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A Genomic and Molecular View of Wood Formation

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Wood formation is a process derived from plant secondary growth. Different from primary growth, plant secondary growth is derived from cambium meristem cells in the vascular and cork cambia and leads to the girth increase of the plant trunk. In the secondary growth process, plants convert most of photosynthesized products into various biopolymers for use in the formation of woody tissues. This article summarizes the new developments of genomic and genetic characterization of wood formation in herbaceous model plant and tree plant systems. Genomic studies have categorized a collection of the genes for which expression is associated with secondary growth. During wood formation, the expression of many genes is regulated in a stage-specific manner. The function

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of many genes involved in wood biosyntheses and xylem differentiation has been characterized. Although great progress has been achieved in the molecular and genomic understanding of plant secondary growth in recent years, the profound genetic mechanisms underlying this plant development remain to be investigated. Completion of the first tree genome sequence (*Populus* genome) provides a valuable genomic resource for characterization of plant secondary growth.

Keywords secondary growth, wood formation, xylem differentiation, lignin, cellulose, hemicellulose, cambium meristem, cell wall

I. INTRODUCTION

Trees play a very important role in sustaining earth's living environment and in providing many essential natural resources for life. Trees display many characteristics that differ from those of herbaceous plants. One of the most prominent features that make trees biologically distinct is the secondary growth initiated from a vascular cambium that gives rise to wood formation.

Wood formation is a unique developmental process, which accounts for most of the approximate 100 billion tons of CO_2 fixed per annum by plants. The wood produced is an essential raw material for human utilization. Understanding wood formation has been of interest in science for more than a century and today we know to a certain extent how wood is formed as illustrated by anatomy, chemistry, biochemistry, physiology, ecology, physics, etc. Generally, wood is considered the dead secondary xylem tissue accumulated in perennial tree plants. More specifically, wood formation is a continuous process of secondary xylem differentiation derived from vascular cambium meristematic cells, which are usually described as a single layer of permanent initiating cells, called *cambial initials*. When the cambial initials divide inwards periclinally, one cell remains in the meristem status while the other, positioned inside, is destined to become a xylem mother cell. The xylem mother cell may in turn proceed to undergo a limited number of cell divisions or without further division, to differentiate into secondary xylem cells (Larson, 1994). Matured secondary xylem tissue, or wood, is composed of several types of shape- and function-specialized cells. These cells include conducting cells, supporting cells, and storage cells. In angiosperms, the conducting cells consist of vessels and the supporting cells of fibers. In gymnosperm species, the tracheids fulfill both functions. Both conducting and supporting cells are thick-walled and elongated along the longitudinal direction of the stem. Storage cells are thin-walled parenchyma cells that usually remain alive to transport and store nutrients for long periods of time. Therefore, the wood formation involves a series of sequential biological events, including cell division, cell function specification (vessel/tracheid, fiber, parenchyma and other cell types), extensive cell enlarging, massive secondary wall thickening, cell aging and death. The wood formation derived from perennial secondary growth is unique to the tree species and represents a functional specialty acquired by tree genomes.

As our knowledge of wood formation is advancing in many aspects, we are fundamentally limited by the knowledge of how wood formation is genetically regulated, and how those mechanisms regulating wood formation can be translated into new technology in order to sufficiently use tree resources in the future. In recent years, genomics is emerging as a new subject of science as well as a powerful technology to understand and investigate complex biological processes. A great deal of progress in genomics studies has been achieved in deciphering the mechanisms of how complex biological processes are controlled in various organisms. Without exception, understanding of wood formation in light of genomics and molecular biology has been rapidly growing. This review summarizes a general view on the current status of genomic and molecular genetics studies on wood formation.

II. RECENT DEVELOPMENTS IN GENOMICS STUDIES OF WOOD FORMATION

Advances in genomics have opened a wide window to look into complex biological processes at the genome level. Genomics approaches analyze, at a genome-wide scale, how genes are expressed in association with various developmental processes, what roles the genes play in growth and development, and in response to environmental conditions and how functioning genes interact with each other in various regulatory networks and signaling circuits. Genomics approaches ultimately enable biological processes to be understood in a comprehensive and systematic way. Wood formation is an essential process in woody plants, and probably, in terms of the scale on which it occurs, one of the most important biological processes to human society and the earth's environment. In the past several years, our understanding of the genomics of wood formation has been advanced through an array of attempts, such as gene expression profiling of Arabidopsis plants induced for secondary growth (Lev-Yadun, 1994, 1997; Busse and Evert, 1999; Zhao *et al*., 2000; Little *et al*., 2002; Oh *et al*., 2003; Chaffey *et al*., 2002; Ko *et al*., 2004; Ko and Han, 2004), sequencing of a large scale of expressed sequence tags (EST) in tree species including loblolly pine, spruce, *eucalyptus* species, *Populus* species, and others (http://www.ncbi.nlm.nih.gov/genomes/PLANTS/PlantList_or.html#EST; http://pinetree.ccgb.umn.edu/; http://www.populus.db.umu.se/; http://www.arborea.ulaval.ca/en/results/est-sequencing.php), gene expression profiling in wood-forming tissues, sequencing of the tree genome (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html), and functional characterization of genes involved in wood biosynthesis. These studies have yielded an array of useful information and resources for the understanding of wood formation.

A. Using Arabidopsis and Cell Culture Systems to Study the Genomics of Xylem Formation

Mainly wood formation involves xylem formation derived from secondary growth that leads to an increase in plant girth

through the activity of the vascular cambium. The secondary growth is different from primary growth that is initiated at apical meristems and gives rise to apical extension of plant body and differentiation of plant organs and tissues. The activities of apical meristems have been studied extensively (Fosket, 1994). In particular, by using Arabidopsis as the model system, many genes involved in regulating the identity and development of apical meristem cells have been characterized (see reviews by Leyser, 2001; Carles and Fletcher, 2003; Fleming, 2005; Jiang and Feldman, 2005). Although secondary growth is a different biological process from primary growth, some genetic mechanisms regulating the activities of apical meristem cells also may show analogies in regulating the vascular cambium activities for xylem development during wood formation (Groover, 2005). As Arabidopsis has been adopted as an excellent system for the study of various plant biological processes (Anderson and Roberts, 1998), attempts have also been made to study secondary growth in Arabidopsis plants because this herbaceous species, under a specific growing condition, can be induced to develop a growth that exhibits many characteristics similar to the secondary growth of tree species (Lev-Yadun, 1994; Ko *et al*., 2004). Furthermore, completion of the Arabidopsis genome sequence has laid a foundation for the genomics study of plant growth, development, and interactions with environmental factors. Thus, it is possible to use the Arabidopsis plant system for the study of some of the genetic mechanisms that underlie secondary growth.

Arabidopsis is naturally an herbaceous species lacking the perennial secondary growth inherent in woody plants. However, when the inflorescences are repeatedly removed during growing time or when the plant is grown under short day conditions, Arabidopsis can develop a stem growth that displays many characteristics similar to secondary growth, such as expansion of stem diameter. This induced secondary growth leads to the formation of a xylem structure in Arabidopsis stems which has an anatomical pattern similar to the secondary xylem in the stem of a hybrid poplar species (*Populus tremula* × *P. tremuloides*) (Chaffey *et al*., 2002). When the gene expression profiles in induced secondary xylem tissue and normal tissue were compared by microarray analysis using the 8.3 K Arabidopsis Genome Array (Affymetrix, Santa Clara, CA), Oh *et al*. (2003) found that 20 percent of the ∼8300 genes were differentially expressed. The promoter structures of the differentially expressed genes were investigated by computational analysis. In those promoter sequences eight putative *cis*-elements were commonly present in the genes that were upregulated in Arabidopsis induced secondary xylem tissue. In another experiment, using the Arabidopsis whole-transcriptome (23 K) GeneChip (Affymetrix), Ko *et al*. (2004) examined the alternation of gene expression profiles in Arabidopsis inflorescence stems during secondary xylem formation induced by cultural manipulation or artificial weight application. They identified 700 genes that were differentially expressed during the transition from primary growth to secondary growth. The expression of 79 of those 700 genes increased 3-fold or higher, and among the 79 genes, the functions of 30 were involved in transcriptional regulation and signal transduction. Moreover, a number of auxin-modulating genes, including four auxin efflux carrier genes, four auxin influx carrier genes, ten *Aux*/*IAA* genes, and eighteen *ARF* genes, were differentially expressed in the induced secondary growth stem. In several studies, auxin was suggested as a factor involved in vascular cambium activities, xylem differentiation and vascular tissue development (Jacobs, 1952; Aloni, 1995; Sachs, 1981, 2000; Little *et al*., 2002; Ye, 2002). Genomic results indicating that the expression of auxin-modulating genes is associated with the transition from primary growth to secondary growth may provide a line of preliminary genetic information to further mechanistic investigations of how auxin regulates secondary growth.

Microarray analysis using the Arabidopsis 24 K GeneChip (Affymetrix) was applied to detect the difference of gene expression in isolated xylem, phloem-cambium, and nonvascular tissues from Arabidopsis hypocotyl segments (Zhao *et al*., 2005). In that study, significantly biased gene expressions were detected in various isolated tissues. Some genes were dominantly expressed in one tissue, xylem, phloem-cambium, or nonvascular tissue. Others were preferentially expressed in two type tissues, such as xylem/phloem-cambium, xylem/nonvascular, or phloem-cambium/nonvascular tissues. In addition, some genes, with known involvement in regulating primary meristem cells, were found to have a biased expression in the phloem-cambium tissue. Those included Class III *HD ZIP* and *KANADI* transcription factors genes, several members of the *G20-like*, *NAC*, *AP2*, *MADS*, and *MYB* transcription factor families.

In addition to already noted Arabidopsis studies, an *in vitro* zinnia cell culture system was established to study cell transdifferentiation from mesophyll cells into protoxylem- and metaxylem-like tracheary elements (Fukuda, 1997). There is a certain degree of similarity between this system and *in vivo* xylem differentiation regarding the processes of secondary cell wall thickening and biosynthesis. Therefore, this system has been used to study the gene expression related to secondary wall biosynthesis. During a 72–96-hour culture period, the zinnia cell transdifferentiation is divided into the following three stages: stage1 (first 24 hours), the functional dedifferentiation; stage 2 (second 24 hours), redifferentiation in the precursor of tracheary elements; stage 3 (next 24–48 hours), tracheary element maturation including secondary wall deposition and cell death. More than 8,000 zinnia cDNA clones were isolated from an equalized cDNA library prepared from cultured cells transdifferentiating into xylem cells. Microarray analysis using these cDNAs revealed several types of gene expression patterns. The genes, of which the expression was transiently induced in culture stage 3, included those related to cell-wall formation and degradation and programmed cell death (Demura *et al*., 2002).

As previously noted, Arabidopsis has been used as a model system to study the genomics of secondary growth when grown under particular growth conditions or treatments. The genomic information collected from the herbaceous Arabidopsis system

provides a line of useful evidence for a general understanding of secondary growth process. However, there are apparent limitations in using this system to comprehensively dissect genomic mechanisms underlying the wood formation caused by natural secondary growth in woody plants (Taylor, 2002). In Arabidopsis stems with induced secondary growth, more layers of xylem cells can be differentiated. Certainly the molecular mechanisms regulating the xylem cell differentiating process could resemble in some degree those occurring in natural secondary growth. However, it is also noticeable that the difference between the induced secondary growth in herbaceous Arabidopsis and the natural secondary growth in woody plants is quite substantial. In woody plants, the secondary growth is initiated from meristematic cambium cells that retain perpetual cell dividing ability in entire lifetime. The secondary growth involves a series of sequential processes from cell division, cell fate termination, cell differentiation, to cell death and cell activity preservation, representing a developmental process beginning with cambial initials and eventually leading to well-organized secondary tissues. The specialized tissues serve many functions in the life of trees, such as nutrient and water conduction, mechanical support, and nutrient and chemical storage. In matured secondary xylem tissue it is remarkable that the ray parenchyma cells can stay alive for a long period of time. Moreover, the secondary growth is determined and regulated by many genetic and environmental factors. For example, mainly tracheid elements were differentiated in gymnosperm plants whereas vessel and fiber cells developed in angiosperm species. Secondary growth displays an annual cycling pattern starting with activation of vascular cambium in the beginning of the growing season and stalling with dormancy of the cambium at the end of the season. In order to systematically understand the process and regulation of plant secondary growth, a wood plant system is required in addition to use of herbaceous Arabidopsis model plants.

TABLE 1
The EST sequence information from tree species

Species	Number of ESTs
Pinus taeda	314,535
Malus × *domestica*	197,774
Picea glauca	104,305
Citrus sinensis	94,895
Populus tremula × *Populus tremuloides*	76,160
Populus trichocarpa	58,146
Populus tremula	37,313
Populus trichocarpa × *Populus deltoides*	33,134
Poncirus trifoliata	28,861
Picea engelmannii × *Picea sitchensis*	28,170
Prunus persica	21,873
Pinus pinaster	18,254
Populus nigra	15,257
Populus deltoides	14,656
Populus trichocarpa × *Populus nigra*	14,281
Citrus clementina	13,942
Populus euphratica	13,903
Populus tremuloides	12,813
Populus × *canescens*	10,446
Populus euramericana	10,157
Citrus × *paradisi* × *Poncirus trifoliata*	8,064
Populus alba × *Populus tremula var. glandulosa*	7,595
Ginkgo biloba	6,254
Citrus aurantium	5,127
Citrus × *paradisi*	4,856
Prunus dulcis	3,864
Citrus reticulata	3,735

B. EST Information in Wood Formation

An expressed sequence tag (EST) database is a collection of short cDNA sequences reverse-transcribed from mRNAs expressed in a specific organism or tissues. A great deal of effort in the past several years has been invested in collecting EST information because these sequences represent the expressed products of the genes functioning in certain tissues under specific conditions. When expressed gene information is collected during wood formation, it provides profiling information to depict a genomic picture of how wood is formed. To date, the EST information has been collected from a number of tree species, especially in wood-forming tissues. Listed in Table 1 is the EST information currently available to the public from tree species, including trees for wood material production and fruit products. In loblolly pine, EST sequences were collected from many tissue-specific cDNA libraries, including root, stem, shoot tip, needle (Laboratory for Genomics and Bioinformatics, Department of Plant Biology, The University of Georgia), embryo (Plant Genomics Group, The Institute for Genomic Research), pollen cone, and xylem (Forest Biotechnology Group, North Carolina State University). There have also been banked abundant EST sequences from *Populus* species and tissues, such as PopulusDB (http://www.populus.db.umu.se) that was built with 121,495 *Populus* EST-sequences collected from 19 cDNA libraries. The sequences represent 24,658 expressed genes from a *Populus* genome, including 11,891 contigs and 12,767 singletons.

Generally homologous genes from different plant species can be assigned a same gene name. Currently, the annotation of tree EST sequences is primarily based on the sequence homology comparison against Arabidopsis gene information. These analyses are able to annotate most EST sequences collected from a tree species. In loblolly pine ESTs, about 90% of the contigs can find apparent homologs in the Arabidopsis sequence, as does a similar percentage of ESTs in *Populus* ESTs (Kirst *et al*., 2003; Sterky *et al*., 1998, 2004). Meanwhile, there exist some ESTs

from tree species for which researchers have been unable to find homologous genes in the model species. The gene function of those ESTs is usually classified as unknown. The sequence similarity or difference between tree species and Arabidopsis has two significant implications. For many biological phenomena, the mechanisms revealed by using an Arabidopsis model system can be applicable to tree species as the same gene may function similarly. On the other hand, the absence of apparent homolog in Arabidopsis for some genes in trees may indicate specialization in tree genomes. Thus, the genomic characterization of those nonhomologous genes could lead to insights into the answer of why the tree is different from Arabidopsis.

1. Gene Expression in Wood Formation

To investigate the genomics of wood formation, gene expression profiles during secondary xylem differentiation have been reported from several laboratories. As the EST sequences from tree species become available, tree-specific cDNA microarrays can be printed for probing the transcripts during wood formation. In a hybrid aspen species, *P. tremula* × *P. tremuloides*, a set of 2085 genes was selected from 2995 unigene clones and was printed on an array (Hertzberg *et al*., 2001). The cDNA array was used to screen the gene expression in differentiating xylem tissue undergoing various stages of wood formation. Samples were collected by micro-sectioning of the vascular cambium and developing xylem zones representing a series of wood-forming stages from meristematic cells, to early cell expansion, to late cell expansion, to secondary wall formation and finally to late cell maturation. The microarray profiling analysis indicated that the gene expression is under strict developmental stage-specific transcriptional regulation. The genes encoding lignin and cellulose biosynthetic enzymes as well as a number of transcription factors are particularly expressed in the secondary wall formation stage. The stage-specific expression was further demonstrated recently in a high-resolution transcript profile. Schrader *et al*. (2004) studied the gene expression map in poplar wood-forming tissue. From the zone of the vascular cambium to the area of matured xylem cells, the wood-forming tissue was serially sampled in 20 μm thick section. The expressed genes in each section were measured using a POP1 poplar cDNA array that included more than 13,000 unique genes assembled from hybrid aspen ESTs. The expression of a number of specific genes was found to be associated with various wood-forming stages from cambium meristem cell division, cell expansion, to cell differentiation. In the cambial zone, Ptt*CLV1*, Ptt*KnOX* and Ptt*ANT* are specifically expressed. As these gene homologs are known for the involvement of apical meristem cell regulation in Arabidopsis (Brand *et al*., 2000; Nakajima and Benfey, 2002; Schoof *et al*., 2000), it is likely that the regulatory molecular networks in apical meristem cells may also be similarly present in cambium meristem cells. A set of genes was found to be associated with cambial cell division. The expression of the Ptt*CYCA1*, Ptt*CDKB2*, Ptt*CYCD3*, Ptt*CKS1* and Ptt*DP-E2F-like* genes is remarkably increased in the dividing vascular cambium zone.

The EST information from loblolly pine has been another valuable genomic resource for studying the wood formation in gymnosperm species that have a wood structure that differs from angiosperm wood, such as *Populus*. Utilizing pine cDNA microarray analysis, a list of candidate genes that may play significant roles in cell wall formation in differentiating pine secondary xylem was identified. Some of these genes seem to be specific to pine, whereas others also occur in model plants, such as Arabidopsis (Allona *et al*., 1998; Whetten *et al*., 2001; Kirst *et al*., 2003). In a detailed analysis of gene expression, the cDNAs that encode six cell wall-associated proteins and a phytocyanin-homologous gene were identified from developing xylem of loblolly pine. The six cell wall-associated proteins include three encoding putative loblolly pine arabinogalactan proteins (AGPs): one was related to the proline-rich protein group, and the other two were related to the glycine-rich protein group and the mussel adhesive protein. All but one of the genes was highly expressed in vascular tissues (Zhang *et al*., 2003). In another study, serial analysis of gene expression (SAGE) was used to quantify gene expression in developing xylem from loblolly pine. According to the results, over 85,000 SAGE tags representing a maximum of 27,398 expressed genes were examined from the developing xylem of the upper trunk, and more than 65,000 tags, representing a maximum of 25,983 expressed genes, were analyzed from the lower trunk. A total of 150,855 tags, representing a maximum of 42,641 different genes, were cataloged in developing xylem (Lorenz and Dean, 2002). The SAGE study provides another line of gene expression information for pine wood formation. Because of short SAGE tags and unavailable pine genome sequence, it is a challenge to fully interpret the results into functioning gene information.

When gene expression during wood formation is profiled, a notable characteristic is the detection of the genes of which the expression is particularly associated with the late differentiation stages, including secondary wall biosynthesis and cell death. The final developing stage in wood formation is cell death after xylem cell maturation. The genes expressed in the late stage of xylem cell development include those functioning in the programmed cell death process. In *Populus*, an EST library from the woody tissues of hybrid aspen (*P. tremula* × *P. tremuloides* T89) stems was constructed (Sterky *et al*., 2004). The woody tissues used for the library construction mainly included the xylem cells with fully developed cell walls. A total of 4,867 EST sequences with an average length of 548 bp were collected from the library. The EST analysis indicated that a large number of previously unidentified transcripts are found in the woody tissue library, suggesting a possibility that the gene function of the newly identified ESTs is related to the late stage of wood formation, such as xylem cell death. In microarray analyses using a *Populus* 25K cDNA array, Moreau *et al*. (2005) further identified two novel extracellular serine proteases, nodulin-like proteins, and an *Arabidopsis thaliana* OPEN STOMATA 1 (*AtOST1*) homolog that could be involved in signaling xylem-cell death. Similarly, in the stage 3 of the *Zinnia* tracheary element culture system, 12

genes encoding proteases, nucleases, or lipases were identified for playing a role in the cell death process (Demura *et al*., 2002).

In attempts to understand wood formation, studies using Arabidopsis plants, and zinnia *in vitro* cell cultures and tree developing xylem tissues have yielded large amounts of gene expression data related to xylem cell differentiation and secondary wall biosynthesis. Many of the highly expressed genes displayed a similar pattern in association with cell wall thickening in the three systems. These genes include *HD-ZIP III* and other transcription factor genes, many of the monolignol biosynthesis genes, and many of the cellulose biosynthase genes. It appears that the function of the common genes is mainly associated with "housekeeping" metabolisms in the downstream process of the cell differentiation (Hertzberg *et al*., 2001; Demura *et al*., 2002; Oh *et al*., 2003; Ko *et al*., 2004; Ko and Han, 2004; Schrader *et al*., 2004; Ehlting *et al*., 2005). Wood formation is initiated from early cell division at a specific tissue location and goes through a series of developing processes to become matured xylem tissue. Many specific genes can certainly be required for the early developing processes. Thus, further and more detailed investigations about the upstream processes are needed to elucidate the full genomic map of the entire wood formation process.

2. *Wood Properties Regulated by Gene Expression*

Wood formation can be regulated by various environmental conditions, yielding the development of characteristic woods with different wood features or properties. Thus, detection of how gene expression is responsive to environmental conditions in the course of wood formation should provide insightful clues about genetic regulation of wood properties. Trees develop "reaction wood" when they suffer from mechanical stress or weight loads generated by, for example, wind and gravity (Sinnott, 1952; Barnett, 1981; Timell, 1986). Reaction wood, namely tension wood, is developed at the upper side of leaning stems or branches in angiosperm trees. The tension wood contains very high cellulose and low lignin. In gymnosperm trees, another type of reaction wood, compression wood is formed at the lower side of leaning stems or branches to react against mechanical stress (Scurfield, 1973; Timell, 1986; Pilate *et al*., 2004a, 2004b).

Gene expression profiles have been studied in the formation of reaction wood in pine (Whetten *et al*., 2001), poplar (Déjardin *et al*., 2004; Sterky *et al*., 2004), and *Eucalyptus* (Paux *et al*., 2005). A total of 5,723 ESTs were cloned from a *P. tremula* × *P. tremuloides* T89 tension wood cDNA library and a total of 10,062 ESTs were cloned from bent *P. tremula* × *P.alba* trees. Analysis of the ESTs cloned from *P. tremula* × *P.alba* trees indicated that five clusters of arabinogalactan proteins, one sucrose synthase and one fructokinase are specific or overexpressed in tension wood. Moreover, using cDNA array analysis, transcript abundance of the 231 genes that are known with preferential expression in differentiating *Eucalyptus* xylem was examined in the course of artificial mechanical stress from 6 hrs to 1 wk. Among them, 196 genes were differentially regulated between control and bent trees. Moreover, some of the differentially regulated genes showed expression patterns in association with the changes of secondary cell wall structure and composition.

Secondary growth in trees is annually regulated by seasonal changes, which leads to the formation of two kinds of wood with distinctive structure and chemical compositions. One is named earlywood, which forms in early growing season, and the other is latewood formed in late growing season. The size of the cells in latewood is usually smaller and its cell wall is thicker as compared with early wood. The content of hemicellulose is significantly higher and lignin content is much lower in latewood than in earlywood (Sewell *et al*., 2002). Using cDNA microarrays that contained 2171 EST cDNA probes selected from loblolly pine xylem cDNA libraries and a shoot tip library, the seasonal variation of gene expression for loblolly pine was recently analyzed (Yang and Loopstra, 2005). The results indicated that the gene expression profiles were different between earlywood and latewood, and this expression difference varied among trees from two seed sources.

Sapwood and heartwood are two different regions in the trunk wood of most tree species. Sapwood is the outermost portion of the xylem tissue and contains living cells, which may conduct sap (water, solutes, and gases) and serve as a reservoir for water, energy, minerals, and solutes. Heartwood is defined as the "dead" central core of the woody axis, which provides passive support to the tree. Gene expression was examined across the stems of 10-year-old *Robinia pseudoacacia* trees (Yang *et al*., 2004). From the samples collected from bark, sapwood, and sapwood-to-heartwood transition zone tissues, a total of 2915 ESTs were cloned and analyzed (Yang *et al*., 2003). Among them, 1304 ESTs matched previously sequenced genes and 909 had significant homology to known genes. Microarray analysis of the EST clones showed that a gene encoding sugar transport had the highest expression in the sapwood, whereas those genes related to flavonoid biosynthesis were upregulated in the sapwood-heartwood transition zone. In another experiment using the same arrays, gene expression profiles in the transition zone were found to display seasonal changes (Yang *et al*., 2004). A group of 293 genes including more than 50% of the secondary and hormone metabolism-related genes on the arrays were found to be upregulated in the summer.

C. Genome Sequence of Tree Species

In the past dozen or so years, a great deal of effort has been devoted to genome sequencing for various organisms. After the first plant genome, the Arabidopsis genome, was sequenced, genome sequencing was undertaken for a number of other plant species. Since trees exhibit many unique characters that are lacking in herbaceous species, a tree genome sequence is required to better understand tree biology as well as comparative genomics (Taylor, 2002). *Populus*, a genus of tree species, has a wide natural distribution and significant ecological and economic value. Many *Populus* species are intensively studied in

genetics and forestry around the world (Stettler *et al*., 1996). *Populus* usually has a relative small genome and takes a short period of time to reach sexual maturity. *Populus* species are also easy to vegetatively propagate and are amenable to tissue culture and genetic transformation. Due to these desirable characters, a species, *Populus trichocarpa*, has been selected for the full genome sequencing; and the approximately 480-million-base-pairs genome has been sequenced 7.5X in depth through a collaborative effort led by the U.S. Department of Energy Joint Genome Institute (JGI). Currently the whole-genome shotgun sequence data is publicly accessible at the website of http://genome.jgi-psf.org/Poptr1/Poptr1.home.html. The genome sequence has so far been partially assembled into contigs and scaffolds. A total of 58,036 gene models have been predicted. Although further effort is required to have the genome sequence completely assembled, the availability of the tree genome sequence opens a wide window to study tree biology as well as wood formation.

In addition to *Populus* genome sequencing, *Eucalyptus*, another important tree genus for fiber material production, has been targeted for genome sequence-decoding. The genome of most *Eucalyptus* species is estimated to be about 600 Mbp on 11 chromosomes. Collaborative efforts from several institutions have been initiated to sequence a *Eucalyptus* genome. Some information about this effort can be found at the website http://www.ieugc.up.ac.za/.

III. FUNCTIONING GENE TOOLBOX FOR THE BIOSYNTHESES OF WOOD FORMATION

As the genomic information about wood formation is accumulating, the functional characterization of the gene toolbox involved in this process has achieved a great deal of progress in recent years. Wood formation includes many enormously active biosynthetic processes during secondary wall thickening, such as cellulose, lignin and hemicellulsoe biosynthesis. Basically, these three polymers consist of more than 95% of the wood's dry weight. Clearly their biosynthesis is the metabolic center in wood formation. Thus, the main efforts on the functional characterization of wood formation genes have been related to cellulose, lignin, and hemicellulose biosynthesis.

A. Lignin Biosynthesis

In past 15 or so years, there has been a great deal of interest in cloning and characterization of the genes controlling monolignol biosynthesis in order to clarify monolignol biosynthetic pathways in trees and other plants. There have been a number of reviews about the advancements of monolignol biosynthesis pathways in plants (see reviews by Whetten and Sederoff, 1995; Whetten *et al*., 1998; Humphreys and Chapple, 2002, Boerjan *et al*., 2003). As enormous variation in lignin content and composition is observed among plant species, tissues, cell types, and even in development stages and environmental conditions, data from the studies using different plant materials display many agreements as well as certain disagreements. Thus, it is debatable whether lignin biosynthesis in all plants follows the exact same pathway or not. To date, most of the genes for monolignol biosynthesis have been known and characterized in various plant species. Figure 1 provides a summarized picture of the main and possible monolignol biosynthesis pathways of wood formation in trees.

1. PAL

Generally, monolignol biosynthesis is considered to start from phenylalanine. The enzyme, phenylalanine ammonia–lyase (PAL) (E.C.4.3.1.5), that catalyzes the conversion of phenylalanine to trans-cinnamic acid, is thought to be the initial step towards monolignol biosynthesis and other phenolic secondary metabolisms derived from primary metabolism. Genes encoding *PAL* have been studied extensively in many plant species, such as various *Populus* species (Osakabe *et al*., 1995; Kao *et al*., 2002), loblolly pine (Whetten and Sederoff, 1991), and many other plant species (Jones, 1984; Ohl *et al*., 1990; Leyva *et al*., 1992; Bate *et al*., 1994; Hatton *et al*., 1995; Kumar and Ellis, 2001). *PAL* exists as a multiple member gene family and the individual members can be involved in different metabolic pathways as suggested by their expression patterns in association with certain secondary compounds accumulated in specific tissue or developmental stage. For example, two *PAL* genes were cloned from quaking aspen and their expression suggested that one is associated with condensed tannin metabolism and the other is involved in monlignol biosynthesis (Kao *et al*., 2002). In the Arabidopsis genome, four *PAL* genes were identified and could be phylogenetically classified into two groups based on sequence similarity (Raes *et al*., 2003). The biochemical activity of all known PALs is verified to specifically catalyze deamination of phenylalanine, but the genetic and physiological function may vary among different *PAL* gene members. The expression of *PAL* genetic function is controlled by various genetic circuits and signaling pathways. The *cis*-element structures in *PAL* gene promoters can be part of the molecular circuit that directs a variety of the *PAL* genetic and physiological functions. In some *PAL* promoters, conserved AC *cis*-elements are identified for regulating the specific expression of the phenylpropanoid genes related to monolignol biosynthesis in the vascular tissues. In other *PAL* member promoters, the *cis*-elements of A box, H box, and G box are found (Cramer *et al*., 1989; Lois *et al*., 1989; Osakabe *et al*., 1995; Leyva *et al*., 1992; Raes *et al*., 2003). Many *cis*-elements have been identified in various members of the *PAL* gene family, however, the function of the *cis*-elements and associated molecular network that regulates the expression of *PAL* gene family for various metabolic pathways remains to be studied.

2. C4H

The conversion of cinnamate to *p*-coumarate is catalyzed by cinamate 4-hydroxylase (*C4H*). *C4H* is a cytochrome P450-dependent monooxygenase, belonging to the *CYP73* family.

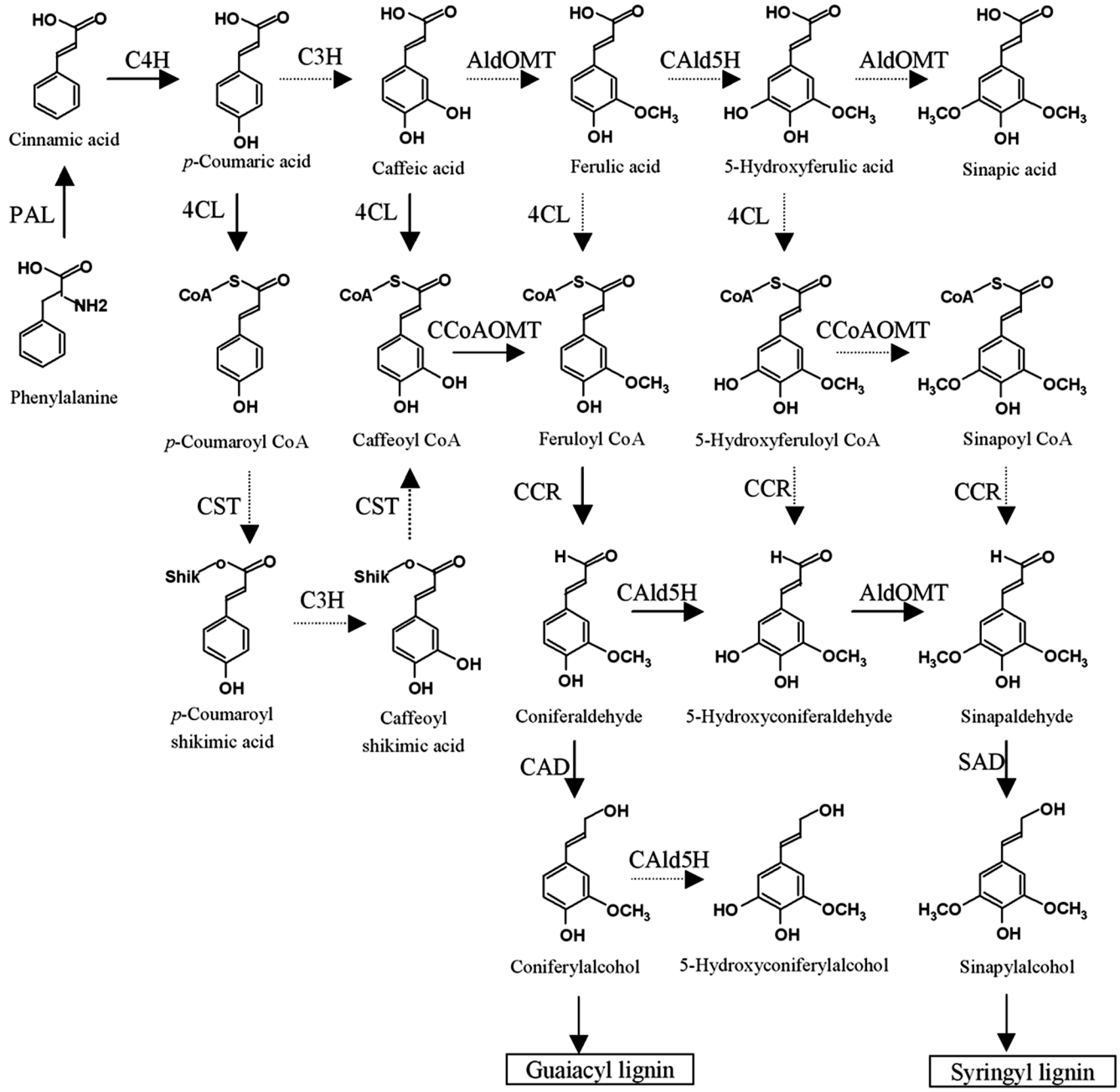

FIG. 1. An overview of the monolignol biosynthesis pathways of wood formation in trees. The main pathways are indicated in solid line arrows and possible pathways in dotted line arrows. PAL, phenylalanine ammonia-lyase; C4H, cinnamic acid 4-hydroxylase; C3H, *p*-coumarate 3-hydroxylase; CST, hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyltransferase; 4CL, 4-coumaroyl-CoA 3-hydroxylase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl coenzyme A reductase; CAld5H, coniferyl aldehyde 5-hydroxylase; AldOMT, 5-hydroxyconiferyl aldehyde *O*-methyltransferase; CAD, cinnamyl alcohol dehydrogenase; SAD, sinapyl alcohol dehydrogenase.

C4H was the first plant P450 gene with its biochemical function characterized in yeast expressed recombinant protein (Teutsch *et al*., 1993; Urban *et al*., 1994, 1997). Similar to PAL, *C4H* is thought to be involved in a number of secondary metabolism pathways in addition to monolignol biosynthesis as *p*-coumarate is an intermediate for biosynthesis of many secondary compounds (Croteau *et al*., 2000). Multiple *C4H* gene members are identified in many plant species; however, only one *C4H* is known in the Arabidopsis genome (Raes *et al*., 2003). The expression study of two *C4H* members in quaking aspen indicated that one is strongly expressed in developing xylem tissues and the other is more active in leaf and young shoot tissues

(unpublished data, Li, Chiang and co-workers). In other species, *C4H* is expressed in a variety of tissues and the expression can be also induced by wounding, light, pathogen attacks and other biotic and abiotic stimuli (Bell-Lelong *et al*., 1997; Raes *et al*., 2003). As the biochemical reaction mediated by *C4H* consecutively follows the reaction that PAL catalyzes, *C4H* and PAL may form an enzyme complex in cells to channel the metabolic flux through the two neighboring reactions (Czichi and Kindl, 1975; Achnine *et al*., 2004). Although the *C4H* is well illustrated for its biochemical function and plays a role in different metabolic pathways which occur in various tissues or cells, the mechanisms that regulate the genetic function of *C4H* gene and its family members are yet unknown.

3. C3H

Although most of the genes encoding the enzymes for the biochemical reaction of each step in the monolignol biosynthesis pathways have been well characterized, the gene for the enzyme that catalyzes *p*-coumarate 3-hydroxylation (*C3H*) is still not fully confirmed. Early biochemical evidence suggested that the reaction is catalyzed by a nonspecific phenolase (EC1.10.3.1), but that suggestion did not receive much support in other studies (Stafford and Dresler, 1972; Boniwell and Butt, 1986; Kojima and Takeuchi, 1989; also see a review by Petersen *et al*. 1999). Recently, an alternative pathway was proposed based on the enzyme activity of *CYP98A3* gene from Arabidopsis (Schoch *et al*., 2001; Franke *et al*., 2002; Nair *et al*., 2002). The proposed alternative suggested that the hydroxylation at the 3-position of the aromatic ring of cinnamic acid does not directly occur on *p*-coumarate, instead, *p*-coumarate is first converted to *p*-coumaroyl CoA ester by 4-cinnamoyl-CoA ligase (4CL), then the CoA ester group of *p*-coumaroyl CoA is exchanged by hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyltransferase (CST) to form *p*-coumaroyl shikimic acid which serves as a substrate of *C3H* to produce caffeoyl shikimic acid. Subsequently caffeoyl shikimic acid reverts back to caffeoyl CoA to push metabolism towards the biosynthesis of monolignols. In trees, a *CYP98* cDNA was cloned from sweetgum and aspen (Osakabe *et al*., 1999), but the postulated genetic and biochemical function in monolignol biosynthesis has not been demonstrated for its role in wood formation.

4. 4CL

Both genetic and biochemical functions of 4-Coumarate Coenzyme A ligase (*4CL*) genes have been clearly demonstrated in association with monolignol biosynthesis (reviewed by Lewis and Yamamoto, 1990; Lee *et al*., 1997; Hu *et al*., 1998, 1999; Harding *et al*., 2002). *4CL* genes usually exist as a family of multiple members in many species. However, different expression patterns of *4CL* members are found in herbaceous and tree species. Four *4CL* genes were detected in the Arabidopsis genome and the expression of each member was regulated differentially in tissues and development stages (Raes *et al*., 2003). In aspen trees, two *4CL* genes were cloned and their expression was clearly distinct, with one in epidermal and leaf tissue and the other specifically in developing xylem tissue (Hu *et al*., 1998; Harding *et al*., 2002). Furthermore, the enzymatic activities of *4CL* members from aspen, loblolly pine, tobacco, soybean, Arabidopsis, and many other species were found to have distinct substrate specificities (Voo *et al*., 1995; Zhang and Chiang, 1997; Hu *et al*., 1998; Lindermayr *et al*., 2003; Schneider *et al*., 2003; Hamberger and Hahlbrock, 2004). Whether the substrate specificity of the *4CL* members relates to different metabolic pathways is unknown. On the other hand, in lignifying tissue, the 4CL enzyme is able to catalyze a variety of cinnamic acid derivatives *in vitro*. Are those substrates factual intermediates of monolignol biosynthesis pathways? From a metabolic economy point of view, however, it is not biologically efficient for lignin biosynthesis *in vivo* to wander through multiple routes in strongly lignifying tissues such as wood formation. Instead a mainstream of the pathway may exist. But it is still unclear which substrate acts as the factual intermediate *in vivo* for the mainstream pathway. As the *4CL* catalytic kinetics vary among species, it is also likely that the mainstream pathway mediated by *4CL* may not be exactly the same in all plant species or tissues. Nevertheless, monolignol biosynthesis is tightly controlled by *4CL*. Suppression of *4CL* expression through antisense technology has repeatedly demonstrated the effectiveness of reducing total lignin content in tree xylem tissue and in other plants (Lee *et al*., 1997; Hu *et al*., 1999; Li *et al*., 2003). In aspen, suppression of *4CL* expression led to more than 55% lignin reduction in wood. Thus, technology aimed at *4CL* suppression could be applied to plant genetic modification for better fiber production and other utilizations.

5. CCoAOMT and COMT or AldOMT

In monolignol biosynthesis, methylation is required at two positions on the aromatic ring of the monolignol unit. One is at the 3-position and the other at the 5-position. The 3-postion methylation leads to guaicyl unit formation and both methylations on the 3- and 5-positions results in a syringyl unit. In early studies, it was postulated that two types of methyltransferases were necessary for the methylations (Higuchi, 1997). Mono-functional *O*-methyltransferase was an enzyme to methylate the 3-position and therefore controlled the G monolignol unit biosynthesis, and bi-functional *O*-methyltransferase that could catalyze both 3- and 5-methylations led to S monolignol unit biosynthesis. However, new molecular genetics studies suggested that there are two kinds of genes encoding for the enzymes that specifically catalyze the 3- and 5-methylation, respectively (Ye *et al*., 1994; Li *et al*., 1999, 2000; Chen *et al*., 2001). Biochemical evidence from the studies using tree material suggests that the 3- and 5-methylations occur at different biosynthesis stages. The 3-methylation occurs on the CoA ester intermediate while the 5-position is methylated at the aldehyde intermediate (Osakabe *et al*., 1995; Li *et al*., 2000). The two genes are: one encoding a caffeoyl-CoA 3-*O*-methyltransferase (*CCoAOMT*) and the other encoding a 5-*O*-methyltransferase

that preferably methylates 5-hydroxyconiferaldehyde. The 5-*O*-methyltransferase was thought to methylate caffeic acid and then was named *COMT* accordingly. However, more recent new evidence indicated that the methylation catalyzed by this enzyme basically occurs at 5-hydroxyconiferaldehyde, therefore the enzyme was renamed *AldOMT* (Li *et al*., 2000). In addition to these two types of OMTs involved in the monolignol biosynthesis in angiosperms, there is another OMT (named AEOMT) that can methylate both hydroxycinnamic acids and hydroxycinnamoyl CoA esters was found in the gymnosperm loblolly pine (Li *et al*., 1997). However, identification of *AldOMT* in gymnosperms has not been reported. It appears that *CCoAOMT* gene plays a predominant role in the gymnosperm lignin biosynthesis. In loblolly pine *CCoAOMT* was detected in a single copy and specifically expressed in developing xylem (Li *et al*., 1999).

6. CCR

It is thought that the reduction of cinnamoyl CoA esters to cinnamaldehydes is the first metabolic step committed to monolignol formation. This step is catalyzed by cinnamoyl-CoA reductase (*CCR*). Many studies of *CCR* activity indicated that five cinnamoyl-CoA esters (*p*-coumaroyl-CoA, caffeoyl-CoA, feruloyl-CoA, 5-hydroxyferuloyl-CoA and sinapoyl-CoA) could be used as substrates (Wengenmayer *et al*., 1976; Luderitz and Grisebach, 1981; Sarni *et al*., 1984; Goffner *et al*., 1994). The *CCR* enzyme purified from *Eucalyptus* xylem tissue was active toward *p*-coumaroyl-CoA, feruloyl-CoA, caffeoyl-CoA and sinapoyl-CoA with approximately equal affinity (Goffner *et al*., 1994). Similar to the native protein, the recombinant *Eucalyptus CCR* protein was also demonstrated to be active with the substrates *p*-coumaroyl-CoA, feruloyl-CoA and sinapoyl-CoA, individually (Lacombe *et al*., 1997). Recently, the characterization of aspen *CCR* recombinant protein indicated that CCR selectively catalyzed the reduction of feruloyl-CoA from the five cinnamoyl CoA esters (Li *et al*., 2005). When *CCR* and *CCoAOMT* were coupled together, the linked reactions constitute the pathways from caffeoyl-CoA ester to coniferaldehyde (Figure 1). In addition, the results also suggested that the neighboring *CCoAOMT* and *CCR* enzymes require different pH environments and compartmentalization *in vivo*. The genes encoding *CCR* in various species appear as a family with multiple members. In the *Populus* genome, there exist 8 CCR-homolog or *CCR*-like gene sequences. At this time it is unclear how each of the CCR-like genes functions in a tree species; are their functions redundant or specialized?

7. F5H/CAld5H

Hydroxylation at the 5-position on the aromatic ring of cinnamic intermediates is a necessary step to biosynthesize S-monolignols. For a long time, this reaction was thought to occur using ferulic acid as the substrate and catalyzed by ferulate 5-hydroxylase (F5H), which is encoded by a P450 protein gene belonging to *CYP84* family. Although forward genetics evidence demonstrated that *F5H* gene is essential for S-lignin formation in Arabidopsis (Meyer *et al*., 1996), it was unable to identify the intermediate on which the 5-hydroxylation biochemically occurs. In tree species, the homologous genes have been cloned from a number of species. The biochemical function of this P450 gene was first demonstrated by expressing a sweetgum *CYP84* gene in yeast (Osakabe *et al*., 1999). The biochemical data suggest that the *CYP84* protein catalyzes 5-hydroxylation using coniferaldehyde, instead of the postulated ferulic acid, as a substrate to produce 5-hydroxyconiferaldehyde. Thus, *F5H* is actually a coniferaldehyde 5-hydroxylase (*CAld5H*). That the 5-hydroxylation occurs on coniferaldehyde is further confirmed with an Arabidopsis *CYP84* recombinant protein (Humphreys *et al*., 1999). According to the biochemical function of this *CYP84* gene, it can be suggested that the S-monolignol biosynthesis pathway is branched out from a guaicyl intermediate at coniferaldehyde. Consistent with this view, 5-hydroxyconiferaldehyde is then methylated by *COMT* or *AldOMT* as described above. The genetic function of *CYP84* is also demonstrated through a reverse genetics approach by overexpression of the gene, which leads to the intensified S-units in lignin (Franke *et al*., 2000; Li *et al*., 2003). Because the lignin with higher percentages of S-unit has a potentially significant value in the pulping economy (Chang and Sarkanen, 1973), overexpression of *CAld5H* gene in trees has great potential to produce desirable wood material for fiber production.

8. CAD and SAD

In gymnosperm wood, coniferyl alcohol is the major monolignol unit while both coniferyl alcohol and sinapyl alcohol are monolignnols in angiosperm wood. The last metabolic step forming these monolignols is reduction of coniferaldehyde and sinapaldehyde. Cinnamyl alcohol dehydrogenase (*CAD*) is believed to catalyze multiple cinnamyl alcohol formations from their corresponding cinnamaldehydes (Lewis and Yamamoto, 1990; Whetten and Sederoff, 1995; Whetten *et al*., 1998). In loblolly pine, *CAD* is a single copy gene and its mutation leads to abnormal lignin formation in wood (MacKay *et al*., 1997; Lapierre *et al*., 2000). When the *Populus* tree was studied for monolignol biosynthesis in wood-forming tissue, in addition to *CAD*, it was found in aspen that another gene, its sequence similar to but distinct from *CAD*, is also associated with lignin biosynthesis (Li *et al*., 2001). The biochemical characterization of the recombinant protein encoded by this gene indicated that the enzymatic activity has specific affinity toward sinapaldehyde, therefore it was named sinapyl alcohol dehydrogenase (*SAD*). Compared with *SAD* enzyme kinetics, *CAD* showed a catalytic specificity towards coniferaldehyde instead. The catalytic specificities of the two enzymes have recently been further verified in protein structure analysis (Bomati and Noel, 2005). Furthermore, it was demonstrated that the expression of *CAD* is associated with G-lignin accumulation while *SAD* was associated with S-lignin formation during xylem differentiation (Li *et al*., 2001). The evidence from molecular, biochemical and cellular characterizations strongly suggest that *CAD* is involved

in G-monolignol biosynthesis and *SAD* in S-monolignol biosynthesis in aspen wood formation. However, a recent genetic study using an Arabidopsis model system suggests a broad CAD function for both G- and S-lignin biosynthesis in the herbaceous species (Sibout *et al*., 2005). It is unclear whether this disagreement is due to the difference in lignin biosynthesis pathways among plant species. Nevertheless, more evidence connecting the biochemical function to its genetic role may be required in order to completely understand how *CAD* and *SAD* genes play a role in monolignol biosynthesis during wood formation.

In analysis of functioning genes involved in monolignol biosynthesis of gymnosperm and angiosperm wood, three genes, *CAld5H*, *AldOMT* and *SAD*, control a line of consecutive metabolic steps and constitute a pathway toward S-monolignol biosynthesis. These three genes have not been known to be present in gymnosperm species that do not synthesize S-lignin. Gymnosperm wood is primarily comprised of tracheid elements, but angiosperm wood contains two types of thickened secondary wall cells, vessel element and fiber cells. Apparently the fiber cell is evolved along with occurrence of angiosperm species. It is known that G-monolignol units are dominant in tracheids and vessels and S-units predominate fiber cells. It can be postulated that monolignol biosynthesis pathway evolution may be correlated with cell type specification in the course of plant evolution; however, this hypothesis remains to be verified.

It is believed that lignin is polymerized at the outside of the plasma membrane in secondary cell walls. Thus, monolignols that are synthesized inside plasma membrane need to be transported across plasma membranes for polymerization. Based on biochemical and cellular evidence, it has been suggested that laccases and peroxidases may be two types of possible enzymes involved in lignin polymerization (Bao *et al*., 1993; Christensen *et al*., 1998; Østergaard *et al*., 2000). However, the convincing genetic evidence to support this suggestion is lacking. As to how lignin is polymerized from monomers, it has been debated for a long time whether lignin is polymerized from monomer units randomly or in a way guided by a specific protein (Ralph *et al*., 2004; Davin and Lewis, 2005). A gene encoding a dirigent protein was cloned and the biochemical results suggested the dirigent protein might play a role in guiding a stereo-specific lignin polymerization (Davin *et al*., 1997). This hypothesis still remains to be confirmed. On the other hand, monolignol glucosides are found in developing xylem tissues in many species but currently it is unclear whether the monolignol glucosides are used as intermediates for monolignol storage or for transportation crossing the plasma membrane (Dharmawardhana *et al*., 1995; Meyermans *et al*., 2000; Steeves *et al*., 2001; Tsuji and Fukushima, 2004). Overall, although there are various studies on the process of monolignol cross-membrane transportation and lignin polymerization, the genetic and molecular evidence is elusive regarding what chemical format is taken for the transportation and the mechanisms of how monolignols are transported to the outside of membrane where they are polymerized into lignin.

B. Cellulose Biosynthesis

In wood, cellulose is the most abundant component, accounting for more than 40% of wood dry weight. The cellulose molecule is a linear β (1, 4) glucan polymer with a relatively simple structure; however, the knowledge about the gene toolbox functioning in the biosynthesis of cellulose is very limited. Due to the inability to determine biochemical activity of cellulose biosynthesis, characterization of this process mainly relies on molecular and genetic approaches. According to genetic studies of the bacterium*Acetobacter xylinum*, a gene *AxCesA1-D1* was identified for likely encoding a cellulose synthase (CesA) catalytic subunit (Ross *et al*., 1991; Delmer, 1999). During cotton fiber development, the expression of two cDNAs was found being well correlated with cellulose fiber formation. The sequence analysis of the cDNAs indicated that they share many sequence characteristic structures with the bacterium *CesA* sequence. These two cDNA clones, named GhCesA1 and GhCesA2, were identified as *CesA* gene clones from plants (Pear *et al*., 1996). The availability of the plant *CesA* sequence information then opened the opportunity to study cellulose biosynthesis in plants. The identification and characterization of cellulose-deficient mutants in Arabidopsis have led to the isolation of a number of *CesA* genes as well as the confirmation of *CesA* genetic function in plants (Arioli *et al*., 1998; Taylor *et al*., 1999, 2000, 2003; Fagard *et al*., 2000; Scheible *et al*., 2001; Desprez *et al*., 2002). Now it is known that in plants *CesA* genes constitute a superfamily consisting of multiple members and encode CesA subunit proteins which are about 900 to 1200 amino acids in length. The CesA subunit protein is conserved with about 8 trans-membrane domains at the N-terminal and C-terminal. A UDP-binding domain including the "D,D,D,QXXRW" motif is featured in the region in front of C-terminal trans-membrane domains. These sequence and structure characteristics are usually considered as CesA protein structure signatures to identify *CesA* genes in DNA sequence analysis. In the Arabidopsis genome, 10 *CesA* genes were identified (Richmond and Somerville, 2000). Among them, three genes, *AtCesA8*, *AtCesA7*, and *AtCesA4* (corresponding to three *irx* mutants: *irx1*, *irx3*, and *irx5*) were suggested to be involved in the cellulose biosynthesis of secondary walls (Taylor *et al*., 1999, 2000, 2003). Another three *CesA* genes, *AtCesA1*, *AtCesA3*, and *AtCesA6* (corresponding to *radical swelling* mutants, *RSW1*, *RSW2*, and *RSW3* mutants), are likely controlling the cellulose biosynthesis in primary cell walls (Arioli *et al*., 1998; Fagard *et al*., 2000; Scheible *et al*., 2001). Analysis of *AtCesA8*, *AtCesA7*, and *AtCesA4* genes in Arabidopsis indicated they are all required, functioning in a coordinative way, for cellulose biosynthesis in secondary walls (Taylor *et al*., 2003). In analysis of rice mutants, three *CesA* genes were characterized for the function in secondary wall biosynthesis (Tanaka *et al*., 2003). In tree species, 9 *CesA* genes are identified in the EST libraries of PopulusDB (Sterky *et al*., 1998; Djerbi *et al*., 2004). Meanwhile, *CesA* cDNAs were also cloned from aspen (Wu *et al*., 2000; Samuga and Joshi, 2002; Kalluri and Joshi, 2003, 2004; Liang and Joshi, 2004) and

loblolly pine (Nairn and Haselkorn, 2005; Shi *et al*., unpublished data). The *P. trichocarpa* genome has been sequenced, for the first time in tree species, to about 7.5 × in depth. By searching the genome sequence data (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html), 18 *CesA* homologous gene sequences can be identified. It has been found that several *CesA* genes are specifically expressed in association with wood formation in various tree species (Wu *et al*., 2000; Samuga and Joshi, 2002; Djerbi *et al*., 2005). However, the mechanisms of how *CesA* genes function in the cellulose biosynthesis of wood formation remains to be elucidated.

In addition to *CesA*s that are required for cellulose biosynthesis, several other genes have been reported for their involvement in synthesizing cellulose. For example, sucrose synthase (SuSy), which converts sucrose to UDP-glucose, is postulated to play a role in providing substrate to CesA for cellulose chain elongation (reviewed by Haigler *et al*., 2001). Cellulose biosynthesis occurs in a protein complex structure, called rosette, on plasma membrane. To date, no rosette complex has been isolated and its structure has not been analyzed. The postulated rosette components may include 6 CesA subunits, and possibly, other elements (Kimura *et al*., 1999; Doblin *et al*., 2002). Sophisticated mechanisms certainly exist to assemble the rosette machinery on plasma membranes and to keep it active in synthesizing cellulose polymer. Therefore, involvement of more gene functions may also be required.

C. Hemicellulose

Hemicellulose is one of the major components in wood. However, the biosynthesis of hemicellulose is difficult to study because hemicellulose polymer is composed of multiple monosaccharide monomers and diverse linkage structures. The genomics data of hemicellulose biosynthesis is limited. There have been several reports about characterization of the genes involved in hemicellulose biosynthesis (Perrin *et al*., 1999; Sarria *et al*., 2001; Faik *et al*., 2002; Vanzin *et al*., 2002). However, those results are mainly from the studies of primary cell wall hemicelluloses, which, regarding to monosaccharide composition and linking structures, are quite different from secondary cell wall hemicelluloses. The flexibility of primary cell walls is essential in order to keep up with cell enlargement of plant growth. The main hemicellulose of the primary wall is xyloglucan in dicot and nongraminaceous in monocot species. Secondary walls are deposited inside primary walls when cell enlargement is completed. Glucuronoxylan is a principal hemicellulose in dicot species along with other minor hemicelluloses such as glucomannan. The hemicellulose constituents in gymnosperm species are mainly galactoglucomannans as well as a small proportion of other hemicelluloses, such as arabinoglucuronoxylan and arabinogalactan (Sjöström, 1993). Synthesizing hemicellulose requires genes encoding glycan synthases and glycosyltransferases, which are responsible for backbone and side chain formation, respectively. Several genes have been identified with the function for controlling the side chain formation of xyloglucan, including Arabidopsis xyloglucan fucosyltransferase (*AtFUT1*) (Perrin *et al*., 1999; Sarria *et al*., 2001; Vanzin *et al*., 2002), α-xylosyltransferase (*AtXT1*) (Faik *et al*., 2002). In hybrid aspen, *P. tremula* × *P. tremuloides*, twenty-five xylem-specific glycosyltransferases belonging to the Carbohydrate-Active EnZYme (CAZy) families (http://afmb.cnrs-mrs.fr/CAZY/) GT2, GT8, GT14, GT31, GT43, GT47, and GT61 and nine glycosidases (or transglycosidases) belonging to the CAZy families GH9, GH10, GH16, GH17, GH19, GH28, GH35, and GH51 were identified by using a functional genomics approach (Aspeborg *et al*., 2005). It is generally believed that cellulose synthase-like (*Csl*) genes may be involved in hemicellulose biosynthesis. The Arabidopsis genome includes *CslA*, *CslB*, *CslC*, *CslD*, *CslE* and *CslG*, 6 families with 30 members (Richmond and Somerville, 2000, 2001). In rice, another 2 *Csl* families, *CslF* and *CslH* were identified in the genome sequence (Hazen *et al*., 2002; http://cellwall.stanford.edu). In recently released *Populus* genome sequence data, 30 *Csl* genes are identified (Li *et al*., unpublished data). Although the *Csl* genomic information is available, the function of these genes is largely unknown. Very recently, a β-mannan Synthase (*ManS*) gene was identified and characterized from the guar seed EST sequence information because the endosperms of guar seed accumulate a large amount of hemicellulose, galactomannan, as a storage carbohydrate (Dhugga *et al*., 2004). This gene belongs to a member of *CslA* family. The functional characterization indicated that *ManS* encodes an enzyme catalyzing β(1,4) linkage formation of mannan. A homologous gene in Arabidopsis (*AtCslA9*) has also been confirmed with β-mannan synthase activity when expressed in insect cells (Liepman *et al*., 2005). *ManS* or *CslA* members constitute the first gene known for hemicellulose backbone biosynthesis. In different plants the backbone structure of hemicellulose varies with sugar composition and linkages. It is a challenging task to identify the genes for their function in hemicellulose backbone biosynthesis.

IV. REGULATION OF WOOD FORMATION

A. Understanding of Regulatory Mechanisms

Wood formation is a unique plant development process derived from secondary growth, mainly occurring in tree species naturally. Although many of the genes involved in the process have been cataloged, the regulatory mechanisms underlying the developing process remain largely to be elucidated. Many cellular and environmental signals are known to be involved in wood formation regulation.

Some early studies suggested that several signaling molecules, such as auxin and cytokinin are associated with the regulation of wood formation, however, molecular and genetic studies about the regulation was not available until recently. The transformation of hybrid aspen (*P. tremula L.* × *P. tremuloides* Michx.) with IAA-biosynthetic *iaaM* and *iaaH* genes resulted in altered wood formation and stem growth patterns, for

example, diameter growth and internode elongation (Tuominen *et al*., 1995). The altered IAA balance in the xylem development of the transgenic aspen was also involved in regulating vessel size, vessel density, and ray cell development (Moyle *et al*., 2002; Schrader *et al*., 2003). The effect of auxin in plants is believed to involve polar auxin transport through specialized carrier proteins. The molecular studies of *PttLAX1–PttLAX3* and *PttPIN1–PttPIN3*, belonging to the AUX1-like family of influx and PIN1-like efflux carriers, respectively, suggests that the auxin transport genes participate in regulating the wood formation (Schrader *et al*., 2003, 2004). Meanwhile, several members of the Aux/IAA gene family that encode possible mediators of the auxin signal transduction pathway were identified from aspen (*P. tremula* L.) (Schrader *et al*., 2004), hybrid aspen (*P. tremula* L. × *P. tremuloides* Michx.) (Moyle *et al*., 2002), and *Eucalyptus* (Paux *et al*., 2004). These genes are auxin inducible and differentially expressed in developmental gradient cells of wood-forming tissues. The expression of the *Aux*/*IAA* genes is down-regulated in transition time from active cambium cells to dormancy (Moyle *et al*., 2002; Schrader *et al*., 2004). Moreover, the formation of tension wood was accompanied by changes in the expression of *AUX*/*IAA* genes in hybrid aspen (Moyle *et al*., 2002) and *Eucalyptus* (Paux *et al*., 2005). These studies suggested that *PttIAA* genes play a role in mediating cambial responses to auxin and xylem development.

Gibberellins (GAs) are another class of plant hormone involved in wood formation. Overexpressing a regulatory gene for GA biosynthesis in hybrid aspen led to the formation of more numerous and longer xylem fiber cells in xylem and an increase in growth rate and biomass (Eriksson *et al*., 2000). A poplar gene, PttRGA1, encoding a repressor of gibberellin responses, was highly upregulated in the dormant vascular cambium (Schrader *et al*., 2004), suggesting the regulatory roles of GA in wood formation.

Moreover, ethylene is also involved in the regulation of wood formation. A gene encoding 1-aminocyclopropane-1-carboxylate oxidase (*PttACO1*) in a hybrid aspen species (*P. tremula* (L.) × *P. tremuloides* (Michx)) was found to be upregulated during secondary wall formation. The PttACO1 catalyzes the conversion of 1-aminocyclopropane-1-carboxylate (ACC) to ethylene. The gravitational stimuli strongly induced the expression of *PttACO1* and ACC oxidase activity in the tension wood-forming tissues, resulting in relatively lower levels of ACC in the tension wood versus the opposite wood (Andersson-Gunnerås *et al*., 2003).

Involvement of zinnia Class III Homeodomain Leucine-Zipper genes in regulation of xylem cell differentiation was recently demonstrated. When ZeHB-10 and ZeHB-12 with a mutation in the START domain are used to transform Arabidopsis, the transgenics shows a higher production of tracheary elements and xylem precursor cells (Ohashi-Ito *et al*., 2005). Another gene is found to play a possible regulatory role in xylem differentiation. A proteoglycan-like factor named xylogen is suggested to mediate local and inductive cell-cell interactions in xylem differentiation in *Zinnia* cells cultured in vitro (Motose *et al*., 2004). The xylogen is a hybrid-type molecule with properties of both arabinogalactan proteins and nonspecific lipid-transfer proteins, located in the cell walls of differentiating tracheary elements. The genetic function of the xylogen genes is indicated in knockouts of Arabidopsis, showing discontinuous veins, improperly interconnected vessel elements and simplified venation.

B. Involvement of MicroRNA in Regulation of Secondary Growth

Recently, a class of small and noncoding RNAs, microRNAs (miRNAs), have been intensively studied for regulatory roles in development, defense and adaptation in eukaryotic organisms by targeting mRNAs for gene-silencing (see reviews by Bartel, 2004; Kidner and Martienssen, 2005). This includes a microRNA family, miR165/166, which has a demonstrated role in regulating vascular cell differentiation by cleaving the transcripts in *Arabidopsis thaliana* of five class III homeodomain-leucine zipper (HD-ZIP) genes. The five genes are *ATHB8*, *CORONA* (*CNA*), *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), and *REVOLUTA* (*REV*) (Emery *et al*., 2003; Juarez *et al*., 2004; Kidner and Martienssen, 2004; Mallory *et al*., 2004b; McHale and Koning, 2004; Zhong and Ye, 2004; Kim *et al*., 2005; Williams *et al*., 2005). *REV* is known to play roles in apical embryo patterning, embryonic and postembryonic shoot apical meristem (SAM) and floral meristem (FM) initiation, lateral organ patterning, vascular development, and plant stature. In conjunction with other genes, *PHB* and *PHV* are involved in regulating various developments, from postembryonic SAM and FM initiation, lateral organ patterning, apical embryo patterning, to meristem size regulation when different genes (*REV* or *CAN*) are partnered. In addition, it appears that *ATHB8* acts redundantly with *CAN* to promote lateral shoot meristem activity (Prigge *et al*., 2005). The five HD-ZIP genes have a common miR165/166 complementary site within the putative sterol/lipid-binding START domain (Rhoades *et al*., 2002). MiR165/166 has been shown to efficiently cleave *PHV* mRNA in wheat germ extracts (Tang *et al*., 2003), *REV* in Arabidopsis (Emery *et al*., 2003), and *CAN*/*ATHB15* in *Nicotiana benthamiana*, wheat germ extract, and in Arabidopsis (Kim *et al*., 2005). When the miR165/166 complementary sites were altered, *PHV*, *PHB* and *REV* mRNA were resistant to the cleavage of miR165/166 (Emery *et al*., 2003; Tang *et al*., 2003; Mallory *et al*., 2004b; Zhong and Ye, 2004). Conversely, in a gain-of-function MIR166a Arabidopsis mutant (*men1*), the decreased transcript levels of *CAN*/*ATHB15*, *PHV* and *PHB* were accompanied by an altered vascular system with expanded xylem tissue and interfascicular region, indicative of accelerated vascular cell differentiation from cambial/procambial cells (Kim *et al*., 2005). Similarly, increased expression of miR166g in a T-DNA insertion Arabidopsis mutant (*jba-1D*) causes a significant reduction in the transcript levels of *PHB*, *PHV* and *CAN*, leading

to morphological defects in shoot apical meristems, stem vasculature, rosette leaves, and gynoecia (Williams *et al*., 2005).

As microRNA is known to be involved in primary vascular differentiation in Arabidopsis, it is also found that microRNA functions in the regulation of secondary growth in tree species (Lu *et al*., 2005). From the developing xylem of *P. trichocarpa* stems, 22 microRNAs have been cloned. They are the founding members of 21 microRNA gene families for 48 microRNA sequences, represented by 98 loci in the *Populus* genome. Of the 21 *P. trichocarpa* microRNA families, ten are not found in the Arabidopsis genome. At this time it is unclear whether the miRNA difference between tree *Populus* and herbaceous Arabidopsis indicates a function specialty in association with the development nature of the two type plants. That all 21 ptr-microRNA families are expressed in developing xylem and/or phloem suggests that they are involved in cambium differentiation activities. On the other hand, their expression patterns are found being distinct. For example, ptr-miR156 and ptr-miR472 also are highly expressed in xylem tissue as well as in leaves, but others (ptr-miR160, 164, 171, 473, 477, 478, 479, and 480) are clearly xylem-tissue-specific with a scant expression in leaves. Furthermore, the miRNA expression is regulated by mechanical stress. The changes of ptr-microRNA transcript abundance are detected in tension-stressed and compression-stressed xylem tissues. For instance, the expression of ptr-miR408 was drastically upregulated in both tension-stressed and compression-stressed xylem tissues. Ptr-miR408 has been demonstrated for cleaving the target genes that encode two plastocyanin-like proteins and one early dehydration-responsive protein. By contrast, the expression of ptr-miR164 is diminished in both tension-stressed and compression-stressed xylem tissues. Ptr-miR164 is suggested to target at five *P. trichocarpa* NAC-domain proteins, which are known for the negative effect on the proliferation and development of certain cells and organs (Aida *et al*., 1997; Xie *et al*., 2000; Laufs *et al*., 2004; Mallory *et al*., 2004a). Another down-regulated miRNA in mechanically stressed woody tissues is ptr-miR171, which has the target genes of *SCLs*. *SCL* is known to regulate cell division and elongation to produce organ cell lineages as a positive response to gravitropism (Di Laurenzio *et al*., 1996; Tasaka *et al*., 1999; Helariutta *et al*., 2000; Nakajima *et al*., 2001). The ptr-miRNAs of which the expression is regulated in tension wood and opposite wood may be candidate regulators regulating wood formation. Further studies on the functional mechanisms of the candidate miRNA genes would lead to insights into understanding wood formation regulations.

V. CONCLUSIONS

Tremendous interest has been dedicated to understanding of plant primary growth; however, plant secondary growth is relatively understudied. The secondary growth in tree species, which gives rise to wood formation, is a major biological process for plant biomass accumulation on the earth, converting photosynthesis-fixed solar energy and carbon into biopolymers and producing various essential natural materials for human utilization. Understanding of this growth process would provide new knowledge for advancement of plant biology as well as lead to the future technology development for plant biomass utilization and wood-related material production.

Recent efforts on understanding of secondary growth involve the genomic and genetic characterizations of xylem formation in herbaceous model plant and tree plant systems. Through gene expression profiling studies, many genes have been identified for their association with the xylem differentiating process. These studies represented a first step towards fully understanding the molecular and genetic mechanisms controlling secondary growth. More detailed and profound evidence is highly desirable. Although herbaceous model plant and cell culture systems are used to study xylem differentiation, understanding of the entirety of secondary growth requires a tree system for dissection.

During wood formation, one of the most noticeable processes is secondary wall thickening and biosynthesis. Secondary walls are mainly composed of cellulose, lignin, and hemicelulose. The biosynthesis of the three polymers has attracted tremendous interest in genetic and molecular studies in past dozens of years. The molecular elucidation of monolignol biosynthesis pathways has been actively advanced, however, illumination of factors regulating how the three polymers are coordinately synthesized in wood is less advanced.

The secondary growth in trees involves a series of sequential biological events, including maintenances of meristem cell entity, cell division, cell fate determination and differentiation, cell enlarging, secondary wall thickening, cell aging and death. However, little is known about the signaling and molecular circuits of the developmental hierarchical system. The available evidence indicates that some factors such as auxin and microRNA may play a part in the hierarchical system but the signaling process is unclear yet. The availability of the *Populus* genome sequence and a number of tree EST databases opens many new opportunities for the genomics and systems biology studies of trees. The new approaches will certainly shed light on depicting a full molecular view of the tree secondary growth.

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