A Weed for Wood? Arabidopsis as a Genetic Model for Xylem Development

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Wood, or secondary xylem, is a water-conductive and supportive vascular tissue highly characteristic of trees. In addition to parenchymatous cells adapted for storage and transport functions, wood is mainly composed of various vertically elongated cell types. These are classified either as tracheary elements or fibers, both of which are characterized with extensive secondary cell wall thickenings. The cell wall characteristics contribute to the properties of wood as a significant raw material for various human applications.

Wood formation occurs during the secondary phase of plant development (Fig. 1). This results from the activity of the vascular cambium, a lateral meristem that is established and functional during the secondary phase. On the other hand, already the primary phase of vascular development, associated with the procambial development of apical meristems, involves xylem production. The formation of both primary and secondary xylem involves a cascade of interesting processes including specification of primary vascular tissue as bundles, cell proliferation within the primary bundles or in the secondary vascular cambium, initiation of xylem differentiation, regulation of cell expansion, deposition of a secondary cell wall, and programmed cell death (Fig. 1). Even as these processes have been extensively documented at the structural level, relatively little is known of the genetic mechanisms behind them.

Although wood formation is an evident characteristic of trees, also many herbaceous plants, including Arabidopsis, develop vascular cambium and form secondary xylem. Thus, Arabidopsis can be considered as a model for the developmental processes underlying xylem development during both primary and secondary phases of development. In this Update we will first review the most recent work related to each developmental process resulting in xylem formation and finally focus on the secondary phase of development to compare wood development in Arabidopsis and trees in light of the most recent molecular data.

SPECIFICATION OF VASCULAR BUNDLES

Primary vascular tissue occurs as bundles interspersed among the surrounding ground tissue. It is first detected as a central cylinder in the embryonic root and hypocotyl and as veins in the cotyledons. After germination this organization is propagated by the apical meristem of the primary root and recapitulated by the secondary root meristems and leaf primordia. On the other hand, collateral vascular bundles are established during stem development by the shoot apical meristem (Fig. 1; for review, see Ye, 2002).

The role of auxin in specifying the vascular bundles has been implicated by early physiological studies that demonstrated the necessity of auxin transport originating from the shoot apex for the formation of vascular tissues (for review, see Aloni, 1987; Sachs 1991). According to Sachs’ canalization hypothesis, a high level of auxin in cell files further enhances their capacity for polar auxin transport and subsequently promotes their differentiation into vascular tissues (Sachs, 1991). This canalization of auxin transport depletes the surrounding cell files from auxin and thus prevents their differentiation.

Strong genetic evidence for the role of polar auxin flow in vascular bundle formation has been provided through identification of genes coding for components involved in polar auxin transport in Arabidopsis (for review, see Muday and Murphy, 2002). One of the genes identified as coding for a putative auxin efflux carrier, PIN1, has a polar localization at the basal ends of procambial and xylem parenchyma cells (Gälweiler et al., 1998). The polar localization of PIN1 is regulated through the activity of GNOM/EMB30, a GDP/GTP exchange factor of ADF-ribosylation factors involved in vesicle trafficking (Steinmann et al., 1999). Recessive mutations that disrupt the PIN1 or GNOM/EMB30 function result in reduction of long-distance transport of auxin from the shoot apex and young leaves (Okada et al., 1991; Geldner et al., 2003). In support of the canalization hypothesis, the gnom/emb30 mutants display discontinuous vascular bundles (Koizumi et al., 2000).

Auxin is believed to initiate a specific signal transduction pathway that specifies the vascular fate in young procambial cells. Although the receptor for auxin remains elusive, the transcriptional control re-
lated to auxin activity is conceptually understood (Gray et al., 2001). In this model, auxin response factors (ARFs) activate or repress the auxin regulated target genes. The activity of ARF transcription factors is repressed by AUX/IAA transcriptional regulators. Auxin promotes the degradation of AUX/IAAs, which leads to derepression of ARFs and activation of auxin response pathway (Gray et al., 2001; Zenser et al., 2001). Expression of AUX/IAA genes is auxin-inducible, which provides a negative-feedback loop for auxin response. The MONOPTEROS (MP) locus was first defined by recessive mutations that disrupt the apical-basal organization of the embryo and the continuity of the vascular strands (Berleth and Jürgens, 1993). Molecular cloning revealed it as one of the first ARFs to be genetically characterized (Hardtke and Berleth, 1998). MP is first expressed in broad domain during embryogenesis and its expression subsequently becomes restricted to vascular tissues, indicating its importance as a key factor controlling auxin mediated specification of vascular fate. A semidominant mutation in the BODENLOS (BDL) locus results in a phenotype similar to mp mutant (Hamann et al., 1999). BDL encodes an AUX/IAA transcriptional regulator and the mutation, located in a conserved degradation domain, is likely to extend its stability against degradation. Since the expression domains of MP and BDL overlap and their products interact, it is quite likely that BDL negatively regulates the MP function (Hamann et al., 2002). Whether other ARF or AUX/IAA genes expressed in the vascular tissue have specific roles during vascular development remains to be determined.

On the other hand, recessive mutations in several genetic loci result in the development of unconnected fragments of vascular tissue (for review, see Turner and Sieburth, 2002; Ye, 2002). This is difficult to explain by the canalization theory but is more consistent with the universal diffusion-reaction hypothesis (Koch and Meinhardt, 1994). It assumes the interaction of two diffusible components: an inducer that promotes specification of vascular bundles, and a yet unidentified inhibitor that prevents the formation of vascular tissue during procambial development. One of the loci associated with vascular continuity, COTYLEDON VASCULAR PATTERN1 (CVP1), was shown to code for an enzyme in sterol biosynthesis, suggesting a possible role for sterol signaling underlying a diffusion-reaction-type mechanism (Carland et al., 2002). Furthermore, recessive mutations in the CONTINUOUS VASCULAR RING (COV1) locus result in a dramatic increase in the amount of vascular tissue at interfascicular regions of the inflorescence stem. Therefore, the COV1 protein is assumed to be involved in maintaining or initiating the ordered patterning of individual vascular bundles within the stem (Parker et al., 2003). COV1 is predicted to be an integral membrane protein, and it could thus be involved in perception or transport of a signaling molecule that negatively regulates the differentiation of vascular tissue as proposed by the reaction-diffusion theory (Parker et al., 2003).

**CELL PROLIFERATION DURING (PRO)CAMBIAL DEVELOPMENT**

Once the vascular bundles are established, they undergo procambial cell proliferation process prior to primary xylem and phloem differentiation development (Fig. 1). On the other hand, a second phase of
cell proliferation occurs during secondary development as the vascular cambium is developing between the primary xylem and phloem (Fig. 1). It is probable that some of the mechanisms underlying cell proliferation during procambial and cambial development are common. The regulatory role of auxin in initiating and promoting vascular cambium growth has been well established, based on classical physiological studies. These experiments with numerous species including Arabidopsis have shown that auxin supply from the shoot apex is required for cambial growth (for review, see Mellerowicz et al., 2001; Little et al., 2002).

Genetic evidence is also available for the role of auxin in (pro)cambial cell proliferation. The pin1 mutants described above display overproliferation of vascular tissue in the bundles adjacent to cauline leaves of the inflorescence stem (Gälweiler et al., 1998). A block in the long-distance transport of auxin from young leaves presumably leads to enhanced auxin concentration and thus to increased xylem proliferation in the vicinity of a leaf. This indicates that auxin may play a key regulatory role in controlling the extent of cell proliferation during procambial development (Gälweiler et al., 1998). Furthermore, overexpression of \textit{ATHB-8}, an auxin-inducible class III HD-ZIP transcription factor, leads to stimulation of procambial and cambial cell proliferation (Baima et al., 2001). The role of auxin in controlling cell proliferation during secondary development is implicated by recessive mutations in the REVOLUTA/INTERFASCICULAR FIBERLESS1 (REV/IFL1) gene (Zhong et al., 1997). The \textit{rev/ifl} mutants are defective in the fascicular cambium activity and in the differentiation of interfascicular fibers in the basal regions of inflorescence stem (Zhong et al., 1997, 1999). REV/IFL, which encodes a class III HD-ZIP transcription factor, is thus necessary for the differentiation of secondary xylem through (inter)fascicular cambial activity in the Arabidopsis inflorescence stems. As both auxin flow and expression of auxin efflux carrier genes are reduced in the \textit{rev/ifl} mutants, Zhong and Ye (2001) suggested that the defect in cambial activity and fiber differentiation is a result of reduced auxin transport.

In addition to auxin, cytokinins are considered necessary for division of procambial and cambial cells (reviewed by Aloni, 1987). Genetic evidence for the involvement of cytokinin signaling in cell proliferation during procambial vascular development is provided by two mutant alleles in the CRE1/WOL/AHK4 locus of Arabidopsis (Mähönen et al., 2000; Inoue et al., 2001). In these mutants, the number of periclinal divisions in Arabidopsis roots is reduced. The CRE1 gene codes for a cytokinin receptor (Inoue et al., 2001) and is expressed in the vascular tissue of the embryonic axis and in the root procambium (Mähönen et al., 2000). Furthermore, the expression of the cytokinin inducible \textit{ARR15} and \textit{ARR16} genes is down-regulated in the loss-of-function \textit{cre1} mutant (Kiba et al., 2002). These observations indicate involvement of a specific cytokinin signal transduction pathway in the regulation of procambial vascular cell proliferation. In addition to auxin and cytokinin related genes, several other gene functions have also been implicated in cell proliferation during vascular development (for review, see Helariutta and Bhalerao, 2003).

**DETERMINATION OF XYLEM IDENTITY**

Cells derived from the procambium and vascular cambium differentiate into either xylem or phloem. In the shoot of Arabidopsis, the structure of vascular bundles is collateral; the xylem develops internally in the stem and adaxially in the leaves, and the phloem, respectively, peripherally and abaxially. Recently, two classes of genes known to be functional in the determination of plant organ polarity were recognized to be additionally directly involved in the regulation of tissue arrangement within the vascular bundles (Emery et al., 2003). The first class is comprised of genes encoding three class III HD-ZIP transcription factors, \textit{PHABULOSA/ATHB14} (\textit{PHB/ATHB14}), \textit{PHAVOLUTA/ATHB9} (\textit{PHV/ATHB9}), and \textit{REV/IFL}. The second class of genes consists of three \textit{KANADI} (\textit{KAN1}, \textit{KAN2}, and \textit{KAN3}) genes, which code for members of the GARP transcription factor family (Eshed et al., 2001).

Gain-of-function mutations in the \textit{REV/IFL} locus result in an amphivasal (xylem surrounding phloem) arrangement within the vascular bundles of the stem (Emery et al., 2003). Also the moderately radialized and adaxialized leaves formed by gain-of-function mutations in the \textit{PHB/ATHB14} locus have an amphivasal anatomy in their vascular bundles, whereas the completely radialized leaves entirely lack the vascular strands or possess only single xylem elements (McConnell and Barton, 1998). Analogously, vascular tissue is lacking in the abaxialized cotyledons formed by ectopic expression of any of the three \textit{KAN} genes. Consequently, a simultaneous loss-of-function mutation in all three \textit{KAN} loci results in amphivasal anatomy of the vascular bundles of the stem (Emery et al., 2003). In contrast to the \textit{kan1 kan2 kan3} triple mutant, the vascular bundles formed in the radialized cotyledons of the \textit{phb phv rev} triple loss-of-function mutant exhibit a reversed, ampicricial (phloem surrounding xylem) arrangement (Emery et al., 2003).

The \textit{PHB/ATHB14}, \textit{PHV/ATHB9}, and \textit{REV/IFL} genes are expressed in the adaxial and the \textit{KAN} genes in the abaxial domains of developing leaves (Eshed et al., 2001; Kerstetter et al., 2001; McConnell et al., 2001; Emery et al., 2003). Based on the contrasting nature of the vascular structure formed by the triple mutants of these two gene classes, it has been postulated that the activity of these two gene classes by mutual antagonism leads to the establishment of collateral arrangement in the vascular bundles of Arabidopsis (Emery et al., 2003). This hypothesis is supported by recent observations on the interaction of the two gene classes. Loss-of-function alleles of \textit{KAN1} and \textit{KAN2} in the double mutant result in the adaxial-
ization of most lateral organs and the expansion of PHV/ATHB9 and REV/IFL expression (Eshed et al., 2001). It was recently shown that the gain-of-function phenotype of REV/IFL can be produced at the level of the mRNA sequence, through an alteration at a micro-RNA165/166 target site (Emery et al., 2003). This implicates the involvement of another, microRNA-mediated, negative regulatory interaction targeted to PHB/ATHB14, PHV/ATHB9, and REV/IFL expression.

The three class III HD-ZIP encoding genes, together with the three KAN genes apparently are functional both in the regulation of the vascular tissue arrangement within vascular bundles and in the regulation of the general polarity (radial stem patterning and dorsiventral leaf polarity) of plant organs. The association between the regulation of the vascular bundle organization and the organ polarity seems however to be quite complex, as the arrangement within the vascular bundles does not always directly correlate with the polar organization of Arabidopsis organs (see above the lack of vascular tissue development in some of the gain-of-function phb mutants and in plants with an ectopic KAN expression).

PHB/ATHB14, PHV/ATHB9, and REV/IFL together with the KANI, KAN2, and KAN3 genes apparently represent a key transcriptional regulatory mechanism underlying the determination of xylem and phloem differentiation inside the vascular bundles. What then determines the spatial specificity of the gene classes? PHB and PHV and REV share a putative steroid binding domain (that is functionally required in REV). It is possible that PHB, PHV, and REV could act as receptors for such ligands (Emery et al., 2003). A specific class of steroids previously linked to patterning of xylem and phloem are the brassinosteroids. Several brassinosteroid biosynthetic mutants show altered organization in the vascular bundles with increased phloem to xylem ratio (Szekeres et al., 1996). Furthermore, brassinosteroids regulate expression of genes homologous for PHB/ATHB14, PHV/ATHB9, and REV/IFL during xylem differentiation in a Zinnia cell culture system (Otashi-Ito and Fukuda, 2003). The exact regulatory connection between brassinosteroids and class III HD-ZIP transcription factors remains to be determined.

Since the expression of class III HD-ZIP/KAN encoding genes is not restricted to vascular tissues, it is likely that they target the expression of another set of genes that has more vascular-specific functions. A recessive mutation in the ALTERED PHLOEM DEVELOPMENT (APL) gene results in ectopic formation of xylem in place of phloem (Bonke et al., 2003). Consistent with a key role in phloem development, APL codes for a MYB-coiled coil transcription factor and is expressed specifically in developing phloem. Furthermore, ectopic expression of APL in the vascular bundle inhibits xylem development. These observations suggest that APL has a dual role both in promoting phloem differentiation and in repressing xylem differentiation during vascular development (Bonke et al., 2003). Whether APL is a target for class III HD-ZIP/KAN regulation remains to be determined. Although the expression of several regulatory genes related to early xylem development has been reported, no mutational evidence for a xylem-specific regulator has yet been shown.

**CELL EXPANSION, CELL WALL DEPOSITION, AND CELL DEATH**

The newly formed xylem cells first undergo a characteristic expansion process. Since expansion occurs before the secondary cell wall is laid down, it seems probable that it shares a mechanistic basis with the expansion of nonvascular cells known to involve local regulation of cell wall biosynthesis and extensibility (for review, see Dolan and Davies, 2004). On the other hand, the hallmark of xylem development is the deposition of a thick secondary cell wall in the elongated cells. This coordinated process involves the deposition of various polymers (cellulose, hemicellulose, lignin, pectin, and various cross-linking proteins). Recent analysis of mutants defective in various aspects of cell wall synthesis has provided important information on the mechanisms related to cell expansion and secondary cell wall biosynthesis during xylem development.

Arabidopsis contains at least 10 CesA genes coding for the catalytic subunits of cellulose synthase, which is a large membrane-bound protein complex (for review, see Dobrin et al., 2002). Some of these genes (IRX1/CesA8, IRX3/CesA7, and IRX5/CesA4) were identified based on recessive mutations that result in collapse of the secondary cell wall of xylem. These genes code for subunits of the enzyme complex during synthesis of the secondary cell wall, whereas certain other CesA genes are active during the synthesis of the primary cell wall. Diversity in the catalytic subunits may account for the distinct compositions of the primary and secondary cell walls. All three gene products are coexpressed in the same cells, and thus are all required for cellulose synthesis in lignified secondary cell walls.

Recent studies have demonstrated the role played by cortical microtubules (CMTs) in regulating the orientation of cellulose microfibrils both during the elongation and secondary wall deposition phases of the developing xylem cells. The three CesA proteins become specifically localized to the site of the secondary cell walls at the same time as the CMTs become visible. Furthermore, CMT arrays are required for maintaining normal CesA protein localization (Taylor et al., 2000; Gardiner et al., 2003). Recessive mutations in the FRAGILE FIBER2 (FRA2) locus result in reduction in both fiber length and wall thickness. FRA2 codes for a katanin-like protein; in animal cells katanin has been shown to possess a microtubulus-severing activity. In accordance with the hypothesis of FRA2 as a microtubulus-organizing protein in plants, the mi-
crotubuli are disorganized in the elongating fra2 cells. Furthermore, disorganization of microtubules is accompanied by an altered orientation of cellulose microfibrils (Burk et al., 2001; Burk and Ye, 2002). Recessive mutations in the FRAGILE FIBER1 (FRA1) locus cause a dramatic reduction in fiber mechanical strength without apparent alteration in cell wall composition (Zhong et al., 2002). The reduced mechanical strength of fra1 fibers correlated with an alteration in the oriented deposition of cellulose microfibrils in fiber cell walls without affecting the organization of CMTs. The FRA1 gene was shown to encode a kinesin-like protein. Since the role of kinesin as a microtubule-binding motor protein is well established, it appears likely that FRA1 mediates the activity of CMT in orienting the cellulose microfibrils during differentiation of xylem cells (Zhong et al., 2002).

The other major polymer in the secondary cell wall of xylem cells is lignin. It is composed of monolignol units and imparts rigidity and impermeability to the cell walls. Arabidopsis has also become an excellent genetic model system for lignin biosynthesis pathway (for review, see Boerjan et al., 2003). In addition to biosynthetic enzymes, the xylem differentiation is associated with specific expression of many cell wall degrading and modifying enzymes. These enzymes may be involved in perforation of the ends of mature vessel members or restructuring primary wall at the time when the secondary wall is laid down.

After identification of specific enzymes, the next step will be to elucidate the underlying regulatory mechanisms during secondary cell wall deposition. A popular approach is functional genomics, based on identification of genes in another xylegenic model system, such as Zinnia or a tree species (Hertzig et al., 2001; Demura et al., 2002). Several regulatory genes, whose expression is correlated with secondary wall formation, have been identified. For example, a member of the pine R2R3 MYB family of transcription factors was shown to regulate the lignin biosynthetic pathway both in Arabidopsis and in loblolly pine (Pinus taeda). PtMYB4 was shown to bind to AC elements in promoters of genes encoding lignin biosynthetic enzymes. PtMYB4 overexpression increased the transcription of enzymes involved in the monolignol-specific portion of the lignin biosynthetic pathway (Patzlaff et al., 2003). In the future, to determine if these MYB transcription factors are necessary for lignification, loss-of-function phenotypes corresponding to these genes should be examined by suppressing several MYB family members in Arabidopsis due to their genetic redundancy.

In the final process of wood development the tracheary elements undergo programmed cell death (for review, see Fukuda, 2000). The cell contents of the tracheary elements are degraded, when hydrolytic enzymes, including proteases and nucleases, are released into the cytosol from the disrupted central vacuole (Fukuda, 2000). Genes coding for proteases potentially involved in the programmed cell death of tracheary elements have been identified from Arabidopsis. Zhao et al. (2000) generated xylem and bark specific cDNA libraries from Arabidopsis root-hypocotyl area and subsequently identified two Cys proteases, XCP1 and XCP2, and one Ser protease, XSP1, which were specifically expressed in xylem. Funk et al. (2002) were able to define that the XCP1 is localized in the vacuoles of differentiating tracheary elements in Arabidopsis, as it would be expected from a protease potentially involved in wood development. Recently it was shown by Ito and Fukuda (2002) that a Zn2+- dependent DNase, ZEN1, has a major role in the degradation of nuclear DNA during programmed cell death of tracheary elements in Zinnia. It was additionally reported that the Arabidopsis genome contains a putative ortholog, BFN1, for this gene. Whether the identified hydrolytic enzymes, proteases and a nuclease, have a function in the process of programmed cell death of tracheary elements in Arabidopsis, remains to be genetically verified.

FUTURE PROSPECTS: WOOD FORMATION IN ARABIDOPSIS

As described above, Arabidopsis is able to undergo secondary growth and subsequently produce secondary xylem in its root (Fig. 1; Dolan et al., 1993; Levey and Yadun, 1994), hypocotyl (Busse and Evert, 1999; Chaffey et al., 2002), and inflorescence stem (Baima et al., 2001) under appropriate growth conditions.

Chaffey et al. (2002) compared the anatomical characteristics of secondary xylem produced by Arabidopsis hypocotyl with those of the wood produced by an angiosperm tree, hybrid aspen (Populus tremula × Populus tremuloides). The wood from Arabidopsis and angiosperm tree contained similar cell types, but an apparent structural difference was the lack of rays, the radial files of parenchyma cells, in Arabidopsis. Although Busse and Evert (1999) observed rays in the secondary xylem of Arabidopsis hypocotyl, they are apparently not a common feature of prolonged secondary growth in Arabidopsis. Therefore, although Arabidopsis is a suitable model for research about secondary xylem formation, there may be some developmental processes characteristic to wood formation in trees that cannot be approached in this system.

For some time, specific microtechniques for dissecting various developmental zones have been applied to the cambial zones of trees. By measuring auxin distribution at fine resolution, Uggla et al. (1996) discovered an auxin gradient is present across the cambial zone of Scotch pine (Pinus sylvestris). This radial gradient, with its peak in the cambial cells, indicates that auxin may act as a positional signal to regulate the area of cell divisions in the cambial zone (Mellerowicz et al., 2001). With the same sectioning technique, Hertzig et al. (2001) created a transcript profile of almost 3,000 genes expressed across the cambial zone of hybrid aspen. Among them Schrader et al. (2003) identified and characterized the homologs of Arabidopsis auxin
transporter genes. They observed gene specific expression patterns for the auxin transporters along the cambial zone. This result suggests that the auxin gradient may be maintained through the differential positioning of specific auxin transporters across the cambial zone.

After a prolonged growth, the secondary development in Arabidopsis is eventually so substantial that isolation of separate secondary tissues, including xylem, phloem-enriched, and nonvascular fractions, becomes possible (Zhao et al., 2000). Oh et al. (2003) utilized the secondary growth in the Arabidopsis root-hypocotyl region to examine the transcriptome profile changes induced in the xylem and bark tissues during secondary development. They observed an elevated expression for several transcription factors, including some members of the R2R3-MYB gene family during secondary phase of vascular development. As described above, some members of the pine R2R3-MYB gene family have recently been implicated in lignin biosynthesis in trees. It will be interesting to see in the future if these genes represent orthologous functions during xylem development in Arabidopsis and tree species.

Kirst et al. (2003) have performed a comparative genomics research about the conservation of genes involved in secondary xylem development in Arabidopsis and conifer trees. They compared expression sequence tags obtained from wood-forming tissues of loblolly pine, to the gene sequences inferred from the Arabidopsis genome. A high level, approximately 90%, of the contigs containing long, high-quality sequences, had an apparent homolog in the Arabidopsis genome. The result indicates that the genetic mechanisms behind the developmental processes of xylem development may potentially be highly conserved between woody and herbaceous seed plants. If a close similarity exists between trees and Arabidopsis, the feasibility for easy identification of Arabidopsis knock-out mutants will be an important resource for wood formation research. Approaches based on natural variation during secondary development between various Arabidopsis accessions may also turn out to be an important avenue to exploit Arabidopsis in wood development research.

ACKNOWLEDGMENTS

We thank Dr. Björn Sundberg for critical reading of the manuscript, anonymous reviewers for excellent suggestions, and the Language Centre of the University of Helsinki for proofreading. We regret that so many elegant studies by many colleagues could not be reviewed due to restricted space.

Received February 2, 2004; returned for revision April 4, 2004; accepted April 5, 2004.

LITERATURE CITED


