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Review

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Molecular Biology of Secondary Growth

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Abstract

Trees have the ability to undergo secondary growth and produce a woody body. This tree-specific growth is affected by the secondary vascular system and the developmental continuum of secondary phloem and xylem. Secondary growth is one of the most important biological processes on earth. Considering its economic and environmental significance, our knowledge of tree growth and development is surprisingly limited. Trees have received little attention as model species in plant science, as most plant biology questions can be best addressed by using herbaceous model species, such as *Arabidopsis*. Furthermore, tree biology is difficult to study mainly due to the inherent problems of tree species, including large size, long generation time, large genome size, and recalcitrance to biotechnological manipulations. Despite all of this, one must rely on trees as models to study tree-specific questions, such as secondary growth, which cannot be studied effectively in non-woody model species. Recent advances in genomics technology provide a unique opportunity to overcome these inherent tree-related problems. Several groups, including our own, have been successful in studying the biology of wood formation with a variety of hardwood and softwood species. In this article, I first review the current understanding of tree growth and then discuss the recent attempts to fully explore and realize the potential of molecular biology as a tool for enhanced understanding of secondary growth.

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Introduction

Plant growth by means of apical (i.e. primary) meristems leads to the development of various sets of primary tissues: epidermis, cortex, vascular bundles, pith, and leaves. In addition to these primary tissues, woody species produce secondary tissue "wood" (i.e. secondary xylem) from vascular cambium (i.e. secondary meristem) (Mauseth, 1998). With their unique ability to undergo secondary growth and produce a woody body, trees comprise over 90% of the terrestrial biomass of the earth and serve as a primary feedstock for biofuel, fiber, solid wood products, and various natural compounds. International demand for wood has grown 36% in the past 25 years (1997 UN Report on World Forests). In order to meet the construction and fiber needs of an expanding population, in consideration of diminishing forestland, future forestry must achieve higher biological productivity of trees and better utilization of forest products.

Many economically important agricultural crops are tree species. Furthermore, forest trees play a prominent role in offsetting the gases released by fossil fuel and thus mitigate the potential negative effects of global warming. For example, forest carbon storage in the coterminous United States increased by 11.3 billion metric tons between 1952 and 1992, an amount that offset about one-quarter of the U.S. carbon emissions for that period (Joyce et al., 1995). A thorough understanding of this process will enable us to exploit the maximum biological potential of trees, arguably the most important biological system on Earth. However, it is surprising that the cellular and molecular regulation of tree growth remains so poorly understood. There are several reasons for the lack of

knowledge on this very important process: 1) difficulty of experimentally observing the processes of wood formation; 2) no real tree model system is available for study; 3) large size and long generation time of trees; and 4) lack of a genetically pure line or readily available mutant population. The recent advances in functional genomics and molecular biological technology provide a unique opportunity to overcome these inherent problems associated with tree studies.

Vascular cambial growth (i.e. Secondary Growth)

In woody plants, the primary vascular tissues of shoots and roots are replaced in later development by secondary vascular tissues, which are produced by a secondary meristem (denoted the vascular cambium). The vascular cambium originates from the pro-cambium, which is derived from the apical meristem. It normally consists of 5 to 15 dividing cells (commonly referred to as cambial initials), and occurs as a continuous ring of cells between the xylem and the phloem throughout the length of fully expanded shoots and roots (the so-called cambial zone) (Larson, 1994; Mauseth, 1998). The cambium has two types of initials: *the fusiform initials* leading to the axial system and *the ray initials* extending to the radial system, or the rays. While the fusiform cambial initials can change into ray initials and *vice versa*, the ratio of fusiform to ray initials in the cambium varies greatly among species, within the same plant at different locations, and following injury (Levyadun and Aloni, 1995). The common values for ray initial proportions range between 10% and 40%. The vascular cambium plays the following crucial roles in tree growth and development (Catesson et al., 1994): 1) promotes an increase in stem diameter by the production of functional vascular elements through tangential (or periclinal) divisions; 2) facilitates stem enlargement and the maintenance of the meristem itself, accomplished by radial (or anticlinal) and transverse divisions; 3) serves as a bridging point for the translocation of nutrients between phloem and xylem; 4) acts as a communication center for the transmission of signals, such as plant growth regulators, in both the axial and radial directions.

Cambial growth results in the production of secondary xylem and phloem elements. This growth is adjusted to the demands of water transport required by the leaf biomass and of the mechanical strength necessary to support the crown and to withstand wind forces (Zimmermann and Brown, 1971). Radial (across the cambium) and longitudinal (along the cambium) transfers of developmental signals and nutrients occur through two distinct routes, the ray initials and the axially elongated fusiform initials. Therefore, maintenance of the relative ratio between the two types of initials throughout the life of the tree is important. The production of new rays through transverse divisions of the fusiform initials and further differentiation of preexisting rays are thought to be regulated by the complex axial and radial flows of developmental signals, such as the polar auxin flow. Nonetheless, regulation of the cambial growth that controls wood production and diameter growth of trees remains to be elucidated.

Contol of secondary growth

Wood formation

Wood is formed by the successive addition of secondary xylem, which differentiates from the vascular cambium. The meristem controls wood production through its organization and its rhythm of activity. Phloem and xylem differentiate radially on each side of the vascular cambium, or the secondary meristem. The cambial derivatives that become phloem expand and differentiate to form a living tissue. On the xylem side of the cambium, the cells first pass through a dividing zone where the xylem mother cells continue to divide, then an expansion zone where the derivative cells expand to their final size, next a maturation zone where lignification and secondary cell wall thickening occurs, and finally through a zone of programmed cell death where all cellular processes are terminated (Chaffey, 1999). The resulting mature secondary xylem includes xylem parenchyma, fibers, vessels, and tracheary elements. The vascular cambium increases the diameter of an axis by periclinal divisions and the circumference of an axis by anticlinal divisions. This development of secondary xylem (i.e. xylogenesis) requires positional information that coordinates the radial pattern of the developmental zones of division (the cambial zone), expansion, and wall formation (Uggla et al., 1996; 1998). Positional information also regulates the cambial growth rate by defining the width of the cambial zone and, therefore, the radial number of dividing cells. Growth regulators such as indole-3-acetic acid (IAA) may serve as morphogens (Wolpert, 1996).

The tree-specific growth is affected mainly by the secondary vascular system and the developmental continuum of secondary phloem and xylem (Chaffey, 1999). Xylem is composed of tracheary elements, parenchyma cells, and fibers. At the end of xylem differentiation, the cells in tracheary elements lose their nuclei and other

contents, leaving a hollow tube that is part of a vessel. As a result of the radial growth and differentiation, the trunk wood of many tree species has two distinctly different regions: sapwood and heartwood. Sapwood is the outermost portion of the xylem tissue and contains living cells, whereas the heartwood is defined as the "dead" central core of the woody axis and provides only passive support to the tree. The amount of sapwood varies according to species, age of trees, growth rate, and environmental conditions (Hillis, 1987). Sapwood (young xylem) has three important functions. These are: 1) to conduct sap (water, solutes, and gases) from the root to all parts of the tree; 2) to provide structural support for the entire tree; and 3) to serve as a reservoir for water, energy, minerals, and solutes. On average, about 10% of the cells in the sapwood are alive (Kozlowski and Pallardy, 1997). The living ray cells in sapwood serve as the source of raw materials for secondary substances. The ray parenchyma may also serve as radial communication channels radially from the cambium through the sapwood, while axial parenchyma cells function largely as storage tissue. Heartwood results from physiological cell death and, therefore, no longer contains living cells. Thus, much of the biological significance of heartwood formation is concerned with metabolic changes and extractive formation at a narrow zone (called "transition zone") adjacent to the heartwood. The reserve materials in the parenchyma cells of the sapwood are used, with sucrose transported via vascular bundles from the leaves, for wood formation and synthesis of heartwood extractives such as condensed tannins, terpenes, flavonoids, lignans, lipids, stilbenes, and tropolones (Burtin et al., 1998; Hillinger et al., 1996a, b; Hillis, 1987; Magel et al., 1994).

The production of wood in trees is determined by the rate of cambial growth. Simultaneous increases in both the radial number of dividing cells and the rate of cell division in the cambial zone will increase productivity. Both cambial growth and the subsequent differentiation of its derivatives are under strict developmental control, which forms typical patterns of wood formation in time and space (Larson, 1994). The formation of secondary vascular tissues is a well-described phenomenon of patterned growth in plants, with both radial and longitudinal components. The quantity and quality of the final wood product is the result of a patterned control of numbers, places and planes of cambial cell division, and a subsequent regulated differentiation of the cambial derivatives into tracheary elements, vessels, fibers, parenchyma, and sieve elements (Mauseth, 1998). This developmental pattern requires temporal and spatial control of gene activity. In order to achieve the patterned growth, every cell must express the appropriate genes in a highly timed manner upon receipt of positional information. This regulation is under strong genetic control (Zobel and Jett, 1995), suggesting the potential for genetic manipulation of cambial activity. Environmental factors, such as temperature and photoperiod, affect the processes of cell production by the cambium, growth of cambial derivatives by expansion, and secondary wall thickening (Antonova and Stasova, 1997).

Hormonal regulation of secondary growth

The plant growth regulator, IAA, is essential for the production of xylem and phloem by the vascular cambium (Little and Sundberg, 1991), while gibberellins (GAs) are required for longitudinal growth (Wang et al., 1995). A change in IAA supply to the vascular cambium results in a change in radial gradient width. The occurrence of a steep radial gradient of IAA across the cambial region of *Pinus sylvestris*, with a peak in the cambial zone (Uggla et al., 1996), suggests that IAA acts as a positional signal from which cambial derivatives interpret their radial position and it also regulates cambial growth rate by determining the radial population of dividing cambial-zone cells. However, there is no evidence to support that IAA in the cambial meristem plays an additional role in controlling rates of cell division. In forest trees, stem-diameter growth is often greatest within the young crown and decreases gradually down the stem, suggesting that the size and arrangement of the crown determine the amount and pattern of growth along the stem. IAA functions as a positional signal for such coordination, which integrates apical growth with production of vascular tissues (Uggla et al., 1998).

Seasonal cycle of secondary growth

The vascular cambium of temperate tree species follows the seasonal cycle of activity and dormancy (Baier et al., 1994). Zhong et al. (1995) studied cambial cell phenology in white ash (*Fraxinus Americana*) in Canada. The cambial cells had no detectable mitoses until April. Ray cambial cells resumed cell division activity in the spring (between 23 April and 1 May), while fusiform cambial cells began activity between 30 March and 23 April. The ray cambial cells entered dormancy on 10 September, when fusiform cambial cells were still dividing occasionally. Following springtime resumption of cambial cell-division activity, the derivatives of cambial fusiform

cells expanded radially to form a zone of primary-walled cells. The primary-walled derivatives on the xylem side of the cambium then underwent terminal differentiation, involving secondary wall thickening, lignification and protoplast autolysis, to become tracheids. Wider-diameter earlywood tracheids were produced in the spring, while tracheids produced later had smaller diameters and thicker secondary walls (latewood). Biochemical studies of the cambial zone in poplar showed that the total amount of pectins per gram of cell wall was rather stable throughout the annual cycle, while the composition of pectin changed dramatically (Baier et al., 1994). Recently, Iliev and Savidge (1999) investigated proteolytic activity in the cambial zone and developing xylem of *Pinus banksiana* over an annual cycle of growth and dormancy. The highest proteolytic activity was observed during the most active period of radial expansion of cambial derivatives, in early spring, before protoplasmic autolysis was initiated in the developing earlywood.

Short photoperiods induce both latewood formation and cambial dormancy in the stem of conifer species (Eklund et al., 1998; Mellerowicz et al., 1992a, b). While the molecular mechanisms regulating these events are still unknown, several explanations have been suggested: 1) short photoperiod increases the concentration of the growth inhibitor abscisic acid (ABA) in the cambial region. Exogenously provided ABA decreases tracheid radial diameter and inhibits tracheid development in conifers (Little and Eidt, 1968; Pharis et al., 1981); 2) it decreases the cambial region concentration of the growth promoter indole-3-acetic acid (IAA); 3) as a result of high rates of the growth and maintenance components of respiration, the O_2 concentration in the cambial region is decreased to the extent that it becomes limiting for IAA action during either the enlargement phase of tracheid differentiation or the division of cambial cells. The O_2 concentrations in the sapwood of field-grown *Picea abies* stems seem to be lower than 5% late in the cambial growing period and that such low O_2 concentrations inhibit IAA-induced proton secretion (Bottger and Hilgendorf, 1988).

A model for secondary growth

We have created a conceptual model of cambial growth that guides our experiments. We expect that cambial activity initially increases in the spring when both photoperiod and temperature increase, and the activity continues into the late fall when the cambial cells enter dormancy. As the environmental conditions change, cambial activities (i.e. growth, dormancy, and transition from growth to dormancy and *vice versa*) will be subjected to act upon that change. This slow and complex event of signal perception and sequential processing of the differentiation steps is likely to be orchestrated by a number of enzymes involved in signal transduction, cell division, primary and secondary metabolite biosynthesis, and programmed cell death. The genes encoding such enzymes are thought to be tightly controlled in each of the cambial growth periods.

Heartwood formation

Transition from sapwood to heartwood

The presence of heartwood is a determining factor for wood quality and influences the utilization of wood in many different ways. For example, it adversely affects forest management decisions by making it difficult to predict the quality of wood available for utilization from the forest inventory. Understanding the biology of heartwood formation will enable us to control the factors that contribute to its formation and thus allow us to make choices of the most suitable forestry practices.

Sapwood is gradually converted to inactive heartwood as its water columns of conducting vessels break due to freezing, wind vibration, tension, wood boring insects, and other factors (Mauseth, 1998). The ultimate fate of the cavities in the broken vessels affects different properties of wood. Trees adopt a mechanism to seal off the empty columns. The adjacent wood parenchyma cells undergo numerous metabolic changes to produce and accumulate in the vessels large quantity of heartwood extractives such as phenolic compounds, lignin, and aromatic substances. These substances inhibit microbial growth. In this process, which occurs in late fall in temperate zones, one annual ring is converted to heartwood each year (Mauseth, 1998).

Formation of heartwood is a form of senescence that is accompanied by a variety of alterations in metabolic conditions. Although the events of senescence have been elucidated at the molecular level during leaf senescence (Miller et al., 1999; Wingler et al., 1998), seed germination (Cercos et al., 1999), and nodule development (Matamoros et al., 1999), the cell maturation and death events occurring during heartwood formation have been difficult to study because of the location and timing of the events. Analysis of global gene expression patterns during the cyclic transition between growth and dormancy may offer a powerful means by which to identify the mechanisms controlling this process. We have generated and annotated over 1,200 expressed sequence tags (ESTs, or single-run

partial sequencing of cDNAs) from a cDNA library constructed from the transition zone of black locust (Han et al., manuscript in preparation).

Timing of heartwood formation

In the temperate zones, heartwood formation occurs in late summer to late fall and at the beginning of dormancy when temperatures remain sufficiently high (above 5℃) for the required cellular reactions (Hillis, 1987). Earlier studies have indicated that heartwood formation occurs at the time of cambial dormancy in pine (Shain and Mackay, 1973), walnut, and cherry (Nelson, 1978). Studies of the cytology and coloration of the extractives suggested that heartwood formation commences in mid-summer and continues into the fall and winter seasons in sugi (Nobuchi et al., 1984a) and black locust (Nobuchi et al., 1984b).

Biochemistry of heartwood formation

During the transformation from sapwood to heartwood, cells undergo metabolic changes that result in increased synthesis of secondary products. These changes involve the consumption of storage carbohydrates and their conversion into heartwood substances such as phenolic compounds (Hillis, 1987; Magel et al., 1991; Magel et al., 1994). Observations lead to the suggestion that heartwood extractives are synthesized *in situ* in the sapwood-heartwood transition zone from the breakdown of starch or from soluble sugars (Magel et al., 1994; Magel and Hubner, 1997). The transition is tightly connected with the degradation of storage lipids and the accumulation of hydrolysis products and intermediates (Hillinger et al., 1996a, b). Light and electron microscopic studies have found that during sapwood-heartwood transition, storage materials such as starch are consumed (Datta and Kumar, 1987; Nair, 1988; Nobuchi et al., 1987). Starch hydrolyzed at the transition zone represents a primary source of hydroxycinnamic acid and flavonoid synthesis (Magel et al., 1994). A period of enhanced metabolic activity has been found at this zone in heartwood-forming species such as Acacia (Baqui and Shah, 1985), black locust (Magel et al., 1991), oak (Ebermann and Stich, 1985), and walnut (Nelson et al., 1981). Maximum oxygen consumption was observed in the sapwood adjacent to the heartwood of black locust, suggesting increased metabolic activity (Holl and Lendzian, 1973). Also, the formation and accumulation of heartwood phenolics coincided with the transformation of sapwood to heartwood in black locust (Magel et al., 1994). Hillis and Hasegawa (1963) noted that 19 days after labeled glucose was administered to the cambial region of *Eucalyptus sieberi*, it was converted to labeled extractives formed at the heartwood periphery. This conversion was also observed in the transition zone of sugi (Higuchi et al., 1969).

Several enzymes have shown increased activity in the transition zone. Elevated levels of phenol-oxidizing enzymes have been observed in the transition zone of *Eucalyptus polyanthemos* (Hillis and Yazaki, 1973). A marked peroxidase activity was observed in the transition zone of *Eucalyptus elaeophora* (Wardrop and Cronshaw, 1962) and *Fagus sylvatica* (Dietrichs, 1964). In addition, Baqui et al. (1979) found that succinate dehydrogenase in *Melia azedarach* was significantly active only in the transition zone. Adenosine triphosphatase, which is implicated in many energy-consuming cellular processes, and lipase were also active in the transition zone. Other enzymes reported to be highly active in the transition zone include catechol oxidase, glucose-6-phosphate dehydrogenase, malic dehydrogenase, maltase, and amylase (Hillis, 1987). Furthermore, Magel et al. (1991) have shown that two key enzymes for flavonoid biosynthesis (chalcone synthase and phenylalanine ammonia-lyase) are highly active in the sapwood-heartwood transition zone of black locust. They investigated the seasonal activities of phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) in the xylem of black locust. They found that PAL was active in the youngest wood near the cambium in April and September, but that it was active in the transition zone in all seasons; CHS was active only in the intermediate wood. From these results they suggested that PAL is involved in lignin biosynthesis in the youngest woods, while it plays a role in flavonoid synthesis in intermediate wood; CHS is only involved in flavonoid synthesis. In addition, the stimulus for biosynthesis of robinetin, a flavonoid with strong antimicrobial activity, apparently occurs in the transition zone in Instsia species (Hillis, 1996).

There are three possible explanations for the increased enzyme activity in the transition zone of mostly dead cells (Hillis, 1987): 1) the enzymes are produced by the heartwood inhabiting microorganisms; 2) the enzymes are encoded by host parenchyma cells and remain active after the host cell death; or 3) the enzymes are made by living host parenchyma cells after heartwood formation. Shain and Mackay (1973) provided indirect evidence indicating that two phenol-oxidizing enzymes that were produced by host parenchyma cells remain active after host cell necrosis. Our laboratory is trying to address these questions pertaining to increased enzymatic activity in the

transition zone by providing information on gene expression patterns during natural and induced heartwood formation.

A model for heartwood formation

While there are no systematic data or observations that can lead to a specific hypothesis, we have created a conceptual model of heartwood formation that guides our experiments. We propose that the ray parenchyma cells at the sapwood-heartwood transition zone undergo a form of programmed cell death. The reserve materials in these cells are converted to secondary metabolites, which may serve as defense chemicals. This slow and complex event of heartwood formation is likely to be orchestrated by a number of enzymes involved in the breakdown of storage materials, secondary metabolite biosynthesis, and senescence. The genes encoding such enzymes are thought to be induced in the transition zone. Our current EST analysis and DNA array hybridization experiments are being carried out to gather molecular evidence that leads to a better understanding of the formation of heartwood and its extractives.

Wood formation studies in non-woody plant species

Processes involved in xylogenesis include cell division and expansion, formation of secondary cell walls, and the autolysis of cell contents. The secondary cell wall is made of cellulose fibers arranged parallel to one another and strengthened by cementing substances such as lignin, hemicellulose, pectin, and proteins (Fukuda, 1996). Furthermore, cell autolysis, a form of apoptosis, requires activation of the expression of degradative enzymes such as nucleases and proteases. A number of genes associated with xylogenesis have been characterized in non-woody model systems, including *Zinnia elegans* (Fukuda, 1996; Ye and Droste, 1996; Ye et al., 1994; Ye and Varner, 1996) and *Arabidopsis* (Arioli et al., 1998; Turner and Somerville, 1997).

Significant advances toward understanding how the cambium meristem differentiates into secondary xylem require extensive studies from all levels of approach histology, molecular biology, biochemistry, and genetics. Although cambial growth, including subsequent wood formation, has been successfully studied at different levels in many tree species, the molecular biology of the process has been lagging behind that of primary growth primarily due to the inherent difficulties of tree biology (discussed above). Therefore, most research on wood formation is carried out using herbaceous model species, such as *Arabidopsis thaliana* (Lev-Yadun, 2001; Lev-Yadun and Flaishman, 2001; Arioli et al., 1998; Perrin et al., 1999) and *Zinnia elegans* (Fukuda, 1996). The root-hypocotyl of *Arabidonsis* produces a relatively large amount of secondary vascular tissue when senescence is delayed by the removal of inflorescences and when plants are grown at low population density (Lev-Yadun, 1994, 1997; Zhao et al., 2000).

Many assumptions about the fundamental biology of tree growth and development have been based upon studies of primary growth systems. While the primary growth systems have been successful in generating a tremendous amount of information on the process (Fukuda, 1996), the relevance of those findings to true xylogenesis within a tree has not been established. Furthermore, studies based on herbaceous model species fall short of providing a knowledge basis for understanding fundamental tree biology, especially heartwood formation. While *Zinnia* and *Arabidopsis* play an important role in developing techniques and ideas which can later be tested in the natural system, studies on the cambial growth of trees should be performed with a true tree system. For instance, the *Zinnia* system presents several clear shortcomings as a model for tree secondary vascular system: 1) the individual cells of the cambium and their derivatives are surrounded by and interconnected with neighboring cells at different stages of development. Such cell-cell interactions, and the positional information related thereto, cannot be studied in the *Zinnia* system, which relies on individual mesophyll cells that are grown in liquid medium; 2) all of the mesophyll cells which differentiate in the Zinnia system become converted to a single cell type, the so-called tracheary element. However, cambial cells give rise to both xylary and phloic derivatives in real trees. Even though xylem and phloem cells differ from each other profoundly in structure and function, the *in vitro* system cannot address the subject of phloem formation, as it does not produce any 'phloem elements'; 3) although the tracheary element produced in vitro has similarities to that found within the primary xylem, its relevance to naturally-produced cells remains to be demonstrated; 4) in the *Zinnia* system, tracheary element differentiation occurs directly from single mesophyll cells without cell division. Those mesophyll cells are consumed in the process of conversion to tracheary elements. On the other hand, the cambial initial cell divides to give rise to one daughter cell, which perpetuates the meristematic state, and another that gives rise to further derivatives; these ultimately differ-

entiate as secondary vascular cells; 5) in the tree secondary vascular system, there is the seasonal cycle of cambial dormancy/activity, which is frequently associated with profound anatomical changes to the cambial cells (e.g. Larson, 1994); and 6) the *Zinnia* system is not suitable to study wood quality traits of economic importance, such as fiber length, radial growth, and physical properties of the wood produced.

Recently, a number of groups, including our own, have been successful in studying the biology of wood formation with a variety of hardwood and softwood species such as black locust, spruce, eucalyptus, horse chestnut, and loblolly pine (Chaffey et al., 1999). However, very few species, including eucalyptus, loblolly pine and black locust, have been studied in earnest to date.

Molecular biology of xylogenesis (i.e. Wood Formation)

Molecular regulation of wood formation

Results from the studies of other model organisms, including animals and plants, indicate that several different regulatory circuits interact in complex ways during development. Various molecular signals are differentially induced by cell-to-cell contacts and relative cell positions, while they are turned on and off in response to environmental cues, nutritional status and/or other long-range stimuli. All of these regulatory steps work by changing the global pattern of gene expression in an individual cell. Thus, the control of cambial activity and derivative differentiation is accomplished by changing the activity of key genes involved in the developmental pathways, which determine the epigenetic state of the vascular cambium. For instance, differentiation will proceed (i.e. it is committed) if only the loci on the cambial genome encoding features specific to vascular development can be expressed. On the other hand, the cambium would be merely competent if the cambial genome could still express other genes. Recently, significant progress has been made in the study of the genes and signaling mechanisms responsible for secondary wall formation, lignin and cellulose biosynthesis (Arioli et al., 1998), and xylem development (Fukuda, 1997). The *Zinnia* mesophyll cell culture has been successfully used to study individual genes expressed at various stages in trans-differentiation of the mesophyll cells into tracheary elements. Several genes that are up-regulated during differentiation have been isolated (reviewed in Fukuda, 1997). Furthermore, a comparison of transcripts produced in differentiating cells with those from freshly isolated mesophyll cells identified tracheary element differentiation-specific transcripts of 13 cDNA clones, three of which share homology with an *O*-methyltransferase and an adenylate kinase. Each of these genes provides a useful molecular marker for cambium differentiation (i.e. wood formation). Study of wood formation at the molecular level using real trees has begun in recent years. Expressed sequence tags (ESTs, or single-run partial sequencing of cDNAs) can provide a relatively rapid means by which to identify genes expressed in wood. A large number of ESTs have been analyzed from the wood-forming tissues of poplar (Sterky et al., 1998), black locust (Han et al., manuscript in preparation), and pine (Allona et al., 1998).

Genes expressed in the transition zone

It is likely that genes encoding the key enzymes for the synthesis of heartwood extractives are expressed in the transition zone during heartwood formation. Higuchi (1997) suggested that such genes are induced in ray parenchyma cells of the transition zone. However, genes expressed during the transformation from sapwood to heartwood have not been characterized. Our laboratory is currently generating ESTs from a cDNA library derived from the transition zone of black locust. The initial sequencing analysis revealed ESTs with significant homology to ATP synthase, chalcone flavonone isomerase, cytochrome b5 DIF-F, deoxychalcone synthase, GTP-binding nuclear protein, helicases, isoflavone reductase-like oxidoreductase, lactoylglutathione lyase, metallothionein, proteasome component C8 (macropain subunit C8), and xyloglucan endotransglycosylase. In this project, our goal is to obtain gene expression profiles from the transition zone of black locust, which is a heartwood-forming legume tree. The expression pattern of genes in the transition zone will provide key insights into how and where the extractives are synthesized. In addition, we plan to identify transition zone-specific promoters during the first year of this project. The promoter regions of such specific genes will be useful in the metabolic engineering of extractive biosynthesis for the production of value-added wood products, as well as in testing the functions of the specific genes.

Proteomics of wood formation

Proteins that are preferentially produced in the developing xylem may play a substantial role in wood formation. Systematic sequencing of proteins upon excision

from two-dimensional polyacrylamide gel electrophoresis (PAGE) facilitates the identification of protein functions and leads to the construction of a protein database. Proteome analysis (two-dimensional PAGE patterns from different tissue, developmental stages, or environmental conditions) permits a fast and simultaneous comparison of variations in the abundance of a large number of proteins, while also providing useful information on posttranscriptional modifications. This proteome analysis has been used to identify the proteins produced in needles and xylem tissue of a gymnosperm tree, maritime pine (Costa et al., 1999). In addition, Vander Mijnsbrugge et al. (2000) performed a comparative two-dimensional PAGE on young differentiating xylem, mature xylem, and bark of poplar harvested at different times of the year. They identified xylem-preferential proteins by comparing the protein patterns from xylem and bark. All of the identified proteins were involved in the phenylpropanoid pathway, and their corresponding ESTs were present in a developing-xylem library from the same poplar clone.

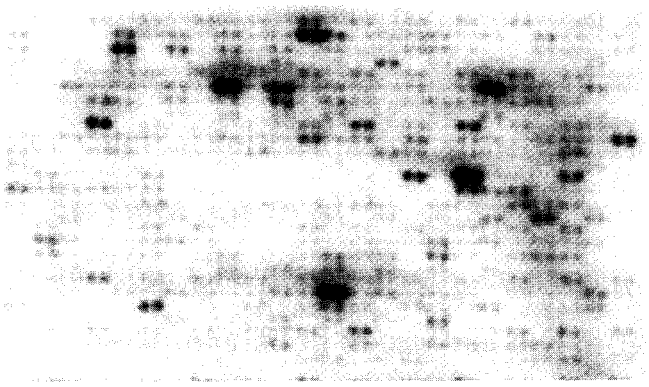
Genomics of wood formation

Genomics is a novel technology that can supplement traditional biological methods, as demonstrated in the successful sequencing of most of the human genome by the Human Genome Project and Celera Genomics. It changes and facilitates the manner in which we acquire and utilize new knowledge of fundamental biological processes in plants (Walbot, 1999; also for a general audience-oriented review, see Hamadeh and Afshari, 2000). DNA array technology provides a simple and economical way to explore the collection of genes that are expressed from genomic DNA (Brown and Botstein, 1999; Duggan et al., 1999; Lockhart and Winzeler, 2000). Furthermore, the global expression data obtained from DNA microarray hybridization experiments can be analyzed using standard statistical algorithms to arrange genes according to similarity in gene expression pattern (Eisen et al., 1998). Such cluster analysis and display systems will facilitate the identification of cambial growth-specific genes and the study of their expression patterns. Despite the increasing significance of understanding wood formation and the rapid advancement of genomics technology, attempts to make use of plant genomics technology for secondary growth studies have not been made.

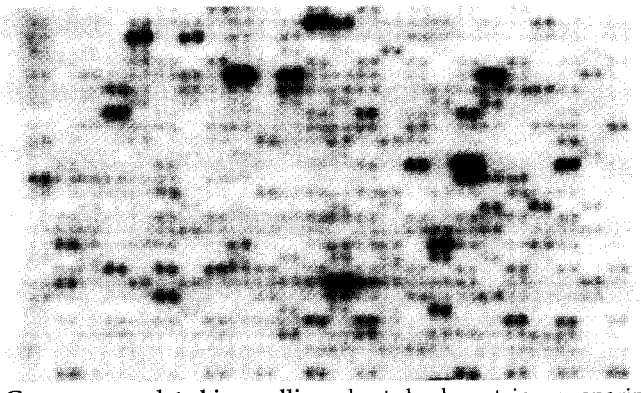
Our laboratory is studying the molecular biology of trunk wood formation and its associated extractives in hardwood species. Such studies are bottlenecked by the limitation of obtaining high quality mRNAs. Isolation of mRNAs from the cells in stem wood is difficult for several reasons: 1) there are few living cells in the transition zone; 2) the presence of high levels of wood extractives reduce the quality and yield of RNA; and 3) RNA must be protected from RNAse contamination during the sawing and carving out of the wood samples. We successfully isolated RNAs from the trunk wood of a 10-year-old tree. High quality cDNA libraries (over 1.0×10^6 pfu) were constructed from sapwood, sapwood-heartwood transition zone, and cambial region of mature black locust. We have sequenced and analyzed more than 3,400 ESTs. About 25% of the ESTs had no homology to sequences in the public databases. Functional classification analysis indicates that primary metabolisms and protein synthesis/processing-related genes are among the most abundant, followed by those for cell wall metabolism, gene expression, and membrane transport in the cambial region; genes encoding secondary metabolite biosynthetic enzymes are among the most abundant in the transition zone library (Han et al., manuscript in preparation). These ESTs are currently being prepared for DNA microarray printing. As a preliminary experiment to test the feasibility of the use of DNA array hybridization experiments, we printed about 700 ESTs from the cambial region of black locust onto nylon membranes using the Biomek 2000 system (Beckman Coulter). The membrane was hybridized with two different DNA probes and the signal was developed (Figure 1). The results confirm that gene expression profile analysis using DNA arrays is suitable for studying differential gene expression associated with secondary growth. The initial data from the sequence analysis and high density DNA array experiments clearly demonstrate that utilizing a genomics approach will provide a unique and powerful means by which the global gene expression changes associated with secondary growth can be investigated. We plan to leverage these initial efforts toward attaining the following goals: 1) understanding the molecular mechanisms of the growth and differentiation of the secondary meristem, 2) identifying genes specifically involved in the process, and 3) making use of this knowledge for the purpose of producing 'super' trees that are capable of adapting more aptly to environmental changes, growing faster, producing higher quality wood, and increasing carbon sequestration and storage.

The prospective

A study of vascular cambium differentiation (i.e. wood formation) culminates in the acquisition of knowledge concerning the hot topic of current developmental



Genes up-regulated in cambial region: metallothionein, phloem-specific potein Veinl, dormancy-associated protein, AUX1-like permease



Genes up-regulated in seedlings: heat shock protein, aquaporin, casein kinase II alpha subunit endopeptidase clp, N-acetyl-gamma-glutamyl-phosphate reductase, photosystem II 10 kd polypeptide precursor

Figure 1. Autoradiogram of DNA arrays containing cambial region ESTs hybridized with cambial region (left) and seedling (right) probes. The blots show differential gene expression between trunk wood and seedling, and between summer and fall in trunk wood. The larger signals indicate higher expression levels, while the spots without signals indicate those genes are not expressed in the probing tissue. The cambial cDNA library and probe was made using a sample collected in July; The seedling probe was from 8-day old *in vitro* germinated random seedlings.

biology - competence and commitment. Vascular cambium differentiation involves the transition of a cambial initial cell from 'competence' to 'commitment' (or determination) status along the pathway of wood formation. In the absence of such changes, the cambial meristematic state is perpetuated. Competence refers to the ability of a cell to develop in an appropriate manner in response to certain developmental cues. On the other hand, commitment may be defined as the 'conditioning' of a cell that enables the transition from one cellular state to another via a defined developmental pathway. Cambial initials are 'competent' since they can differentiate into xylem or phloem tissues. However, as the cambial derivatives become removed both physically and developmentally from the initials, their further development becomes limited until a point is reached in which they are 'committed' to a single developmental pathway. "How does a single cell type (i.e. the cambial intial) give rise to so many distinctive end points?" remains a key question to be answered in current developmental biology.

Identification of molecular markers associated with the processes along the pathway of cambium differentiation will facilitate our understanding of wood formation. Within each cell file of the cambial zone, there are initial cells that both perpetuate the meristematic state and produce daughter cells that ultimately give rise to the secondary vascular tissues. Few anatomical and ultrastructural markers for hardwood initial cells are currently available (Catesson et al., 1994; Larson, 1994). Furthermore, no suitable 'molecular markers' for vascular differentiation and for cambial initials per se (Chaffey, 1999) have been reported. The change in activity of some enzymes involved in cell wall metabolism could provide indications of the initial differentiation. Changes in pectin composition have been studied in the cambial zone and inner bark of poplar in relation to cambial cell differentiation and the seasonal cycle (Baier et al., 1994; Guglielmino et al., 1997). The results indicate that such changes might represent early markers of both the determination of cambial derivatives to differentiate into either phloem or xylem and the transition from activity to rest or from rest to activity (Guglielmino et al., 1997).

Use of genomics technology in the study of the molecular mechanisms controlling wood formation should facilitate identification of the novel genes that are differentially expressed and/or have biological significance in secondary growth. Characterization and functional assignment of the newly identified genes will be the most logical challenge that follows the gene discovery effort. Due to the inherent problems of long generation time and large size, most of the gene disruption approaches that have been effective with herbaceous model species will not be applicable to tree species. However, gene inactivation by either antisense- or co-suppression will be a powerful approach to assess the biological function of the genes. The novel genes can be manipulated to increase wood production, which is responsible for producing more terrestrial biomass and stored chemical energy than any other biological process. Furthermore, biotechnological manipulation of the biochemical processes involved in wood

formation can lead to significant changes in the properties of the wood produced. For example, over-expression of the C1/R transcription factor (Bruce et al., 2000) may increase the level of heartwood extractives, resulting in enhanced decay-resistance of solid wood products.

In addition, our ability to manipulate the occurrence of cambial dormancy in many temperate fruit crops is of particular economical significance with regard to the control of their winter hardiness. The single most important factor limiting the range and distribution of plants is cold temperature. Examples of the severity of cold injury in the United States in recent years are the November 11 freeze of 1940 in the Midwest, the late November freeze of 1950 in Michigan which killed most of the peach trees in the state, the November 1955 and December 1964 freezes in the Pacific Northwest which killed 2.5 million fruit trees and injured 4 million trees in New York state (Flore and Howell, 1987). Florida has experienced 4 severe freezes since 1979 which resulted in over 81,921 ha of oranges being killed, reducing Florida acreage by 25% (Parsons, et al 1986). A large proportion of this damage was caused by improper acclimation or hardening. As plants become dormant, hardiness increases. It reaches a maximum in winter and then gradually decreases (deacclimation) as warmer temperatures occur toward the end of dormancy in the late winter and early spring. Short days (Sakai, 1968; Howell and Weiser, 1969) have been implicated as the trigger for stage 1 hardening in the shoot tips and the cambium. Acclimation can be induced by short days (Flore, 1987). The regulation of cambium activity would greatly affect the hardening process.

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