Plant Molecular Biology **47:** 239–274, 2001.

Unravelling cell wall formation in the woody dicot stem

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Key words: expressed sequence tag, hybrid aspen, *Populus*, vascular cambium, wood formation, xylem cell wall

Abstract

Populus is presented as a model system for the study of wood formation (xylogenesis). The formation of wood (secondary xylem) is an ordered developmental process involving cell division, cell expansion, secondary wall deposition, lignification and programmed cell death. Because wood is formed in a variable environment and subject to developmental control, xylem cells are produced that differ in size, shape, cell wall structure, texture and composition. Hormones mediate some of the variability observed and control the process of xylogenesis. High-resolution analysis of auxin distribution across cambial region tissues, combined with the analysis of transgenic plants with modified auxin distribution, suggests that auxin provides positional information for the exit of cells from the meristem and probably also for the duration of cell expansion. Poplar sequencing projects have provided access to genes involved in cell wall formation. Genes involved in the biosynthesis of the carbohydrate skeleton of the cell wall are briefly reviewed. Most progress has been made in characterizing pectin methyl esterases that modify pectins in the cambial region. Specific expression patterns have also been found for expansins, xyloglucan endotransglycosylases and cellulose synthases, pointing to their role in wood cell wall formation and modification. Finally, by studying transgenic plants modified in various steps of the monolignol biosynthetic pathway and by localizing the expression of various enzymes, new insight into the lignin biosynthesis *in planta* has been gained.

Abbreviations: 4CL, 4-coumarate:coenzyme A ligase; ACC, 1-aminocyclopropane-1-carboxylate; C3H, coumarate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; CAD, cinnamyl alcohol dehydrogenase; CCoA3H, coumaroyl-coenzyme A 3-hydroxylase; CCoAOMT, caffeoyl-coenzyme A *O*-methyltransferase; CCR, cinnamoyl-coenzyme A reductase; COMT/AldOMT, caffeate/5-hydroxyconiferaldehyde *O*-methyltransferase; DDC, dehydrodiconiferyl alcohol; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EST, expressed sequence tag; F5H/CAld5H, ferulic acid/coniferaldehyde 5-hydroxylase; FCC, fusiform cambial cell; G, guaiacyl; GA, gibberellin; GCA, O^3-β-D-glucosyl-caffeic acid; GSA, O^4-β-D-glucosyl-sinapic acid; GVA, O^4-β-D-glucosyl-vanillic acid; H, *p*-hydroxyphenyl; IAA, indole-3-acetic acid ; IDDDC, isodihydrodehydrodiconiferyl alcohol; MIOP, *myo*-inositol oxidation pathway; NMR, nuclear magnetic resonance; PAL, phenylalanine ammonia-lyase; PCBER, phenylcoumaran benzylic ether reductase; PCD, programmed cell death; PME, pectin methyl esterase; QTL, quantitative trait locus; RCC, ray cambial cell; RE, radial expansion; S, syringyl; SAM, *S*-adenosyl-L-methionine; SHMT, serine hydroxymethyltransferase; TE, tracheary element; XET, xyloglucan endotransglycosylase

Introduction

Wood is a product of the vascular cambium (Larson, 1994), a lateral meristem that develops in conifer and most dicot land plants but that contributes substantially to plant biomass only in perennial tree species. Lignified xylem cells, which are functionally competent in water transport and mechanical support, are formed by cambial derivatives that undergo terminal differentiation ended by the autolysis of the cell protoplast. In this review, we will focus on the organismal approach to study wood formation or xylogenesis

Table 1. Most common poplar species and their systematic classification (http://willow.ncfes.umn.edu/silvics_manual/volume_2/populus/populus.htm).

Section	Species
Leuce (aspen type)	*P. grandidentata* (Michx.)
	P. alba (L.)
	P. tremula (L.)
	P. tremuloides (Michx.)
Aigeiros (cottonwood or poplar type)	*P. deltoides* (Bartr. ex. Marsh.)
	P. sargentii (Dode)
	P. fremontii (Wats.)
	P. nigra (L.)
Tacamahaca (balsam poplar type)	*P. balsamifera* (L.)
	P. maximowiczii (Henry)
	P. trichocarpa (Torr. & Gray)
	P. angustifolia (James)
Leucoides (swamp poplar type)	*P. heterophylla* (L.)
Turanga	*P. euphratica* (Olivier)

using functional genomics in a dicot tree. First, the cellular process of wood formation and the variability in wood properties brought about by developmental and environmental stimuli are described. Second, some aspects of hormonal control of wood formation are highlighted. Finally, the current progress in understanding the biosynthesis of the carbohydrate skeleton and lignification of the xylem cell wall is presented. The aim is to put detailed knowledge obtained from less complex systems, such as the xylogenic *Zinnia* cell culture (Fukuda, 1992, 1997; Milioni *et al.*, 2001, this issue), *Arabidopsis* (Baima *et al.*, 1995: Turner and Somerville, 1997; Taylor *et al.*, 1999; Zhong and Ye, 1999), and suspensions of primary walled cells (Takeda *et al.*, 1996; Kakegawa *et al.*, 2000; Thompson and Fry, 2000), into an organismal perspective. This aspect is important to understand fibre biogenesis in a tree and is a basis for the use of genetic engineering and marker-assisted selection to modify wood properties according to human needs.

It is not our intention to cover the extensive older literature on wood development. For this, the reader is referred to the appropriate reviews. Instead, our aim is to highlight current developments and concepts. The literature on poplar has been preferentially reviewed because poplar has emerged as a model tree for research on wood formation.

Populus as a model

A woody perennial model species is needed if we are to understand such phenomena as shoot dormancy, adaptations to deep frost including the deciduous habit, the presence of juvenile and mature phases, and the extensive secondary growth. Poplar (*Populus* spp.) combines the advantages of being a suitable model for experimental research with its economic importance as a forestry species (Klopfenstein *et al.*, 1997). The genus *Populus* belongs to the Salicaceae family and comprises about 30 species that are native to the Northern hemisphere and are classified into five sections (Table 1). *Populus* offers several advantages as a model tree: it grows fast, is easy to propagate and can be transformed (Klopfenstein *et al.*, 1997). Transformation protocols based on *Agrobacterium*-mediated gene transfer have been successfully applied in the sections *Leuce, Tacamahaca* and *Aigeiros* (Kim *et al.*, 1997; Han *et al.*, 1997, 2000). In addition, *Populus* species have a small genome of 1.1 pg/2C, i.e. 550 Mb (Dhillon, 1987; Bradshaw and Settler, 1993; Wang and Hall, 1995), which is only ca. 5-fold larger than that of *Arabidopsis thaliana*, facilitating map-based approaches. Genetic maps have been made for several *Populus* species (Bradshaw *et al.*, 1994; Cervera *et al.*, 2001; Yin *et al.*, 1999) and can be used for quantitative trait locus (QTL) analysis and map-based cloning (Wu and Stettler, 1994, 1997; Wu *et al.*, 1997, 1998; Wu, 1998; Frewen *et al.*, 2000). The detection of QTLs for wood properties is of particular interest.

High-throughput screening methods for wood properties such as computer tomography X-ray densiometry and pyrolysis molecular beam mass spectrometry will increase our knowledge on the variability and genetics of wood characters (Tuskan *et al.*, 1999).

These advantages have given us and others the incentive to initiate functional genomics programs in *Populus*. Our approach is based on large-scale expressed sequence tag (EST) sequencing (Sterky *et al.*, 1998a; http://www.biochem.kth.se/PopulusDB/). Initially, ESTs were obtained from a cDNA library of the cambial region of hybrid aspen (*P. tremula* L. × *P. tremuloides* Michx.), here denoted the cambium library, and a cDNA library of differentiating xylem of black cottonwood (*P. trichocarpa* Torr. & Gray), denoted the xylem library. More recent sequencing from various tissues and organs within the Swedish initiative has currently increased the number of ESTs to 30 000. These libraries are mines for finding genes based on homology searches. About half of the ESTs from the cambium and xylem libraries correspond to genes for which no function has been described in any other system (Sterky *et al.*, 1998a). Several of these genes are apparently expressed at a high level and may represent novel enzymes (Table 2). Genes related to cell wall formation are represented by 4% and 7% of the ESTs from the cambium and xylem library, respectively. The functional analysis of genes involved in wood formation requires knowledge of the cellular and developmental context in which they operate, which is described briefly below.

Wood cell wall formation: a morphological perspective

The meristematic stage: the cambium

Xylogenesis is initiated in the vascular cambium. The term cambium is defined here as a tissue comprising meristematic cells organized in radial files, which give rise to the secondary xylem and phloem (Larson, 1994; Figure 1). Conceptually, each file contains one initial cell that remains in the meristem, cells that are destined to become phloem, called phloem mother cells, and cells destined to become xylem, called xylem mother cells. The initial cells set the pattern of meristem organization by regulating the number of radial files through anticlinal divisions and by establishing the direction of intrusive tip growth resulting in the spiral fibre orientation that is frequently seen in wood (Wloch and Polap, 1994). It has been suggested that the initials function as a reservoir of genetically sound cells (stem cells) by keeping the frequency of periclinal cell division low and that most cell divisions occur in the mother cells (Gahan, 1988); however, experimental evidence supporting this idea is scarce. The initials and the mother cells are cytologically identical (except for a small difference in length) and there is no evidence for their determination (reviewed by Larson, 1994; Savidge, 2000). Because most published data do not distinguish between the initials and the mother cells of the cambium, the term cambial cells will be used hereafter to denote both the initials and the mother cells.

Whereas the main function of the cambium is cell division and setting out patterns for differentiation similar to other meristems, several aspects are unique to the vascular cambium. Unlike apical meristems, the cambium is a complex tissue containing two morphologically distinct cell types: axially elongated fusiform cambial cells (FCC) and somewhat isodiametrical ray cambial cells (RCC) (Figure 1). These cells give rise to the axial and horizontal cell systems in the secondary xylem and phloem. The identity of cambial cells is determined by positional cues rather than by cell lineage, because the interconversion between a FCC and a RCC is a common phenomenon (reviewed by Iqbal and Ghouse, 1990; Larson, 1994). Most divisions of FCC (90% in *Acer pseudoplatanus* (L.); Catesson, 1964) are periclinal, i.e. new cells are added within a radial file towards either the secondary xylem or the secondary phloem (Figure 1B). The wall that needs to be formed at each periclinal division of a FCC is the largest possible partition within the cell unlike in other cell types where it is usually the smallest possible partition. To cope with such a task, the rate of biosynthesis of the cell plate, the middle lamella, the plasma membrane and the primary cell wall must be exceptionally high in the rapidly growing cambium. This tissue may therefore be the richest source of mRNA and proteins involved in these biosynthetic processes. Indeed, ultramicroscopy of FCC has revealed all features characteristic for high rates of protein biosynthesis and secretory activity (Catesson, 1990).

The speed of new periclinal wall formation in the FCC of *Pinus strobus* (L.) varies from 47 to 105 μm/h, as estimated by the progress of the phragmoplast. This makes the time of completing the phragmoplast movement ca. 20–40 h in this species (Wilson, 1964). Shorter FCCs of dicot trees would probably require slightly shorter times. Nevertheless, the formation of

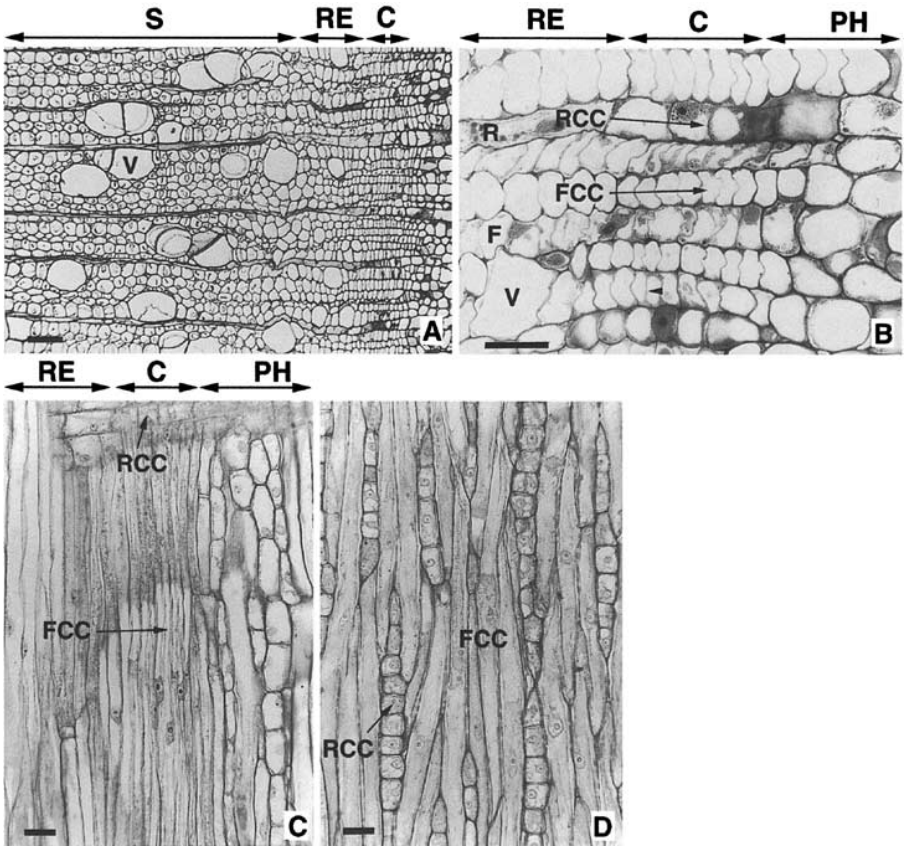

Figure 1. Vascular cambium of hybrid aspen. A. Overview of the cambial region in a transverse section. B–D. Transverse (B), radial (C) and tangential (D) views of the cambium. FCC, fusiform cambial cell; RCC, ray cambial cell; C, cambium; R, ray; RE, the zone of radial cell expansion; S, the zone of secondary wall deposition; PH, phloem; V, vessel element; F, fibre. The arrowhead in B points to a recent periclinal division. Sectioned material provided by Laurence Puech and Siegfried Fink. Bars: A = 50 μm, B, C and D = 20 μm.

the periclinal cell plate and later on the primary cell wall probably limits the frequency of cell divisions. The shortest average duration of a cell cycle for a population of FCCs across the cambium has been estimated to be 7–11 days by counting the number of newly formed cells within a defined period in various conifer species (Mellerowicz *et al.*, 1992; Larson, 1994). Therefore, when high rates of cell formation are required, trees must increase the size of the cambial cell population. Indeed, a good correlation has been observed between the number of cambial cells and the rate of xylem cell formation (Gregory, 1971; Uggla *et al.*, 1998).

Periclinal divisions create the additional difficulty of guiding the phragmoplast and the newly formed cell plate towards the upper and lower ends of a FCC. Furthermore, the new cell plate must traverse the large central vacuole present in FCCs. Sinnott and Bloch (1940) were the first to observe a rim of the cytoplasm in the plane of the future cell plate in vacuolated cells, which they called a phragmosome. The phragmosome has been also found in FCCs and it contained longitudinally oriented microtubules that probably function as guides for the movement of the phragmoplast in the FCCs (Goosen-de Rao *et al.*, 1984). Vesicles containing cell wall material are guided along perpendicularly oriented microtubules of the phragmoplast and coalesce at the equatorial plane to release their contents, which form the cell plate. The cell plate is probably made of callose initially and of cellulose at a later developmental stage, as observed in apical meristems and in tobacco BY2 cells (Northcote *et al.*, 1989; Samuels *et al.*, 1995; Vaughn *et al.*, 1996; Sonobe *et al.*, 2000). Xyloglucan,

Table 2. Assembled clusters that correspond to the highest expressed genes from the cambium and the xylem libraries. Total number of ESTs in each cluster and their frequency in each library is given. Reprinted from Sterky *et al.* (1998a) with permission.

Putative gene identification	Number of ESTs	%
Cambial-region ESTs		
Cyclophilin	30	0.62
Unknown (I)	28	0.58
Translationally controlled tumour protein	26	0.54
Unknown (II)	23	0.48
Blue copper protein	21	0.44
ADP ribosylation factor (I)	21	0.44
HMG protein 1	17	0.35
ADP ribosylation factor (II)	16	0.33
Developing-xylem ESTs		
Nodulin	17	1.93
Laccase	14	1.59
Unknown	10	1.13
S-adenosyl-L-methionine synthase	8	0.91
Elongation factor 1-α	7	0.79
14-3-3-like protein	7	0.79

rhamnogalacturonans, and arabinogalactans have also been immunolocalized to the developing cell plates in these systems. The nascent periclinal walls of FFCs are very resistant to ethylenediaminetetraacetic acid (EDTA) and dimethylsulfoxide (DMSO) extraction, indicating that they do not contain acidic pectins or cross-linking glycans but are rather of cellulosic nature with a high content of methylated pectin (Catesson, 1989, 1990; Catesson *et al.*, 1994; Chaffey *et al.*, 1997b). The merge of the nascent periclinal wall with the pre-existing radial wall is accompanied by local digestion of the radial cell wall until the middle lamella is reached (Catesson and Roland, 1981). The zone containing acidic pectins in the middle lamella of the radial wall then becomes continuous with the middle lamella of the newly formed tangential walls.

The production of xylem cells displaces the cambium centrifugally (outwards) necessitating its extension in girth. This process is accomplished by anticlinal divisions of cambial initials, where the newly formed cell wall is placed radially, thus initiating new radial cell files. The number of newly formed radial files always exceeds the number of new files needed. Therefore, most new files are lost from the meristematic population, which is accomplished by differentiation of their initial cells to xylem elements (reviewed by Larson, 1994). This mechanism may be important for the elimination of somatic mutations from the meristematic population, a feature probably important for long-lived organisms such as trees (Gahan, 1988; Klekowski and Godfrey, 1989). Anticlinal divisions play an important role in regulating the length of fusiform initials, and indirectly also fiber length (Larson, 1994). This results from the oblique (pseudotransverse) orientation of the newly formed radial wall, which leads to the formation of daughter cells that are shorter than their mother cell. The length of these fusiform initials then gradually increases over several periclinal divisions via intrusive tip growth until it is reduced again by the next anticlinal division. The average frequency of anticlinal divisions for individual initials may be as low as one every two years up to several divisions per year (reviewed by Larson, 1994).

Meristematic activity is often regarded as an important determinant of growth rate. Considering the functioning of the vascular cambium, two aspects appear to be important for the rate of wood production: (1) the number of xylem mother cells, which depends on whether the initials form xylem or phloem mother cells and how fast the xylem mother cells exit from the meristematic zone, and (2) the duration of the cell cycle in xylem mother cells. Each of these aspects may be individually targeted to maximize the rate of wood production.

Stages of wood differentiation: cell expansion and secondary wall deposition

In *Populus*, identical FCCs give rise to three cell types: vessel elements, fibres, and axial parenchyma, whereas identical RCCs produce two cell types: contact and isolation ray cells. How and when cell fate becomes established is one of the most intriguing issues of xylogenesis. In several dicot species, including poplar, cell wall properties vary among cambial cells and their immediate derivatives (i.e. xylem or phloem, vessel elements or fibres), as determined by their differential susceptibility to extraction with solvents that solubilize pectin and matrix glycans. This suggests that cell fate has already been determined at this early stage (Catesson and Roland, 1981; Catesson, 1989; Catesson *et al.*, 1994). However, the exact molecular nature of these differences is not yet fully understood and molecular markers for the various cell types are not yet available. The absence of plasmodesmatal connections between vessel elements and other

cell types during the early stages of differentiation has been suggested as a mechanism of determination of these cells (reviewed by Barnett, 1995). Careful investigations in hybrid aspen, however, have now revealed plasmodesmatal connections between vessel elements and other cell types, but their frequency was very low (K. Pickering and J. Barnett, personal communication). After xylem mother cells have left the meristem, they enlarge while in the primary walled stage. This phase corresponds to the radial expansion (RE) zone (Figure 1). It is in this zone where vessel elements and fibres clearly display different morphological characteristics.

The zone of radial cell expansion

All cells in the RE zone undergo enlargement, with cell types differing in the extent, polarity, and type of enlargement. Fibres and axial parenchyma cells expand primarily in the radial direction. In this case, their radial walls expand uniaxially. Vessel elements, in addition to radial expansion, may undergo substantial tangential growth that is accomplished by the uniaxial extension of radial walls and the lateral displacement of adjacent cells. No growth of the tangential wall is observed except for the tangential walls of contacting vessel elements (Catesson, 1989; Barnett, 1992; Catesson *et al.*, 1994). In contrast, the end walls of vessel elements and axial parenchyma cells undergo multiaxial expansion. Fibre elongation is achieved by intrusive tip growth and requires local wall biogenesis and dissolution of the middle lamellae between neighbouring cells. In *P. deltoides* (Bartr. ex Marsh.), fibre elongation by ca. 1.5-fold was observed during their differentiation from FCCs (Kaeiser, 1964), but in many dicot species the length of the xylem fibres exceeds several-fold that of FCCs. Tip growth is already evident in the cambium, but is most intense in the RE zone (Wenham and Cusick, 1975). It has been suggested that the formation of Ca^{2+}-bound pectins in the middle lamella of neighbouring cells may limit the penetration of the intrusively growing fibre tip (Catesson *et al.*, 1994; Guglielmino *et al.*, 1997a). Ray cells elongate radially by uniaxial wall expansion. Remarkably, the basic pattern of cell arrangement between the different cell types of the xylem is largely conserved despite their different patterns of expansion.

Because the degree of enlargement varies among cell types, mechanisms must exist that differentially regulate cell turgor pressure and/or cell wall plasticity. Numerous observations in dicot trees, including poplar, indicate that changes in the amount and composition of pectins play an important role in radial cell expansion (Roland, 1978; Catesson and Roland, 1981; Catesson, 1989; Barnett, 1992).

Secondary wall deposition

All xylem cells in *Populus* form a secondary cell wall that is deposited when the radial expansion is completed. In both conifers and dicots, the orientation of cellulose microfibrils in the primary wall is usually random or longitudinal (reviewed by Funada, 2000). The deposition of the secondary wall is marked by the formation of a dense array of helical, almost transverse cellulose microfibrils, which limit the further radial expansion, as demonstrated in *Abies sachalinensis* (Masters) (Abe *et al.*, 1997). In tracheids of conifers and in fibres of dicot trees, successive layers of the secondary wall have orderly arranged cellulose microfibrils that form a helicoidal or semi-helicoidal structure (Abe *et al.*, 1995; Prodhan *et al.*, 1995; Awano *et al.*, 2000; Funada, 2000). The first formed S1 layer has a flat, almost transverse microfibril angle that gradually changes clockwise, as seen from the cell lumen, to the longitudinal arrangement that characterizes the thickest S2 layer. Finally, the S3 layer is formed after an abrupt reorientation of the microfibrils back to the transverse helix. The changes in microfibril orientation during the formation of successive cell wall layers are always paralleled by the reorientation of cortical microtubules (Abe *et al.*, 1995; Prodhan *et al.*, 1995; Chaffey *et al.*, 1997a, 1999; Funada, 2000). Drugs that disrupt the microtubules, such as colchicine, abolish the normal pattern of secondary wall thickening (Torrey *et al.*, 1971). These observations support the hypothesis that microtubules control the orientation of cellulose microfibrils in xylem cells. Successive changes in microtubule density and orientation have also been observed in developing fibres of hybrid aspen (Figure 2; N. Chaffey, P. Barlow and B. Sundberg, unpublished).

In *Populus*, the formation of the secondary cell wall starts first in vessel elements and their contact cells (Murakami *et al.*, 1999). The vessel elements have a three-layered secondary cell wall similar to that of the fibres, but the S2 layer is proportionally thinner (Harada and Côté, 1985). The ray cells also develop secondary walls with three S layers (Fuji *et al.*, 1979; Harada and Côté, 1985; Murakami *et al.*, 1999). Contact ray cells develop a tertiary wall, called the protective layer, over the secondary lignified wall after autolysis of the contacting vessel element (Benayoun, 1983). The protective layer remains non-lignified

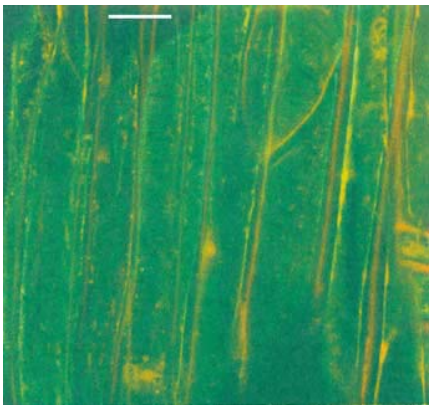

Figure 2. Reorientation of microtubules during fibre differentiation in hybrid aspen. The radial section through the cambial region shows fibres at successive stages of secondary wall deposition. Microtubules are stained by indirect immunofluorescence. The vascular cambium is to the left. Illustration provided by Nigel Chaffey. Bar = 20 μm.

(Murakami *et al.*, 1999), and is rich in cross-linking glycans (Fujii *et al.*, 1981). It forms the tyloses in non-functional vessels.

Longitudinal co-ordination of vessel differentiation

The formation of cell contacts and perforations as well as the differentiation of the vessel members must occur in a coordinated fashion to ensure root-to-shoot water transport. Hundreds of vessel elements may be joined end-to-end to form a functional vessel in wood (reviewed by Butterfield, 1995). Pits and perforations become evident in expanding vessel elements as microtubule-free areas (Chaffey *et al.*, 1999; Funada, 2000). Secondary walls develop around pits and perforations and subsequently lignify, but no secondary wall deposition or lignification is observed in the pit/perforation area (Butterfield 1995). Whereas simple pits develop over pre-existing plasmodesmata in primary pit fields, the bordered pits develop without any apparent relation to the plasmodesmatal connections (Barnett and Harris, 1975; Yang, 1978). Before the protoplast of a vessel element is autolysed, the primary wall that closes the perforation appears swollen, presumably because of local wall restructuring and enzymatic hydrolysis (Butterfield, 1995). However, no specific enzymatic activity responsible for the formation of the perforation has been identified. In species that have simple perforation plates, including *Populus*, the nature of vessel end walls is very different from that of the lateral walls (Benayoun *et al.*, 1981). Almost all end wall material, except for the pectins in the middle lamella, is DMSO-extractable, indicating that the end walls contain mostly cross-linking glycans and very little cellulose. The non-cellulosic material is removed first during the formation of the perforation plate, leaving webs of naked cellulose microfibrils that can sometimes be observed by microscopy (Butterfield, 1995). The subsequent steps are not well documented and probably occur quickly. The fibrilar rim of the primary wall may remain after autolysis of the vessel element and its final removal is possibly accomplished by the surge of the transpiration stream. Within one vessel, the primary wall forming the perforation plates might still be present in some elements while it is already removed in others (Butterfield, 1995).

Lignification and programmed cell death

Lignification starts in vessel elements and contact ray cells that have secondary cell walls, whereas fibres and isolation cells lignify later (Murakami *et al.*, 1999). Lignin is first detected in the middle lamella, particularly at cell corners, when cells have completed the deposition of the S1 layer (Bailey, 1954; Terashima *et al.*, 1993). Lignification progresses inwards during the S2 layer formation, concomitantly with cellulose, mannan and xylan deposition. Lignin deposition is most intense when the S3 layer is formed and it progresses towards the cell lumen until all the wall layers are lignified.

Lignin composition changes during cell differentiation in relation to the distance from the cambium; the closer to the cambium, the more *p*-hydroxyphenyl (H) and guaiacyl (G) units are present in lignin, whereas the further away from the cambium, the more syringyl (S) units are incorporated (Terashima *et al.*, 1979, 1993; Yoshinaga *et al.*, 1997b). Thus, cells that complete lignification closer to the cambium would have more H and G lignin. The composition of lignin affects the interaction between lignin and the carbohydrate components of the cell walls. The H and G lignin interact with pectins, while the S lignin form links with cross-linking glycans (Fukuda and Terashima, 1988). In lignified cells, pectin is difficult to extract, stain and label with pectin-specific antibodies (Guglielmino *et al.*, 1997a; Hafren *et al.*, 2000), but it is still present as demonstrated by the presence of radiolabelled galacturonan upon feeding with radiolabelled pectin precursors (Imai and Terashima, 1992). When lignification is completed, vessel elements undergo programmed cell death (PCD), which involves the hydrolysis of the protoplast. Contact ray cells, which

produce secondary cell walls and lignify concomitantly with the vessel elements, do not initiate PCD, indicating that PCD is regulated independently from secondary cell wall formation and lignification.

Cell types: wall properties and chemical composition

Within the wood-forming tissues, cell wall composition differs between the primary- and secondary-walled stage as determined by the analysis of bulked tissue samples. Cell walls derived from the cambium, and the primary-walled developing xylem and phloem tissues of *P. tremuloides* contain 47% pectin, 22% cellulose, 18% matrix glycans, 10% protein, and 3% other material (Simson and Timell, 1978a). In contrast, the secondary-walled wood from *P. nigra* (L.) is composed of 48% cellulose, 23% matrix glycans, 19% lignin, and 10% other material (McDougall *et al.*, 1993). Some changes in dry mass composition associated with the transition from primary- to secondary-walled stage are listed in Table 3. The most noticeable change, however, is the decrease in cell wall water content. This is related to a decrease in wall porosity as a result of the tight packing of cellulose microfibrils and the heavy cross-linking of lignin that fills all available space and concomitantly displaces water (Fujino and Itoh, 1998).

In terms of cell types, 33% v/v of poplar wood is composed of vessel elements, 53–55% of fibres, 11–14% of ray parenchyma and less than 1% of axial parenchyma (Panshin and de Zeeuw, 1980). These cell types differ in wall structure and chemical composition as observed in other dicot trees. For example, vessel elements in oak contain more mannose, xylose, rhamnose, and arabinose but less glucose and galactose than do fibres (Yoshinaga *et al.*, 1993). In several other species, vessel elements contain more polyphenols (Watanabe *et al.*, 1997) and have a lower S/G ratio of lignin monomers when compared to fibers and ray parenchyma cells (Fergus and Goring, 1970; Saka and Goring, 1985; Yoshinaga *et al.*, 1993, 1997a, b). Within a complex tissue such as wood, techniques such as immunolabelling with specific antibodies and lectins (Wojtaszek and Bolwell, 1995; Knox, 1997), UV microspectrometry (Goto *et al.*, 1998) or Fourier transformed infrared microspectroscopy (McCann and Roberts, 1994) are required to determine the precise chemical composition of individual cells.

Variation in wood cells and their wall properties: positional and environmental controls

Because wood is formed in a variable environment and subject to developmental control, xylem cells are produced that differ in size, shape, cell wall structure, texture, and chemical composition. These aspects have to be taken into account when genetically modified trees are compared or the effects of various treatments on wood formation are studied. Below, some important sources of within-tree variation are described.

Juvenile/mature wood and longitudinal variability

During the first years of cambial growth, relatively rapid cellular changes take place within the cambium, which are reflected in its derivatives. For example, the length of the FCCs increases from one year to the next, as deduced from the progressive increase in the length of xylem cells derived from these cells (Hejnowicz and Hejnowicz, 1958). Wood produced during this period is called juvenile. In addition to the shorter cell length, juvenile wood is characterized by a lower crystallinity of the fibers, a larger microfibril angle, thinner secondary walls, a higher density of vessels (number per mm^2), and a lower proportion of latewood than the mature wood produced in the subsequent years (reviewed by Zobel and van Buijtenen, 1989; Kroll *et al.*, 1992; Parresol and Cao, 1998). In poplar, the juvenile wood is produced during the first 5 to 10 years of cambial growth (Liese and Ammer, 1958), when the cambium is situated close to foliated branches.

As a consequence of the gradual transition from juvenile to mature wood, a longitudinal variation is observed in fiber and vessel length along the tree trunk in *Populus* (Kaeiser and Stewart, 1955; Hejnowicz and Hejnowicz, 1958; Boyce and Kaeiser, 1961), similar to other tree species. In a thorough analysis of xylem cell length in a single trunk of *P. tremula*, Hejnowicz and Hejnowicz (1958) found that there was no basipetal increase in the degree of intrusive tip growth of the fibers. Therefore, the observed basipetal increase in fibre length can be explained by a comparable increase in the length of the FCCs. They also observed that the FCC length was primarily determined by cambial age rather than by its distance from the ground (Hejnowicz and Hejnowicz, 1958). Similarly, the radial diameter of vessel elements increases basipetally along the trunk and centrifugally from pith to bark (Kroll *et al.*, 1992). Thus, the xylem cell volume increases concomitantly with cambial age.

Table 3. Chemical composition of cell walls in poplar xylem.

Stage of xylem development	Component (% dry weight)	Composition	Reference
Primary-walled stage	Pectins (47%)	galacturonic acid, galactose, arabinose and rhamnose in a molar ratio 5:2:2:1 Main chain: rhamnogalacturonan [(1→4)-D-galacturonosyl-(1→2)α-L-rhamnosyl-]. Side-chains branching from rhamnose units at carbon 4 contain: (1→4)β-D-galactan and (1→5)-L-arabinan, with occasional terminal arabinose, or a fucose	Simson and Timell, 1978a,c
	Cellulose (22%)	(1→4)β-D-glucan, degree of polymerization 4200	Simson and Timell, 1978a,d
	Xylan (11%)	(1→4)β-D-xylan	Simson and Timell, 1978a
	Xyloglucan (6%)	3 species giving an average molecular mass of 62 kDa Main chain: (1→4)β-D-glucan Side-chains (70% of the glucose residues are substituted): xylose residues, some of which are further linked to a terminal galactose, galactose–galactose or galactose–fucose	Simson and Timell, 1978a;b
	Glucomannan (1%)	(1→4)β-D-glucan, (1→4)-β-D mannan oligomeric units	Simson and Timell, 1978a
	Proteins (10%)	Various structural proteins and cell wall bound enzymes	Simson and Timell, 1978a
Mature wood (secondary wall plus the middle lamella and the primary wall)	Cellulose (43–48%)	(1→4)β-D-glucan, degree of polymerization 9300	Goring and Timell, 1962; Panshin and de Zeeuw, 1980; McDougall *et al.*, 1993
	Xylan (18%–28%)	Main chain: (1→4)β-D-xylan Side-chains: (1→2)β-4-*O*-methyl-α-D-glucuronic acid, acetyl and arabinosyl residues	Jones *et al.*, 1961, Bolwell, 1993; Panshin and de Zeeuw, 1980; McDougall *et al.*, 1993
	Glucomannan (5%)	(1→4)β-D-glucan, (1→4)β-D-mannan oligomer units in a molar ratio 1:2	Sultze, 1957; Northcote, 1972; Panshin and de Zeeuw, 1980
	Pectin and xyloglucan (3%)	As in primary-walled stage	Panshin and de Zeeuw, 1980
	Lignin (19–21%)	H, G and S units	Panshin and de Zeeuw, 1980; McDougall *et al.*, 1993

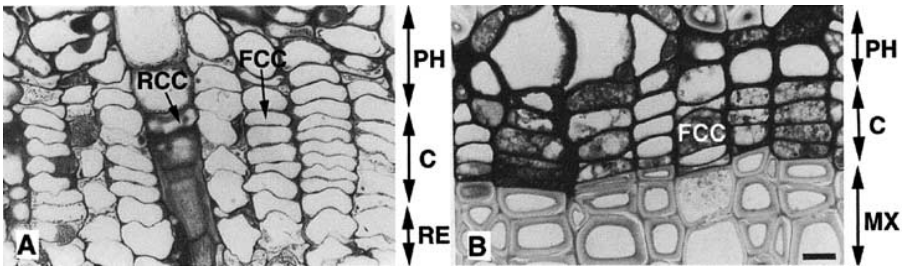

Figure 3. Active (A) and dormant (B) cambium of *P. tremula*. Note the thickened cell walls and the absence of the RE zone in the dormant cambium. MX, mature xylem; other labels as in Figure 1. Bar = 10 μm.

Seasonal variability

Both in conifers and dicot trees, cambial cell walls are thick during the dormant period and thin during the period of active growth (Figure 3) (Catesson, 1964; Riding and Little, 1984; Funada and Catesson, 1991; Chaffey *et al.*, 1998). The question arises whether this thickening represents storage of material that could be metabolized in spring. Storage walls are typically observed in seeds and contain cross-linking glycans and pectins that serve as metabolic reserves during seed germination (Brett and Waldron, 1996). In contrast to seed storage walls, cambial walls of *Aesculus hippocastanum* (L.) display a multi-layered 'herring bone' structure during dormancy, as visualized by transmission electron microscopy, indicating the deposition of layers of helicoidal microfibrils (Chaffey *et al.*, 1998). This is clearly different from the random orientation of microfibrils in the cell walls of the active cambium. Moreover, the cortical microtubules were found to be axially oriented during dormancy and randomly during the period of active growth, conforming to the pattern observed for the microfibrils (Chaffey *et al.*, 1998). Thus, the cellulose microfibrils appear to be one of the components of the thickened cell walls. In poplar, dormant cambial cell walls are enriched in glucan- and xylan-containing hot-water-extractable polysaccharides (Baïer *et al.*, 1994; Vietor *et al.*, 1995; Ermel *et al.*, 2000).

The seasonal cycle of cambial activity and dormancy correlates also with changes in the nature of pectins. Dormant cambium contains less hot-water-extractable pectin than active cambia (Baïer *et al.*, 1994; Ermel *et al.*, 2000). Studies on outdoor-grown *P.* × *euramericana* (Dode Guinier) and greenhouse-grown hybrid aspen (under natural photoperiod and temperature) indicate an increase in pectin methylation during cambial growth and an up-regulation of the demethylating enzyme, pectin methyl esterase

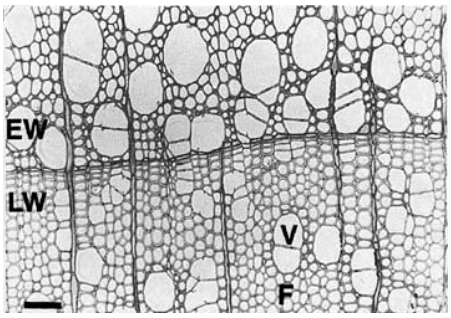

Figure 4. Variability in the secondary xylem of *P. tremula* at the latewood/earlywood boundary. Note the change in size and density of vessel elements. EW, earlywood; LW, latewood; other labels as in Figure 1. Bar = 50 μm.

(PME), during dormancy (Baier *et al.*, 1994; Ermel *et al.*, 2000; Follet-Gueye *et al.*, 2000). Interestingly, a basic isoform of PME, which was active at pH 7.0 but not at pH 6.1, was specifically found in the dormant cambium (Micheli *et al.*, 2000a). A similar basic PME isoform, active at pH 7 to 11, was shown to be specifically induced by the dormancy-breaking chilling treatment in cedar seeds (Ren and Kermode, 2000). It is possible that the B4 form of hybrid aspen PME is involved in the chilling response required to break cambial dormancy (Olsson and Little, 2000).

The time during the growing season at which cambial derivatives are formed affects their properties, thus creating variability within each annual ring. This variability is referred to as the earlywood/latewood continuum. The earlywood and the latewood are formed during the first part and at the end of the growing season, respectively. In poplar, diffuse porous wood is formed that is characterized by a gradual decrease in vessel size and density from earlywood to latewood within an annual ring and by an abrupt increase at the annual ring boundary (Figure 4). Several other features distinguish earlywood and latewood in

Populus. Xylem cell length increases from a minimum in earlywood to a maximum in latewood and sharply decreases at the annual ring boundary (Bissett and Dadswell, 1950; Liese and Ammer, 1958; Hejnowicz and Hejnowicz, 1958). This pattern is explained both by the progressive elongation of FCCs during the growing season before the onset of anticlinal cell divisions in late summer and by the more extensive intrusive fibre tip growth during latewood differentiation. Cell wall crystallinity increases from earlywood to latewood (Parresol and Cao, 1998). In *Fagus crenata* (Bl.), the lignin composition follows a seasonal pattern in vessel elements different from that in fibres (Takabe *et al.* 1992). Within each growing season, the S/G ratio increases in vessel elements whereas it decreases in fibres.

Gravity effects: tension wood

Leaning stems and branches of most dicot trees, including poplar, develop tension wood at their upper side. The extent of tension wood formation correlates with the degree of leaning (Ohta, 1979). Upright-growing stems usually form scattered bands or even isolated tension wood fibers, probably because of bending by wind or growth adjustment (Kaeiser, 1955). Kroll *et al.* (1992) found that 22–63% of the fibres in straight trunks of *P. balsamifera* (L.) had characteristics of tension wood fibres and these characteristics were more often seen in earlywood than in the latewood and in upper parts of the trunk than in the lower parts.

Tension wood contracts longitudinally and pulls the stem back to the vertical position. It is characterized by a high rate of cell production on the tension wood side, which may lead to stem eccentricity (Ohta, 1979). At the same time, wood formation on the opposite side of the stem is inhibited. Other anatomical features include the reduction in vessel size and density and the formation of gelatinous instead of ordinary fibres (Araki *et al.*, 1982). These fibres form a gelatinous cell wall layer (G layer) over the secondary wall inside the cell. In *Populus*, the G layer is formed over the partially developed S2 layer (Figure 5.; Nobushi and Fujitta, 1972; Araki *et al.*, 1982). It is composed of almost pure, high-crystalinity cellulose and has a low lignin content (Norberg and Meier, 1966; Bentum *et al.*, 1969). The microfibril orientation is longitudinal and parallel to the microtubules (Nobushi and Fujitta, 1972; Fujita *et al.*, 1974). The length of gelatinous fibres does not differ significantly from normal fibres (Kaeiser and Stewart, 1955).

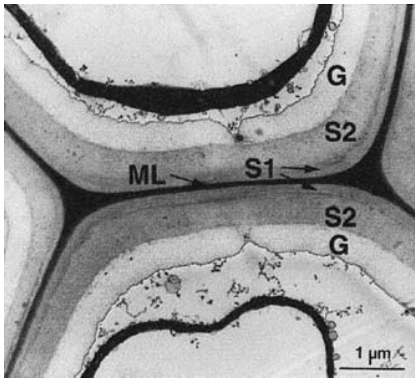

Figure 5. Ultrastructure of developing gelatinous fibres of hybrid aspen. Note the G-layer inside the S2 wall layer. ML, compound middle lamella. Illustration provided by Kathryn Pickering and John Barnett.

Hormones as molecular transducers of positional and environmental information for xylem cell formation in poplar

Plant hormones are key regulators in development and pattern formation, and environmental stimuli often act on plant growth by modulating the hormonal balance. When applied exogenously, plant hormones have been observed to affect most aspects of cambial growth, such as cell division, cell expansion, final cell morphology, the induction of differentiation into different cell types, and cell wall chemistry (Little and Savidge, 1987; Aloni, 1991; Little and Pharis, 1995; Sundberg *et al.*, 2000). This demonstrates the importance of hormones as endogenous regulators of xylem formation and implies that they are involved in controlling at least some aspects of the variation in wood properties described above. However, in most experiments with exogenous hormones, the resulting internal hormone balance in the experimental tissue is not known. This complicates the interpretation of the observed effects of hormonal treatments, and has resulted in conflicting views about the role of hormones in xylem formation. Here we will highlight recent progress on the role of hormones in intact tissues by using modern analytical techniques and molecular tools.

The auxin gradient and its role in cambial cell division and expansion

Auxin is a key signal in xylogenesis. It is the only plant hormone that on its own is sufficient to induce differentiation of vascular elements when applied to plant tissues (Roberts, 1988). Auxin can induce tracheary element (TE) differentiation without prior cell division when applied to *Zinnia* cultures (Fukuda and Komamine, 1980). In contrast, the differentiation of auxin-induced TEs *in planta* is often preceded by cell division (Sussex *et al.*, 1972; Phillips and Arnott, 1983), similar to normal development of vascular bundles. During secondary growth, auxin depletion results in a dedifferentiation of FCC to axial parenchyma cells by transverse divisions (Evert and Kozlowski, 1967; Evert *et al.*, 1972; Savidge, 1983). Thus, a continuous supply of polarly transported auxin is required to maintain the identity of FCC. Numerous experiments have also demonstrated that the application of auxin to cambial tissues stimulates xylem production, i.e. cambial cell division (Sundberg *et al.*, 2000). In a few cases, the indole-3-acetic acid (IAA) level that is induced in the cambial region after exogenous feeding through the polar transport system has been quantified. Both in Scots pine (Sundberg and Little, 1990; Wang *et al.*, 1997) and hybrid aspen (B. Sundberg, unpublished data), feeding with different IAA concentrations resulted in a range of internal IAA levels that was within the variation found in intact plants and showed a positive correlation with the extent of xylem formation. It was further demonstrated that the endogenous supply of IAA in intact conifer shoots was sub-optimal for xylem production, indicating that it plays a regulatory role (Sundberg and Little, 1990).

High-resolution analysis of endogenous IAA in Scots pine and hybrid aspen has demonstrated steep radial concentration gradients across developing xylem and phloem (Uggla *et al.*, 1996; Tuominen *et al.*, 1997). The IAA concentration peaked in the cambium and its most recent derivatives, and reached low levels in maturing xylem and phloem (Figure 6). The visualization of an auxin gradient across developing tissues supports the idea that auxin has a function in positional signalling; cambial derivatives would develop according to their position along the auxin gradient and neighbouring cell files will receive the same information and develop in synchrony. According to this idea, auxin would modify cambial growth by influencing developmental patterns (Sundberg *et al.*, 2000). The cambium would be positioned within a

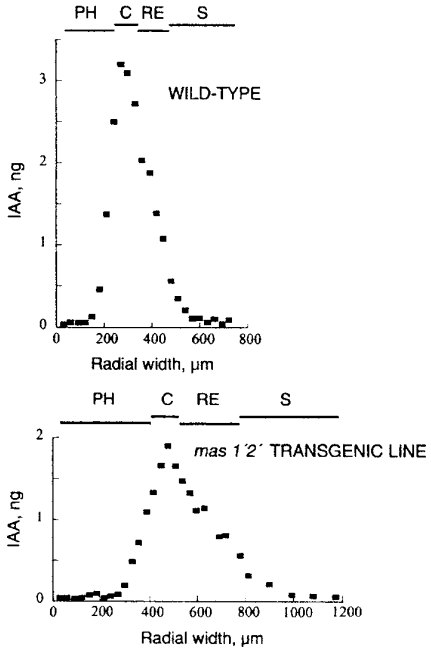

Figure 6. Radial distribution of IAA across the cambial region of hybrid aspen. Each data point represents the IAA content in 1 cm^2 of a 30 μm thick, tangential section. Horizontal bars indicate the approximate position of developmental zones. The IAA distribution is modified by the expression of IAA biosynthetic genes from *Agrobacterium tumefaciens* under the control of the mannopine 1′2′ promoter in transgenic poplar. The altered distribution correlates with an altered developmental pattern in the xylem (see text for discussion). Labels are as in Figure 1. Figure redrawn from Tuominen *et al.* (1997).

window of high auxin concentration and the radial population of meristematic cells would be determined by the width of the appropriate IAA concentration window. Accordingly, in Scots pine, xylem cell production is related to the meristem size and the auxin distribution pattern (Uggla *et al.*, 1998). It is clear, however, that auxin is not the only player in providing positional signalling to the cambial meristem. Cells at the phloem side of the meristem remain meristematic at a lower auxin concentration than cells at the xylem side. Thus, the auxin gradient by itself does not provide enough information to position either xylem and phloem mother cells or the cambial initial. The recent demonstration of steep concentration gradients of soluble carbohydrates across the cambium (Uggla *et al.*, 2001), together with accumulating evidence in favour of sugar sensing in plants (Sheen *et al.*, 1999), provide substantial evidence to the concept that auxin/sucrose ratios determine the positioning of the cambium (War-

ren Wilson and Warren Wilson, 1984). Moreover, the idea that auxin distribution sets the radial limits of the meristem does not exclude the possibility that the amplitude of auxin concentration within the critical window is important for the rate of cell cycling of the meristematic cells.

In a series of experiments with hybrid aspen, the auxin biosynthetic genes from *Agrobacterium tumefaciens* were expressed under the control of different promoters, with the aim of altering the auxin balance in the cambial region (Tuominen *et al.*, 2000a). Surprisingly, neither the strong 35S promoter nor any other promoter used induced an increase in the IAA level comparable to that previously obtained in tobacco and petunia (Klee *et al.*, 1987; Sitbon *et al.*, 1992). The most conspicuous phenotype resulted from the expression of the IAA biosynthetic genes under the mannopine synthase $1'2'$ promoter (Tuominen *et al.*, 1995). These trees were smaller than wild-type trees and had an altered development of xylem tissue. Increased IAA levels were only found in the root tips and mature leaves, whereas IAA levels in the apex and stem internodes were similar or lower than those in wild-type. The ectopic expression of the IAA biosynthetic genes in the stem was however demonstrated by the fact that the apical dominance was maintained after decapitation. A detailed examination of the auxin balance in stem tissues of these trees showed a wider radial distribution but with lower concentrations across the cambial region, compared to wild-type plants (Figure 6) (Tuominen *et al.*, 1997). The lower concentration was explained by a decrease in the supply of IAA originating from endogenous genes because of the smaller size of the transgenic trees, whereas ectopic production of IAA induced its wider distribution. The altered IAA balance was interpreted to cause the wider developmental zones of division and expansion observed in the transgenic trees, and to support the idea that IAA has a function in positional signalling. In spite of the wider cambium, the number of cells in the meristem remained the same and the cell production rate was lower, possibly as a result of the lower IAA concentration. When the IAA biosynthetic genes were expressed under the *rolC* promoter from *Agrobacterium rhizogenes*, a 35–40% increase in IAA concentration in the cambial meristem was obtained, but the pattern of radial IAA distribution was unaffected (Tuominen *et al.*, 2000). In this case, no difference in xylem development or anatomy was observed, and the rate of xylem production was similar in transgenic and wild-type trees. This result strengthens the importance of auxin distribution in controlling cambial growth patterns. The small increase in auxin concentration that was induced did not affect cell cycling rate in this experiment.

Cambial derivatives will cease to divide, but continue to expand during their exposure to decreasing IAA concentrations when developing along the auxin gradient. Conceivably, the width of the gradient influences the width of the RE zone, and therefore the duration of expansion, whereas the IAA concentration within this zone can be expected to influence the rate of cell expansion. In conifer trees, both the rate and the duration of expansion have been shown to be important factors that determine the final radial size of the tracheids (Dodd and Fox, 1990). Exogenous feeding experiments in both conifers and angiosperm trees have demonstrated that auxin stimulates xylem cell expansion up to an optimal concentration, while a further increase in the exogenous auxin concentration will reduce the radial size of the derivatives (Sheriff, 1983; Zakrzewski, 1991). Whether the endogenous concentration to which the developing xylem cells are exposed, is sub- or supra-optimal for the rate of radial expansion is unknown. The transgenic hybrid aspen plants that express the IAA biosynthetic genes under the mannopine synthase $1'2'$ promoter had radially wider fibres. These fibres had developed in a lower IAA concentration present across a wider radial zone that corresponded to the RE zone (Figure 6). Thus, the increase in radial fibre size was interpreted to be a consequence of a longer duration of fibre expansion. In this context, it should be emphasized that fibres and vessel elements do not develop synchronously: vessels mature earlier than fibres (Murakami *et al.*, 1999). This observation again highlights the fact that the auxin gradient only provides some of the positional information required for xylem development.

Other hormones involved in cambial cell division and expansion

The interpretation of the role for endogenous IAA in cambial growth is complicated by the fact that other hormones often act in synergy with auxin. In particular, gibberellins (GAs) will stimulate meristematic activity and xylem fibre elongation when applied together with auxin (Digby and Wareing, 1966). The role for GAs in wood formation and their potential in tree biotechnology has recently been demonstrated by over-expression of a GA-20 oxidase in hybrid aspen (Eriksson *et al.*, 2000), resulting in a 20-fold increase

in the biologically active GA1 and GA4. In line with data from exogenous applications, the transgenic trees exhibited an increased longitudinal and radial growth as well as an increased xylem fibre length.

Although cytokinins have a well-established function in cell division, their role in cambial growth is far from clear (Little and Savidge, 1987; Little and Pharis, 1995). Accurate information on endogenous cytokinin levels in the cambial region of dicot trees is not available. Mass spectrometry quantification in Scots pine revealed nanomolar concentrations in samples comprising the combined tissues of the cambial region from both dormant and actively growing trees (Moritz and Sundberg, 1996). Interestingly, zeatin, which is supposedly a biologically active cytokinin, was only putatively identified and present only in trace amounts. With an auxin concentration in the micromolar range in the same tissues (Uggla *et al.*, 1996), the endogenous auxin/cytokinin ratios would differ strongly from, for example, the ratio used for TE induction in the *Zinnia* system.

Exogenous ethylene stimulates cambial cell division, possibly by increasing auxin levels through interaction with auxin transport (Eklund and Little, 1996). The concentration of endogenous ethylene in cambial tissues of intact plants cannot be easily assayed because of its gaseous nature. Measurements of ethylene emanating from isolated cambial regions may just reflect the availability of the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) or wound-induced ethylene, and should be interpreted with caution. Several recent observations from aspen provide additional evidence for ethylene signalling in cambial growth processes. ACC was identified by mass spectrometry in developing xylem tissues (J. Hellgren, T. Moritz and B. Sundberg, unpublished). ESTs for ACC synthase, ACC oxidase and putative ethylene receptors are present in the cambium library. Only one ACC oxidase gene was found to be expressed in the cambial region, and its mRNA was mainly present in developing xylem at the stage of secondary wall formation (S. Andersson, S. Regan, and B. Sundberg, unpublished). The cellular localization of ACC and ACC oxidase has not yet been established, but it is of particular interest to investigate whether ethylene production takes place in the symplast or in the apoplast, because this will be an important factor determining the ethylene balance in the cambial region.

Induction of xylem cell differentiation

The xylogenic nature of plant hormones has been extensively investigated in cell cultures, callus, and explants (Roberts, 1988). The absolute requirement for auxin in TE induction is without question. Other plant hormones often act in synergy with auxin when applied exogenously, but frequently the observed effects vary between different experimental systems. This can be explained by differences in the resulting hormonal balance in the experimental tissues, which is unknown in most cases. In many systems, including *Zinnia* cultures, cytokinin is needed for TE induction. When cytokinin is not required, it seems likely that the experimental tissue already contains a sufficient amount of cytokinins. Early studies showed that the number of auxin-induced TEs decreased when ethylene biosynthesis and response inhibitors were applied (Miller and Roberts, 1984), pointing to a role for ethylene in xylogenesis. However, the addition of ethylene has never been demonstrated to be required for TE induction, as is the case for auxin and cytokinin. Possibly, IAA induces the necessary levels of ethylene through the well-established induction of ACC synthase (Abel *et al.*, 1995), but conclusive evidence for the role of ethylene in xylogenesis is still lacking. GAs have been observed to stimulate the production of both phloem and xylem fibres when applied together with auxin to intact tissues (Digby and Wareing, 1966; Aloni, 1979), but evidence for a requirement for GAs in xylem cell differentiation is lacking.

Formation of secondary walls

Most data on the role of plant hormones in secondary wall formation concern the involvement of auxin and ethylene in lignification. Auxin has been shown to enhance lignification in primary phloem fibres of *Coleus* (Aloni *et al.*, 1990), and in secondary xylem of tobacco (Sitbon *et al.*, 1999). However, at least in the latter case, ethylene production was also increased, and ethylene is well known to induce several enzymes involved in lignin biosynthesis (Sitbon *et al.*, 1999). The endogenous auxin concentration was found to be low in lignifying xylem of Scots pine and hybrid aspen (Uggla *et al.*, 1996; Tuominen *et al.*, 1997) but this observation does not preclude its role in secondary wall formation. Other effects of exogenous hormones on cell wall biosynthesis, such as cytoskeleton organization, induction of genes and enzyme activities, may be direct, particularly in the case of ethylene. However, many of the observed effects of applied hormones are

likely to be inductive and result from their primary role in determining developmental fate, cell division, and cell expansion. For example, the induction of TE in *Zinnia* cell cultures requires the presence of IAA and cytokinin only for a very short inductive period prior to any visible sign of differentiation (McCann *et al.*, 2000).

Hormones and variation in wood properties

Little is known about the molecular mechanisms that determine the formation of the different wood types, and about the role of hormones in this process. Many aspects of the spatial and seasonal variability in wood formation have been suggested to be under the control of auxin concentration. However, information on endogenous IAA levels to support this idea is scarce and, when available, does not support traditional views (Sundberg *et al.*, 2000). For instance, the lack of radial expansion during latewood formation has been suggested to result from decreased auxin levels (Larson, 1969). However, high-resolution analysis revealed an increase in auxin concentration during the transition from earlywood to latewood in Scots pine (Uggla *et al.*, 2001). The radial distribution of auxin was narrowed in accordance with the decrease in the width of the RE zone, which is typical of latewood development (Whitmore and Zahner, 1966; Dodd and Fox, 1990). Whether the altered auxin distribution is a cause or a consequence of the altered pattern of development is not known, but it supports the view that auxin is a developmental signal in cambial growth.

The formation of tension wood is an interesting case of a developmental switch that results in an increased formation of xylem having gelatinous fibres with an altered secondary wall composition and structure (see above). From experiments with exogenous auxin and auxin transport inhibitors, it has been proposed that tension wood is induced by auxin deficiency (Little and Savidge, 1987). However, high-resolution analysis of endogenous auxin revealed that the auxin concentration in cambial derivatives developing into tension wood is not significantly affected compared to that in normal xylem development (J. Hellgren and B. Sundberg, unpublished). Instead, the IAA amount and, hence, the width of the radial IAA distribution across the developing xylem tissues was increased. This is in accordance with IAA being a positional signal that stimulates growth by acting on meristem size. Ethylene production is stimulated during tension wood formation and has been suggested to play a role in this process (Little and Savidge, 1987). In support of this observation, the expression of ACC oxidase is strongly induced in hybrid aspen during tension wood formation (S. Andersson, S. Regan and B. Sundberg, unpublished). However, exogenous ethylene, while stimulating radial growth, has not been observed to induce gelatinous fibres. Taken together, both ethylene and IAA may be involved in growth stimulation accompanying tension wood formation rather than in the induction of gelatinous fibres. Finally, it should be mentioned that exogenous GAs have been observed to induce tension wood characteristics in Japanese cherry (Baba *et al.*, 1995). The endogenous GA balance during tension wood formation is unknown.

Biosynthesis of wall carbohydrates in poplar xylem cells

Precursors for carbohydrate components of the cell wall

UDP-D-glucose is a precursor of all carbohydrates in the cell wall (Gibeaut, 2000; Reiter and Vanzin, 2001, this issue). It is provided either from the reaction (sucrose + UDP → UDP-D-glucose + fructose), catalysed by sucrose synthase (SuSy), or by the reaction catalysed by UDP-glucose pyrophosphorylase (glucose-1-P + UTP → UDP-D-glucose + PP). cDNAs coding for both enzymes are present in the cambium library (Table 4). Correlative evidence indicates that SuSy plays an important role in xylem cell wall formation. The gene is highly expressed in the cambial region as judged from the high frequency of the corresponding ESTs in the cambium library. In addition, the enzymatic activity of SuSy in the developing xylem is correlated with the intensity of xylem wall formation in poplar (Sauter, 2000) and *Robinia pseudoacacia* L. (Hauch and Magel, 1998). In developing xylem of Scots pine, the enzymatic activity of SuSy exhibits a minor peak in the cambium, whereas the highest activity is found in the zone of secondary wall formation (Uggla *et al.*, 2001). This pattern of activity supports a function for the membrane-associated isoforms of SuSy in providing UDP-D-glucose directly to cellulose synthase (Winter and Huber, 2000; Haigler *et al.*, 2001, this issue).

UDP-glucose dehydrogenase, which converts UDP-D-glucose to UDP-D-glucuronic acid, is a key enzyme that regulates the flux of UDP-glucose into

Table 4. Enzymes related to cell wall polysaccharide precursor biosynthesis corresponding to ESTs from the cambial region and developing xylem (Sterky *et al.*, 1998a, 1998b).

Enzyme	Library	Accession		
α,α-trehalose-phosphate synthase (UDP-forming) (UDP-glucose-glucosephosphate glucosyltransferase (EC 2.4.1.15)	Cambium	AI163996		
Diphosphate–fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.90)	Xylem	AI162647	AI163266	
dTDP-glucose 4-6-dehydratase (EC 4.2.1.46)	Cambium	AI161839	AI162550	AI162558
		AI162651	AI163474	AI163546
		AI163581	AI164673	AI165131
		AI165272	AI165288	AI166047
	Xylem	AI166408	AI166808	
Fructokinase (EC 2.7.1.4)	Cambium	AI165611	AI165646	AI166765
		AI162548		
	Xylem	AI162846		
GDP-mannose pyrophosphorylase (EC 2.7.7.13)	Cambium	AI161856	AI162882	AI163264
		AI165115	AI165373	AI166098
myo-inositol 2-dehydrogenase (EC 1.1.1.18)	Cambium	AI163525	AI164586	
myo-inositol-1(or 4)-monophosphatase (EC 3.1.3.25)	Cambium	AI162400	AI165680	
Phosphoglucomutase (EC 5.4.2.2)	Cambium	AI162727	AI165714	
		(AF097938)		
6-phosphogluconate dehydrogenase (EC 1.1.1.44)	Cambium	AI162532	AI165699	
Phosphomannomutase (EC 5.4.2.8)	Cambium	AI163799	AI162548	AI162846
		AI163832		
Putative sucrose phosphatase (EC 3.1.3.24)	Cambium	AI165881		
Putative UDP-galactose transporter	Cambium	AI165948	AI166124	AI166211
Reversibly glycosylated polypeptide-1	Cambium	AI161671	AI163874	
Sucrose synthase (EC 2.4.1.13)	Cambium	AI162073	AI162587	AI163208
		AI163479	AI163591	AI163699
		AI163955	AI164134	AI164295
		AI164415	AI164510	AI164858
		AI165555	AI165740	AI165766
		AI166066		
	Xylem	AI166709		
UDP-glucose 6-dehydrogenase (EC 1.1.1.22)	Cambium	AI164180	AI162135	AI163328
			(AAF04455)	
		AI166238	AI166122	
	Xylem	AI166760		
UDP-glucose pyrophosphorylase (EC 2.7.7.9)	Cambium	AI161980		

several other nucleotide-sugars that are substrates for cross-linking glycans and pectin precursors synthesized in the Golgi apparatus (Figure 7; Tenhaken and Thulke, 1996; Seitz *et al.*, 2000). The high abundance of UDP-glucose dehydrogenase transcripts in the cambial region tissues (Table 4) indicates that UDP-D-glucuronic acid may be largely formed directly from UDP-D-glucose. The sugar interconversion reactions also include dehydrogenation, decarboxylation and epimerization reactions that are discussed in detail by Reiter and Vanzin (2001, this issue). Transcripts corresponding to enzymes involved in some of these reactions have been found in the wood-forming tissues of hybrid aspen (Table 4), whereas enzymatic activities for UDP-D-galactose 4-epimerase, UDP-L-arabinose epimerase and UDP-D-glucuronate decarboxylase have been detected in the cambial region of *P.* × *euramericana* cv. Robusta (Dalessandro and

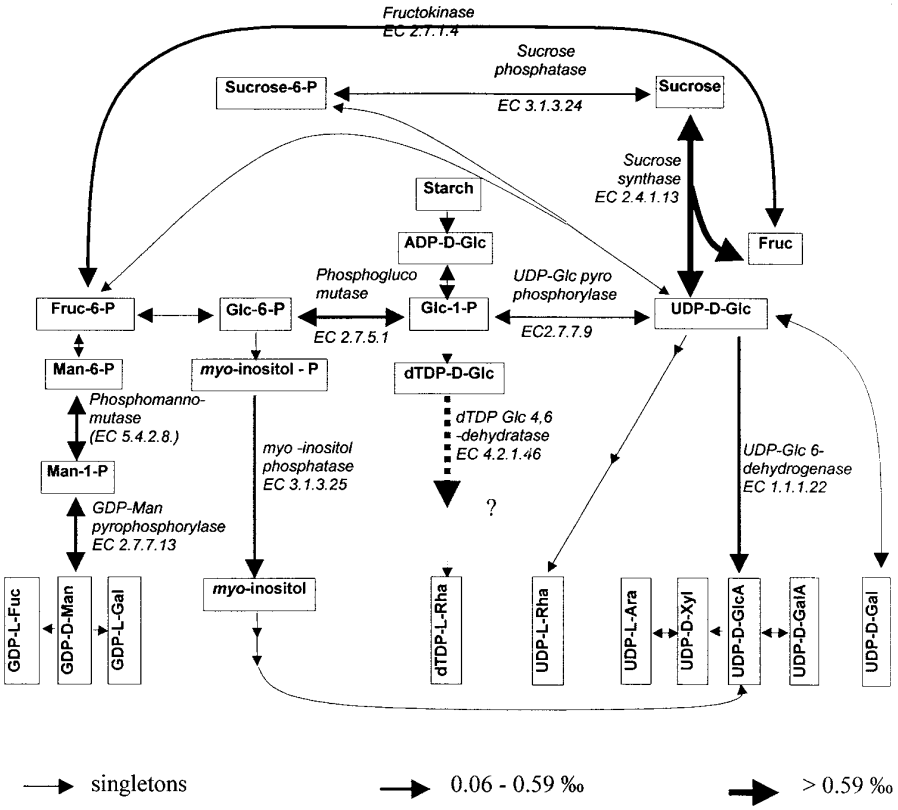

Figure 7. Biosynthesis of cell wall polysaccharide precursors showing enzymes for which corresponding ESTs have been found in the xylem and cambium EST libraries. The thickness of the arrows relates to the abundance of each EST. The dTDP-D-glucose pathway does not have a precedent in plants, and thus the idenity of dTDP Gluc 4,6-dehydratase should be considered as tentative.

Northcote, 1977). The composition of glucomannan, synthesized presumably by a single glucomannan synthase, is dependent on the ratio of GDP-D-glucose to GDP-D-mannose in pea (Piro *et al.*, 1993). These results suggest that it will be possible to genetically engineer wall carbohydrate polymers in trees by modifying sugar pools similarly as monolignol pools have been altered to affect lignin composition (see below).

The reversibly glycosylated polypeptide 1 has been proposed to be involved in sugar transport into the Golgi lumen and in biosynthesis of non-cellulosic polysaccharides (Dhugga *et al.*, 1997; Saxena and Brown, 1999). ESTs for the nucleotide sugar transporter-like protein and the reversibly glycosylated polypeptide 1 have been found in the cambium library (Table 4). Several Golgi membrane-bound glycosyltransferases have been identified and are thought to synthesize cell wall matrix components (see Perrin *et al.*, 2001, this issue). Some candidates for glycosyltransferases from poplar are listed in Table 4.

Another pathway that can be involved in sugar interconversion is the *myo*-inositol oxidation pathway, providing UDP-activated sugars from the *myo*-inositol (Figure 7; Loewus and Murthy, 2000). The presence in the cambium library of ESTs for *myo*-inositol-1-phosphatase and phosphoglucomutase, the enzyme that provides glucose-6-phosphate to *myo*-inositol, indicates that this pathway operates in the cambial region tissues.

Secretion: the role for RHOs

Pre-formed matrix carbohydrates and probably also lignin precursors are secreted to the wall via exocytosis. Because numerous vesicles are usually observed near to the wall, the process of fusion itself has been suggested to be a limiting step that regulates the rate of wall biosynthesis (Northcote, 1989). In pollen tubes,

a small GTPase, denoted RHO-related GTPase from Plants 1 (ROP1) and belonging to the RHO family of GTPases (Valster *et al.*, 2000), plays a regulatory role in vesicle exocytosis and pollen tube enlargement (Zheng and Yang, 2000). Antibodies raised against a related protein, RAC1, labelled the cell plate and Golgi compartments in tobacco BY2 cells (Couchy *et al.*, 1998). Interestingly, genes coding for similar proteins, called *RAC13* and *RAC9,* were specifically up-regulated in cotton fibres during the transition from primary to secondary wall secretion (Delmer *et al.*, 1995). Transient expression of a dominant negative *rac13* mutant or antisense suppression of *RAC13* in soybean and *Arabidopsis* cell cultures decreased the H_2O_2 secretion, whereas ectopic over-expression caused an increase in H_2O_2 secretion (Potikha *et al.*, 1999). The presence of H_2O_2, in turn, was correlated with secondary wall formation and cellulose biosynthesis in cotton. This raises the question whether RHO proteins play a role in cell wall formation in the secondary xylem. Transcripts of *RHO* genes are exceptionally abundant in the cambium EST library. Over 58 (1%) ESTs that encode small GTPases could be found and 25 of them had a BLAST similarity score of 200 or more to RAC13. Such a high similarity indicates that homologues of RAC13 are present in poplar and that they are particularly highly expressed in the cambial region. Analysis of the expression of these proteins will tell us whether they are candidates to be regulators of the primary to secondary wall transition in poplar.

Expanding walls in developing xylem

Cellulose microfibrils coated with xyloglucan and linked by xyloglucan bridges form the main load-bearing component of the primary wall in dicot plants (Hayashi, 1989; Carpita and Gibeaut, 1993; Nishitani, 1998; Whitney *et al.*, 1999; Cosgrove, 2000). Thus, the expansion of the wall depends heavily on the ability to cut the xyloglucan bridges and/or break the hydrogen bonding in the xyloglucan-cellulose interaction. Xyloglucan endotransglycosylases (XETs), endoglucanases and expansins are the most important proteins involved in these processes (McQueen-Mason, 1997; Cosgrove, 2000; Darley *et al.*, 2001, this issue). Only preliminary evidence exists for a role for these proteins in secondary xylem development.

Among the ESTs from differentiating xylem of pine, Allona *et al.* (1998) have found two *XET* genes that were differentially expressed in upright and bent trees. Three different cDNAs encoding XET have been found in the cambium library of hybrid aspen (Mellerowicz *et al.*, 2000), all belonging to the *XET* subfamily I (Nishitani, 1997). Immunolocalization with antibodies against one of these XET proteins indicated that this particular XET was present in cells engaged in wall biosynthesis of secondary xylem and phloem, but it was not detected in xylem RE zone (V. Bourguin, and E. Mellerowicz, unpublished). These data suggest that the poplar XET is involved in the incorporation of newly synthesized xyloglucan into the existing cross-linking glycan-cellulose network and possibly in wall restructuring in long-lived xylem cells. It is still unknown which *XET* genes play a role in radial expansion of cambial derivatives.

The hydrolysis of xyloglucan by xyloglucan-specific glucanases also might contribute to cell expansion (Matsumoto *et al.*, 1997). Endoglucanases are enzymes that catalyse the endo-hydrolysis of (1→4)-β-D-glucan. They can also be involved in the degradation of non-crystalline cellulose (Ohmiya *et al.*, 2000) or in the biosynthesis of cellulose during growth (del Campillo, 1999). ESTs encoding two different endoglucanases have been found in the cambium library of hybrid aspen, all belonging to the glycosyl hydrolase family 9 (Mellerowicz *et al.*, 2000). Both ESTs were singletons and preliminary sequence comparisons suggest that one corresponds to a soluble, and the other to a membrane-bound enzyme. The role of endoglucanases in wood formation is hypothetical. They may be involved in the regulation of fibre length by acting on cell elongation during primary growth and thus affecting the length of the procambial cells. Longer procambial cells would give rise to longer FCCs and, hence, longer fibres and vessel elements in the secondary xylem. This is supported by the analysis of transgenic *P. tremula* plants over-expressing the *Arabidopsis* endoglucanase *CEL1* gene under the control of the CaMV 35S promoter, which have significantly longer internodes as well as longer fibers (Shani *et al.*, 1999).

Expansins are cell-wall-bound proteins that mediate acid growth and are considered the main agent regulating cell wall rheological properties (McQueen-Mason, 1997; Cosgrove, 1997, 2000). They are proposed to intercalate between the cellulose microfibrils and the xyloglucan polymers in the primary cell wall and thus to disrupt the hydrogen bonding between them. However, an enzymatic activity of expansins has not yet been documented. Interest in the role of expansins in xylem formation has been stimulated by

the discovery that transcripts of three xylem-specific expansins were localized either to the apical or to the basal tip of axial xylem parenchyma cells in the primary xylem of *Zinnia* (Im *et al.*, 2000). The meaning of this peculiar localization needs to be further investigated. ESTs for two expansin genes have been identified in the hybrid aspen cambium library, both corresponding to α-expansins (Mellerowicz *et al.*, 2000). One of these ESTs is abundantly present in the library and is specifically expressed in the xylem mother cells and expanding xylem cells, whereas the other is expressed at a low level in the cambium closer to the phloem (M. Grey-Mitsumune and E.J. Mellerowicz, unpublished). Thus, these expansins may be involved in the radial expansion and/or tip growth of FCCs and developing xylem fibre cells.

The stiffening of the wall during cell differentiation may be related to the cross-linking of extensin (and other hydroxyproline-rich proteins) with the cellulose-xyloglucan-pectin network, which locks the microfibrils in place (Carpita and Gibeaut, 1993; Brett and Waldron, 1996). In addition, the incorporation of Ca^{2+} into pectins causes their gelation and makes the wall unstretchable. Parallel neighbouring chains of demethylated (acidic) pectins can ionically bind Ca^{2+} to form an 'egg-box' structure that stiffens the wall. Acidic pectins, on the other hand, while not engaged in Ca^{2+} bonding, lower the pH of the cell wall and therefore may stimulate the activity of wall-bound hydrolases and expansins that are important for wall plasticity (McQueen-Mason, 1997). Thus, the status of pectin methylation, regulated by PME, is an important factor in controlling wall plasticity. Pectin methylation and the composition of the side-chains are developmentally regulated in the cambial region of *Populus*. Labelling with the monoclonal antibodies JIM5 and JIM7, which distinguish homogalacturans with low and high levels of methyl esterification, respectively (Knox *et al.*, 1990), indicates that radial walls of FCC and expanding xylem cells are rich in pectins of both kinds and that the degree of methylation decreases during xylem development (Guglielmino *et al.*, 1997a). This decrease in pectin methylation is accompanied by a shift in the localization of PME from the Golgi (in the cambium) to the cell wall (in the RE zone), as judged from immunolabeling with polyclonal antibodies against flax PME (Guglielmino *et al.*, 1997b). This observation led the authors to propose that the cambium can synthesize but not secrete PME. Micheli *et al.* (2000a) found wall-bound and soluble PME activities at pH 6.1 in the cambium and a soluble PME activity in the RE zone. Ten PME isoforms have been detected in the cambial region of hybrid aspen by isoelectric focusing and activity assays at pH 7 (Micheli *et al.*, 2000a, b). The expression of several isoforms seemed to be developmentally regulated. Five putative *PME* cDNAs have been cloned from the hybrid aspen cambium library (Micheli *et al.*, 2000b). Their deduced molecular mass was 34 kDa and the deduced pI was between 9 and 10. It will be important to find out which gene corresponds to which isoform and to establish whether the different isoforms are transcriptionally or post-transcriptionally regulated and differentially secreted to the cell wall.

Monoclonal antibodies have been made that recognize specific side-chains of rhamnogalacturonan I, namely LM5 and LM6 that recognize (1→4)-L- or D-galactosyl residues and 5 to 6 units of (1→5)α-L-arabinan, respectively (Jones *et al.*, 1997; Willats *et al.*, 1998, 1999, and 2001, this issue). In hybrid aspen, the arabinan epitope was found in phloem cells, particularly in the sieve tubes, in the cambium and in the RE zone, whereas the galactan epitope was restricted to the cambium and the RE zone (Ermel *et al.*, 2000). Whereas the antibodies clearly differentiate between cell populations with different destinies, i.e. differentiating to either xylem or phloem tissues, they do not differentially mark meristematic and expanding cells as was found for carrot suspension cultures (Willats *et al.*, 1999).

Pectins determine porosity and thus appoplastic exclusion limits of primary walls. Therefore, a degradation of the pectin network may make walls more accessible to other modifying enzymes (Carpita and Gibeaut, 1993). Pectin metabolism appears to play an important role in xylem cell development. Pectate lyase hydrolyses demethylated pectin (pectate). In *Zinnia*, a pectate lyase (*ZePel*) mRNA was found to be associated with vascular tissue by *in situ* hybridization technique and the gene was up-regulated in the xylogenic cell culture at the early stage of induction, before the formation of secondary cell walls (Domingo *et al.*, 1998). Poplar EST clones similar to *ZePel* are very abundant in the cambium and xylem EST libraries (Sterky *et al.*, 1998a). Another enzyme involved in pectin degradation is endopolygalacturonase (pectinase). It is encoded by a multigene family of at least 19 members in *Arabidopsis* and is ubiquitously expressed (Torki *et al.*, 2000). Four ESTs corresponding to pectinase have been found in the xylem and cambium libraries.

Cellulose biosynthesis

Cellulose is synthesized by plasma membrane-bound enzyme complexes that can be seen as rosettes in freeze fracture preparations of plant cells (Mueller and Brown, 1980; Brett, 2000). Rosettes are also found in developing primary and secondary xylem (Haigler and Brown, 1986; Fujino and Itoh, 1998). Cellulose synthase has been recently immunolocalized to the freeze-fractured rosette complex of *Vigna angularis* (Kimura *et al.*, 1999). At least 10 cellulose synthase genes are present in *Arabidopsis*, all of them carrying the type A catalytic domain and therefore called *CesA* genes (Holland *et al.*, 2000; Richmond and Somerville, 2001, this issue). Some of them, such as *RSW*1 and *PRC*1, are involved in primary wall biosynthesis (Arioli *et al.*, 1998; Fagard *et al.*, 2000), whereas others are active in secondary wall biosynthesis (*IRX3* and *IRX1*) (Taylor *et al.*, 1999; Turner *et al.*, 2001, this issue). Recently, the *CesA* genes have been cloned from the wood-forming tissues of tree species, including *Populus* (Sterky *et al.*, 1998a; Wu *et al.*, 2000) and pine (Allona *et al.*, 1998).

Two *Populus* genes, *PtCesA*1 and *PtCesA*2 (also called *PtCesA*) from *P. alba* (L.) × *P. tremula* and *P. tremuloides*, respectively, are similar to the cotton *CesA* genes involved in secondary wall formation (Holland *et al.*, 2000; Wu *et al.*, 2000). *In situ* hybridization showed that *PtCesA2* is expressed specifically during secondary wall biosynthesis in developing protoxylem, metaxylem, and secondary xylem, but that the expression is not detectable in the phloem fibers that also develop a secondary cell wall (Wu *et al.*, 2000). Thus, the *CesA* genes have cell-specific expression patterns. Curiously, the *PtCesA2* promoter drives GUS expression in tobacco in developing primary and secondary xylem cells in upright-growing stems, but upon bending of the stem, the expression is induced in the phloem fibres of the convex (upper) and down-regulated in the xylem of the concave (lower) side of the stem. This indicates that the *PtCesA2* promoter responds to mechanical and/or gravitational stimuli. It would be of interest to investigate whether this gene is involved in tension wood formation in gravistimulated stems of trees. Four different cDNAs encoding CesA have been found in the cambium and xylem libraries (Mellerowicz *et al.*, 2000). One of these ESTs was present in many copies and probably represents the major cellulose synthase involved in xylem cell wall formation.

Other carbohydrates present in the secondary wall

Pectin biosynthetic enzymes are down-regulated during secondary wall formation in the xylem (Bolwell and Northcote, 1981; Bolwell *et al.*, 1985; Fukuda, 1992; Gregory *et al.*, 1998), but pectins are still present in the lignified walls of mature xylem cells (Imai and Terashima, 1992). The strong reaction with the JIM7 antibody indicates the prominent presence of methylated pectin in the middle lamellae and primary cell wall of Scots pine tracheids and ray cells, as well as in the membranes of bordered pits (Hafren *et al.*, 2000). Reaction with the JIM5 antibody was weaker but showed a similar pattern, indicating the presence of acidic pectins in the same areas.

The occurrence of xyloglucan in the secondary wall has not been well established. In cotton fibres, the amount of 24% KOH-extractable xyloglucan was positively correlated with cell elongation and decreased to 20% of its maximum when the secondary wall was formed (Shimizu *et al.*, 1997). Surprisingly, *XET* mRNA has been detected at all stages of fibre development in cotton, including the stage of secondary wall formation. The monoclonal antibody CCRC-M1 that recognizes fucosyl side-chains that are typically present in the xyloglucan (Puhlmann *et al.*, 1994), has been used to immunolocalize xyloglucan in *Zinnia* TEs (Stacey *et al.*, 1995). Xyloglucan was found in the cell wall layer corresponding to the primary wall, but not in secondary wall thickening. However, large amounts of xyloglucan were secreted into the culture medium and were present in the protoplasts of tracheary elements undergoing secondary wall deposition.

Xylan synthesis seems to be specifically up-regulated during secondary wall formation. A membrane-bound UDP-D-xylose-xylan transferase activity was shown to increase in *Zinnia* cultures and differentiating secondary xylem of several dicot species during secondary cell wall formation (Suzuki *et al.*, 1991; Bolwell and Northcote, 1981, 1983; Gregory *et al.*, 1998). Correspondingly, xylan deposition increases in the secondary walls of tracheary elements when compared to the primary walls (Ingold *et al.*, 1988; Northcote *et al.*, 1989). In *Zinnia*, xylan deposition depends on the undisturbed synthesis of cellulose (Taylor and Haigler, 1993). This observation, together with the spontaneous binding of xylan to cellulose microfibrils (Reis *et al.*, 1994), indicate that xylan coats cellulose microfibrils in secondary walls, similarly to the xyloglucan coating of microfibrils in primary

walls. By using immuno-field emission scanning electron microscopy, Awano *et al.* (2000) have detected an association of xylan with the thick cellulose microfibrils in the S1 and S2 layers, but not with the thin (5 nm) microfibrils present in the S1 layer of fibres of beech wood. Immuno- and enzymatic labelling experiments have demonstrated that xylan is not uniformly distributed within the wall in wood cells of dicot plants (Vian *et al.*, 1992; Awano *et al.*, 1998, 2000). Labelling was particularly strong in the outer S2 layer and it appeared to increase in the S1 layer during S2 layer deposition (Awano *et al.*, 1998, 2000). It was suggested that the newly synthesized xylan might migrate to the deeper wall layers and associate there with uncoated cellulose microfibrils.

Xylanases are (1→4)β-D-xylan endohydrolases that are able to attack intact polymers of xylan. A xylanase cDNA has been found in the cambium library of hybrid aspen (Sterky *et al.*, 1998a). The corresponding protein belongs to the glucan hydrolase family 10 (Mellerowicz *et al.*, 2000), and is similar to that found in *Arabidopsis,* in which the xylanase-catalytic domain is preceded by an additional domain of unknown function. However, the function of this protein in *Arabidopsis* is still unknown. The plant xylanases studied so far appear to be involved in wall degradation in connection with seed and pollen germination, aerenchyma formation, or fruit ripening (Bragina *et al.*, 1999; Benjavongkulchai and Spencer, 1986; Bih *et al.*, 1999). Wall turnover (Gorshkova *et al.*, 1997) or restructuring may also require a xylanase activity and the hybrid aspen xylanase might be involved in any of these activities.

Lignification of xylem cell walls in poplar

Lignin is a heterogeneous phenolic polymer that is mainly present in the secondary thickened cell wall. It is essential for mechanical support and plays a role in defence towards pathogens. Because of its hydrophobic nature, it provides impermeability to tracheary elements and therefore allows the transport of water and solutes through the vascular system. Numerous efforts have been made over the past decade to understand the lignification process because of its economical relevance; lignin is considered as a negative factor in paper-making and limits the digestibility of fodder crops.

Lignin is mainly derived from the dehydrogenative polymerization of three different hydroxycinnamyl alcohols (or monolignols), *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Figure 8). These monolignols give rise to the H, G, and S units of the lignin polymer, respectively, and differ from each other only by their degree of methoxylation. The content and composition of lignin are known to vary among taxa, tissues, cell types, and cell wall layers and to depend on the developmental stage of the plant and the environmental conditions (Côté, 1977; Campbell and Sederoff, 1996; He and Terashima, 1991; Joseleau and Ruel, 1997; Monties, 1998). The overall lignin heterogeneity is thought to result from

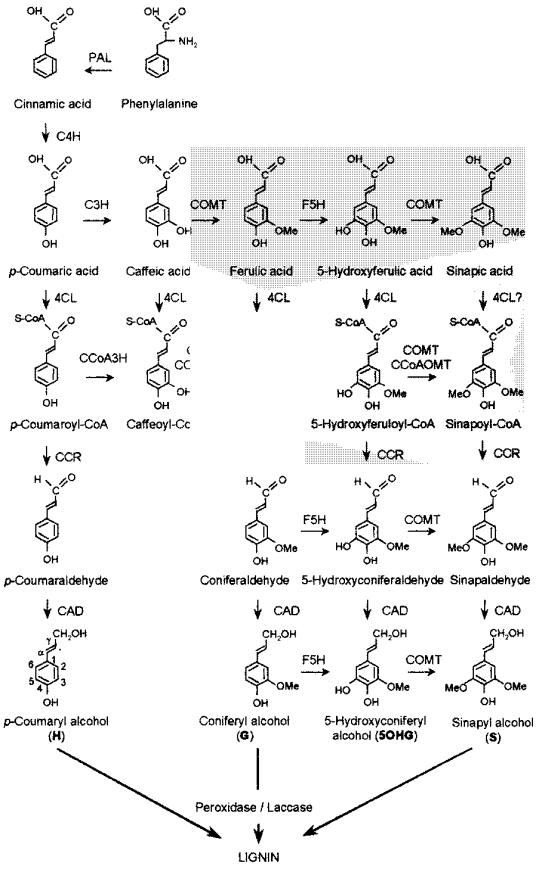

Figure 8. Monolignol biosynthesis pathways. The enzymatic steps shown in the grey box probably do not play a major role *in vivo*. CAD, cinnamyl alcohol dehydrogenase; C3H, coumarate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; CCoA3H, coumaroyl-coenzyme A 3-hydroxylase; CCoAOMT, caffeoyl-coenzyme A *O*-methyltransferase; CCR, cinnamoyl-coenzyme A reductase; COMT, caffeate/5-hydroxyconiferaldehyde *O*-methyltransferase; F5H, ferulic acid/coniferaldehyde 5-hydroxylase; 4CL, 4-coumarate:coenzyme A ligase; PAL, phenylalanine ammonia-lyase.

the spatio-temporal and conditional regulation of the genes involved in the lignin biosynthesis pathway (Campbell and Sederoff, 1996; Chen *et al.*, 2000).

The biosynthesis of the lignin precursors proceeds through the common phenylpropanoid pathway, starting with the deamination of phenylalanine to cinnamic acid. Further enzymatic reactions include hydroxylations of the aromatic ring, the methylation of selected phenolic hydroxyl groups, the activation of cinnamic acids to cinnamoyl-CoA esters, and the reduction of these esters to cinnamaldehydes and further to cinnamyl alcohols (Figure 8). Despite the increasing knowledge on the biochemical properties of the enzymes involved in monolignol biosynthesis, the precise order in which these reactions occur is not yet fully understood and many uncertainties remain concerning the *in vivo* role of the respective enzymes in the monolignol biosynthesis pathway. Also little is known on the storage form, the intracellular transport through the cell wall as well as the mechanisms of polymerization of lignin precursors. Recently, it has been demonstrated by *in vitro* enzymatic assays that the hydroxylation and methylation reactions occur preferentially at the cinnamaldehyde and the cinnamyl alcohol level (Figure 8) (Chen *et al.*, 1999; Humphreys *et al.*, 1999; Osakabe *et al.*, 1999; Li *et al.*, 2000; Matsui *et al.*, 2000).

Genes and cDNAs for most of the known enzymes of the monolignol biosynthesis pathway have been cloned (reviewed by Baucher *et al.*, 1998; Christensen *et al.*, 2000) and ESTs for all of these enzymes are present in the xylem and cambium libraries of hybrid aspen (Sterky *et al.*, 1998a). As reviewed below, it is now possible to affect lignin content and composition by genetic modification of the expression of these genes (Table 5). The results obtained have made it possible to redraw the lignin biosynthesis pathway that had been described for many years in biochemistry textbooks (for recent reviews see Baucher *et al.*, 1998; Whetten *et al.*, 1998; Grima-Pettenati and Goffner, 1999; Boudet, 2000). Furthermore, it has been demonstrated that the lignin polymer can be built with different types of phenylpropanoid units (Sederoff *et al.*, 1999; Jouanin *et al.*, 2000; Kim *et al.*, 2000; Ralph *et al.*, 2001). Consequently, for the same overall amount of lignin, different chemical properties of the polymer can be obtained.

Genetic modification of lignin biosynthesis in poplar

Down-regulation of phenylalanine ammonia-lyase (PAL) or cinnamate 4-hydroxylase (C4H) in transgenic tobacco has been shown to dramatically reduce lignin content (Sewalt *et al.*, 1997), but because these enzymes are involved in early steps of the phenylpropanoid pathway, pleiotropic effects could be expected. Indeed, alterations in development were observed; the PAL-down-regulated plants were stunted and had curled leaves. The walls of secondary xylem cells were thinner and contained markedly less lignin than those of wild-type, as monitored by either histochemical staining of cross-sections with toluidine blue or by UV-fluorescence (Elkind *et al.*, 1990; Bate *et al.*, 1994). Interestingly, PAL activity is decreased in plants down-regulated for C4H, indicating a feedback regulation by the product of PAL, cinnamic acid (Blount *et al.*, 2000).

Transgenic poplars with a 95% (Van Doorsselaere *et al.*, 1995), a 78% (Tsai *et al.*, 1998), and a more than 97% (Jouanin *et al.*, 2000) reduction in caffeate *O*-methyltransferase (COMT/AldOMT) activity in the developing xylem have been generated. This enzyme has recently been demonstrated to use preferentially 5-hydroxyconiferaldehyde as substrate *in vitro* (Figure 8; Li *et al.*, 2000). A reduction in Klason lignin content (by 17%) has been detected only in the transgenic lines described by Jouanin *et al.* (2000), showing that COMT activity has to be decreased strongly to affect lignin content. Consistent with the substrate specificity of COMT described by Li *et al.* (2000), the lignin composition of the COMT-down-regulated poplars is characterized by a drastic decrease in the amount of S units and by the incorporation of an unusual lignin monomer, 5-hydroxyguaiacyl (5OHG), as demonstrated by thioacidolysis and nuclear magnetic resonance (NMR) experiments (Van Doorsselaere *et al.*, 1995; Lapierre *et al.*, 1999; Jouanin *et al.*, 2000; Ralph *et al.*, 2001). Modifications in the frequency of the linkages between monomers (lower frequency of β-*O*-4 ether linkages and a higher proportion of biphenyl (5-5) and phenylcoumaran (β-5) carbon-carbon linkages) indicated an increase in the degree of condensation of the lignin in the transgenic plants (Lapierre *et al.*, 1999). Moreover, a novel dimer corresponding to an α-β-diether structure involving a G unit and a 5-OHG unit has been identified by thioacidolysis (Jouanin *et al.*, 2000). Furthermore, a lower amount of free phenolic groups in β-*O*-4-linked S and G units has been detected

Table 5. Poplar lignin mutants obtained by genetic engineering.

Gene	Residual enzyme activity, %	Lignin content	Lignin composition	References
P. tremula × *P. alba*				
COMT	5	no changes	S/G decreased G increased, 5OHG	Van Doorsselaere *et al.* (1995); Lapierre *et al.* (1999)
	<3	reduced	S/G decreased 5OHG	Jouanin *et al.* (2000)
CAD2	30	slight decrease	more aldehydes	Baucher *et al.* (1996); Lapierre *et al.* (1999)
CCR	–	reduced	S/G increased	J.-C. Leplé, C. Lapierre and W. Boerjan, unpublished
CcoAOMT	10% protein amount	reduced	S/G increased	Meyermans *et al.* (2000)
	30	reduced	–	Zhong *et al.* (2000)
LAC	–	no changes	no changes	Ranocha *et al.* (2000)
F5H	–	–	S/G increased	Franke *et al.* (2000)
POX	800	no changes	no changes	J.H. Christensen, C. Lapierre, and W. Boerjan, unpublished
P. tremuloides				
COMT	28	no changes	S/G decreased, 5OHG more coniferaldehyde	Tsai *et al.* (1998)
4CL	10	reduced	no changes	Hu *et al.* (1999)

S/G, syringyl/guaiacyl; POX, peroxidase; LAC, laccase; other abbreviations as in Figure 8; – , not determined.

in the lignin of COMT-down-regulated poplars, resulting in a lower chemical reactivity of the lignin (Lapierre *et al.*, 1999; Jouanin *et al.*, 2000). In accordance with these structural changes in lignin, the wood of the transgenic trees is more resistant to Kraft delignification (Lapierre *et al.*, 1999; Jouanin *et al.*, 2000).

Caffeoyl-CoA *O*-methyltransferase (CCoAOMT) preferentially catalyses the methylation of caffeoyl-CoA over 5-hydroxyferuloyl-CoA *in vitro* (Inoue *et al.*, 1998; Martz *et al.*, 1998; Meng and Campbell, 1998; Grimmig *et al.*, 1999; Li *et al.*, 1999; Maury *et al.*, 1999). Transgenic poplars with 10% residual CCoAOMT protein amount have a 12% reduced Klason lignin content and an increased incorporation of *p*-hydroxybenzoic acid into the lignin, as shown by NMR (Meyermans *et al.*, 2000). Following a similar approach, Zhong *et al.* (2000) obtained transgenic poplars with a 40% reduction in Klason lignin content. Both in transgenic tobacco (Zhong *et al.*, 1998) and poplar (Meyermans *et al.*, 2000; Zhong *et al.*, 2000) down-regulated for CCoAOMT, the decreased lignin content was due to a decrease in both S and G lignin units, confirming the role of CCoAOMT in the biosynthesis of both S and G lignin. Also, the lignin was characterized by a higher S/G ratio. By diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS), the lignin of CCoAOMT down-regulated poplars was shown to be less cross-linked (Zhong *et al.*, 2000). This decrease in lignin content did not affect the morphology and the growth of the transgenic poplars. Furthermore, down-regulation of CCoAOMT causes an accumulation of methanol-extractable O^3-β-D-glucosyl-caffeic acid (GCA), O^4-β-D-glucosyl-vanillic acid (GVA), and O^4-β-D-glucosyl-sinapic acid (GSA) (Meyermans *et al.*, 2000). Feeding experiments have shown that GCA and GSA are storage or detoxification products of caffeic acid and sinapic acid, respectively. These results provide *in vivo* evidence that the pathway for the methylation/hydroxylation reactions of the lignin precursors does not occur at the cinnamic acid level and that sinapic acid is not a precursor for S lignin.

In vitro studies have revealed that ferulic acid 5-hydroxylase (F5H/CAld5H) catalyses preferentially the hydroxylation of coniferaldehyde over ferulic acid (Humphreys *et al.*, 1999; Osakabe *et al.*, 1999). This enzyme has been shown to be a major regulatory step in the determination of lignin monomer composition. The lignin of *Arabidopsis f5h* mutants is devoid of S units, whereas transgenic *Arabidopsis* overexpressing *F5H* have a lignin almost entirely built of S units (Chapple *et al.*, 1992; Meyer *et al.*, 1998; Jung *et al.*, 1999; Marita *et al.*, 1999). Similarly, transgenic poplars over-expressing the *Arabidopsis F5H* under the control of the cinnamate 4-hydroxylase (*C4H*) promoter have a lignin enriched in S units (Franke *et al.*, 2000).

4-coumarate:CoA ligase (4CL) catalyses the formation of CoA thioesters of hydroxycinnamic acids. No activity towards sinapic acid has been found (Allina *et al.*, 1998; Hu *et al.*, 1998), indicating, in addition to the results of Humphreys *et al.* (1999), Osakabe *et al.* (1999), Matsui *et al.* (2000) and Meyermans *et al.* (2000), that sinapic acid is probably not an important precursor for sinapyl alcohol. A more than 90% reduction in 4CL activity has been obtained in transgenic poplar by the antisense strategy (Hu *et al.*, 1999). This modification has led to a 45% reduction in lignin amount in the xylem. On the other hand, a 15% increase in cellulose was detected. The monomeric composition of lignin as determined by thioacidolysis, as well as the frequency of the main dimeric linkages in lignin (β-O-4, β-5, β-β, and α-keto-β-aryl ethers) as determined by NMR, were similar to those of control plants. An increase in non-lignin cell wall constituents, such as *p*-coumaric, ferulic, and sinapic acid, was evidenced by gas chromatography-mass spectrometry analysis (Hu *et al.*, 1999). In agreement with the data of Zhong *et al.* (2000), these results show that it is possible to largely reduce lignin amount in trees without affecting overall plant viability.

Cinnamoyl-CoA reductase (CCR) catalyses the reduction of the hydroxycinnamoyl-CoA esters to their corresponding aldehydes. Transgenic tobacco down-regulated for CCR have a decreased lignin content, an increased S/G ratio as well as an orange-brown coloration of the xylem (Piquemal *et al.*, 1998). Several chimeric *CCR* constructs under the control of the double *CaMV 35S* promoter have been used to transform poplar. Some transgenic lines showed an orange coloration, a decreased lignin content, and a higher S/G ratio in the lignin, similar to the results obtained in transgenic tobacco plants down-regulated for CCR (J.-C. Leplé, C. Lapierre and W. Boerjan, unpublished).

Cinnamyl alcohol dehydrogenase (CAD) catalyses the last step in the monolignol biosynthesis pathway, which is the reduction of cinnamaldehydes to cinnamyl alcohols. A reduction of CAD activity has been described in tobacco (Halpin *et al.*, 1994; Hibino *et al.*, 1995, Yahiaoui *et al.*, 1998), alfalfa (Baucher *et al.*, 1999), poplar (Baucher *et al.*, 1996) and in a pine mutant (MacKay *et al.*, 1997) and has been associated with the presence of an increased amount of aldehydes in the lignin. The incorporation of cinnamaldehydes in the lignin of tobacco lines down-regulated for CAD has been demonstrated by Kim *et al.* (2000). In poplar, a 10% lower Klason lignin content has been detected in the wood of the transgenic lines (Lapierre *et al.*, 1999). In addition, the lignin has more free phenolic groups in G and S units, an important parameter that contributes to the solubility of lignin (Lapierre *et al.*, 1999). The wood of down-regulated CAD poplars is more easily delignified than control wood, as shown by the lower kappa number after chemical Kraft pulping (Baucher *et al.*, 1996; Lapierre *et al.*, 1999).

Peroxidases are widely believed to be responsible for the final condensation of cinnamyl alcohols to form lignin. However, the high redundancy in genes (more than 60 peroxidase genes have been identified from the *Arabidopsis* genome sequence; Tognolli *et al.*, 2000) and in functions (low substrate specificity) are the main obstacles to identify a peroxidase isoform that is specifically involved in lignification (reviewed by Christensen *et al.*, 2000). Two anionic peroxidase isoenzymes of poplar, shown to be preferentially expressed in developing xylem and to oxidize syringaldazine (a lignin monomer analogue; Goldberg *et al.*, 1983) have been purified and characterized (Christensen *et al.*, 1998). A cDNA corresponding to one of the syringaldazine-oxidizing peroxidases (PXP 3-4) has been cloned. Transgenic poplars that over produce this peroxidase under the control of the *CaMV 35S* promoter have up to 800-fold higher peroxidase activities in the developing xylem. However, no differences in lignin content or S/G composition were detected in this tissue (J.H. Christensen, C. Lapierre and W. Boerjan, unpublished). In contrast, tobacco lines with 10-fold higher peroxidase activities are characterized by a higher lignin content in the leaves, but also by a reduced growth and a browning of wounded tissues (Lagrimini *et al.*, 1990; Lagrimini, 1991).

Other enzymes believed to polymerize monolignols are the laccases, but the precise role played by these enzymes is unclear. Transgenic yellow poplar (Dean *et al.*, 1998) and poplar (Ranocha *et al.*, 2000) down-regulated for laccase did not have alterations in their lignin content. However, an increase in soluble phenolics has been detected in poplar, suggesting a possible role for laccase in the oxidation of simple phenolics leading to cross-linking in the cell wall (Ranocha *et al.*, 2000).

The altered expression of genes coding for MYB-related (Tamagnone *et al.*, 1998) and LIM-related (Kawaoka *et al.*, 2000) transcription factors has been shown to affect the expression of genes involved in the phenylpropanoid biosynthesis pathway and to affect lignin content and the accumulation of phenolics in transgenic tobacco plants. The *Arabidopsis* mutant *ectopic lignification in the pith (elpi)* shows ectopic deposition of lignin in the pith (Zhong *et al.*, 2000). ELPI is thought to act as a repressor of lignin biosynthesis in the pith because the expression of *PAL*, *CCoAOMT* and *CCR* are induced in the *elpi* mutant.

Temporal and spatial expression of lignin biosynthesis genes

The temporal and spatial expression of a number of genes from the lignin biosynthesis pathway has been studied. A strong activity of the *PAL* promoter has been detected in the developing xylem, in the endodermis as well as in flower tissues of tobacco (Kawamata *et al.*, 1997). *In situ* localization and protein gel blot analysis have shown that *PAL* and *C4H* are expressed in the vascular bundles, the epidermis and the ovules and that the expression of both genes is closely co-regulated in parsley (Koopmann *et al.*, 1999). The *C4H* promoter confers expression in all tissues in *Arabidopsis*, but predominantly in the vascular bundles (Bell-Lelong *et al.*, 1997). Genes coding for two isoforms of 4CL, isolated from poplar, are differentially expressed, one being specifically expressed in developing xylem and probably involved in lignin biosynthesis whereas the other, in the epidermis, is most probably associated with the biosynthesis of non-lignin phenylpropanoids (Hu *et al.*, 1998). Promoter-GUS and *in situ* hybridization studies in poplar have enabled the localization of *CAD* gene expression in the vascular tissues, the periderm and the cambium (Feuillet *et al.*, 1995; Hawkins *et al.*, 1997; Regan *et al.*, 1999). Immunolocalization studies have shown that COMT is strictly expressed in lignifying cells of the phloem and the xylem. In the developing xylem, COMT is expressed in all cell types (C. Chen and W. Boerjan, unpublished).

Zhong *et al.* (2000) have immunolocalized CCoAOMT in all cell types of the developing xylem. In contrast, Chen *et al.* (2000) have shown by promoter-*GUS* analyses and immunolocalization studies that in poplar xylem, CCoAOMT is preferentially expressed in developing vessels and in contact ray cells, whereas it is barely detectable in isolation ray cells and developing xylem fibres (Chen *et al.*, 2000). As a consequence, down-regulation of CCoAOMT in transgenic poplar is expected to affect lignin biosynthesis in a cell-specific manner. This cell-specific effect could indeed be visualized by UV microscopy that showed an increased fluorescence specifically in the vessel cell walls (Meyermans *et al.*, 2000). Upon bending of the stem, however, the cell-specific expression is lost, and CCoAOMT becomes expressed in all cell types of developing xylem, i.e. fibres, vessel elements, contact and isolation rays cells (Chen *et al.*, 2000).

Subcellular localization of enzymes involved in lignin biosynthesis

Little is known about the mechanisms by which lignifying cells regulate the flux of phenylpropanoid metabolites into the different end products. Metabolic labelling experiments have suggested the channelling of lignin precursors by multi-enzyme complexes (Stafford, 1981; Hrazdina, 1992; Rasmussen and Dixon, 1999). PAL has been localized to the cytoplasm, to Golgi-derived vesicles and to secondary wall thickenings (Smith *et al.*, 1994; Nakashima *et al.*, 1997) and C4H has been shown to be associated with the endoplasmic reticulum membrane and Golgi vesicles (Smith *et al.*, 1994). CAD has been localised in the cytoplasm, on Golgi-derived vesicles, and on secondary wall thickenings (Nakashima *et al.*, 1997; Samaj *et al.*, 1998). CCoAOMT and COMT have been localized in the cytoplasm, not associated with any membranes (Ye, 1997; Chen *et al.*, 2000; C. Chen and W. Boerjan, unpublished).

New tools to study cell wall formation in wood

The vascular cambium is an inaccessible tissue and therefore not easily studied. Large organisms, such

as trees, however, have the advantage that significant amounts of developing xylem can be obtained for molecular and chemical characterization. In addition, samples from defined developmental stages of xylogenesis can be obtained by tangential cryosectioning. Consecutive sectioning, in combination with microanalytical techniques, has been used to visualize patterns of gene expression, enzyme activities, amino acids, carbohydrates and plant hormones across developing wood tissues (Uggla and Sundberg, 2001). The exciting perspective is now to combine microanalysis with the emerging high-throughput techniques for large-scale analysis of gene and protein expression in wood forming tissues.

Large-scale EST sequencing of hybrid aspen has provided the necessary tools for global expression analysis of genes during wood formation. Within the Swedish consortium (http://www.biochem.kth.se/PopulusDB), a poplar microarray containing some 3000 unique cambial region ESTs has been produced. To take full advantage of this technology, a 3′ cDNA target amplification protocol has been established and validated that enables transcript profiling from submilligram amounts of plant tissue (Hertzberg *et al.*, 2001). This technique has now been used in combination with tangential cryosectioning to visualize global gene expression patterns during xylogenesis from cambium to maturing xylem. The next generation of poplar microarrays currently under production will consist of about 10 000 unique EST sequences selected from a range of poplar cDNA libraries.

However, the expression of genes is not necessarily proportionally related to the abundance of the corresponding proteins. Therefore, the integration of both mRNA and protein data will give a more comprehensive view on xylogenesis. The identification and quantification of the proteins present in a particular tissue or in a particular developmental or environmental condition is possible through the developments in mass spectrometry combined with two-dimensional protein gel electrophoresis. Matrix-assisted laser desorption-ionization/time-of-flight (MALDI-TOF) mass spectrometry and tandem mass spectrometry are powerful high-throughput methods for protein identification, provided the DNA or protein sequences already exist in databases (Chalmers and Gaskell, 2000). In addition, the recent development of protein arrays will definitively allow to link genomics with proteomics (Ge, 2000). In this system, fully active proteins are spotted onto membranes that can be used for several purposes, such as the study of interactions with nucleic acids, proteins, and other ligands.

The identification of proteins that are highly abundant in developing xylem tissue, through two-dimensional protein gel electrophoresis, has been initiated in poplar (Vander Mijnsbrugge *et al.*, 2000) and pine (Plomion *et al.*, 2000). The most abundant soluble protein on two-dimensional protein gels of poplar xylem has been shown to have phenylcoumaran benzylic ether reductase (PCBER) activity *in vitro* (Gang *et al.*, 1999). PCBER reduces dehydrodiconiferyl alcohol (DDC) to isodihydrodehydrodiconiferyl alcohol (IDDDC) and is thus involved in lignan biosynthesis. The function of another abundant protein found in developing poplar xylem, a serine hydroxymethyltransferase (SHMT) homologue, is still unknown. Interestingly, ESTs for PCBER and SHMT are highly abundant in both the poplar and pine xylem EST libraries. Our sparse knowledge on the biological role of these abundant proteins indicates how little we know about important processes in wood formation. Other highly abundant spots correspond to five *S*-adenosyl-L-methionine (SAM) synthetases, one COMT and two CCoAOMT, all playing important roles in lignin biosynthesis. For all of these proteins, the corresponding ESTs are abundant in the xylem EST library too. It is conceivable that poplar genomics and proteomics, combined with the *Zinnia* xylogenic system and with reverse and forward genetics approaches in *Arabidopsis,* will result in the identification of marker genes for wood formation, in the discovery of new genes and their function, and in novel insight into biological processes of wood formation and of growth and development of woody species.

Acknowledgements

This work was supported by the Swedish Wood Ultrastructure Research Centre (WURC), the Foundation for Strategic Research (SSF) and the Belgian Fonds National de la Recherche Scientifique (FNRS). We thank N. Chaffey and L. Kleczkowski for critical reading of parts of the manuscript, K. Pickering, J. R. Barnett, S. Fink, L. Puech, P. Barlow and N. Chaffey for providing original figures and materials for illustrations, and K. Olofsson for technical assistance with photography.

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