcosset 2006). Other types of MAS include gene tagging (Bernatzky and Mulcahy 1992; Woeste et al. 1996b; Wang et al. 2004), identification of parents or progenitors of phenotypically valuable offspring (Akerman et al. 1995; Grattapaglia et al. 2004; Blenda et al. 2006), and whole genome selection backcross breeding (Kubisiak et al. 1997).

Forest tree breeders and conservation geneticists would like to be able to use the large amount of population genetic data now available to understand, conserve, and utilize the enormous phenotypic and adaptive variation of wild populations. Unfortunately, variability at neutral genetic markers and QTL cannot be simply translated into a measure of adaptive variation (Geburek 1997; Karhu et al. 1996; McKay and Latta 2002; Gonzalez-Martinez et al. 2006). Spitze (1993) defined a parameter Q_{st}, that is analogous to Wright's (1951) F_{st}, to describe the partitioning of quantitative genetic variation (not phenotypic variation) within and among subdivided populations. The "magnitude of the difference between Q_{st} and F_{st} can be used to infer the degree of local adaptation" of a population (McKay and Latta 2002; Storz 2002). In a meta-analysis, McKay and Latta (2002) found that populations can maintain substantial adaptive differences in spite of high levels of gene flow (a feature common in many hardwood forest species). Consequently, populations can be markedly different for adaptive traits, but have small differences in allele frequencies at QTL. The comparison of Q_{st} and F_{st} has been used to identify clonal variation in a population of fish and mammals (Storz 2002; Rogers and Bernatchez 2005), but the method requires careful application to avoid pitfalls (Waldmann et al. 2005). In addition to traditional common garden experiments and QTL, a number of new approaches for understanding the nature of quantitative phenotypic variation in natural populations have emerged, including the application of spatial analysis to patterns of genetic diversity (Escudero et al. 2003), an approach now called landscape genetics (Manel et al. 2003), association genetics (Neale and Savolainen 2004), and linkage disequilibrium mapping (see Ehrenreich and Purugganan 2006 and citations therein).

Association genetics (AG) is similar to QTL approaches and is more amenable to hardwoods because it does not rely on a structured pedigree, but instead analyzes the variation within an entire population (Neale and Savolainen 2004). Landscape genetics attempts to identify spatial patterns such as clines, isolation by distance, and discontinuities, and associate them with landscape or environmental features. In effect, the tools of molecular genetics are combined with biogeography and landscape ecology. Landscape genetics maps variation in allele frequency and correlates them with current or previous ecological variability or landscape features (Manel et al. 2003). Linkage disequilibrium (LD) mapping is an alternative to QTL for mapping adaptive genes. Originally developed for human genetics (Pritchard and Przeworski 2001), LD mapping is based on the LD of polymorphisms within a population with other polymorphisms that have functional effects. LD mapping can be applied to wild, unstructured, and unpedigreed populations, but its effectiveness is primarily determined by the rate of LD decay, which varies across species and, potentially, populations (Gonzalez-Martinez et al. 2006). Expression mapping is another technique for

identifying loci responsible for adaptive variation. Expression mapping produces eQTL based on whole genome arrays. The eQTL is a marker interval associated with transcriptional differences (Gibson and Weir 2005). The trend in genetic analysis is toward sequence-based markers such as ESTs or markers derived from ESTs, and single nucleotide polymorphisms (SNPs; Rafalski 2002), markers for which sequence variation can potentially be directly linked to phenotypic variance. The capacity of modern sequencing technology to generate genotypic data using SNPs and ESTs has led to what has been called a phenotype gap (Miflin 2000) that can only be filled by the expansion of phenomics. Whether LD mapping, QTL or SNP-based haplotyping approaches to MAS and molecular breeding are as successful as simple recurrent phenotypic selection may depend on what is learned about the genetic structure of quantitative variation (Morgante and Salamini 2003). A strictly quantitative approach to the conservation of forest tree genetic resources has also been described (Yanchuk 2001).

In Vitro Culture

Research on *in vitro* culture of hardwood species in a tree improvement program is usually conducted with the ultimate goal of clonally propagating mature, elite genotypes and producing plants on their own roots. Micropropagation of these genotypes may provide genetically uniform material for breeding and seed orchards, plantations, production of improved transgenic trees, and trees on their own roots may be more productive than grafted trees. However, rooting and acclimatization of microshoots of different genotypes on a commercial scale are limitations in the micropropagation of some temperate hardwood tree species (e.g., *Juglans nigra*, *Quercus rubra*, and *Castanea dentata*). *In vitro* culture can also be utilized for the development of regeneration systems (adventitious or embryogenic) for genetic modification, *in vitro* selection for disease and pest resistance, conservation of germ plasm (cryopreservation) of endangered or threatened tree species, and understanding the basic physiological and biochemical mechanisms involved in tree growth and development. *In vitro* culture may also provide a model system to study wood formation (Leitch and Bossinger 2004).

Micropropagation. Micropropagation can be defined as the *in vitro* clonal propagation of plants from shoot tips or nodal explants, usually with an accelerated proliferation of shoots during subcultures (Schaeffer 1990). Micropropagation of valuable temperate hardwood tree species has been successful using explants originating from seeds, seedlings, and young trees. These protocols, developed with juvenile explants, can then be useful in the development of *in vitro* propagation systems for mature, selected trees. Several species of valuable hardwoods have been successfully propagated using explants from grafted (mature scions) plants or mature trees. Most commercial-scale micropropagation of hardwood tree species has been successful with species or cultivars selected for nut production, rootstock quality, or ornamental characteristics (e.g., fall foliage, shape, hardiness) and not specifically for timber quality. Sycamore maple (*Acer pseudoplatanus*) can be micropropagated via a photoautotrophic system (Hennerty et al. 2001) and from stump sprouts (Rohr and Hanus 1987). *Alnus glutinosa* (European black alder) was micropropagated using shoot tips taken from fruit-bearing branches of a sexually mature tree (Lall et al. 2005). *Alnus cordata* (Italian alder) can be propagated using axillary bud explants from rooted stem cuttings originating from mature mother trees (Barghchi 1988). Cultivars of silver birch (*Betula pendula*) are commercially micropropagated, and protocols using mature tissue have been reported (Sarkilahti 1988; Chalupa 1989; Jones et al. 1996). *Castanea dentata* (American chestnut) was propagated from stump sprouts of a mature tree (Xing et al. 1997). European chestnut (*Castanea sativa*) is more responsive to micropropagation and rooting when basal shoot explants are taken from mature trees (Sanchez and Vieitez 1991; Sanchez et al. 1997; Fernandez-Lorenzo et al. 2005) or when serial grafting is used to reinvigorate a mature chestnut (Giovannelli and Giannini 2000). Mature American beech (*Fagus grandifolia*) can be propagated using shoot tips from root sprouts and dormant buds (Barker et al. 1997). Several researchers have reported on the factors influencing micropropagation of mature *Fagus sylvatica* (Nadel et al. 1991a, b; Meier and Reuther 1994; Meena and Ahuja 1996). Propagation of several selected clones of *Fraxinus excelsior* (European ash) was successful when buds were taken from grafted (mature scion) plants (Douglas 2001; Hennerty et al. 2001). Microshoots were also achieved from a mature ash tree (*F. excelsior*), and shoot buds developed when compound leaves from these microshoots were cultured (Hammatt 1994). *Fraxinus angustifolia* (narrow-leaved ash) has been micropropagated from mature shoot tips and nodal explants (Perez-Parron et al. 1994). Micropropagation of mature Persian walnuts has been pursued since 1984, with recent propagation reports for *Juglans regia* selected for nut production, rootstock, or timber production (Dolcet-Sanjuan et al. 2004; Vahdati et al. 2004). Progress with this species should be applicable to improving the *in vitro* propagation of mature black walnut (*J. nigra*; Stefan 1989; van Sambeek et al. 1997). A genotype effect was reported with sweetgum (*Liquidambar styraciflua*) micropropagation from mature selections (Sutter and Barker 1985). Wild cherry (*Prunus avium*) can be propagated from buds, shoot tips, and root suckers of mature trees (Hammatt and Grant 1993; Harrington et al. 1994;

Pevalek et al. 1994; Hammatt and Grant 1997; Hammatt et al. 1998; Hammatt 1999; Durkovic 2006). Mature clones (grafted plants) of *P. serotina* (black cherry) have been micropropagated and field tested (Tricoli et al. 1985; Maynard 1994). Several *Quercus* species (*Q. petraea*, *Q. robur*, *Q. rubra*, and *Q. suber*) have been micropropagated from mature explants (San-Jose et al. 1990; Romano et al. 1992; Juncker and Favre 1994; Sanchez et al. 1996; Chalupa 2000; Vidal et al. 2003). Black locust (*R. pseudoacacia*) can be propagated using dormant vegetative buds, shoot tips, and nodal segment explants of mature trees (Davis and Keathley 1987a, b; Kamlesh et al. 1995; Han et al. 1997; Nakatsubo et al. 2003). Plantlet regeneration has been achieved with *Tilia cordata* (small-leaved linden) using buds from mature trees (Youn et al. 1988; Mala et al. 2001). *Tilia platyphyllos* (large-leaved linden) has been propagated from mature trees by axillary shoot proliferation from nodal segments and shoot tips (Chalupa 2003). American elm (*Ulmus americana*) was micropropagated from nodal segments taken from shoot sprouts of root cuttings of a 36-yr-old American elm selection (Chanon et al. 1997). The use of micropropagated plants as rejuvenated stock for cutting propagation is becoming a standard for the economical cloning of hardwood trees. Although progress continues to be made in the micropropagation of mature temperate hardwood tree species, further research is needed to clonally multiply, root, and acclimatize these species efficiently on a commercial scale.

Somatic embryogenesis. In plant culture, somatic embryogenesis is the process of embryo initiation and development from vegetative or nongametic cells (Schaeffer 1990). Most somatic embryos (SE) are initiated using juvenile tissue (immature and mature zygotic embryos). There have been numerous journal reports, book chapters, and proceedings published on the successful initiation of SE from temperate hardwood tree species (too numerous to do the subject justice in this review). Table 1 highlights recent advances (the last 10 yr) in somatic embryogenesis of some important temperate hardwood species (*see* also reviews by Wilhelm 2000; Merkle and Nairn 2005; Nehra et al. 2005). Induction frequency of SE can be low or high, but maintenance of embryogenic cell lines, maturation, conversion, and acclimatization of plants at high frequency from these SE can be problematic for several species. It is also well known that genotype influences the induction of SE. Long-term maintenance of repetitive embryogenic cultures, progress in plantlet regeneration, and initiation of SE from mature tissue could provide a means for mass propagation and genetic modification of superior timber species.

Adventitious shoot regeneration. Adventitious has been defined as the development from unusual points of origin, such as shoots or root tissues from callus, or embryos from sources other than zygotes (Schaeffer 1990). Adventitious shoot production is undesirable for clonal propagation because of the possibility of somaclonal variation. However, advances in the development of protocols for adventitious shoot regeneration, rooting, and acclimatization of plants will be applicable for the genetic modification and improvement of selected timber tree species. Adventitious shoots of sycamore maple (*A. pseudoplatanus*) can be regenerated from zygotic embryo explants and plants acclimatized under high humidity (Wilhelm 1999). European birch (*B. pendula*) has been regenerated from leaf explants from a mature tree (Leege and Tripepi 1993). Immature and mature seeds were used for adventitious regeneration of plants of *Fraxinus americana* and *F. excelsior* (Bates et al. 1992; Tabrett and Hammatt, 1992). Regeneration of plants through bud or shoot organogenesis from mature embryonic explants was achieved with *F. angustifolia* (Tonon et al. 2001). Plants have been regenerated from leaves and internodal sections of cultivars of *P. avium* (Bhagwat and Lane 2004; Matt and Jehle 2005). Plants regenerated from leaves of *in vitro* shoot cultures of *P. serotina* survived acclimatization and overwintering in cold storage (Espinosa et al. 2006). Barghchi and Chi (1998) reported the regeneration of plants from various explant types from seedlings of *R. pseudoacacia* grown *in vitro*. Lyyra et al. (2006) regenerated black willow (*S. nigra*) plants from unexpanded inflorescence explants excised from dormant buds of mature trees.

Cryopreservation. Cryopreservation is the ultralow temperature (−196°C) storage of cells, tissues, embryos, or seeds (Schaeffer 1990), where biochemical and most physical processes are completely arrested. Cryopreservation involves multiple steps to be successful (choice of material, pretreatment, freezing, storage, thawing, and post-treatment handling) for each species. Cryopreservation has several advantages in a tree improvement program, such as the long-term storage of valuable germ plasm, pollen, genetically transformed lines, and recalcitrant seeds, and it also allows propagation of elite genotypes throughout the year. A recent report describes the procedures most commonly used in the cryopreservation of crops and forest trees (Panis and Lambardi 2005). Ryynanen and Aronen (2005) reported no genetic or phenotypic changes in the short- and long-term culture and cryopreservation of birch (*B. pendula*). Pecan (*Carya illinoinensis*) pollen stored for 1–13 yr in liquid nitrogen showed no diminished viability, and the morphology of pollen grains and the germ tube was normal compared to freshly collected pollen (Sparks and Yates 2002). Plants of *C. sativa* can be recovered after cryopreservation of *in vitro* grown shoot apices using vitrification (Vidal et al. 2005). Shoot tips of ash (*F. excelsior*) grown *in vitro* were successfully cryopreserved and a mean regrowth

Table 1. Recent advances (1996–2006) in somatic embryogenesis of some important temperate hardwood species

Species	Explant tissue	Results or Information	References
Betula pendula	SE	Bioreactor development	Hvoslef-Eide et al. 2005
Carya illinoinensis	Immature ZE	Field and Molecular evaluation	Vendrame et al. 2000
Castanea dentata	Ovules; immature ZE	Somatic seedling production	Andrade and Merkle 2005
Fagus sylvatica	Immature ZE, embryogenic callus	SE; plants	Naujoks 2003; Vieitez et al. 2003
Fraxinus angustifolia	Immature ZE	Plants; synchronous SE	Tonon et al. 2001a, b
Juglans nigra	Cotyledons of immature seeds	SE	Bosela et al. 2004; Steger and Preece 2003
Juglans regia	Immature ZE; mature embryos	SE; plants; flowering; desiccation	Breton et al. 2004; Dumanoglu 2000; Kaur et al. 2006; Sanchez-Zamora et al. 2006; Tang et al. 2000
Quercus petraea	SE	Inhibition of phenolic biosynthesis	Cvikrova et al. 2003
Quercus robur	Leaves (mature tree); ZE	Plants; encapsulation; physiology; histology, RAPD analysis	Chalupa 2000; Prewein and Wilhelm 2003; Prewein et al. 2006; Valladares et al. 2006; Zegzouti et al. 2001
Quercus rubra	Cotyledons of immature seeds	SE	Bosela et al. 2004
Quercus suber	Leaves (mature tree); SE	SE; plants; ploidy stability; SE; histology; heat shock proteins	Garcia-Martin et al. 2005; Loureiro et al. 2005; Puigderrajols et al. 2000, 2002.
Robinia pseudoacacia	Mature seeds	SE; embryogenic callus	Barghchi and Chi 1998
Tilia cordata	Immature cotyledonary embryos	Anatomical SE development	Karkonen 2000
Tilia platyphyllos	Zygotic embryos	SE; plants	Chalupa 2003

See also Merkle and Nairn 2005; Nehra et al. 2005; and Wilhelm 2000. References cited within these papers and citations in previous reviews are not reported here.
SE=somatic embryos; ZE=zygotic embryos

of 67% was achieved for selected mature trees (Schoenweiss et al. 2005). Somatic seedlings were regenerated from *L. tulipifera* cultures stored in liquid nitrogen for 48 h (Vendrame et al. 2001). Verleysen et al. (2005) reported the successful cryopreservation of *R. pseudoacacia* via vitrification and encapsulation-dehydration. Seeds of *T. cordata* show 65–75% seedling emergence after freezing in liquid nitrogen, if seeds are dried to 11–20% moisture content and scarified before freezing (Chmielarz 2002). Dormant buds of three elm species (*U. glabra*, *U. laevis*, and *U. minor*) were collected in nine European countries and successfully cryopreserved (Harvengt et al. 2004). The cryopreservation of these 444 elm clones had no negative effect on the viability and regrowth potential of frozen buds.

Genetic Transformation

Genetic engineering provides an opportunity to modify tree species to enhance productivity and increase resistance to diseases (Powell et al. 2006), insects, and environmental stress, thereby complementing conventional breeding and selection programs. The tree improvement strategy involves both short- and long-term measures for ensuring an immediate and sustained supply of quality planting stock. The potential also exists to allow precision improvement of individual traits in forest trees without losing the unique combination of traits in the parental line. Several major goals for the genetic improvement of temperate hardwood tree species are the development of genotypes having traits such as time to maturity, resistance to biotic and abiotic stress, and desirable tree growth and wood quality. Gene transfer in several hardwood species has been recently and thoroughly reviewed and will not be duplicated here (Merkle and Nairn 2005; Nehra et al. 2005). Only recent advances or species not covered in these reviews will be discussed.

Species of *Alnus*, mainly *A. incana* and *A. glutinosa*, were reported to be amenable to transformation via electroporation of protoplasts. An in-depth study of the transformation parameters suggested that higher voltages applied during electroporation in the presence of higher DNA concentra-

tions of the plasmid produced more β-glucuronidase (GUS) activity from fewer surviving cells (Seguin and Lalonde 1988). Use of the *Agrobacterium tumefaciens* strain Ach5 for the transformation of nine clones of *Alnus* was demonstrated by Mackay et al. (1988). Valjakka et al. (2000) developed a protocol for transferring the *nptII* and *RbcS* genes into *B. pendula* using particle bombardment. Genotypic variation played a significant role in influencing DNA delivery. Only a single clone could be transformed and regenerated with the *RbcS* gene with a transformation frequency of 6 % as confirmed by Southern analysis; whereas only transgenic callus developed from the rest of the clones. Critical parameters for optimal gene delivery using the Helios™ gene gun for *B. pendula* were studied by Helenius et al. (2000). Genetic constructs contained the luciferase (LUC) and GUS genes. Helium pressure and the size of the gold particles played a crucial role in transferring DNA into the cells of the leaf explants. Significant transient gene expression was observed using 0.04 μg DNA per shot. An insecticidal peptide gene was transferred into birch using leaf, stem, and leaf stalk segments with GUS as the reporter gene (Zhan et al. 2001). GUS analysis revealed that 34 % of kanamycin-resistant plants had GUS activity. A co-inoculation methodology was used for the transformation of silver birch (Aronen et al. 2002). Two strains, 82.139 and C58C1, both with the pGUSINT, were used to co-inoculate seedlings and *in vitro* shoot cultures. It was observed that the higher the concentrations of the 82.139 strain in the inoculation mixture, the greater the gall and shoot formation frequencies under greenhouse and *in vitro* conditions. Although no transgenic plant recovery could be obtained, shoots that regenerated from the infected explants showed the integration of the T-DNA as verified by polymerase chain reaction (PCR). Lemmetyinen et al. (2004) showed that flowering could be prevented in *B. pendula* through the use of the *BARNASE* gene under the control of the *BpMADS1* promoter. Inflorescences did not form or aborted early in the transformed plants. Inflorescences that did develop were without stamens or pistils. However, the construct caused unwanted changes in vegetative development such as, bonsai-like growth, increased branching, absence of axillary buds, and darkening of leaves. Pecan genotypes were evaluated for the production of transformed plants using *A. tumefaciens* strain EHA101 containing the APH3'II and GUS genes (McGranahan et al. 1993). Variations in GUS activity were observed between two induction media and kanamycin concentrations did not affect the recovery percentage of transformed embryos. The study also confirmed that a variation exists among pecan genotypes toward transformation and production of transgenic embryos, and somatic embryos of pecan can serve as potential target tissue for transformation. Further contributions toward improvement of pecan breeding and development will be associated with the identification of genes for insect and disease resistance, increased yield, and nut quality (Vendrame and Wetzstein 2005).

Advancement in chestnut transformation was mainly observed by choosing marker, reporter genes, and alternate explants such as pollen. Polin et al. (2006) used a construct with three genes (*gfp*, *bar*, and oxalate oxidase) for transformation of American chestnut somatic embryos. This was the first report on the successful regeneration of transgenic American chestnut somatic embryos with normal plant development. The expression of the oxalate oxidase gene was detected in one transgenic line. Later, transgenic plantlets were successfully acclimatized and two were transferred into the field (Maynard, personal communication). When pollen was used as the target explant for gene transfer (Fernando et al. 2006) the main objective was to use viable transgenic pollen for artificial pollination and fertilization of receptive female flowers. A pBIN *35S-mgfp5-ER* plasmid construct was used and the DNA was transferred via particle bombardment. Because transgenic pollen was the goal, parameters that influenced transformation mainly target distance and helium pressure, and the developmental stage of pollen was optimized utilizing GFP expression. Conventional chestnut breeding requires superior cultivars, and genetic engineering offers a potential means to overcome factors that limit its breeding. Because the demand for the crop has surpassed the supply, there is an immediate need to develop insect- and disease-resistant genotypes, and high-yielding *Castanea* clones (Vieitez and Merkle 2005). One other temperate hardwood species that required transfer of fungal resistance genes, other than chestnut, was elm. Successful transformation of Chinese elm (*Ulmus parvifolia*) was reported by Aziz et al. (2003) using hypocotyl-derived callus as explants and phosphinothricin as the selection agent. Production of buds and shoots differed from one callus explant to another. However, eight out of ten putatively transformed tissues displayed amplification of the *bar* gene as confirmed by PCR analysis. Newhouse et al. (2006) developed a leaf piece-based transformation system for American elm. Transgenic elm plantlets containing an antimicrobial peptide gene (Powell et al. 1995, 2000) were regenerated (Powell, personal communication). Because walnuts are difficult to root using conventional methodologies, *Agrobacterium rhizogenes* was used to infect microcuttings of *J. regia* (Falasca et al. 2000). Infected cuttings showed a high degree of rhizogenesis. Bacteria were present in the roots and the roots were chimeric. Similarly, *rol*ABC genes were transferred into a hybrid walnut (*J. hindsii* × *J. regia*) rootstock to improve the rooting potential (Vahdati et al. 2002). Transgenic subclones budded onto *J. regia* seedling rootstocks resulted in growth of trees with reduced internode length and increased lateral branching with wrinkled leaves.

After grafting scions of *J. regia* onto transformed and nontransformed cuttings, the rooting potential was studied and compared to the controls. The transformed cuttings showed poor rooting potential both *in vitro* and in the greenhouse, despite the fact that the *rol*ABC genes altered the growth characteristics and produced a fibrous root system. Influence of the selection agent kanamycin at low and stringent frequencies on somatic embryo production in black walnut (*J. nigra*) was studied by Bosela et al. (2004). The presence of kanamycin in the selection medium reduced the proliferation of embryos and a large number of chimeric secondary embryos were obtained. Fewer than 10 % of the initial secondary embryos were wholly transgenic and they were essential for the initiation of stable transgenic lines. Emphasis is being placed on *J. regia* transformation for nutritional enhancement, altering oil and fat composition, and improvement of shelf life (Dandekar et al. 2002). Although *J. regia* transformation has become routine through a somatic embryogenesis regeneration system, the industry's acceptance of transgenic scion production has decreased (Mehlenbacher 2003). Genetic engineering of *J. regia* has resulted in the production of walnuts resistant to codling moth, crown gall disease, and commercially important rootstock problems (Dandekar et al. 2005). An understanding of walnut physiology and metabolism has presented additional opportunities for improving timber and kernel traits.

Susceptibility of *P. avium* to six different wild-type *Agrobacterium* strains was analyzed by Brasileiro et al. (1991). Micropropagated shoots infected with the nopaline strains C58, 84.5, and 82.139 developed tumors, which subsequently produced shoots. These shoots were of two different types and those with a normal morphology rooted, whereas the abnormal shoots did not root. Meristem-tips propagated *in vitro* from microshoots of *P. avium* (sweet cherry) cv. Summit were transformed via particle bombardment using the plasmid pUC 18 *basta-gus* (Druart et al. 1998). Most of the bombarded meristems produced transformed shoots, which were cloned by axillary branching. Transformation status of the shoots was observed through GUS reaction on leaves or shoots. Buds and rosettes developing from the meristems and stems from the clones also showed transient GUS activity. Another target gene for transformation was the *ipt* gene that was transferred into callus lines of *P. avium* (Grant et al. 1998). Among the several transformed callus lines obtained, only one line developed shoots with an abnormal morphology, but was observed to produce nopaline. Pratesi et al. (2004) studied the competence of various tissues to *Agrobacterium* infection for two genotypes of *P. avium*. Stem tissues were more amenable to transformation than were petioles and leaves, and post-inoculation culture conditions influenced GUS expression notably in the leaf explants. The use of wild cherry for silviculture and timber production has been reduced considerably because of the breeding of available stock for fruit production and ease of collection (Burley 2004). Trees selected for timber will need to exhibit different growth features such as minimal branching, vigorous apical growth, and less susceptibility toward bacterial canker. In addition, the genetic improvement of *Prunus* genotypes that can tolerate adverse biotic and abiotic conditions would help growers and at the same time deliver products much appreciated by consumers. An encouraging feature throughout the transformation studies in *Q. suber* was the advantage of somatic embryogenesis for the routine production of transgenic plants (Sánchez et al. 2005). As evaluated by PCR, 5.8 % of surviving pro-embryos were GUS-positive after infection with *A. tumefaciens*. A salt-tolerant gene, betaine aldehyde dehydrogenase (*badh*), from *Atriplex hortensis*, was transferred into black locust via *A. tumefaciens* using callus as explants. PCR and Southern analysis revealed the integration of the transgene into the genome of regenerated plants and the surviving plants exhibited an increase in NaCl resistance (Xia et al. 2004).

Transgenic Hardwoods in the Field

The number of field trials of transgenes expressed in hardwoods is dwarfed by the number for transgenic crops such as maize and canola. Despite considerable regulatory hurdles for transgenic trees, reports from trials of transgenic hardwoods have appeared over the past two decades or so. Aside from poplar, the most transgenic research in field trials has been with silver birch. Silver birch clones expressing *rol* and *aux* genes under the regulation of endogenous promoters showed alterations in anatomy, morphology, and physiology when grown in a greenhouse (Piispanen et al. 2003). In the field, silver birch expressing chitinase IV (from sugar beet) were no more resistant to leaf spot disease than control plants, but did have improved resistance to birch rust (Pasonen et al. 2004). The same gene (chitinase), when expressed in birch, affected the numbers of some soil-dwelling, leaf-decomposing organisms, but not others (Kotilainen et al. 2004). Silver birch has also been used to show that barnase constructs can be used to ablate flowering, reducing the risk of transgene spread and altering carbon allocation to vegetative growth (Lannenpaa et al. 2005). Insect resistance and disease resistance are common goals for transgenic technology, and both have been achieved in *Juglans* (Leslie et al. 2001; Escobar et al. 2002). American chestnut transgenic plantlets containing the *Oxo* gene (Polin et al. 2006) have also been planted in the field (Maynard, personal communication). Research into the control of Dutch elm disease in English

elm (*Ulmus procera*) has led to some of the most advanced applications of transgenic technologies to forest trees (Gartland et al. 2005). Field trials of transgenic American elm (*U. americana*) are also underway (Powell, personal communication). The potential for using transgenic hardwoods in phytoremediation has also been demonstrated (Rugh et al. 1998).

Current Challenges and Future Opportunities

New knowledge of genes for important economic traits. With the completion of the first tree genome sequencing project with *Populus* spp. in 2004 and the potential for comparative genetics with other hardwood species, new knowledge of tree genes and their function will enable researchers to gain a better understanding of the genes for economic traits in fine hardwoods. In particular, genes associated with heartwood formation, insect and disease resistance, precocity, branching, straightness, and specialty traits such as phytoremediation will hasten both classical and molecular breeding efforts. For example, with heartwood formation, on average, 12 to 13 yr of sapwood is produced before heartwood production. For both lumber and veneer production, additional steps are needed to treat sapwood to induce colorization that closely resembles that found in heartwood. The potential exists to reduce energy inputs and processing steps if trees could be produced with significantly greater heartwood-to-sapwood than found in native trees.

The case for increased insect resistance exists with the Emerald ash borer (EAB), an exotic insect introduced through shipping channels to Detroit, MI ports, that is currently devastating native U.S. ash populations. It appears that no native populations have genetic resistance. Chemical controls are being tested and deployed, but will only be a means of protecting urban trees, not forest trees. To date, parasitic fungi and insects have not been identified in the US. Thus, this may be the first case where the use of transgenic insect-resistant ash may be publicly accepted. It has been reported that the potential economic impact is over $80 M, partly as a result of the wide use of ash as an urban street tree, in many cases, one of every three urban trees planted. The use of *Bt* toxins is undergoing laboratory testing (Meilan, personal communication) and two toxins have shown promise after preliminary testing. Pijut (unpublished results) is developing tissue culture and transgenic technologies that will allow the insertion of genes for insect resistance and the multiplication of these trees for testing the efficacy and stability of the transgenes. Although it has never caused increased resistance to *Bt* in insect populations (Christou et al. 2006) except in laboratory conditions (Tabashnik et al. 2003), it has been theorized by some (Christou et al. 2006; Ferry et al. 2006) that single *Bt* transgenes could quickly lead to *Bt* resistance in insect populations. To minimize this possibility, it has been proposed that inserting multiple *Bt* genes would limit enhanced insect resistance because selection would need to occur at multiple loci instead of one locus. By adding a second *Bt* transgene the chance of producing resistant insect populations is significantly reduced (Jackson et al. 2004). To reduce the chance of inducing resistance to *Bt*, novel insecticidal proteins are being tested. These biological compounds can cause increased binding of insecticidal proteins, function with novel transmembrane carrier proteins, or produce novel toxins from other insect pathogens, e.g., *Photorhabdus* and *Xenohabdus* spp.

Exotic diseases, including butternut canker, also threaten extinction of important hardwood species. In the case of butternut, the existence of native resistant trees is very limited, and a few dark-bark phenotypes with apparent resistance have been identified. Although some vigorous butternut-type trees have been identified, in most cases these are hybrid trees with Japanese walnut, a species introduced in the U.S. in the 19th century. It is suspected that these hybrids are harboring resistance genes to the canker while displaying vigorous growth typical of F1 hybrids of *Juglans* spp. Collection and screening of putatively resistant trees, breeding, clonal multiplication, and forest restoration holds promise to prevent butternut extinction. Many private landowners desire multiple uses of their hardwood forests. Many times, timber production ranks far below that of wildlife viewing and wildlife habitat. These landowners would achieve desired ecological characteristics of newly planted forests decades earlier if forest geneticists had greater control over precocity. Efforts are underway (Woeste, personal communication) to select early flowering genotypes that could speed mast production in young plantations. If achieved, landowners could benefit from increased wildlife habitat while not sacrificing timber production and quality.

Rapid breeding. It has long been recognized that classical breeding of fine hardwoods is limited by long reproductive cycles. Except in cases where physiological manipulations are used, many of the fine hardwoods do not consistently flower for several decades when grown from seed, thus severely limiting the number of generations of genetic improvement that can be achieved in a breeder's career. Thus, any molecular or physiological tools that can quicken this cycle would offer significant benefit. Woeste, Struve, and Coggeshall (personal communication) are testing root restriction and trellising as tools to induce early flowering on seedlings and grafted trees of black walnut. These methods have the potential to reduce the time for breeding generations to a decade or less, thus doubling the amount of

genetic improvement that can be achieved in the traditional timeframe. Molecular methods such as determining QTL have the potential to track genes for economic traits in breeding populations, thus increasing the efficiency of breeding and selection. Although not widely practiced, technologies are available to link molecular markers to genes for both economic and adaptive traits in hardwoods. In theory, this could reduce the time required and size of progeny tests that are associated with tree breeding both for production of sustainable families and clones. In addition, association mapping can give us clues to genes associated with adaptive traits, such as those that will be important for adaptation of species to climate change.

Genomics. For those in research and development of gene sequencing technologies, engineers in the field are working toward the goal of sequencing genomes for $1,000. It is envisioned that one day, single genomes can be quickly sequenced to uncover underlying genetic makeup that predisposes that individual to genotypic differences within a species. Although this will be a few years away, important advances have recently been made to significantly reduce labor needed to sequence a genome and the associated cost. One such technology uses emulsion-based PCR with solid-phase sequencing (Marusina 2006). With these recent advances, costs have been decreased by a hundredfold. In the next decade, it could become possible to economically sequence any species of interest in short order. Methods are available to pyramid transgenes that allow for insertion and expression of multiple genes for both similar and dissimilar traits. If those transgenes prove to have stable expression over time, the value to transgenic technology will increase from the ability to improve single traits to improvement of multiple traits at one time.

Obstacles to clonal multiplication and transformation. For the most part, it is still difficult to transform elite genetic lines and adult plant material. For transformation of clonal materials, this presents some unique obstacles. By the time that genetic tests have been completed and selections are made, clonal lines will have entered their adult phase of growth. Some tools are available, such as tissue culture, grafting, and tree decapitation that partially rejuvenate selected trees, as well as cryopreservation, but success to date is still limited. For most hardwood species, successful transformation is still limited to seedling stocks. Molecular tools are available that will allow the elucidation of the regulation of genes that control the phase change from juvenile to mature growth and at the same time help us to understand how maturity restricts somatic cell duplication.

Regulation of transgenic trees. As of this date, no transgenic tree, except for virus-resistant papaya, has been given regulatory approval that would allow commercial deployment. In this case, without virus resistance, commercial papaya production in Hawaii was going to eventually cease. Risks of transgenic papaya were going to be minimal because of natural barriers to gene flow external to the Hawaiian Islands. Another important factor was that owners of intellectual property that needed to be considered with this crop were willing to cooperate with all parties and waive their rights to financial gain from deployment. In the future, the ability to deploy new transgenic forest trees that are minor crops could benefit from this precedent. Otherwise, the cost of intellectual property or the ability to use it may be too high a hurdle that will prevent deployment.

The Animal Plant Health and Inspection Service is working with other government regulators (Food and Drug Administration and Environmental Protection Agency) and the interested public to craft rules that will guide data collection and analysis, disclosure, and potential regulatory approval. Meanwhile, scientists are performing studies to assess risks and benefits associated with release of various classes of transgenics that will help guide the regulatory community. One generally accepted means to reduce the risks of transgenic deployment is flowering control. It is expected that most deployed transgenic trees will need flowering control to receive regulatory approval. It is interesting to note that one case where it may not be required is with transgenic resistance to EAB. If it continues to be the case that resistance is not found in the native ash population and effective biological controls are not identified, the public might accept the flow of resistance genes into the native population to regenerate ash in forests with resistance to EAB. Despite control of gene flow or sound reasons for transgene flow to occur, the public may still resist acceptance of transgenic trees in the near future despite regulatory framework and sound scientific data on efficacy.

The use of flowering control in transgenic trees will require the use of clonal production methods. With some species, this does not appear to be an insurmountable hurdle. With other hardwood species, maturation is a severe limitation that leads to recalcitrance. This hurdle could be overcome by identifying the genetic basis of maturation, which might lead to rejuvenation of explants that would be more amenable to tissue culture manipulations.

Individual tree identity and bioinformation. One challenge, although not biological, but equally important to hardwood breeders, is the inventory and categorical classification of breeding stock. Taking advantage of new engineering breakthroughs, Woeste (personal communication) is developing radio frequency identification tags for use in both identifying individual trees and for storage of important genetic data for efficient recall. When developed and refined, the breeder will be able to attach microscopic tags

to parent trees that will retain important historical growth records for instant recall. Another associated benefit will be the ability of the landowner to track inventory data and individual tree identity, the latter which becomes important in cases of timber theft. Further, these tags could also be utilized in commercial log inventory, allowing the tracking of inventory from the forest stand, to the log yard, and to the sawmill.

Conclusions

The improvement of temperate hardwoods for reforestation and plantations has advanced considerably in the past decade, but progress still lags behind that of agronomic crops. Trees are valuable resources and provide environmental services that must be managed and enhanced for productivity in a sustainable fashion. Breeding, genetic modification, propagation, and deployment of trees with traits such as disease and pest resistance, improved wood quality, reproductive changes, superior growth, form, and timber characteristics will help in the establishment, management, preservation, and production of valuable hardwood species for future generations. Over the next few decades, these new technologies promise to enhance and expand the toolkit available to the tree improvement specialist.

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