Genomics, Domestication, and Evolution of Forest Trees

R. SEDEROFF,¹ A. MYBURG,² AND M. KIRST³

¹Forest Biotechnology Group, North Carolina State University, Raleigh, North Carolina 27695; ²Department of Genetics, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, 0002, South Africa;

³School of Forest Resources and Conservation, University of Florida, Gainesville, Florida 32611

Correspondence: ron_sederoff@ncsu.edu

The forests of the world continue to be threatened by climate change, population growth, and loss to agriculture. Our ability to conserve natural forests and to meet the increasing demand for fuel, biomass, wood, and paper depends on our fundamental understanding of tree growth and adaptation (FAO 2001; Fenning and Gershenzon 2002; Campbell et al. 2003; Gray et al. 2006). Our knowledge of the unique biology of trees will be greatly advanced through the application of genomics. The purpose of this chapter is to describe this emergent genomic paradigm as it is being applied to trees.

Genomics provides a platform to learn the relationships of genes and phenotypes and to integrate molecular and quantitative genetics for the analysis of adaptive traits (Frewen et al. 2000; Wullschlegel et al. 2002; Brunner et al. 2004). Genomics provides answers to fundamental questions of tree biology and addresses practical problems in tree plantations, forest management, and conservation. Genomic association of natural allelic variation with improved growth and wood properties is being incorporated into tree-breeding programs through allele-specific evaluation and selection (Neale 2007; Grattapaglia et al. 2009). The following are four main areas of genome-related investigation in forest trees.

1. **Fundamental biology of growth, metabolism, and development.** Genomic analysis elucidates the mechanisms regulating the vascular cambium, the lateral meristem (stem cell layer) that produces wood. There is also considerable interest in the control of flowering and dormancy, because of the adaptive significance of these traits. Abundant flowering and short growing seasons reduce wood yield and biomass. The ability to induce flowering can accelerate breeding.
2. **Abiotic stress.** Our understanding of the mechanisms of tree responses to abiotic stress can be expanded by genomic analysis. Stresses may be due to water and temperature or the effects of environmental chemistry or environmental pollution. Abiotic stress is a major consequence of climate change, and the understanding of the genetic architecture of stress responses is essential for predicting adaptation in forests.
3. **Pests and pathogens.** Understanding the interactions of trees with their symbionts, pathogens, and pests is important for predicting the biological consequences of climate change and for developing more tolerant plantation trees. Trees, pathogens, and pests are co-evolving systems that often determine the fates of their ecosystems. Pest and pathogen interactions can now be explored at a genomic level to ameliorate ecosys-

tem threats (Steiner and Carlson 2004; Ralph et al. 2006a,b; Whitham et al. 2006).

4. **Adaptation and evolution.** Genomics provides valuable information about genetic diversity of trees, its distribution, and effects on adaptation and evolution (Neale and Ingvarsson 2008). This knowledge is being applied to forest management and conservation in the context of past history and predicted change in climate.

Molecular genetic studies of forest trees have followed three main approaches: (1) Comparative analysis of trees with *Arabidopsis* and model crops has been used to identify functional genetic conservation and to expand our understanding of the similarities and differences between woody and herbaceous plants. (2) Transgenic technology in trees has allowed a reverse genetics approach, using activation or suppression of gene expression to investigate function. (3) Forward genetics approaches, based on quantitative and qualitative analysis of natural variation, have been used to understand the genetic architecture and molecular basis of specific traits.

FOREST TREE DIVERSITY AND DOMESTICATION

In contrast to many of our herbaceous crops that have been bred and selected for thousands of generations, forest trees are essentially undomesticated. They are also characterized by high genetic diversity and high genetic load (Hamrick et al. 1992; Remington and O'Malley 2000). An exception, red pine, exhibits low genetic diversity, attributed to a bottleneck in its recent evolution (DeVerno and Mosseler 1997). Clones of quaking aspen (*Populus tremuloides*) are found in massive monoclonal stands, consisting of tens of thousands of genetically identical trees, all connected through a massive underground root system. One such clone has been described as the largest single plant and potentially the largest organism on earth (Mitton and Grant 1996).

The high levels of genetic diversity can be exploited for tree breeding (Zobel and Talbert 1984). Trees used for plantation forestry are very much like their natural counterparts. Early domestication in annual crops such as maize is thought to have involved relatively small numbers of genetic events (Doebley et al. 1997; Doebley and Lukens 1998), followed by rapid changes in allele frequencies at loci that affect plant architecture and biochemistry (Lev-Yadun et al. 2002; Jaenicke-Després et al. 2003; J. Doebley, pers. comm.). Major genetic changes of a kind that have accompanied domestication of cereals, for example, have not yet occurred in forest trees. It is not yet clear what kind of genetic changes might become associated with tree domestication (Boerjan 2005).

WOOD DEVELOPMENT AND EVOLUTION

Wood provides the basis for the mechanical support and water transport that allow trees to attain great age and size. Wood formation (xylogenesis) takes place through cell division and differentiation of the vascular cambium, a secondary lateral meristem in the stems, branches, and roots of woody plants. Cambial initials divide and differentiate into a vertical support and water transport system consisting of tracheids in gymnosperms or fibers and vessels in angiosperms. A lateral transport system is predominantly composed of rays (Larson 1994). Growth rate slows as trees age, thought to be a consequence of the physiological burden of increasing size (Mencuccini et al. 2007). With abundant water and nutrients, absence of biotic and abiotic stresses, and competition for light, trees may have the potential to grow as high as 120 m (Koch et al. 2004). The current tallest tree—a costal redwood—(*Hyperion*) is 115 m. Records of *Eucalyptus regnans*

from the 19th century exceed this height by more than 30 m.

Vascular plants are derived from a rootless leafless freshwater filamentous alga in the genus *Cooksonia*, ~400 million years ago (mya) (Chaloner and Macdonald 1980). Five extant plant lines derived from this early ancestor are separated into two major groups, the angiosperms and the gymnosperms (Fig. 1). The five lines are the Gnetales, cycads, ginkgos, conifers (all gymnosperms), and angiosperms (Qui et al. 2007). Angiosperms and gymnosperms last shared a common ancestor ~300 mya (Bowe et al. 2000). The angiosperms include the largest number (250,000+) and the most diverse number of species, with both herbaceous and woody plants, and include all the major food crops. The gymnosperms include only ~1000 species, but a small number of those dominate many of the Northern hemisphere's temperate forests. All gymnosperms are woody plants.

Small numbers of genetic changes may be involved in the transition between herbaceous and woody plants. DNA sequence relationships support the relatively recent evolution of woody plants found on islands descending from continental herbaceous founders (Böhle et al. 1996; Helfgott et al. 2000). *Arabidopsis thaliana*, typically small, fast growing, and herbaceous, can be induced to form secondary xylem (wood) by delaying flowering and senescence (Lev-Yadun 1994; Dolan and Roberts 1995; Chaffey et al. 2002). The genes that determine the woody growth habit appear not to be unique to woody plants but are likely to be common genes, regulated in different ways (Groover 2005). A suite of transcription factors that regulate secondary cell wall formation in xylem vessel and fiber cells have recently been characterized in *Arabidopsis* (Zhong et al. 2008) and may perform similar functions in woody plants.

Figure 1. Simplified phylogeny of the major groups of woody plants. Species for which genome sequencing has been completed, or is under way, are indicated by DNA helices. The position of the Gnetales in the seed plant phylogeny has not been resolved. Some studies place Gnetales as a sister group to the Coniferales or the Pinaceae, but a phytochrome phylogeny placed it at the base of the gymnosperms (Chaw et al. 2000; Magallón and Sanderson 2002; Schmidt and Schneider-Poetsch 2002; Burleigh and Mathews 2004; de la Torre et al. 2006).

APPLICATION OF GENOMICS IN FOREST TREES

Although they are large, unwieldy, and have long generation times, many features of trees make them suitable for genomic studies. Trees provide excellent material for genome-wide studies of adaptation and evolution in natural populations. Genomics has also provided insight into the molecular basis of tree growth (Kirst et al. 2004a; Poke et al. 2005; Grattapaglia et al. 2009), development (Hertzberg et al. 2001; Schrader et al. 2004; Ko et al. 2006), adaptation (Yang and Loopstra 2005), and the evolution of herbaceous and woody growth habits (Kirst et al. 2003; Tuskan et al. 2006). The molecular basis of wood formation, the chemical diversity of woody plants, and the basis for extensive longevity in forest trees are topics being addressed. Genomics has also provided a paradigm for the investigation of the molecular basis of complex traits in forest trees, by associating genotype with phenotype. A more complete understanding of this relationship leads to predictive models of growth and adaptation of individual organisms and, perhaps, of species interactions and ecosystems.

Traditionally, forest genetics was built on three approaches: (1) studies of natural variation, particularly, for population and quantitative genetics, (2) micropropagation directed to plantation forestry, and (3) traditional biochemistry for studies of enzymes and metabolites, particularly for secondary metabolism. Early genetic studies of trees focused on quantitative trait variation in populations, using long-term crosses for the purpose of improving breeding material (Zobel and Talbert 1984). Genetic

studies of trees focused on isozymes and population genetics (Adams et al. 1992; White et al. 2007).

The advent of DNA-based molecular markers led to the first genomic maps of forest trees (Bradshaw et al. 1994; Devey et al. 1994). Genetic analysis was greatly facilitated by the simplified segregation in the haploid megagametophyte of conifers and by the availability of polymerase chain reaction (PCR)-based molecular markers. This led to single-tree genetic maps (Tulsieram et al. 1992; Grattapaglia and Sederoff 1994; O'Malley et al. 1996) and quantitative trait mapping, e.g., to identify loci that control host resistance to fusiform rust disease in pines (Wilcox et al. 1996). Dozens of genetic maps have since been made for forest trees (Kirst et al. 2004b; <http://dendrome.ucdavis.edu>).

The next major advance in forest genomics came with the advent of automated DNA sequencing, which enabled high-throughput expressed sequence tag (EST) analysis in trees (Allona et al. 1998; Sterky et al. 1998, 2004; Kirst et al. 2003). A large number of ESTs are now available for forest tree species (Table 1). Microarrays developed from ESTs have been used for functional genomic studies for many of these species.

Gene transfer technology has been developed for many broadleaf species and for some conifers, particularly pine and spruce. The best-developed transformation methods are for poplars. Gene transfer experiments have shown that specific genes can have major effects on wood properties and control of flowering (Strauss et al. 1995; Chiang 2006). Many genes encoding enzymes of the monolignol biosynthetic pathway affect the chemical composition of wood (Chiang 2006).

Table 1. Some Tree Genomes Being Studied

Species	Common name	Genome size (Mbp) ^a	ESTs in NCBI ^b (GenBank)	Physical map (Mbp)	Genome sequence	Properties of interest
<i>Castanea mollissima</i>	Chinese chestnut	800	none	in progress	none	disease resistance
<i>Cryptomeria japonica</i>	Sugi	10,824	19,605	none	none	timber
<i>Eucalyptus camaldulensis</i>	River red gum		none	none	in progress	biomass and pulp
<i>Eucalyptus grandis</i>	Flooded gum	640	1,574	in progress	in progress	biomass and pulp
<i>Eucalyptus globulus</i> (incl. subspecies)	Blue gum	564	3,951	none	none	biomass and pulp
<i>Ginkgo biloba</i>	Ginko	19,462	6,432	none	none	plant evolution and medicine
<i>Gnetum gnemon</i>	Melindjo	3,793	4,323	none	none	plant evolution
<i>Picea abies</i>	Norway spruce	18,228	10,217	none	none	timber
<i>Picea glauca</i>	White spruce	19,796	132,624	none	none	biomass and adaptation
<i>Picea sitchensis</i>	Sitka spruce		139,570	none	none	biomass, adaptation and disease resistance
<i>Pinus elliottii</i>	Slash pine	22,834	150	none	none	timber and pulp
<i>Pinus taeda</i>	Loblolly pine	21,658	329,469	in progress	none	biomass and adaptation
<i>Pinus pinaster</i>	Maritime pine	23,863	27,288	none	none	timber and pulp
<i>Pinus radiata</i>	Monterey pine	21,560	151	none	none	timber and pulp
<i>Pinus sylvestris</i>	Scots pine	27,244	1,689	none	none	timber and pulp
<i>Populus trichocarpa</i>	Black cottonwood	485	89,943	410	draft sequence	model tree
<i>Pseudotsuga menziesii</i>	Douglas fir	18,669	18,142	none	none	adaptation
<i>Quercus robur</i>	Oak	907	1,439	none	none	environmental adaptation
<i>Robinia pseudoacacia</i>	Black locust	2,933	637	none	none	heartwood formation

^aGenome sizes are only listed where published estimates exist.

^bNumbers of ESTs listed in NCBI for each species in September 2007.

Comparative genetics has been a powerful tool for forest trees by the identification of tree candidate genes affecting related traits in the better-characterized model systems. This is possible because of the substantial sequence conservation (Kirst et al. 2003) and functional homology (Böhlenius et al. 2006) among genes of vascular plants. Genomic approaches can identify important candidate genes for molecular breeding or genetic engineering. Genes associated with variation in wood chemistry (lignin), wood density, and growth rate have been identified using such approaches (Kirst et al. 2004a; Gonzales-Martinez et al. 2006a; Li et al. 2006).

The most intensively studied forest trees are pines, spruces, poplars, and eucalypts (Table 1; Fig. 1). Within each of these genera, several species and hybrid combinations are widely planted because of their fast growth, broad adaptation to site, and superior wood properties. Many are grown as exotics in different parts of the world. Poplar has emerged as the model system for molecular studies in trees (Bradshaw et al. 2000; Taylor 2002) because of its rapid growth rate, facile propagation from cuttings, well-developed cell and tissue culture, transformability, small genome size, saturated genetic maps, and genome sequence (Tuskan et al. 2006). A chestnut genome is being characterized to advance restoration of American chestnut (*Castanea dentata*), a species highly valued in the 19th century that was essentially lost due to a devastating fungal blight (Steiner and Carlson 2004).

Poplar is a good model tree, but it is not widely planted compared to the pines and the eucalypts. Eucalyptus species and their hybrids are the most widely planted hardwoods. Draft sequencing of the genome of *Eucalyptus camaldulensis* is in progress at the Kazusa DNA Research Institute (<http://www.kazusa.or.jp/e/index.html>), and the Department of Energy (DOE) Joint Genome Institute expects to complete a draft of the genome sequence of *Eucalyptus grandis* (the rose gum) in 2010 (Myburg et al. 2008). Genomic resources developed for eucalypts include large-insert bacterial artificial chromosome (BAC) genomic libraries, ESTs, and high-resolution genetic maps (<http://www.eucagen.org>). Similar genomic resources are being developed for other species with the goal of genome sequencing, for example, pine, spruce, and chestnut (Table 1).

Pines and spruces are the most widely planted gymnosperms. Full-genome sequencing is a challenge in conifers due to their large genome sizes and the high proportion of repeated sequences (Kamm et al. 1996; Kossack and Kinlaw 1999; Schmidt et al. 2000; Ahuja and Neale 2005). Genome sizes of conifers range from 6500 Mb to ~37,000 Mb. The loblolly pine genome, estimated at 21,500 Mb, is about seven times larger than the human genome. A BAC library (6x) of loblolly pine has been constructed (Plomion et al. 2007), and pilot scale BAC sequencing projects are under way in the United States and Canada. With new high-throughput sequencing methods (Margulies et al. 2005; Wicker et al. 2006), draft sequences of a pine and a spruce genome are likely to be initiated soon.

INFERRING FUNCTIONAL ROLES FOR GENES IN TREES

Forward Genetics

In model plant systems such as *Arabidopsis*, it is possible to induce and identify mutations in most genes and to infer phenotypic and functional relationships with genes in other plants (Alonso and Ecker 2006). In most forest tree populations, the frequency of recessive mutations is high, but analysis of mutations is limited by lethality, redundancy, and difficulty in the detection of quantitative phenotypes. Due to limited selfing and long juvenility, it is difficult to make heterozygous mutations homozygous in forest trees. Some mutations can be detected in the haploid megagametophytes that surround the embryo in the seed of gymnosperms. For example, a null mutation in the monolignol biosynthetic gene *Cad* was discovered in megagametophytes and was subsequently mapped, sequenced, and characterized (MacKay et al. 1997; Ralph et al. 1997; Gill et al. 2003).

Forward genetics approaches such as population-wide association genetics are feasible in trees because of the high levels of genetic diversity in natural populations and breeding populations (Neale and Savolainen 2004). Experimental populations derived from interspecific hybrids have even higher levels of variation and high levels of linkage disequilibrium (Grattapaglia et al. 1996).

Another forward genetics approach that has been successful in trees is activation tagging (Busov et al. 2003), i.e., using random transgenic insertions of strong enhancers to create dominant hypermorphic phenotypes. When an enhancer is inserted near the transcriptional start site of a gene, ectopic expression of the gene may give rise to an unusual phenotype (Fig. 2). Activation-tagged trees can be screened in the field, and altered phenotypes can be detected by inspection or aerial photography. In hybrid poplar, dominant variants occurred at a frequency of >1% and included a dwarf caused by overexpression of GA 2-oxidase, the major gibberellin catabolic enzyme (Busov et al. 2003).

Figure 2. Activation tagging in trees. Explants of leaves or stems are cocultivated with *Agrobacterium* containing a pUC19-based vector carrying multiple (4x) (cauliflower mosaic virus) CaMV 35S enhancer sequences. Ectopic activation of genes is detected in replicated field trials showing altered growth and developmental traits in some insertion lines.

Reverse Genetics

For more than a decade, where candidate genes have been identified, specific transgene expression has been modified in forest trees using antisense or cosuppression (Hu et al. 1999; Li et al. 2003). More recently, suppression by RNA interference (RNAi) (Wang and Waterhouse 2002) has been used in forest trees to perform functional tests. RNAi suppression has been used to test for stability of transgene suppression in trees in the field over time (Meilan et al. 2002; Li 2006). RNAi was used in grey poplar (*Populus canescens*) to suppress isoprene synthase and demonstrate that isoprene protects leaves from thermal and oxidative stress (Behnke et al. 2007). Coleman et al. (2008a) used RNAi suppression of *p*-coumaroyl-CoA 3' hydrolase (C3H) in hybrid poplar to verify the role of C3H in lignin biosynthesis. The perturbation of lignification reduced tree growth and compromised vascular integrity (Coleman et al. 2008b).

A Novel Method to Test Candidate Genes in Wood Formation: Somatic Sector Analysis in Developing Stem Tissues

The lateral secondary meristem of trees is abundant and accessible and has been used to test gene function by direct transformation of cambial cells in planta (Van Beveren et al. 2006). A segment of bark is carefully removed to provide access to cambial cells that are then inoculated with *Agrobacterium* containing the GUS marker and a candidate gene. The bark is replaced and cambial growth resumes. Subsequently, stably transformed cambial initials differentiate, giving rise to transformed xylem and/or phloem sectors identified by GUS staining. In this way, the role of a β -tubulin gene (*EgrTUB1*) in microfibril angle (MFA) formation was demonstrated in *Eucalyptus* (Spokevicius et al. 2007). The transformed wood segments were macerated, and MFA was determined microscopically from the angle of the pit apertures in the secondary cell walls relative to the long axis of the fibers. MFA is a function of the orientation of cellulose microfibrils in the secondary walls of wood fiber cells and is a widely used indicator of the strength and dimensional stability of wood.

Which Tree Genomes Are Being Studied?

Many of the traits studied in forest trees are quantitative and are affected by multiple genes of varying degrees of effect. The genetic dissection of complex traits into defined components has been advanced greatly by the development of genetic mapping in trees. The first step in the analysis of the genetic basis of complex traits has been the localization of regions of the genome, quantitative trait loci (QTLs) that harbor factors with significant effects on the phenotype. The second step is the identification of one or more specific genes or regulatory sequences in each QTL interval responsible for the effect. Many kinds of genomic data can be analyzed as quantitative molecular phenotypes (Brem et al. 2002; Schadt et al. 2003). Transcript abundance, protein abundance, enzyme

activity, and metabolite concentrations vary quantitatively in genetically diverse individuals or populations and may be regulated by alleles in one or more genes.

QTL mapping was initially considered likely to fail in forest trees, due to the complexity of growth and adaptation associated with long lifetimes (Strauss et al. 1992). However, tree growth (Bradshaw and Stettler 1995; Grattapaglia et al. 1996, 2009; Plomion et al. 1996; Verhaegen et al. 1997; Wu 1998; Kaya et al. 1999) and wood properties (Grattapaglia et al. 1996; Sewell et al. 2000, 2002; Ukrainetz et al. 2007) have been successfully studied using QTL mapping approaches. A great many quantitative traits in trees appear to be oligogenic with substantial proportions of the phenotypic variance attributable to a few genetic loci (Table 2). QTL mapping also provides a way to integrate various forms of genomic data to make associations of alleles and/or transcripts, with other quantitative traits or phenotypes, provided there is sufficient statistical power to validate such relationships (Beavis 1998; Yvert et al. 2003; Kirst et al. 2004b).

Growth rate has been the single most important trait in tree breeding and plantation forestry. Multiple QTLs for growth have been identified within species of *Populus*, *Eucalyptus*, and *Pinus* (Table 2) (Grattapaglia et al. 2009). Genetic differences are more readily detected in interspecific hybrids. In F_1 progeny, it is possible to investigate variation within each pure-species parent, whereas an F_2 cross provides detection of interspecific polymorphisms segregating in the F_1 parent. Maturation may induce a progressive shift in the genetic control of height growth, providing an explanation for the low juvenile/mature correlation for height growth in pines.

Eucalyptus is grown widely by vegetative propagation leading to deployment of specific clones for high-quality pulp properties. QTL analysis was performed in an *E. grandis* $\times$ *urophylla* F_1 hybrid progeny set for traits affecting vegetative propagation (Grattapaglia et al. 1995). QTLs were detected for rooting ability, stump sprouting, and shoot fresh weight. Similarly, genetic dissection of vegetative propagation traits was performed for *E. tereticornis* and *E. globulus* (Marques et al. 1999).

The genetic architecture of chemical and physical properties of wood also shows oligogenic structure. In an *E. grandis* study (Grattapaglia et al. 1996), QTLs for wood-specific gravity were identified, and in loblolly pine, QTLs were identified for MFA and wood-specific gravity (Sewell et al. 2000). QTLs for chemical properties of wood have been described in *Eucalyptus* (Kirst et al. 2004a) and in conifers (Sewell et al. 2002; Ukrainetz et al. 2007). QTLs have been identified for lignin content and composition and hemicellulose composition.

It had been assumed for many years that host resistance to disease in trees should be controlled by many genes of small effect because of the long lifetime of the host and the far shorter generation time of the fungal pathogens. White pine blister rust resistance in sugar pine, controlled by a single locus (Kinloch et al. 1970; Devey et al. 1995), had been viewed as an exception. The resistance phenotype of seedlings could be genetically mapped using the haploid maternal genetics of the megagametophytes (Devey et al. 1995).

Table 2. Diversity of Traits for Which QTLs Have Been Mapped in Forest Tree Genomes (Some Examples)

Species	Type of cross and size of population ^a	Trait	Number of QTLs ^b	Proportion phenotypic variance explained ^c	Reference
<i>Populus trichocarpa</i> × <i>P. deltoides</i>	interspecific F ₂ (n = 55)	stem volume (age 2 yr)	2	44.7%	1
		spring bud flush	5	84.7%	1
<i>Eucalyptus grandis</i>	half sib (n = 300)	volume growth	3	13.7%	2
		wood-specific gravity	5	25.7%	2
<i>Eucalyptus urophylla</i> × <i>E. grandis</i>	interspecific F ₁ (n = 200)	wood density at 38 mo	4	26.4%	3
		vigor at 38 mo	6	39.8%	3
		height:diameter ratio at 38 mo	4	31.2%	3
<i>Pinus taeda</i>	full sib (n = 172)	percentage latewood	5	28.6% ^d	4
		microfibril angle	5	44.8% ^d	4
		wood-specific gravity	9	56.0% ^d	4
<i>Pinus pinaster</i>	full sib (n = 202)	water use efficiency (δ ¹³ C)	4	51.4%	5
		diameter growth (ring width)	2	42.9%	5
<i>Pinus sylvestris</i>	full sib (n = 94)	height, frost hardiness	12	9%–23%	6
<i>Pinus taeda</i>	full sib (n = 172)	PLS of cell wall chemistry (e.g., α-cellulose, galactan, lignin) ^e	8	52.7%	7
<i>Populus trichocarpa</i> × <i>P. deltoides</i>	interspecific F ₂ (n = 167)	response to drought (Abscission)	5	43.0%	8
<i>Populus deltoides</i> × <i>P. nigra</i> and <i>P. deltoides</i> × <i>P. trichocarpa</i>	interspecific F ₁ (n = 152 and n = 202)	metabolite concentration (15 flavonoid compounds)	4	24%–44%	9
<i>Quercus robur</i>	full sib (n = 120)	water logging tolerance traits (e.g., hypertrophied lenticels, leaf epinasty)	5	56.0% ^d	10
<i>Populus trichocarpa</i> × <i>P. deltoides</i>	interspecific F ₂ (n = 285)	stem height under elevated CO ₂	2	12.2% ^d	11
		stem diameter under elevated CO ₂	4	18.1% ^d	11
<i>Eucalyptus nitens</i>	full sib (n = 296)	foliar terpene (α-pinene)	3	33.3% ^d	12
		foliar terpene (trans-pinocarveol)	4	30.6% ^d	12
<i>Eucalyptus globulus</i>	full sib (n = 112)	leaf damage (<i>Mycosphaerella</i> leaf disease)	2	52.0%	13

^aHalf-sib families were typically from two-generation pedigrees (seed parent and progeny, grandparents unknown), whereas full-sib families were typically from three-generation pedigrees (at least one grandparent known).

^bNumber of significant QTLs detected for each trait.

^cTotal proportion of the phenotypic variance explained by the QTLs.

^dSum of the phenotypic variance explained by individual QTLs is given where multipoint estimates were not reported. These totals may be inflated relative to multipoint estimates of the joint effects of the same QTLs.

^ePLS (Projection to latent structures) was used to identify shared variance components of several cell wall chemistry traits.

References: (1) Bradshaw and Stettler 1995; (2) Grattapaglia et al. 1996; (3) Verhaegen et al. 1997; (4) Sewell et al. 2000; (5) Brendel et al. 2002; (6) Lerceteau et al. 2000; (7) Sewell et al. 2002; (8) Street et al. 2006; (9) Morreel et al. 2006; (10) Parelle et al. 2007; (11) Rae et al. 2007; (12) Henery et al. 2007; (13) Freeman et al. 2008.

Host resistance to fusiform rust disease in loblolly pine, long thought to be a complex quantitative trait, was shown to be a simple gene-for-gene pathosystem once genome mapping was applied (Wilcox et al. 1996). Similarly, in poplar, host resistance to *Melampsora medusae* leaf rust was mapped to a single locus in a hybrid poplar F₂ pedigree (Newcombe et al. 1996). The severity of rust in the field and in growth-room experiments was associated with Mmd1, a single factor with a major role in resistance. More recently, two QTLs were found to explain 52% of the phenotypic variation in leaf damage of *E. globulus*, by the fungal pathogen *Mycosphaerella cryptica* (Freeman et al. 2008). Therefore, the genetic basis of host disease resistance in forest trees appears to be similar to that of annual crops.

Even when genome sequence is available, as it is for poplar, QTL regions often include hundreds of genes, and the identification of a specific candidate is not obvious. Associations can be made, however, using functional data, such as transcript abundance in combination with

QTL data, or from direct allele–trait associations in large populations in high linkage equilibrium.

Transcriptomics

Microarrays have been used to study transcriptome changes in trees during adaptation to biotic and abiotic stress, development, wood formation, maturation, and dormancy. Even relatively small numbers of ESTs in libraries from pines, spruces, eucalypts, and other species have permitted a broad scale of gene expression studies defining specificity and coordinated control. Microarrays have also been used in combination with genetic markers to map the controlling factors that regulate variation in expression of specific genes, called expression QTLs (eQTLs) (Kirst et al. 2004a, 2005).

Adaptive stress responses. Expression profiles of loblolly pine seedlings subjected to drought reflected

photosynthetic acclimation to mild stress and photosynthetic failure during severe stress (Watkinson et al. 2003). Distinctive responses to acclimation were found for transcripts encoding heat shock proteins (e.g., HSP70 and HSP90), late embryo-abundant proteins (e.g., LEA g3), as well as enzymes of the aromatic acid and flavonoid biosynthetic pathways. Many of the responses have clear functional relationships to drought.

The response of Scots pine (*Pinus sylvestris*) root tissue to invasion by *Heterobasidium annosum*, the root and butt rot pathogen of forest trees, has been investigated using transcript profiling (Adomas et al. 2007). In general, the total number of expressed genes decreases over time of infection, but genes were up-regulated for the phenylpropanoid pathway (shikimate, lignin, and flavonoid pathways) and for oxidases (peroxidases and laccases). In a related study (Adomas et al. 2008), Scots pine transcript abundances were compared after infection with fungi of different trophic strategies, the saprotrophic fungus *Trichoderma auriviride*, the pathogen *Heterobasidium annosum*, and the mutualistic ectomycorrhizal symbiont *Laccaria bicolor*. The diverse fungi elicited different transcriptional responses. The results of challenge by *L. bicolor* indicate substantial similarities among infection by ectomycorrhiza in angiosperms, with some differences in timing and regulation (Heller et al. 2008). The similarity of expression profiles to the responses known in agricultural crops suggests that angiosperms and gymnosperms may have similar responses to invasive pathogens.

Barakat et al. (2009) used high-throughput sequencing to compare transcripts of susceptible American chestnut (*Castanea dentata*) and resistant Chinese chestnut (*Castanea mollissima*) induced in response to chestnut blight (*Cryphonectria parasitica*) infection. A large number of genes associated with resistance to stresses were expressed in canker tissues, some differentially expressed in canker tissues of the two species.

cDNA microarrays were also used to study genome-scale changes in transcript abundance in hybrid poplar leaves following herbivory by forest tent caterpillars (Ralph et al. 2006a). The up-regulated genes included many involved in plant defense (e.g., endochitinases), octadecanoid and ethylene signaling (e.g., lipoxygenase), secondary metabolism, and transcriptional regulation. Similarly, large-scale changes in the transcriptome were observed in Sitka spruce (*Picea sitchensis*) following either budworm or weevil attack (Ralph et al. 2006b).

Transcript changes in development. Variation in transcript abundance was studied during somatic embryogenesis of Norway spruce (*Picea abies*) to investigate early differentiation in gymnosperms (van Zyl et al. 2002; Stasolla et al. 2004). Early phases of normal embryo development are characterized by a precise pattern of induction, repression, followed by induction (up, down, and up again) as cells differentiate from proembryos to early embryos (repression), and then to late embryos (induction).

Transcriptome changes during adventitious root development (induced by an auxin pulse) in lodgepole pine (*P. contorta*) were monitored during initiation, meristem for-

mation, and root elongation (Brinker et al. 2004). During initiation, transcript abundance reflected cell wall weakening and cell replication. During root initiation, a PIN-HEAD/ZWILLE-like protein (with a potential role in meristem formation) was up-regulated, whereas auxin related genes were down-regulated. During meristem formation and root differentiation, transcript changes indicated that the roots had become functional in water transport and auxin transport. Expression patterns therefore provided information on general physiological responses and specific genes that may regulate those processes.

Wood formation. Microarrays have been used to investigate the variation in transcript abundance associated with wood formation (Hertzberg et al. 2001; Yang et al. 2003; Geisler-Lee et al. 2006). Wood-forming tissue has a complex, highly regulated transcriptome, with abundant expression of genes dedicated to secondary cell wall biosynthesis (Hertzberg et al. 2001; Kirst et al. 2003, 2004a; Yang et al. 2003; Paux et al. 2005; Lu et al. 2005; Geisler-Lee et al. 2006). A microarray study in *Eucalyptus* suggested a negative correlation of tree growth and lignification at the transcript level (Kirst et al. 2004a). Transcript abundance of lignin biosynthetic genes was highest in slow-growing trees, along with increased lignin content and abundance of syringyl lignin subunits. The most elegant use of microarray technology combined transcriptome analysis with tangential cryosectioning of the developmental gradient of differentiating xylem in poplar (Hertzberg et al. 2001) to identify stage-specific gene expression patterns. Transcripts for biosynthetic enzymes of lignin and cellulose as well as potential regulators of wood formation were found to be under stage-specific transcriptional control.

An analysis of homology and tissue specificity of transcripts for carbohydrate-active enzymes (CAZymes) in poplar and *Arabidopsis* (Geisler-Lee et al. 2006) compared glycosyl transferases, glycoside hydrolases, carbohydrate esterases, polysaccharidelyases, and expansins in both species. *Populus* has ~1600 CAZyme genes compared to ~1000 for *Arabidopsis*. This difference may be the result of a recent genome-wide duplication event in the poplar genome (Tuskan et al. 2006) and greater emphasis on carbohydrate biosynthesis in woody tissues of poplar. Woody tissues have abundant CAZyme transcripts related to cell wall formation but low levels associated with starch formation.

Variation in gene expression has been studied in pines and poplars during growth seasons and over winter dormancy. In European aspen (*P. tremula*), seasonal growth and cambial dormancy involve extensive remodeling of the transcriptome (Schrader et al. 2004). The complexity of transcripts is greatly reduced in the dormant state; however, a minimal level of transcripts for some cell cycle regulators is maintained. Transcript abundance for starch enzymes increases during dormancy. Less-extensive studies in pine showed changes in abundance of transcripts for genes of cell wall biosynthesis associated with earlywood and latewood (Egertsdotter et al. 2004; Yang and Loopstra 2005).

The trunk wood of trees is differentiated into sapwood (involved in vertical transport) and heartwood, which is

rich in chemical extractives, composed of waxes, fats, and resins. RNA obtained from the sapwood/heartwood transition zone shows transcript profiles that are more abundant for genes in flavonoid biosynthesis and many genes of unknown function (Yang et al. 2003).

Integration of quantitative and molecular genomics. Mapping QTLs provides valuable information regarding the genetic structure of complex traits and identifies genomic regions that contain genes with polymorphisms affecting traits of interest in a particular cross. This kind of information has led to the identification of positional candidate genes underlying quantitative traits in plants (Morgante and Salamini 2003); however, few genes have been identified in this way. Association of genes and QTLs requires high-resolution genetic maps, identification of genes by sequence and location, and verification by direct gene transfer. Such experiments are difficult and slow even in the most tractable forest tree species. Attempts to clone a disease resistance gene from *Populus* are hindered by low recombination around the resistance locus (Stirling et al. 2001). The large amount of DNA per recombination unit in conifer genomes (e.g., 12 Mb/cM in pines) makes the identification of genes by positional cloning appear to be infeasible. However, the recombination rate per chromosome in conifers is similar to that in *Arabidopsis*, despite a difference in genome DNA content of nearly two orders of magnitude. The density and spacing of genes per recombination unit will ultimately be a critical factor determining the success of map-based cloning. Information on gene spacing and crossover distribution has not yet been obtained for any gymnosperm, although exploratory BAC sequencing is under way for spruce and pine.

Association studies in tree populations. In many organisms, including humans, association genetics has become an established approach to detect genetic variants that may affect phenotypic variation. Large populations are screened

for associations of specific markers and phenotypes. Single-nucleotide polymorphisms (SNPs) are abundant in plant genomes and have been used to find associations (Rafalski 2002; Gupta et al. 2005; Buckler et al. 2009).

Associating genes and traits in trees is challenging because of the high levels of genetic diversity, large genomes, substantial effects of environment on phenotypes, and the life history of trees (Table 3) (Neale and Savolainen 2004). Only a few specific alleles in an entire genome may be expected to have detectable quantitative effects for any given phenotype. Association genetics has been applied to wood property traits (Thumma et al. 2005; Gonzalez-Martinez et al. 2006a) and the drought stress-response (Gonzalez-Martinez et al. 2006b). In loblolly pine, a study of 58 SNPs in 20 wood and drought-related candidate genes found association of allelic variation in α -tubulin with earlywood microfibril angle (Gonzalez-Martinez et al. 2006a). Association was also found for SNPs in two genes in the lignin biosynthetic pathway. Cinnamyl alcohol dehydrogenase (CAD) alleles were associated with earlywood-specific gravity, and specific alleles of the 4-coumarate CoA-ligase (*4CL*) gene were associated with percentage of latewood. In *Eucalyptus*, allelic variants of cinnamoyl CoA reductase (CCR) were associated with variation in microfibril angle (Thumma et al. 2005). Detection of gene effects at the population level in trees therefore appears to be feasible, but much larger numbers of genes and alleles need to be evaluated.

Association genetics has strengths and limitations. The power of the method depends on the size of the experimental population, heritability of the trait, number and effects of contributing genes, and allelic frequencies for these genes. Also critical for association genetics is the amount and distribution of linkage disequilibrium (LD), which determines the number of markers and the experimental approach (genome-wide scans vs. gene-based assays) used to find marker-trait associations (Gratta-paglia et al. 2009). LD decays rapidly in pines with average r^2 values of

Table 3. Genes and Markers Identified in Association with Quantitative Traits in Forest Tree Species

Gene/allele or marker	Location (genic/intergenic)	Trait	Species	Detection approach	Population	Reference
<i>cad/cad-n1</i>	genic (exon)	wood density	loblolly pine (<i>Pinus taeda</i>)	n.a.	n.a.	Wu et al. (1999)
<i>RCI2</i> /marker unknown	unknown	wood density	<i>E. grandis</i> and <i>E. globulus</i>	QTL/genetical genomics	pseudo-backcross hybrid population	M. Kirst et al. (unpubl.)
<i>ccr</i> /SNP20 and SNP21	genic (intron)	microfibril angle	<i>E. globulus</i>	LD mapping	unrelated gentotypes ($n = 290$)	Thumma et al. (2005)
<i>sams-2</i> /M44	genic (intron)	earlywood-specific gravity	loblolly pine (<i>Pinus taeda</i>)	LD mapping	unrelated gentotypes ($n = 435$)	Gonzales-Martinez et al. (2006a)
<i>cad</i> /M28	genic (exon)	earlywood-specific gravity	loblolly pine (<i>Pinus taeda</i>)	LD mapping	unrelated gentotypes ($n = 435$)	Gonzales-Martinez et al. (2006a)
<i>lp3-1</i> /Q5	genic (UTR)	percentage of latewood	loblolly pine (<i>Pinus taeda</i>)	LD mapping	unrelated gentotypes ($n = 435$)	Gonzales-Martinez et al. (2006a)
α -tubulin/M10	genic (intron)	earlywood microfibril angle	loblolly pine (<i>Pinus taeda</i>)	LD mapping	unrelated gentotypes ($n = 422$)	Gonzales-Martinez et al. (2006a)

Abbreviations: (*cad*) Cinnamyl alcohol dehydrogenase; (*ccr-1*), cinnamoyl CoA reductase 1; (*sams-2*) S-adenosylmethioninesynthetase 2; (*lp3-1*) water-stress-inducible protein 1; (n.a.) not applicable.

0.2 for sites within 2 kb of each other (Brown et al. 2004). Similar rates of decay have been reported in *Eucalyptus* (Thumma et al. 2005) and *Populus* (Ingvarsson 2005). At such low levels of LD, there should be little correlation between adjacent genes, and an association of a gene and a trait implies a direct effect. However, to achieve such resolution, multiple, well-spaced markers are needed, preferably for all genes. New ultra-high-throughput DNA sequencing technologies (Wicker et al. 2006) enable the discovery of many SNPs in large numbers of tree genes, paving the way for genome-wide gene-based association genetics in trees. A recent study in *Eucalyptus* (Novaes et al. 2008) shows that very large numbers of informative SNPs can be readily obtained using this technology.

Genetical genomics in forest trees. Quantitative genetic analysis of transcript abundance and trait variation in segregating populations can identify functional associations of genes and traits, providing a powerful complement to association genetic studies. This approach, called genetical genomics (Jansen and Nap 2001), is based on the expectation that transcript level variation should be correlated with trait variation where allelic polymorphisms affect gene expression. Many genes show significant variation in transcript abundance associated with segregating genotypes, which can be considered quantitative traits and therefore be mapped as eQTLs. Studies in yeast, *Drosophila*, mice, maize, humans, and forest trees (Brem et al. 2002; Wayne and McIntyre 2002; Schadt et al. 2003; Yvert et al. 2003; Kirst et al. 2004a; Bystrykh et al. 2005; Hubner et al. 2005) revealed large numbers of specific genes with heritable variation in transcript abundance. Transcript abundance is measured in mapping pedigrees by microarray analysis, and gene–trait associations are inferred by the colocation of the resulting eQTLs and trait QTLs.

eQTLs provide information about gene regulation in two ways. If a site regulating transcript variation is coincident with the location of the gene encoding the transcript, then variation of transcript abundance is inferred to be *cis* regulated, (i.e., the controlling element is within or very near to the gene itself). If the eQTL is located elsewhere in the genome, gene expression is inferred to be regulated in *trans*, by a polymorphism in another molecule, presumably RNA or protein. A *trans*-acting eQTL shared by several genes (giving rise to an “eQTL hot spot”) may indicate polymorphism in a transcription factor that regulates many genes coordinately.

Besides transcript profiling tools, genetical genomics in trees requires large-scale determination of phenotypes such as wood chemistry, tree physiology and disease resistance. High-throughput nondestructive measurement systems of wood chemistry by near-infrared (NIR) spectroscopy (Schimleck et al. 2000; Yeh et al. 2005) have been developed that allow genetic dissection of wood properties in large populations. Gene expression arrays are now available for several tree species (Hertzberg et al. 2001; Yang et al. 2003; Ralph et al. 2006a; EUCAGEN [www.eucagen.org]; NSF-GLPEP 2007 [http://compbio.dfc.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=pine]). The costs of phenotyping and microarray analysis for large populations and the estab-

lishment of new large experimental populations may be the most serious limitations for application of genetical genomics.

We have studied a wide interspecific cross of *E. grandis* × *globulus* species that are planted for rapid growth (*E. grandis*) and high wood density (*E. globulus*) (Myburg 2001; Myburg et al. 2003, 2004). QTLs were identified for many traits, including wood density, growth rate, and wood properties (Myburg 2001; Kirst et al. 2004a). At least two chromosomal regions showed a significant correlation with lignin content (Fig. 3). A segment from the *E. globulus* parent on linkage group 3 (LG3) was associated with decreased lignin relative to the *E. grandis* allele, and a site on LG7 associated increased lignin and a segment from *E. globulus*. Segregating eQTLs were detected for large numbers of genes (Kirst et al. 2005). eQTLs were detected for 41% of 2304 genes represented on a microarray, of which 77% had a single eQTL. For the remaining 23%, more than one significant eQTL was detected.

eQTL analysis has also allowed us to identify a candidate gene for wood density in this *Eucalyptus* hybrid population (Myburg 2001; Kirst et al. 2004a). The abundance of one gene transcript, a *Eucalyptus* cDNA that has homology with the *RCI-2* gene of *Arabidopsis* (Jarillo et al. 1994), was strongly associated with wood density (Fig. 4, left). Several other transcripts were associated with diameter growth rate and lignin properties and appeared

Figure 3. QTL mapping in progeny of heterozygous trees. (Top) Schematic representation of the use of dominant molecular markers to track the inheritance of parental chromosome segments in segregating progeny and associate the presence of alternative alleles with differences in trait values. The strength of the association is represented by the QTL graph (thick red line), which is a likelihood ratio (LR) test statistic for the difference in trait means of progeny with alternative marker alleles. QTL allele effects (relative to the trait mean) are indicated by red arrows. (Bottom) Example of a QTL scan for lignin content in the genome of a F_1 hybrid of *E. grandis* × *globulus* backcrossed to a unrelated *E. grandis* individual (i.e., trait variation in F_2 pseudo-backcross progeny (Myburg 2001; Kirst et al. 2004a). Significant and suggestive composite interval mapping LR thresholds (solid and broken lines) correspond to approximate experiment-wise $\alpha = 0.05$ and $\alpha = 0.3$, respectively. The additive effect of putative QTLs is indicated below the LR plot and shows the magnitude and direction of the effect of the *E. globulus* allele relative to that of the *E. grandis* allele in the F_1 hybrid. The 11 linkage groups are shown end to end, and positions of framework markers are indicated by triangles (Δ) along the x axis.

Figure 4. Association of variation in transcript abundance of the *RCI-2* homolog of *Eucalyptus* with wood density in an *E. grandis* × *globulus* hybrid F_2 progeny. (Left) Correlation analysis identified a significant association between *RCI-2* relative expression level and wood density measured in the F_2 population ($r^2 = 0.28$, $p < 0.0001$). (Right) Colocalization of the *RCI-2* gene locus, *RCI-2* eQTL (black line), and wood density QTL (gray line) on the hybrid parent genetic map further defined *RCI-2* as a strong candidate for regulation of the trait variation. The arrow indicates the genetic location of *RCI-2*.

to be coordinately controlled (Kirst et al. 2004a). Among the genes most strongly and negatively associated with growth were several genes encoding enzymes for monolignol biosynthesis. The strongest correlation with growth was found for *Cald5H* ($r^2 = 0.38$), known to regulate the angiosperm-specific branch of monolignol biosynthesis forming sinapyl alcohol (Chiang 2006).

The association of transcript variation and trait variation can be confirmed by evaluating the colocalization of the genes, the eQTL regulating transcript level, and the QTL(s) for the trait of interest. In the case of the *RCI-2* transcript, all three (the gene, eQTL, and wood density QTL) were colocalized (Fig. 4, right), suggesting that the *RCI-2*eQTL affects *cis* regulation of the gene itself. In the case of the monolignol genes (Kirst et al. 2004a), the polymorphisms affecting their transcript levels (i.e., the eQTLs) did not colocalize with the genes, but they appeared to exert coordinate control. The shared eQTLs (eQTL hot spots) may be *trans*-acting regulators of the lignin biosynthetic pathway.

Under appropriate conditions, eQTLs can explain a larger component of the total phenotypic variance than traditional marker-based trait QTLs. In the case of *RCI-2*, the eQTL based on transcript level variation accounted for 28% of the total phenotypic variance, whereas the marker-based QTL in the same region accounted for only 11% of the variance. Phenotypic traits are subject to additional developmental and environmental variation that affects their transcript levels. Transcript level measurements can therefore have greater power to detect genetic associations than markers alone.

It has not yet been possible to determine the nature of the eQTLs associated with lignin biosynthesis and growth in the *E. grandis* × *globulus* backcross pedigree in the absence of genome sequence. Preliminary genetical genomics studies in F_2 hybrid progeny of *P. trichocarpa* and *P. deltoides* succeeded in identifying candidate genes that colocalize with drought resistance QTLs (Street et al. 2006).

In the near future, we may expect genetical genomics and association genetics to uncover many associations of specific genes with disease resistance, insect resistance,

climatic adaptation, wood properties, and carbon allocation in both hardwoods and conifers.

CONCLUSIONS

Genome Content and Evolution

Research should focus on obtaining fundamental information on gene content, gene location, and gene function in key species through comparative genomics. A pressing goal will be the ability to infer functional gene orthology across the major taxa of higher plants. Comparison of the genomes and transcriptomes of herbaceous and woody plants will help to elucidate the genetic basis of the woody growth form and perennial plant life. A major objective should be the *de novo* sequencing of genomes within the gymnosperms and a great many of angiosperm tree genomes to gain a detailed understanding of genome evolution in trees.

Population Genomics

Further integration of molecular and quantitative research tools will come from studies of quantitative variation in populations and, in particular, where associations can be made directly between alleles and phenotypes on a genomic scale. New methods for high-throughput phenotyping of traits and the physical and chemical properties of cells and tissues will make more associations possible. Applications may be expected in both plantations and natural populations. Comparisons of populations within and between related species will provide new insights into the evolution of many woody plant genera.

Metabolomics

The characterization of large numbers of metabolites from cells and tissues (Fiehn et al. 2000) would be of great value for forest trees to study chemical diversity and to associate metabolites with genes. Such associations

could aid in assigning function to unknown genes, many of which may be involved in secondary metabolic pathways that may be less readily studied in model plants. Specific applications of global metabolite profiling will be particularly important for understanding interactions of insect pests and the chemical defenses of tree hosts (Henery et al. 2007).

Environmental Genomics

Environmental genomics (also called Ecogenomics) is an emerging area of tree biology (Whitham et al. 2006). The application of genomics to the breeding or engineering of a blight-resistant American chestnut (Steiner and Carlson 2004; Wheeler and Sederoff 2009) aimed at species restoration is a model for many endangered tree species where both conservation and genetic intervention may be needed. Such species include Fraser fir, lodgepole pine, American beech, sugar pine, and American elm. Trees are desirable for bioremediation with the added advantage of carbon sequestration. Recently, transgenic poplar plants expressing a cytochrome P450 were shown to increase metabolism and remove halogenated hydrocarbons from hydroponic solution (Doty et al. 2007).

Woody Biomass for Energy

Large efforts in tree genomics will be directed to the improved use of woody biomass for fermentation to ethanol or for thermal conversion to electrical energy. The successful use of trees as bioenergy crops will require increased forest tree productivity and changes in wood biochemistry that will make cellulose from wood more accessible for saccharification and fermentation. Alternatively, wood with high lignin content is a superior source of thermal energy, and efforts may also be directed to produce fast-growing high-content lignin trees. A substantial gap exists between the current information and what is needed to describe the relationship between genotype and phenotype by predictive models for important traits such as biomass production in forest trees.

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R. Sederoff, A. Myburg and M. Kirst

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