*Journal of Experimental Botany*, Vol. 64, No. 1, pp. 11–31, 2013
doi:10.1093/jxb/ers287 Advance Access publication 16 November, 2012

Journal of Experimental Botany
www.jxb.oxfordjournals.org

DARWIN REVIEW

# Xylem tissue specification, patterning, and differentiation mechanisms

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Received 5 July 2012; Revised 11 September 2012; Accepted 24 September 2012

## Abstract

**Vascular plants (Tracheophytes) have adapted to a variety of environments ranging from arid deserts to tropical rainforests, and now comprise >250 000 species. While they differ widely in appearance and growth habit, all of them share a similar specialized tissue system (vascular tissue) for transporting water and nutrients throughout the organism. Plant vascular systems connect all plant organs from the shoot to the root, and are comprised of two main tissue types, xylem and phloem. In this review we examine the current state of knowledge concerning the process of vascular tissue formation, and highlight important mechanisms underlying key steps in vascular cell type specification, xylem and phloem tissue patterning, and, finally, the differentiation and maturation of specific xylem cell types.**

**Key words:** *Arabidopsis*, fibre, secondary cell wall, lignification, tracheophytes, tracheary element, xylem specification, xylem differentiation.

## The vascular tissue differentiation trajectory

Mature vascular tissues consist of highly specialized cell types that generally arise from discrete populations of undifferentiated progenitor cells located in meristem (stem cell) niches. Root and shoot apical meristems are established during embryo development, whereas lateral meristems (procambium and vascular cambium) appear at later stages of development and result from hormone-driven cellular recruitment and re-differentiation processes. For example, vascular tissues in plant leaves (leaf veins) are derived from procambial cells, which are vascular precursor cells. Procambial cells can arise through the *de novo* differentiation of parenchyma cells in a process that not only commits each recruited cell to a vascular cell fate, but also generates adjoined files of such cells (Fig. 1) (Esau, 1965*a*, *b*; Mattsson *et al*., 2003; Scarpella *et al*., 2004, 2006; Wenzel *et al*., 2007). Parenchyma cells that are committed to a procambial cell fate can be visually distinguished in leaf blades by their elongated cell shape and their alignment in discrete cell files among otherwise isodiametric ground tissue cells. The processes of procambial cell specification and organization into continuous procambial strands appear to be tightly linked developmentally, and both are thought to be the product of the canalization of auxin fluxes in the developing leaf, as discussed later.

Once formed, individual procambial cells can undergo periclinal divisions (parallel to the plane of cell elongation), ultimately giving rise to the procambium tissue, from which specialized xylem and phloem cells are subsequently formed (Fig.1). However, a subset of cells within the procambium remains in an undifferentiated state, positioned between the differentiating xylem and phloem tissues. These cells function as vascular stem cells and enable the prolonged formation of vascular tissues in rapidly elongating or expanding organs such as young stems and leaves during primary plant growth.

The bulk of primary plant growth occurs from two other populations of stem cells, located in the shoot apical meristem (SAM) and root apical meristem (RAM). All new shoot and root structures are ultimately derived from these two meristems, but the formation of these new organs is also tightly coordinated with procambium formation and the specification of vascular tissues

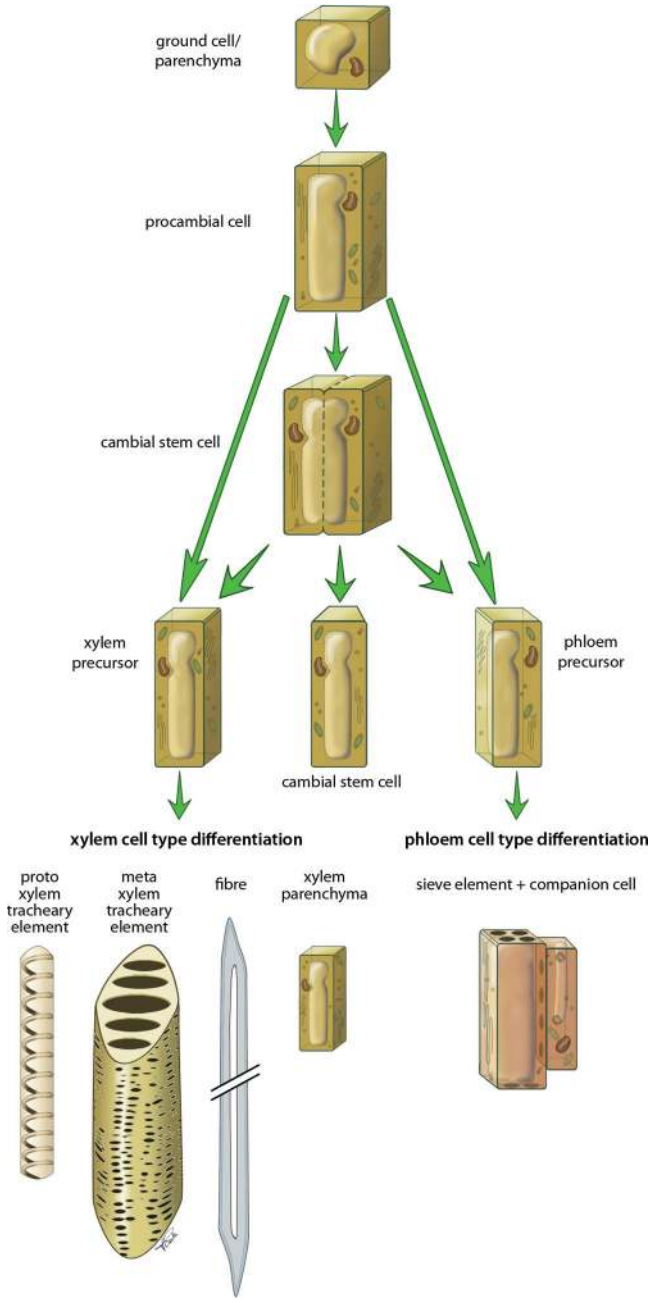


**Fig. 1.** Overview of procambial/cambial cell specification and xylem/phloem cell differentiation. Procambial cells can form by the *de novo* differentiation of parenchyma cells, or by division of existing procambial cells during primary growth, thereby forming the procambium. The vascular cambium and associated cambial cells are derived from the procambium during the transition to secondary growth, at which point the nomenclature of 'procambial' cells no longer applies and 'cambial' cells is used instead. In woody plants, the cambial cells are further categorized as ray initials or fusiform initials (not shown here). Cambial/procambial cells differentiate into either xylem or phloem cell types, as shown. Note: different cell types are not to drawn to scale.

(Bayer *et al*., 2009). Finally, in woody plants (e.g. trees) where a pattern of secondary growth becomes prominent, a fourth population of specialized stem cells develops from the procambium to form the vascular cambium, a lateral meristem from which extensive secondary xylem (wood) is formed. Thus, the procambium provides a source of vascular stem cells during primary growth, while the vascular cambium and associated cambial cells perform an analogous role during secondary growth as the plant continues to grow and mature.

Differentiation of vascular tissues from the procambium/vascular cambium follows two different developmental pathways to produce xylem and phloem (Fig. 1). Mature xylem tissues are composed of three main cell types: xylem tracheary (vessel) elements, xylary fibres, and xylem parenchyma cells (Fig. 1). Tracheary elements, which facilitate water and solute transport between organs, and fibres, which provide structural support for the plant, both possess thick secondary cell walls. Xylem parenchyma cells lack well-defined secondary cell walls and are implicated in a variety of biological processes, including aiding the lignification of secondary cell walls in neighbouring vessel elements and fibres (discussed later in this review). All three cell types are formed from xylem precursor cells, which are derived from procambial/cambial cells, but only tracheary elements and fibres undergo the extensive secondary cell wall formation that typifies xylogenesis.

Tracheary elements formed during early and later stages of plant and vascular development are structurally distinguished as protoxylem and metaxylem (Fig. 1). Protoxylem tracheary elements form during primary plant growth and deposit localized annular or helical secondary cell wall thickenings, reinforcement patterns that allow these cells to continue to elongate within actively growing areas of the plant. As vascular tissues mature and primary growth ceases, relatively larger metaxylem tracheary elements are formed. These are marked by a distinctive pitted or reticulate pattern of secondary cell wall deposition. Unlike the protoxylem wall thickenings, this pattern of secondary cell wall deposition does not allow continued cell elongation, and thus the shape of the metaxylem precursor cells is reflected in the radial and axial dimensions of the mature metaxylem vessels. As a final stage of differentiation, both protoxylem and metaxylem tracheary elements undergo programmed cell death (PCD), resulting in a continuous system of adjoining hollow cells that function in water/solute transport.

In contrast to the patterned deposition of secondary cell walls in xylem tracheary elements, xylary fibres develop a thick, evenly deposited secondary cell wall (Fig. 1). During fibre development, PCD is delayed, allowing for more extensive thickening and lignification of secondary cell walls, consistent with a primary role for this cell type in providing structural support. The developmental commitment to formation of specific xylem cell types has recently been shown to be under the control of several key transcription factors, and the identification of these genes has provided important tools to further our understanding of xylem differentiation (discussed later).

Procambial/cambial stem cells are also the source of phloem precursor cells, which differentiate into specific phloem cell types. Mature phloem tissues consist of sieve tube elements, phloem companion cells, and, in many cases, phloem fibres. Sieve tube elements function to transport metabolites from source tissues, such as leaves, to metabolic sinks, such as roots and seeds. During differentiation, sieve tube elements lose most

of their organelles, including the nucleus, but they remain physiologically alive. Sieve tube elements are cytoplasmically coupled to companion cells through numerous plasmodesmata, and the companion cells can thereby maintain the metabolic competency of the sieve tube elements, and also 'load' molecular cargo into them for long-distance transport. Relative to the recent progress in understanding the regulation of xylem development, far less is known about the specific regulatory factors involved in the developmental commitment to phloem cell fates, such as sieve tube or companion cell formation.

The economic importance of secondary growth and wood formation has focused considerable research attention on the function of the vascular cambium in tree species, but this question has also been actively studied in *Arabidopsis thaliana*. Although this herbaceous plant does not normally undergo extensive secondary growth, two regions of vascular cambium—zones of fascicular cambium and the neighbouring zones of interfascicular cambium—are found within the *Arabidopsis* inflorescence stem. Despite their proximity to each other and their apparent similarity, these two cambial niches have different developmental origins. The fascicular cambium is derived from the procambium that developed within the original vascular tissue as it was formed during the primary growth of the stem. It is not surprising, therefore, that radial differentiation of fascicular cambial cells gives rise to the full range of both xylem and phloem cell types. The inflorescence stem fascicular cambium can thus be considered functionally analogous to the circumferential vascular cambium of woody plants. The interfascicular cambium, on the other hand, is thought to arise through the *de novo* recruitment of interfascicular parenchyma cells as primary growth in the stem slows. It represents a specialized vascular meristem that gives rise exclusively to the structurally important interfascicular fibres.

In contrast to the bundled arrangement of vascular tissues and intervening cambial cells in *Arabidopsis* inflorescence stems, the true stem (hypocotyl) of *Arabidopsis* forms a circumferential vascular cambium that can also undergo substantial secondary growth under certain conditions to form so-called '*Arabidopsis* wood' (Lev-Yadun, 1994; Busse and Evert, 1999; Chaffey *et al*., 2002). Examination of cross-sections of this 'secondary growth' hypocotyl reveals an axial tissue arrangement of large metaxylem tracheary elements dispersed among thick xylem fibres, similar to that observed in woody stems of trees (Chaffey *et al*., 2002). The most prominent difference between the secondary xylem in true woody plant stems and in these *Arabidopsis* 'secondary' stems is the lack of well-defined xylem rays and the determinate growth habit in the latter. Despite these differences, *Arabidopsis* provides a useful model for understanding the process of secondary growth in woody plants.

### *What triggers vascular differentiation?*

The phenomenon of recruiting existing parenchyma cells for differentiation into files of procambial precursors is observed both during normal leaf vein development and in response to rupture of existing vascular strands (Jacobs, 1952; Sachs, 1969; Sauer *et al*., 2006). Early experiments established that ectopic application of the hormone auxin [indole-3-acetic acid (IAA)] was sufficient to trigger the specification of vascular tissue, including proliferation of cambial cells and final differentiation of vascular cell types (Kraus *et al*., 1936; Jacobs, 1952; Sachs, 1969). In 1981, Sachs proposed the 'canalization of auxin flow hypothesis' as a model for the auxin-mediated formation of vascular tissues (Sachs, 1981). In this model, channels of preferential auxin flow are created when a series of cells gradually become specialized for directional auxin transport. Once established, these channels effectively drain auxin from surrounding cells, resulting in localized concentration of auxin within distinct cell files, and this shift in auxin distribution was hypothesized to subsequently induce vascular tissue formation. The 'canalization of auxin' model thus provided a single mechanism that could account for both the initial specification and the physical contiguity of developing vascular tissues.

Recent molecular genetic studies have experimentally confirmed the accumulation of auxin within developing procambial cells (Aloni *et al*., 2003; Mattsson *et al*., 2003), but while auxin is still considered an essential factor for triggering vascular tissue specification, it has also become clear that other plant hormones also perform important functions during vascular tissue development (reviewed in Dettmer *et al*., 2009). For instance, both cytokinin and auxin are required for efficient trans-differentiation of mesophyll cells in *in vitro* tracheary element (xylem vessel) differentiation systems (Fukuda and Komamine, 1980). This experimental induction system has proven to be a productive platform for identifying genes required for tracheary element differentiation (Demura and Fukuda, 1993; Demura *et al*., 2002; Kubo *et al*., 2005), and has also helped uncover a role for the brassinosteroid class of hormones as initiators of the final stages of the differentiation process (Yamamoto *et al*., 1997, 2001).

Mutant screens performed in *Arabidopsis* have led to the identification of numerous mutants defective in vascular tissue formation and/or patterning, and many of these have proven to be defective in the biosynthesis or signal transduction of auxin and other hormones (reviewed in Berleth *et al*., 2000; Fukuda, 2004; Cano-Delgado *et al*., 2010). Of particular note is the identification of genes involved in auxin signalling and transport, which are two key processes facilitating the proposed canalization of auxin. For example, the *MONOPTEROS* (*MP*) gene is a member of a transcription factor gene family encoding auxin response factor (ARF) proteins, which are involved in regulating auxin-responsive gene expression (Guilfoyle and Hagen, 2007). Loss-of-function *mp* mutants have pleiotropic phenotypes, including a highly reduced leaf vein system and misaligned tracheary elements in *Arabidopsis* inflorescence stems and leaves (Berleth and Jurgens, 1993; Przemeck *et al*., 1996). *mp* mutants also have severely attenuated levels of expression of the *PINFORMED1* (*PIN1*) gene (Wenzel *et al*., 2007) that encodes a plasma membrane-localized auxin efflux protein (Galweiler *et al*., 1998). Asymmetric localization of PIN1 in plant cells is thought to establish a directional auxin flow (reviewed in Petrasek and Friml, 2009). In the *Arabidopsis* leaf, *PIN1* and *MP* are co-expressed during very early stages of procambial cell specification, and their spatial pattern of expression gradually changes from initially broader domains to a single file of cells, as predicted for positive feedback onto the auxin canalization process (Scarpella *et al*., 2006; Wenzel *et al*., 2007).

In addition to promoting vascular tissue formation, auxin accumulation in discrete populations of plant cells triggers a variety of other developmental processes (Benkova *et al*., 2003). The canalization of auxin flow may therefore be thought of as a general mechanism for regulating localized auxin distribution, but the specific factors that interpret these auxin gradients, and respond by promoting a specific developmental process, remain largely unidentified. An important contribution to this area of research was the identification of the link between *MP*-mediated procambial cell specification and *AtHB8* function. *AtHB8*, a member of the class III homeodomain leucine zipper (*HD-ZIP*) transcription factor family, is co-expressed with *MP* and *PIN1* during vascular cell specification, and functions as a positive regulator of procambial and cambial cell proliferation, as well as xylem differentiation (Baima, 1995; Baima *et al*., 2001; Mattsson *et al*., 2003; Scarpella *et al*., 2006; Wenzel *et al*., 2007). Donner *et al*. (2009) identified a regulatory element in the promoter of *AtHB8* to which MP binds and thereby regulates the auxin-responsive gene expression of *AtHB8* in procambial cells. Because *AtHB8* function promotes procambial cell proliferation and subsequent procambium formation, the integration of this transcription factor within the MP–PIN-mediated canalization model provides one example of how general auxin gradients could be translated into a specific developmental output (Fig. 2B). *HD-ZIP* family members have been implicated in a variety of developmental processes, including modulation of polar auxin transport, regulation of the radial patterning of vascular tissues, and differentiation of specific xylem cell types. In the following section, we examine the way in which auxin, *HD-ZIP* genes, and other factors interact to govern the developmental patterning of vascular tissues and the maintenance of cambial stem cells.

## The cambial stem cell niche and radial patterning of vascular tissues

Despite the apparent uniformity of the stem cell population within each cambial niche (procambium and vascular cambium), differentiation programmes operating on this pool of precursor cells simultaneously convert some of them into either developmentally distinct xylem or phloem cell types (Fig. 2A). Phloem typically forms at the face of the cambial cell population oriented toward the abaxial surface of the leaf, and at the outward-facing surface of the stem, whereas xylem forms in the adaxial and inward-facing position in leaves and stems, respectively. As a result, vascular differentiation results in a tissue organization pattern in which xylem, cambium, and phloem occupy specific radial positions within developing organs and stems. The factors which determine the radial polarity of the vascular cambium are not fully understood, but differential gradients of several plant hormones (cytokinin, gibberellin, and auxin) across the cambium are hypothesized to play a role in both cambium cell proliferation and phloem/xylem cell fate determination (Loomis and Torrey, 1964; Uggla *et al*., 1996; Tuominen *et al*., 1997; Moyle *et al*., 2002; Israelsson *et al*., 2005; Matsumoto-Kitano *et al*., 2008; Nieminen *et al*., 2008; Hejatko *et al*., 2009). Several studies have concluded that shoot-to-root polar auxin transport occurs in the cells associated with the procambium and vascular cambium, and that auxin levels peak along the radial axis within cambial and differentiating xylem cells (Savidge, 1988; Galweiler *et al*., 1998; Uggla *et al*., 1998; Mattsson *et al*., 1999; Schuetz *et al*., 2007; Nilsson *et al*., 2008). It is hypothesized that the activity of key transcriptional switches that promote either xylem or phloem cell fate within the vascular stem cell niche is being influenced by the balance of specific hormones generated within any particular procambial/cambial cell. Because several *Arabidopsis* mutants defective in the radial patterning of vascular tissues also have defects in the radial (abaxial/adaxial) patterning of leaves/flowers, these switches appear to be integrated into the broader radial patterning mechanisms responsible for organizing overall plant organ morphology. Analysis of these patterning mutants has identified members of the KANADI and class III *HD-ZIP* gene families as important regulators of the overall abaxial/adaxial polarity of all shoot lateral organs, as well as the radial patterning of vascular tissues.

### *The KANADI/HD-ZIP/miRNA165/166 nexus*

One clade of the GARP family of transcription factors consists of four *KANADI* genes (*KAN1*, *2*, *3*, and *4*), which are expressed in abaxial domains of developing shoot lateral organs, as well as in phloem tissues throughout the plant (Kerstetter *et al*., 2001; Emery *et al*., 2003). On the other hand, five class III *HD-ZIP* transcription factors (*AtHB15/CORONA*, *PHABULOSA*, *PHAVOLUTA*, *REVOLUTA/INTERFASCICULAR FIBRELESS*, and *AtHB8*) are expressed in complementary domains, and function to specify adaxial cell fate of shoot lateral organs, in addition to promoting meristem function and xylem tissue formation (Fig. 2A) (Emery *et al*., 2003; Prigge *et al*., 2005; Ilegems *et al*., 2010). The *KAN* and *HD-ZIP* gene families play several roles in plant growth and development, but for the purpose of this review we will focus on their contribution to the formation and patterning of vascular tissues.

Within each of these gene families, individual family members function somewhat redundantly, although mild radial patterning defects can be detected in some single loss-of-function mutants (Eshed *et al*., 2001; Emery *et al*., 2003). Quadruple mutants of the *KAN1*, *2*, *3*, and *4* genes, however, produce a striking amphivasal (xylem surrounding phloem) radial pattern of vascular tissue organization in both leaves and stems (Emery *et al*., 2003) and *kan* loss of function has been correlated with the expansion of HD-ZIP gene expression domains (Eshed *et al*., 2001). Triple loss-of-function mutants of the *HD-ZIP* genes, *REVOLUTA* (*REV*), *PHABULOSA* (*PHB*), and *PHAVOLUTA* (*PHV*), in contrast, possess an amphicribal (phloem surrounding xylem) radial pattern that is postulated to correspond to an expansion of *KAN* gene expression domains (Emery *et al*., 2003). Although *KAN* genes are expressed in phloem tissues, phloem formation itself is not dependent on *KAN* function because phloem tissues are still formed in *kan1–kan4* quadruple mutants (Ilegems *et al*., 2010). However, proliferation of cambial-like cells is observed in the hypocotyls of *kan1–kan4* quadruple mutant seedlings, suggesting that *KAN* may function as a negative regulator of procambium/cambium formation, in addition to regulating the organization of vascular tissues (Ilegems *et al*., 2010).

Consistent with the idea that *KAN* genes mediate repression of procambium/cambium formation, ectopic *KAN1* expression

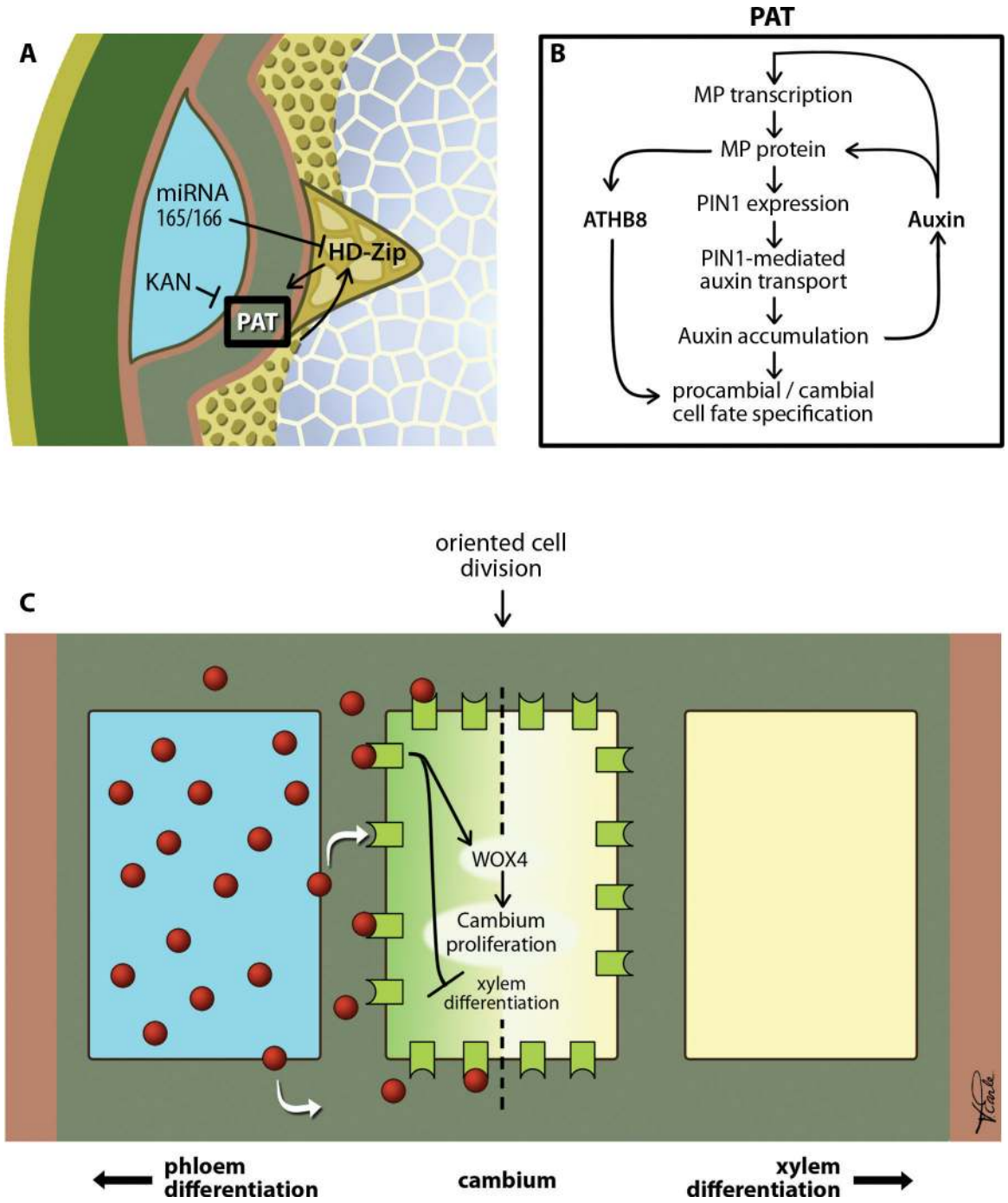


**Fig. 2.** Radial patterning and polar auxin transport (PAT). (A) Cross-section of the fascicular cambium region of an *Arabidopsis* inflorescence stem showing the epidermis and cortex in green, phloem tissue in blue, vascular cambium in khaki, xylem tissue in yellow, and pith cells in pale blue. In this model, phloem-expressed KANADI (KAN) genes restrict PAT to the cambium and developing xylem regions, while HD-ZIP genes positively influence PAT and promote xylem development. The expression domains of HD-ZIP genes are maintained by auxin and by post-transcriptional gene silencing mediated by phloem-expressed microRNA 165 and 166. (B) The molecular model for the canalization of auxin and subsequent PAT. Auxin-mediated activation of *MONOPTEROS* (*MP*) transcription and MP protein function promotes the expression of the PIN1 auxin transporter and the HD-ZIP gene *AtHB8*. PIN1-mediated auxin transport continues to activate MP, forming the basis of a positive feedback loop that strengthens the canalization of auxin into discrete files of cells. Throughout this process, MP-mediated activation of AtHB8 promotes procambial cell differentiation and procambium formation. (C) CLE/TDIF peptides (red spheres) originating from phloem cell types diffuse throughout the apoplast and are sequestered by the PXY/TDR receptor (green rectangles) which is specifically expressed in cambial cells. The polar activation of PXY/TDR signalling leads to cambial cell polarization and results in periclinal division. PXY/TDR signalling also promotes WOX4-mediated cambial cell proliferation and promotes stem cell fate by inhibiting xylem differentiation.

(*KAN1-OE*), either ubiquitously or from a procambium-specific promoter, was found to abolish all vascular tissue formation (Eshed *et al*., 2001; Kerstetter *et al*., 2001; Ilegems *et al*., 2010). In *KAN1-OE* plants, the expression of *HD-ZIP* genes was also severely reduced, again demonstrating the antagonistic interaction between these two gene families. In addition to defective xylem and phloem formation, the expression of both *PIN1:GFP* (green fluorescent protein), a marker gene for auxin-mediated procambium formation, and the synthetic auxin reporter *DR5:GFP*, was severely reduced in the procambium of developing *KAN1-OE* embryos and seedlings (Ilegems *et al*., 2010). Reflecting a reciprocal relationship, ectopic *PIN1:GFP* expression has been observed in developing embryos of the *kan1,2,4* triple mutants (Izhaki and Bowman, 2007). Interestingly, the inhibition of vascular tissue formation in plants misexpressing *KAN1* could be suppressed by co-overexpression of *PIN1* from the 35S promoter, suggesting that at least part of *KAN1* function could involve directly regulating *PIN1* transcription (Ilegems *et al*., 2010). In addition to *PIN1*, expression of the *PIN3*, *PIN4*, and *MP* genes was also rapidly reduced in response to inducible *KAN1-OE* expression, indicating that KAN may function as a general repressor of auxin transport and signalling.

Despite the apparent reciprocity of function, KANADI and HD-ZIP proteins do not appear to interact directly to limit each other's expression; instead, it has been suggested that their mutually antagonistic expression domains are derived from their differential regulation of auxin signalling and transport (Pekker *et al*., 2005; Izhaki and Bowman, 2007; Ilegems *et al*., 2010). Several lines of evidence associate auxin transport/signalling with *HD-ZIP* gene function. Expression of *HD-ZIP* genes is stimulated by ectopic auxin application, and *in planta* expression patterns of *HD-ZIP* genes are correlated with endogenous auxin gradients. Basipetal auxin transport in *revoluta/ifl* loss-of-function inflorescence stems is reduced to ~30% of wild-type levels and the expression of two auxin transport genes (*PIN3* and *PIN4*) is severely reduced (Zhong and Ye, 2001). The existing evidence thus demonstrates that *HD-ZIP* gene expression is positively regulated by auxin, and that *HD-ZIP* activity positively promotes polar auxin transport (PAT).

In addition to auxin-based regulation of *HD-ZIP* gene expression, microRNAs (miRNAs) 165 and 166 directly regulate the stability of *HD-ZIP* transcripts. *HD-ZIP* genes and their associated miRNAs represent ancient gene families, that are conserved in all tracheophytes, indicating that this post-transcriptional control mechanism probably became an important regulator of *HD-ZIP* function early in plant evolution (Floyd and Bowman, 2004; Floyd *et al*., 2006). The expression domains of miRNAs 165 and 166 overlap considerably with those of *KAN* genes in *Arabidopsis* phloem tissues and abaxial leaf domains, but, unlike the *KAN* genes, these miRNAs can directly regulate *HD-ZIP* function. Thus, overexpression of miRNA 165 or 166 leads to enhanced degradation of *HD-ZIP* transcripts, thereby phenocopying *HD-ZIP* loss-of-function mutants (Kim *et al*., 2005; Williams *et al*., 2005; Zhou *et al*., 2007). In addition, dominant mutant alleles of *HD-ZIP* genes have been isolated that possess mutations in a conserved miRNA-binding site that is present in the transcripts of all five *HD-ZIP* family members. These dominant *HD-ZIP* mutants display amphivasal phenotypes similar to that seen in *KANADI* loss-of-function mutants, which suggests that miRNA-mediated regulation of *HD-ZIP* transcripts may be more functionally relevant than antagonistic KAN function (McConnell and Barton, 1998; McConnell *et al*., 2001; McHale and Koning, 2004; Zhong and Ye, 2004; Ohashi-Ito *et al*., 2005).

A model integrating *KANADI*, *HD-ZIP*, and miRNA 165/166 in a regulatory network that promotes vascular tissue patterning and differentiation is shown in Fig. 2A. In this model, *KAN* genes inhibit *PIN* gene expression in abaxial phloem-forming tissues, thereby negatively regulating canalization of auxin flow and thus limiting efficient auxin transport to procambial/cambial and differentiating xylem cells. *HD-ZIP* genes, on the other hand, positively regulate canalization of auxin by activating the expression of genes that promote auxin transport (PINs and ARFs) and thus promote procambium/cambium formation. The expression of *HD-ZIP* genes is, in turn, activated by auxin, thus completing a positive feedback cycle (Fig. 2A) (Ilegems *et al*., 2010). In this model, phloem-expressed miRNAs further define *HD-ZIP* gene expression domains by limiting *HD-ZIP* transcript stability in the phloem-forming regions of the procambium/cambium.

It is noteworthy that miRNA-mediated regulation of *HD-ZIP* genes was also recently found to be an important determinant for specifying particular xylem cell types, at least in *Arabidopsis* roots. A radial gradient of miRNA 165 accumulation was shown to control the formation of protoxylem versus metaxylem by negatively regulating *HD-ZIP* transcript stability in a dose-dependent manner (Carlsbecker *et al*., 2010). It will be interesting to establish whether a similar mechanism functions in the procambium/cambium of plant shoots. More research effort is also needed to clarify the exact biological function of the individual *HD-ZIP* gene family members, and to determine how endogenous expression of miRNAs 165 and 166 is integrated with other mechanisms controlling vascular tissue patterning and differentiation.

### *The WOX/PXY/CLE41/44 signalling module*

Another key regulator of vascular cambium function and patterning is a diffusion gradient of peptide signalling molecules originating from differentiating phloem cells. *CLE41*, *CLE42*, and *CLE44*, three members of the *CLAVATA3/EMBRYO SURROUNDING REGION-related* (*CLE*) gene family, encode proteins that are post-translationally processed to form 12-amino acid signalling peptides. These peptides were initially identified as 'tracheary element differentiation inhibitory factors' (TDIFs) isolated from *Zinnia in vitro* tracheary element induction cultures, but they were subsequently shown both to promote cell division and specifically to inhibit xylem differentiation *in planta* (Ito *et al*., 2006).

The three *CLE* genes (*CLE41*, *42*, and *44*) that possess TDIF activity (*CLE/TDIF*) are expressed and processed into peptide signalling molecules in phloem cells, and the resulting peptides subsequently diffuse into the apoplast to reach neighbouring cells (Fig. 2C) (Hirakawa *et al*., 2008). Previous studies identified the CLE peptide CLAVATA3 (CLV3) as an important signalling molecule in regulating stem cell proliferation in the SAM, where extracellular CLV3 is recognized by the apoplastic domain of the leucine-rich repeat receptor-like kinase (LRR-RLK) CLAVATA1

(Schoof *et al*., 2000; Lenhard and Laux, 2003; Ogawa *et al*., 2008). The product of another LRR-RLK gene, *Phloem intercalated with XYlem/TDIF Receptor* (*PXY/TDR*), has also been identified as a functional receptor for CLE/TDIF peptides (Fig. 2C) (Hirakawa *et al*., 2008).

The *PXY/TDR* gene was initially identified based on the breakdown of the radial organization of vascular tissues in *pxy* loss-of-function mutants, and was found to be specifically expressed in the procambium/cambium (Fisher and Turner, 2007). The spatial arrangement of CLE/TDIF peptide production in phloem cells, and of *PXY/TDR* receptor expression in the procambium/cambium, sets up a signalling polarity that was shown to be an important factor in maintaining the radial organization of vascular tissues. Etchells and Turner (2010) proposed that periclinal division of cambial cells is regulated by the polarization of cambial cells as a result of differential CLE/TDIF ligand binding (Fig. 2C). Consistent with this model, either ubiquitous or xylem-specific CLE/TDIF misexpression resulted in loss of periclinally oriented cell division in the cambium and subsequent breakdown of radial xylem/phloem organization (Etchells and Turner, 2010).

In addition to establishing the polarity of cell division, CLE/TDIF peptide signalling through the PXY/TDR receptor stimulates procambial/cambial cell proliferation and also specifically inhibits xylem differentiation. One direct outcome of PXY/TDR signal transduction is up-regulation of the expression of the *WOX4* (*WUSCHEL-related HOMEOBOX*) transcription factor (Hirakawa *et al*., 2010). *WOX4* is specifically expressed in the procambium/cambium stem cell niche where it functions to stimulate cell proliferation (Hirakawa *et al*., 2010; Ji *et al*., 2010; Suer *et al*., 2011). However, *wox4* loss-of-function mutants are not totally impaired in procambial/cambial cell proliferation, indicating that additional factors are participating in this process. Furthermore, *WOX4* activity does not inhibit xylem differentiation, which indicates that an unidentified factor functions downstream of CLE/TDIF-PXY/TDR signalling to inhibit cell differentiation (Hirakawa *et al*., 2010).

Ectopic CLE/TDIF peptide application, or overexpression of CLE/TDIF-encoding genes, results in a remarkable proliferation of cells within existing vascular tissues, and these cells express the procambial/cambial cell fate marker gene, *AtHB8* (Hirakawa *et al*., 2008; Whitford *et al*., 2008). The expression of several auxin transport genes and the synthetic auxin reporter *DR5:GUS* was similarly enhanced (Whitford *et al*., 2008). While the role of auxin as a potent regulator of cambial cell proliferation has been extensively documented, recent results now point to a convergence of auxin and CLE/TDIF signalling. When auxin accumulation in wild-type *Arabidopsis* inflorescence stems is induced by local inhibition of auxin transport, or by ectopic application of auxin, stimulation of cambial cell proliferation is observed (Mattsson *et al*., 1999; Suer *et al*., 2011). However, neither *pxy* nor *wox4* mutants responded to local auxin transport inhibition or auxin application with enhanced cambial cell proliferation, although cambial cell proliferation is not completely abolished in the untreated mutants (Suer *et al*., 2011). On the other hand, in the inverse situation, the CLE/TDIF-mediated cambial cell proliferation observed in wild-type plants is dependent on functional auxin signal transduction. Thus, no stimulation of cambial cell formation was observed upon ectopic application of CLE/TDIF to *mp* mutant plants, which are impaired in auxin signalling and transport (Whitford *et al*., 2008). These studies have demonstrated that auxin and CLE/TDIF–PXY/TDR signalling converge to regulate the identity and proliferation of cambial stem cells.

Because auxin has been shown to be essential for effective trans-differentiation of tracheary elements in both plant cell cultures and intact plants, it is not immediately obvious how auxin can, at the same time, promote cambial stem cell proliferation. Perhaps when CLE/TDIF is recognized by PXY/TDR in the procambium/cambium, the combination of both auxin and CLE/TDIF signalling provides the specific conditions to promote cambial stem cell proliferation, whereas in the absence of CLE/TDIF signalling, auxin signalling could converge with other pathways to promote the differentiation of different xylem cell types.

## Differentiation of xylem cell types

Secondary cell wall formation and subsequent PCD are two critical steps in the maturation of xylem tracheary elements and fibre cells. Interest in the biosynthesis and deposition of secondary cell walls has been spurred recently by the search for plant-based feedstock sources for the generation of biofuels. Studies in *Arabidopsis* have identified a network of transcription factors that regulate the expression of numerous genes directly involved in the biosynthesis of secondary cell walls and in PCD. Many of these transcription factors have apparent orthologues in economically important tree species such as eucalyptus, pine, and poplar, but the functional characterization of these secondary cell wall-associated genes in trees has been greatly facilitated by exploiting the more experimentally accessible genetic tools available in the *Arabidopsis* system (Demura and Fukuda, 2007; Zhang *et al*., 2010; Zhong *et al*., 2010*a*).

For example, application of whole-genome microarray analysis to *Arabidopsis* suspension culture cells differentiating into tracheary element-like cells has allowed the identification of numerous genes, both known and novel, likely to be involved in secondary cell wall formation and PCD (Kubo *et al*., 2005). Through these and other studies, several key transcriptional switches have been identified that are able to recapitulate the entire differentiation process for specific xylem cell types, when ectopically expressed (Kubo *et al*., 2005; Yamaguchi *et al*., 2010*a*). These ‘master regulator’ genes are thought to be positioned near the top of transcriptional cascades that control the xylem differentiation process, and direct it to specific end-points.

### *Initiation of tracheary element differentiation*

*VND7* and *VND6* belong to a seven-member gene family encoding VND (*VASCULAR-RELATED NAC DOMAIN*) transcription factors whose expression is spatially and temporally correlated with tracheary element differentiation (Kubo *et al*., 2005). Expression of *VND7* and *VND6* is specifically localized to developing protoxylem and metaxylem tracheary elements, respectively, and inducible or constitutive expression of these transcription factors in transgenic plants results in ectopic

protoxylem tracheary element (*VND7*) or metaxylem tracheary element (*VND6*) trans-differentiation, even in highly specialized cell types such as stomata, trichomes, and root hairs (Kubo *et al*., 2005; Yamaguchi *et al*., 2010*a*). Although single VND loss-of-function mutants are not defective in tracheary element differentiation, indicative of functional redundancy with the VND family, the use of dominant repression constructs for *VND7* and *VND6* resulted in defective protoxylem and metaxylem formation in *Arabidopsis* roots (Kubo *et al*., 2005).

Analysis of the transcriptional profiles of plants ectopically expressing either *VND7* or *VND6* genes revealed that a wide array of genes are differentially expressed in response to VND activity, and the respective gene expression lists also showed significant overlap (Ohashi-Ito *et al*., 2010; Yamaguchi *et al*., 2010*b*, 2011; Zhong *et al*., 2010*b*). The spatial patterns of expression of the *VND6* and *VND7* genes partially overlap in developing vascular systems, and the encoded proteins were shown to interact physically through the formation of homo- or heterodimers (Kubo *et al*., 2005; Yamaguchi *et al*., 2008, 2010*b*). Taken together, these data are consistent with a substantial degree of common function for the two genes, but the developmentally distinct consequences of their overexpression make it clear that *VND6* and *VND7* also play unique roles in specifying either protoxylem or metaxylem cell fates. The remarkable ability of *VND7* and *VND6* to trigger execution of the entire tracheary element developmental programme places these genes at, or near the top of, the transcriptional cascade regulating xylem tracheary element differentiation (Fig. 3). While tracheary element formation is apparently unaffected in *vnd6* or *vnd7* plants (Kubo *et al*., 2005), analysis of higher order mutants of these genes and other members of the VND gene family would be predicted to generate a pronounced tracheary element formation phenotype.

Yamaguchi *et al*, (2010*b*) recently described the *VNI2* (*VND-INTERACTING 2*) transcriptional repressor, a protein that can bind to VND proteins and has been shown to inhibit *VND7*-mediated gene transcription. *VNI2* expression precedes that of *VND7* in procambial cells, and the VNI2 protein is targeted for degradation when *VND7* expression is required for protoxylem tracheary element differentiation (Yamaguchi *et al*., 2010*b*). Although VNI2 degradation unleashes the VND7 transcriptional programme in nascent tracheary elements, *VNI2* expression persists in neighbouring xylary parenchyma cells, which suggests that *VNI2* may be inhibiting these cells from differentiating (Yamaguchi *et al*., 2010*b*). However, the functionality of the VNI2–VND7 interaction is probably more complex, because ectopic expression of VNI2 using a tracheary element-specific promoter reduced, but did not abolish, tracheary element differentiation. Identification of other components interacting with the VNI2–VND7-containing transcriptional complex could provide useful insights into the spatial and temporal specificity that allows upstream transcription factors to regulate the expression of different groups of genes involved in tracheary element differentiation. Establishing whether and how these putative complexes might be post-translationally modified to differentially regulate the expression of specific downstream genes will also be an interesting future challenge.

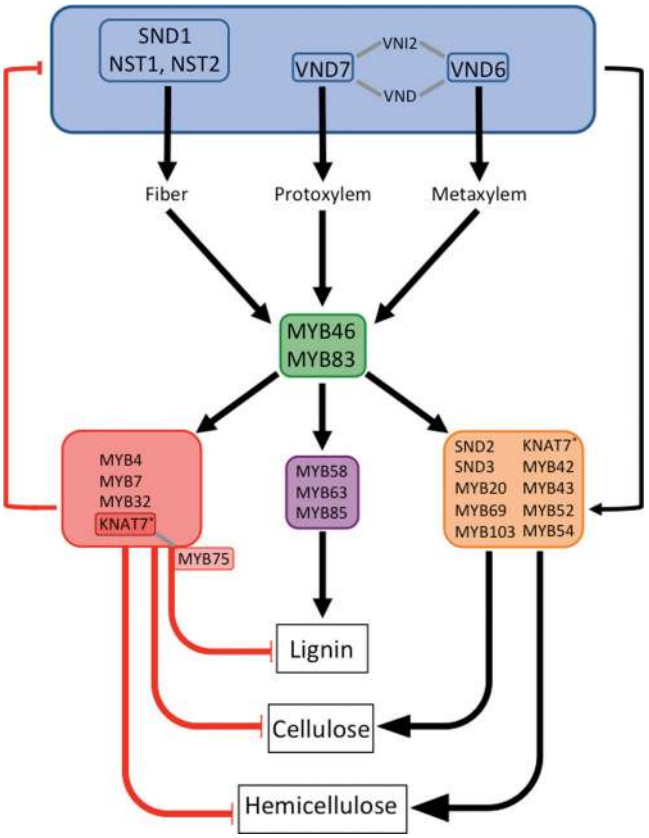


**Fig. 3.** An overview of the transcriptional network regulating secondary cell wall biosynthesis in *Arabidopsis thaliana*. The master switches for fibre (SND1, NST1, NST2), protoxylem (VND7), and metaxylem (VND6) differentiation (in blue) initiate the transcriptional network and directly activate several transcription factors, including the two core transcription factors, MYB46 and MYB83 (in green). The MYB46/83 node activates the expression of a plethora of other transcription factors, which promote lignin biosynthesis (in purple), cellulose and/or hemicellulose biosynthesis (in orange), or function as negative regulators (in red) of secondary cell wall formation. MYB4, MYB7, and MYB32 have been documented to repress the expression of master switches such as SND1, but can also directly or indirectly repress the expression of genes involved in secondary cell wall biosynthesis. *KNAT7 has been reported as both a positive and negative regulator of different genes involved in secondary cell wall biosynthesis. Although MYB75 has not been shown to be targeted by the transcriptional network, a MYB75–KNAT7 transcriptional complex may function as a negative regulator of lignin biosynthesis.

### *Initiation of fibre differentiation*

Key transcription factors that function as master regulators for differentiation of fibres, such as the interfascicular and xylary fibres in the *Arabidopsis* stem, have also been identified. Loss of function at the *HD-ZIP* gene *REVOLUTA/INTERFASCICULAR FIBRELESS* (*REV*) largely eliminates interfascicular fibre formation, but because ectopic expression of *REV* does not result in ectopic differentiation of fibre-like cells, it is thought to act

as an indirect regulator of fibre differentiation, perhaps by promoting polar auxin transport (Zhong *et al*., 1997; Zhong and Ye, 1999). In contrast, ectopic expression of the NAC domain-containing transcription factor *SND1/NST3* is sufficient to elicit ectopic differentiation of non-vascular cell types into fibre-like cells (Zhong *et al*., 2006; Ohashi-Ito *et al*., 2010). Like *VND6* and *VND7*, *SND1* regulates a transcriptional cascade that ultimately activates the specific genes necessary for secondary cell wall biosynthesis (Fig. 3). However, in contrast to *VND6* and *VND7*, *SND1* does not appear to activate the expression of genes involved in PCD, a feature that is consistent with the prolonged period of fibre development relative to that of tracheary elements (Zhong *et al*., 2006; Ohashi-Ito *et al*., 2010). Interestingly, ectopic overexpression of *SND1* results in uniform deposition of secondary cell walls, in contrast to the helical or pitted secondary cell walls observed in protoxylem and metaxylem tracheary elements (Zhong *et al*., 2006; Ohashi-Ito *et al*., 2010).

*SND1* functions redundantly with two other NAC transcription factors, *NST1* and *NST2*, to control secondary cell wall formation of all fibre types in *Arabidopsis* (Mitsuda *et al*., 2005, 2007; Zhong *et al*., 2006, 2007*b*). Single loss-of-function mutants of *snd1* or *nst1* have no observable phenotype, but developing fibres in the *snd1nst1* double mutant retain the general fibre cell shape without developing secondary cell walls (Mitsuda *et al*., 2007; Zhong et al., 2007*b*). This double mutant phenotype is also consistent with the fibre-specific expression for *SND1* and *NST1* (Mitsuda *et al*., 2005, 2007; Zhong *et al*., 2006).

The development of inducible expression/activation systems for the *VND6*, *VND7*, and *SND1* genes, coupled to comparative transcriptome analysis, has uncovered both overlapping and distinct target genes for these three transcription factors, including numerous other transcription factors (Fig. 3) (Yamaguchi *et al*., 2008, 2010*b*, 2011; Ohashi-Ito *et al*., 2010; Zhong *et al*., 2010*b*). In particular, several members of the MYB family of transcription factors appear to be crucial targets whose activity amplifies the transcriptional network and thereby promotes secondary cell wall formation. Although *VND6*, *VND7*, and *SND1* share significant functional overlap in their ability to activate the downstream transcriptional network and the general metabolic machinery required to form secondary cell walls (Ohashi-Ito *et al*., 2010; Zhong *et al*., 2010*b*), specific factors that establish the pattern of deposition of the secondary cell wall in different xylem cell types have yet to be identified.

### *The secondary cell wall transcriptional network converges on MYB46 and MYB83*

The secondary cell wall NAC domain transcription factors (*VND6*, *VND7*, *SND1*, *NST1*, and *NST2*) are thought to activate the transcriptional network for secondary cell wall formation by binding to SNBE (*S*econdary wall *N*AC-*B*inding *E*lement) regulatory regions in the promoters of target genes (Zhong *et al*., 2010*b*). SNBE elements were initially identified in the promoter of the *MYB46* transcription factor, and were subsequently shown to be the promoter regions necessary for activating the expression of several other transcription factors involved in secondary cell wall formation (Zhong *et al*., 2010*b*). Although the SNBE regulatory element is highly degenerate, a synthetic promoter consisting of six SNBE repeats was able to drive expression of reporter genes specifically in developing xylem and fibre tissues in *Arabidopsis* (Zhong *et al*., 2010*b*).

The transcriptional network activated by the secondary cell wall NAC genes appears to be particularly dependent on the *MYB46* and *MYB83* genes, whose promoters each contain several SNBE promoter elements and are thought to be direct targets of secondary cell wall NAC genes (Fig. 3) (Zhong *et al*., 2007*a*; Ko *et al*., 2009; McCarthy *et al*., 2009). Double *myb46 myb83* knockout mutants are defective in secondary cell wall formation, and double mutant seedlings arrest growth and development shortly after germination, consistent with their importance in the network (McCarthy *et al*., 2009). Ectopic secondary cell wall formation is observed in plants misexpressing MYB46 or MYB83, which can be positioned epistatically upstream of numerous other transcription factors regulating secondary cell wall formation (Fig. 3) (Zhong *et al*., 2007; Ko *et al*., 2009; McCarthy *et al*., 2009). The 'seedling lethal' phenotype of *myb46 myb83* plants is reminiscent of phenotypes observed in other mutants in which vascular tissue formation is completely abolished or the integrity of secondary cell walls is severely compromised (Kerstetter *et al*., 2001; Brown *et al*., 2009; Wu *et al*., 2009, 2010; Ilegems *et al*., 2010; Yamaguchi *et al*., 2010*b*). Since no obvious phenotypes were observed in single *myb46* or *myb83* loss-of-function mutants, this functional redundancy may help ensure that secondary cell wall formation, which is integral to the proper functioning of tracheary elements and fibres, always proceeds.

Figure 3 provides a current view of this transcriptional network. In this model, a master switch, such as *SND1*, activates the expression of *MYB46* and *MYB83*, but also other genes, including *SND3*, *MYB103*, and *KNAT7*, which do not appear to make as prominent a contribution to the extension of the network (Zhong *et al*., 2008; Ko *et al*., 2009). It is possible that the *MYB46* and *MYB83* gene products help maintain the expression of some of the other genes that are initially directly targeted by the secondary cell wall master switches. Numerous transcription factors, including *SND2*, *SND3*, *MYB103*, *MYB85*, *MYB52*, *MYB54*, and *MYB69*, are thought to operate downstream of *MYB46* and *MYB83* in the transcription network (Fig. 3) (Zhong *et al*., 2007*a*; Ko *et al*., 2009; McCarthy *et al*., 2009). Dominant-negative repression of each of these downstream genes resulted in reduced secondary cell wall thickening of both interfascicular fibres and xylary fibres, but not in tracheary elements, despite the ubiquitous expression of these dominant-negative constructs (Zhong *et al*., 2008). This observation points to intrinsic differences in the processes by which secondary cell wall formation is regulated in tracheary elements and fibres (Zhong *et al*., 2008).

In protoplast transfection assays, *SND2*, *SND3*, and *MYB103* constructs were able to activate the promoters for genes involved in cellulose biosynthesis, such as *CesA8*, while *MYB52* and *MYB54* constructs activated promoters for genes involved in cellulose, xylan, and lignin biosynthesis, indicative of a more general role for these two MYBs in regulation of cell wall biosynthetic enzymes (Zhong *et al*., 2008). In addition to these downstream targets, a lignin-specific subgroup of transcriptional regulators were also identified as targets of MYB46 and MYB83 (discussed later).

These studies have provided an initial roadmap for transcriptional regulation of cell wall deposition, but many questions remain to be addressed. While protoplast-based promoter activation assays can yield important indications of functional relationships between transcription factors and their putative targets, they fail to capture the cellular and developmental context in which the relevant secondary cell wall-related processes are unfolding. Experimental strategies that enable these relationships to be probed within intact developing tissues will therefore be needed. It will also be important to establish the functional relationships between different transcription factors that appear to share common targets.

### *Transcriptional activation and repression*

The expression of four transcription factors (*KNAT7*, *MYB4*, *MYB7*, and *MYB32*) that function as transcriptional repressors is influenced by *MYB46* and/or *MYB83* (Ko *et al*., 2009; McCarthy *et al*., 2009). For instance, expression of the *KNAT7* transcription factor is directly activated by *SND1*, but also by *MYB83* (Zhong *et al*., 2008; Ko *et al*., 2009). *KNAT7* expression has been localized to the vascular system of all plant organs, as well as interfascicular fibre and cortex regions of inflorescence stems and hypocotyls (Zhong *et al*., 2008; Li *et al*., 2012). *KNAT7* has been shown to function as a transcriptional repressor in protoplast transfection assays, and candidate gene reverse transcription–PCR (RT–PCR) experiments have revealed that, in the absence of *KNAT7* function, the expression of two secondary cell wall cellulose synthase genes, a number of hemicellulose biosynthesis genes, and the majority of lignin biosynthesis genes is increased (Li *et al*., 2012). This suggests that *KNAT7* functions as an important modulator of the output of the transcriptional network, which generally functions to activate the transcription of genes involved in secondary cell wall biosynthesis.

The concept of *KNAT7* functioning as a negative regulator of secondary cell wall deposition is consistent with the thinner secondary cell wall phenotypes observed in *Arabidopsis* fibres in response to either *KNAT7* overexpression or overexpression of *KNAT7* dominant repression constructs designed to repress the transcription of *KNAT7* target genes (Brown *et al*., 2005; Zhong *et al*., 2008; Li *et al*., 2011, 2012). However, while *knat7* loss-of-function mutants form thicker secondary cell walls in interfascicular fibres, as predicted for a negative regulator, they also develop 'irregular xylem' phenotypes, an outcome associated with thinner secondary cell walls in xylary fibres and tracheary elements. This combination of cell wall phenotypes suggests that *KNAT7* function may be more complex than initially thought. Because the KNAT7 protein has been shown to interact physically with several other transcription factors in both plant and heterologous systems (Bhargava *et al*., 2010; Li *et al*., 2011), the tissue-specific effects on secondary cell wall formation resulting from manipulation of *KNAT7* expression could reflect local differences in the composition of transcriptional complexes in any particular tissue type. Definition of the scope and functional consequences of protein–protein interactions within the secondary cell wall transcriptional control network, as well as the role of post-translational modification in modulating those interactions, is just beginning to be explored.

In addition to activating *KNAT7* expression, *MYB46* activates the expression of the *MYB4*, *MYB7*, and *MYB32* genes. These function as potent transcriptional repressors that can reduce both their own expression and the expression of the *SND1* upstream 'master switch' (Fig. 3) (Ko *et al*., 2009; Zhong *et al*., 2010*b*; Wang *et al*., 2011). *MYB4* and *MYB32* also function to repress lignin biosynthesis, with *MYB32* negatively regulating general phenylpropanoid biosynthesis genes (Preston *et al*., 2004) and *MYB4* specifically suppressing the expression of the *C4H* gene, which encodes the first committed step in the phenylpropanoid pathway (Jin *et al*., 2000). The apparent similarity in function between these three negative regulators, and the fact that they belong to the same subgroup of MYB transcription factors, has raised the question of whether they are functionally redundant (Jin *et al*., 2000). Although a survey of *MYB4*, *MYB7*, or *MYB32* target genes has not been reported, we can hypothesize that these MYBs may collectively function to fine-tune the activity of other transcription factors that are involved in activating expression of numerous secondary cell wall biosynthetic enzymes. The incorporation of a negative regulator node into the known transcriptional network, as illustrated in Fig. 3, provides a potential negative feedback mechanism that may be an important homeostatic control for the overall network.

### *Cellulosic secondary cell wall*

Although variable between different plant species and cell types, the secondary cell walls of flowering plants are predominantly composed of cellulose (~40–50%), accompanied by lesser amounts of hemicelluloses (~20–30%) and lignin (~25–30%), with only small amounts of pectin and proteins (Mellerowicz and Sundberg, 2008; Dejardin *et al*., 2010). Many of the genes involved in the biosynthesis of each of the three main components have been identified, and the transcriptional network described in Fig. 3 targets most of these known genes. The majority of studies that have attempted to identify downstream target genes for specific transcriptional regulators within the network have combined inducible activation/expression systems with whole-genome transcriptome analysis, or assessed expression of candidate target genes by RT–PCR. However, relatively few studies have demonstrated direct activation of specific genes through use of chromatin immunoprecipitation, electrophoretic mobility shift assays, or protoplast transfection systems (Jin *et al*., 2000; Zhong *et al*., 2007*a*, 2008; Ko *et al*., 2009; McCarthy *et al*., 2009; Zhou *et al*., 2009; Yamaguchi *et al*., 2010*b*; Zhao *et al*., 2010*b*; Wang *et al*., 2011). With the exception of three MYB genes (*MYB58*, *MYB63*, and *MYB85*) that seem to be relatively specific activators of lignin biosynthesis, the general trend appears to be that any particular transcription factor in the network is capable of activating the expression of multiple genes involved in cellulose, hemicellulose, and/or lignin biosynthesis. Although several studies have reported that genes involved in the biosynthesis of cellulose/hemicellulose and lignin are expressed concurrently during xylem cell differentiation (Ehlting *et al*., 2005; Zhao *et al*., 2005), the diversity of tissues and developmental states being sampled in most cases precludes clear resolution of the timing of these events. Histochemical studies have indicated that lignification of the secondary cell wall generally occurs after the initial

deposition of the cellulosic components, and that it is initiated in a spatially distinct manner, beginning with the lignification of the middle lamella (Saka and Thomas, 1982; Donaldson, 2001). One of the future challenges will be to understand how the temporal pattern of biosynthesis and deposition of the different components of the secondary cell wall is established.

Secondary cell wall formation must also be spatially regulated, even within one cell. Comparative studies of the patterns of secondary cell wall deposition in xylem tracheary elements in many different vascular plants have shown that the patterning of secondary cell wall deposition in these cells is a highly conserved process (Esau, 1965*a*, *b*; Meylan and Butterfield, 1972). The fact that cellulose makes up the bulk of the secondary cell wall implies that regulation of spatial patterning must be intimately linked to regulation of the biosynthesis and deposition of cellulose microfibrils in the cell wall.

Much of what has been learned about cellulose biosynthesis has been gleaned from studies on cellulose deposition in primary cell walls, but there appear to be significant parallels between primary and secondary cell wall cellulose synthesis. In both cases, cellulose synthesis complexes (CSCs) composed of 36 CesA (cellulose synthesis A) isoform subunits catalyse the linear polymerization of glucose molecules, a process that is also thought to drive the lateral movement of CSCs within the plasma membrane (reviewed in Taylor, 2008). Distinct subgroups of *CesA* genes appear to be involved in cellulose biosynthesis in primary or in secondary cell walls. In *Arabidopsis*, the products of the *CesA4*, *CesA7*, and *CesA8* genes appear to function non-redundantly to catalyse cellulose biosynthesis in secondary cell walls. All three proteins were reported to be necessary to form the hexameric rosette-shaped secondary cell wall-associated CSCs whose form appears very similar to that of CSCs engaged in primary cell wall biosynthesis (Haigler and Brown, 1986; Taylor, 2008).

Nevertheless, secondary cell wall cellulose synthesis differs in some important respects from the primary cell wall-associated process. The length of individual cellulose chains ranges from 500 to 2000 glucose molecules in primary cell walls, but can extend to >10 000 glucose molecules in secondary cell walls (Joshi and Mansfield, 2007). It would be informative to determine whether the processivity of secondary cell wall CSC polymerization is significantly higher than that of primary CSCs, but limitations in the optical resolution of deeply buried xylem cell types have so far precluded the use of live cell imaging to assess the velocities of fluorescent protein-labelled secondary CSCs in plasma membranes accurately (Wightman *et al*., 2009). The spatial distribution of secondary cell wall CSCs appears to be correlated with sites of wall deposition, since high densities of CSCs have been localized to specific plasma membrane domains immediately below the developing secondary cell walls (Wightman *et al*., 2009).

The delivery and maintenance of CSCs in specific plasma membrane domains of developing tracheary elements is influenced by the subtending cortical microtubule array (Wightman and Turner, 2008, 2010). In *Arabidopsis* root protoxylem, microtubule bundles oriented along the borders of the developing secondary cell wall thickenings have been hypothesized to help organize functional CSCs at particular regions of the plasma membrane (Wightman and Turner, 2010). Live cell imaging using fluorescently tagged CesA proteins has revealed Golgi localization of CesA proteins, as well as actin cable trafficking of CesA-containing Golgi vesicles that reportedly 'pause' in the region of secondary cell wall thickenings (Wightman and Turner, 2008, 2010). Since similar Golgi 'pause events' during primary cell wall synthesis seem to coincide with the insertion of CesA-containing vesicles into the plasma membrane, the 'pause' events observed during secondary cell wall synthesis could, by analogy, reflect specific localization of the CSCs to the secondary cell wall thickenings (Crowell *et al*., 2009).

In contrast to the linear cellulose polymer, hemicelluloses are branched cell wall matrix polysaccharides composed of backbones of glucose, mannose, or xylose, substituted to various degrees by other sugars. Hemicelluloses are synthesized in the Golgi, and hemicellulose-containing vesicles may be trafficked to specific plasma membrane domains during secondary cell wall formation in a manner similar to that shown for CSCs. In *Arabidopsis*, several Golgi-localized glycosyltransferases have been identified that are involved in the biosynthesis of the xylose sugar backbone, and in the addition of the glucuronic acid side groups of glucuronoxylan. Glucuronoxylan is the most abundant hemicellulose found in the secondary cell walls of dicotyledonous plants and is thought to function as the major cellulose cross-linking component in secondary cell walls. Defects in xylan synthesis result in collapsed or 'irregular xylem' (IRX) phenotypes, indicating the important contribution these molecules make to the structural integrity of secondary cell walls (reviewed in Scheller and Ulvskov, 2010; Doering *et al*., 2012).

## Lignification and programmed cell death

Lignification of the largely cellulosic secondary cell wall makes a major contribution to the functionality of mature tracheary elements and fibres. This phenolic polymer imparts both increased structural stability and water impermeability to the cell wall, and failure of lignin biosynthesis or polymerization often leads to IRX phenotypes similar to those observed in cellulose- or hemicellulose-deficient mutants (Jones *et al*., 2001; Hoffmann *et al*., 2004; Sibout *et al*., 2005; Berthet *et al*., 2011). Because of its importance to the integrity of the vascular system, and the severe consequences of ectopic lignification in non-vascular tissues, the process of lignification is tightly controlled, both spatially and temporally. In protoxylem, for example, lignin polymerization is restricted to the narrow helical or annular zones of secondary cell wall deposition. This provides a degree of wall rigidification while leaving intervening areas of the wall still capable of expansion (Fig. 4). At the other extreme, total lignification of the massive secondary cell walls in fibre cells must be closely coordinated with the loss of metabolic capacity in these cells as they undergo PCD.

### *Regulation of monolignol biosynthesis*

Lignin formation has been intensively studied for several decades and, as a result, most of the enzymatic machinery involved, and the corresponding genes, have been well characterized. Attention

has recently been focused on the regulation of the pathway; particularly, how it is integrated into the larger secondary cell wall transcriptional network. The lignin biosynthesis pathway can be divided into two parts. The first is the general phenylpropanoid pathway, a multistep reaction sequence that generates precursors not only for synthesis of lignin monomers (monolignol alcohols), but also for the synthesis of other phenylpropanoid compounds such as flavonoids, tannins, phenolic esters, and acids (Hahlbrock and Scheel, 1989). The general phenylpropanoid pathway feeds into the more specific monolignol biosynthetic pathway that provides substrates for the enzymes encoded by members of the *CCR*, *COMT*, *F5H*, and *CAD* gene families (reviewed in Boerjan *et al*., 2003; Vanholme *et al*., 2008).

The regulation of monolignol biosynthesis and subsequent lignin polymer formation is a complex process that is influenced by a variety of developmental, physiological, and environmental cues (Zhao and Dixon, 2011). Therefore, different layers of regulatory mechanism(s) are likely to have evolved to respond to specific situations. Transcriptional regulation appears to play a major role in controlling lignin biosynthesis, and several transcription factors are known to affect the transcription of the corresponding genes directly or indirectly (reviewed in Zhong and Ye, 2009). *MYB75* was previously found to function as an activator of flavonoid biosynthesis, based on the phenotype of the *PAP1-d* enhancer trap mutant that has elevated *MYB75* expression and increased anthocyanin content (Borevitz *et al*., 2000). However, subsequent analysis of the *myb75* loss-of-function mutant uncovered cell wall thickness phenotypes similar to those seen in the *knat7* mutant, and both mutants were found to have increased expression of secondary cell wall biosynthesis genes (cellulose, hemicellulose, and lignin) (Bhargava *et al*., 2010; Li *et al*., 2012). *KNAT7* and *MYB75* share partially overlapping tissue expression domains in developing xylem tissues and can physically interact with each other in yeast two-hybrid and bimolecular fluorescence complementation assays (Zhong *et al*., 2008; Bhargava *et al*., 2010; Li *et al*., 2012). KNAT7 and MYB75 may therefore form a transcriptional complex (Fig. 3) that acts to modulate carbon allocation into secondary cell wall biosynthesis, and may also repress secondary cell wall formation, especially in cortical cells adjacent to interfascicular fibres (Bhargava *et al*., 2010).

Constitutive expression of *MYB58*, *MYB63*, or *MYB85* leads to ectopic lignification of plant cells, but not to ectopic cellulose or xylan deposition, which indicates that these transcription factors specifically regulate lignin biosynthesis/deposition (Zhong *et al*., 2008, 2009). *MYB58* and *MYB63* directly activate the expression of nearly all the genes involved in the lignin biosynthetic pathway, and both genes are thought to bind at AC regulatory elements (also known as H-boxes or PAL boxes), conserved regulatory elements found upstream of most lignin biosynthetic genes (Ohl *et al*., 1990; Zhong and Ye, 2009).

The one lignin biosynthetic gene that is not activated by either *MYB58* or *MYB63* is the cytochrome P450 enzyme, ferulate-5-hydroxylase (*F5H*), which sits at a key branch point in the monolignol biosynthesis pathway. The lignin polymer is typically dominated by two monomer structures in angiosperm species, those with a 3'-methoxy-4'-hydroxy ring substitution pattern (contributing to guaiacyl, or 'G-lignin') and those with a 3',5'-dimethoxy-4'-hydroxy pattern (contributing to syringyl, or 'S-lignin') (Fig. 4). Within the plant, the lignin polymer composition (S/G ratio) differs dramatically between different lignified cell types. For instance, xylem vessels or tracheary elements have secondary cell walls primarily composed of G-lignin (Fig. 4, blue colour), while fibres, such as *Arabidopsis* interfascicular fibres, are S-lignin rich (Fig. 4, magenta colour). The ability of the F5H enzyme to catalyse the 5'-hydroxylation of the phenylpropanoid ring enables the biosynthesis of the sinapyl alcohol monolignol (and, thus, of S-lignin), which has led to the hypothesis that the *F5H* gene should be preferentially expressed in interfascicular and xylary fibres.

In agreement with this model, the *SND1* master switch from *Arabidopsis*, which is expressed in S-lignin-rich interfascicular fibres, was shown to directly activate the expression of reporter genes driven by the *Medicago truncatula F5H* promoter in transient protoplast expression assays (Zhao *et al*., 2010*b*). However, no characterized SND1-binding motifs were found in the *Medicago F5H* promoter (Zhao *et al*., 2010*b*), and it was recently shown that AtSND1 was unable to activate the corresponding *Arabidopsis F5H* promoter in similar assays (Öhman *et al*, 2012). Consistent with this result, the overall *cis*-element structure of the *Arabidopsis F5H* promoter was found to be distinctly different from that of the homologous *Medicago F5H* promoter. At the same time, loss of function at the *AtMYB103* locus was correlated with a marked suppression of *F5H* expression in *myb103* plants. Although significant sequence divergence also exists between the MtNST1 and SND1 coding sequences (Zhao *et al*., 2010*a*, *b*), loss of function of the *Medicago NST1* (*MtNST1*) gene results in defective interfascicular fibre differentiation in *Medicago* stems similar to that observed in *snd1/nst1* double mutants in *Arabidopsis*.

### Monolignol export

The metabolic reactions required for monolignol biosynthesis are believed to operate in the cytosol, or perhaps in the region of the cytosol directly associated with the endoplasmic reticulum (Boerjan *et al*., 2003; Vanholme *et al*., 2008). However, in order for monolignols to participate in polymerization to form the final lignin structure, they must move from their site of synthesis, across the plasma membrane to the cell wall. How this export is accomplished remains unclear. The results of recent studies are inconsistent with vesicle-mediated trafficking of monolignols to the cell wall (Kaneda *et al*., 2008). On the other hand, the small size of monolignols, and their demonstrated ability to partition into the membrane of synthetic lipid disks, supports the idea that monolignols could potentially exit the cell by passive diffusion (Boija and Johansson, 2006; Boija *et al*., 2007). In this model, monomer export would be driven by the concentration gradient between the cytosol, where monolignols are being actively synthesized, and the cell wall matrix, where they are rapidly polymerized into lignin. However, only low levels of monolignol diffusion across the membrane of plasma membrane vesicles have been reported (Miao and Liu, 2010). Since the rate of simple diffusion of the monolignols across the plasma membrane would have to be very high to account for the rapid and extensive lignification occurring in the maturing secondary cell wall,

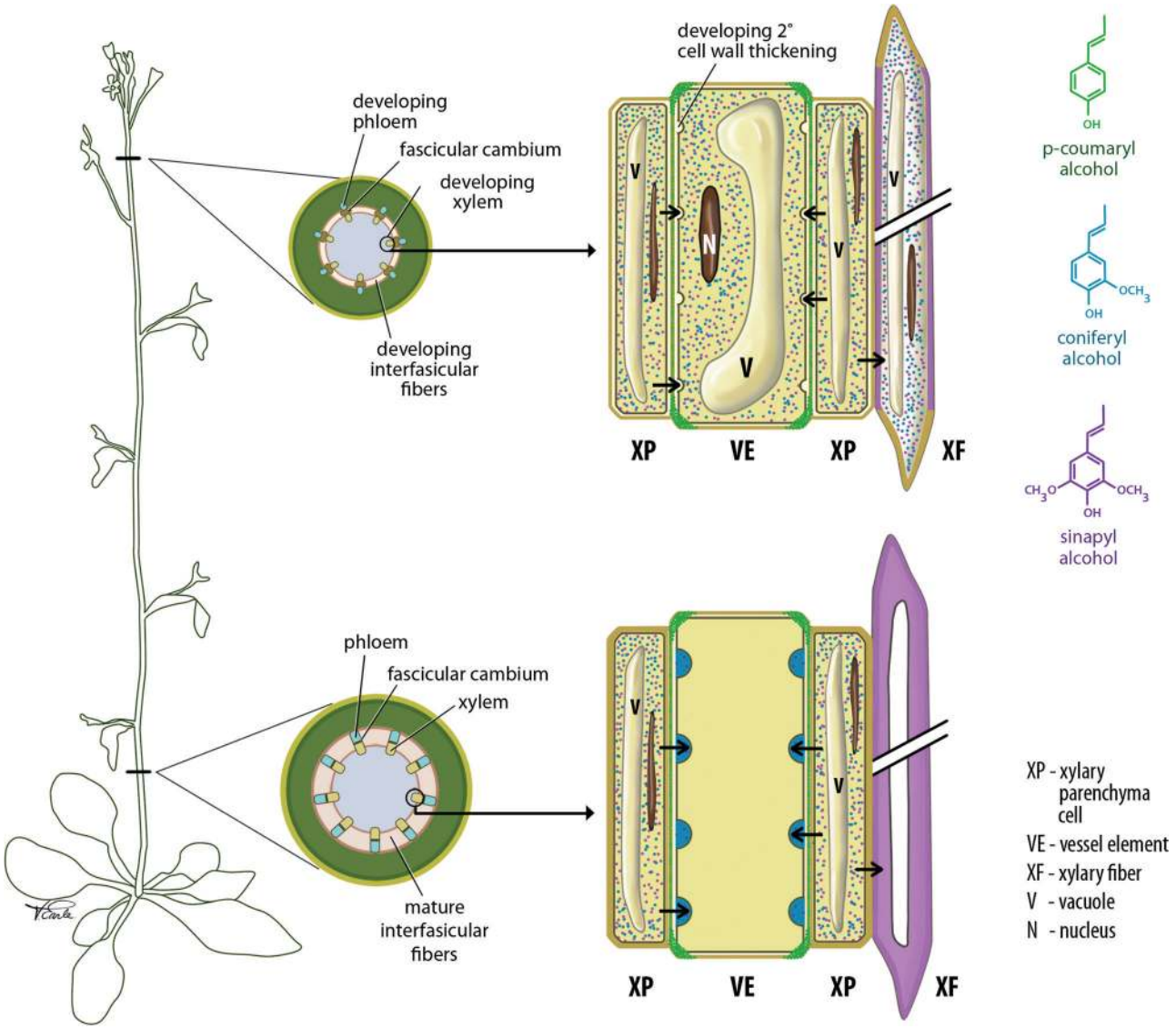


**Fig. 4.** The developmental progression of xylem cell types in the *Arabidopsis* inflorescence stem. A diagrammatic representation of the morphology of vessel elements (VE), xylary fibres (XF), and xylem parenchyma cells (XP) near the apex and at the base of the stem. Monolignols are indicated by coloured spots in the cytosol, while solid colour within cell walls indicates the location of H-rich (green), G-rich (blue), and S-rich (magenta) lignin. Vessel element lignification progresses from initial H-lignin deposits in the middle lamella and cell corners to accumulation of G-lignin-rich secondary cell wall thickenings over the course of development, while fibres form a more massive S-lignin-rich secondary cell wall. Both vessel elements and xylary fibres ultimately undergo programmed cell death, as indicated by the lack of cell contents of these cells near the base of the stem. Arrows from the xylem parenchyma cells to the vessel elements and xylary fibres indicate the putative metabolite contribution of neighbouring cells to the lignification process and demonstrate how lignification may proceed post-mortem.

alternative models postulating that monolignol export to the cell wall should occur via plasma membrane-localized transporters have also been put forward (Kaneda *et al*., 2008; Li and Chapple, 2010; Simmons *et al*., 2010). If appropriately configured at the plasma membrane, such putative transporters could not only meet the metabolite flux demands but could potentially account for the spatial precision with which lignin is deposited in the cell wall of different cell types (e.g. protoxylem versus metaxylem tracheary elements).

The identification of a monolignol transporter protein from among the hundreds of active transporters encoded in a plant genome would fill a significant gap in our understanding of the lignification process. Miao and Liu (2010) provided some insight into this question by testing the ability of plasma membrane vesicles derived from *Arabidopsis* seedlings to export monolignols. Transport of the coniferyl alcohol monolignol into these vesicles was shown to be primarily energy dependent, in keeping with the active transport hypothesis. Disruption of trans-membrane pH or potential gradients with pharmacological inhibitors did not affect the observed monolignol transport, but treatment with chemicals known to act as ABC (ATP-binding cassette) transporter inhibitors, such as vanadate or nifedipine,

greatly reduced monolignol accumulation in these vesicles (Miao and Liu, 2010).

No specific transport protein has yet been identified, however, and the plasma membrane vesicles used in the study of Miao and Liu (2010) were isolated from *Arabidopsis* seedlings in which only a small proportion of tissues would be undergoing lignification. Because several ABC transporters have been shown to facilitate the export of a wide range of low molecular weight, hydrophobic substrates, including auxin (Yazaki, 2006; Verrier *et al*., 2008), it is possible that the capacity to export monolignols is a general feature of multiple ABC-type transporters. The extent to which such functional promiscuity would be biologically relevant to lignification remains to be established.

A set of candidate ABC transporters for monolignol export was previously identified based on their co-expression with phenylpropanoid biosynthesis genes in developing *Arabidopsis* inflorescence stems (Ehlting *et al*., 2005), but subsequent loss-of-function mutant analysis did not uncover phenotypes associated with defective lignification (Kaneda *et al*., 2011). Instead, several of the loss-of-function ABC transporter mutants examined did display polar auxin transport defects (Kaneda *et al*. 2011). This observation is consistent with the transporter multifunctionality mentioned above, and because local auxin concentrations play a key role in determining vascular cell fate, the ability of these particular transporters to transport auxin might account for the observed correlation between expression of the corresponding genes and increasing inflorescence stem lignification (Ehlting *et al*., 2005). In light of such functional redundancy, and the size of the ABC transporter gene family (129 members in *Arabidopsis*), it is uncertain whether higher order mutant analysis would be capable of uncovering phenotypes consistent with defective monolignol export.

### *Lignin polymerization*

Once in the cell wall, monolignols are oxidatively polymerized through the process of radical combinatorial coupling (reviewed in Boerjan *et al*., 2003). The oxidation of the monolignol molecule is thought to be catalysed by one or more peroxidases and/or laccases, and the resulting radical is then coupled with a phenoxy radical on the growing lignin chain, through a process referred to as endwise polymerization (Morreel *et al*., 2004; Mechin *et al*., 2007). A recent study showed that laccases *LAC4* and *LAC17* are necessary for normal lignification of *Arabidopsis* fibre cell walls and, to some extent, of tracheary elements (Berthet *et al*., 2011). Expression of the *LAC4* gene was also found to be up-regulated in response to overexpression of the *MYB58* transcription factor, which suggests that *MYB58* could be activating genes involved in both monolignol biosynthesis and polymerization (Zhou *et al*., 2009). Unfortunately, the catalytic promiscuity and large gene families of peroxidases and laccases make it difficult to establish functional relationships between the activity of specific gene products and the spatiotemporal pattern of lignin deposition during xylem development. Many other aspects of the lignin polymerization process also remain unclear, including the physical nature of the association between lignin and other cell wall polymers, control of the spatial patterning of lignin deposition in the wall, and the relationship between the metabolic supply of G- and S-type monolignols and the composition of the final polymer.

In *Arabidopsis*, tracheary elements have secondary cell walls composed primarily of G-lignin while the walls of the interfascicular fibres are S-lignin rich (Fig. 4) (Chapple *et al*., 1992; Meyer *et al*., 1998). While we might, by analogy, expect the xylary fibre cell wall also to be S-lignin rich, recent data in poplar suggest that the walls of xylary fibres close to, or surrounded by, tracheary elements have a lignin composition that is intermediate between the lignin of G-rich tracheary elements and S-rich fibres (Gorzsas *et al*., 2011).

Spatial variability in lignin deposition was also revealed by a series of elegant autoradiography studies in pine, poplar, Japanese cedar, and Japanese black pine, demonstrating that the first stage of lignification during xylem development involves the incorporation of a mixture of H-lignin (dominated by 4-hydroxy ring structures) and G-lignin in the middle lamella and cell corners (Fujita and Harada, 1979; Takabe *et al*., 1981, 1985; Terashima and Fukushima, 1988). Since this pectin-rich area of the cell wall is hydrophilic, whereas lignin is hydrophobic, it has been hypothesized that the deposition of the lignin polymer in the middle lamella and cell corners may displace or modify pectin. In the next phase of wall lignification, the primary cell wall and outer layers of the secondary cell wall are lignified primarily with G-lignin (Terashima and Fukushima, 1988). The last stage of lignin deposition is directed to the innermost layer of the secondary cell wall (Fujita and Harada, 1979; Takabe *et al*., 1981, 1985), and in fibres it is largely S-lignin that is formed at this stage (Terashima and Fukushima, 1988). If these temporal and spatial patterns are consistent across higher plant taxa, it is clear that highly integrated intracellular mechanisms must exist that focus both the genetic and metabolic resources of developing xylem cells on formation of the final cell wall structures.

### *Programmed cell death and lignification models*

Secondary cell wall formation and patterned deposition is tightly regulated in specific xylem cell types, but much about the fine regulation of the transcriptional network remains unknown. During fibre development, the thick secondary cell wall is formed over an extended period, whereas tracheary element differentiation progresses quickly from secondary cell wall deposition to PCD, the final stage of differentiation. The expression of several genes functionally associated with PCD is correlated with xylem development (Zhao *et al*., 2000), particularly with tracheary element differentiation (Funk *et al*., 2002; Ito and Fukuda, 2002; Avci *et al*., 2008) and some [e.g. *XYLEM CYSTEINE PROTEASE1* and *2* (*XCP1* and *XCP2*); *BIFUNCTIONAL NUCLEASE1* (*BFN1*)] have also been shown to be directly activated by both the *VND7* and *VND6* transcription factors (Ohashi-Ito *et al*., 2010; Zhong *et al*., 2010*b*). In contrast, *SND1* has not been shown to regulate the expression of genes mediating PCD, which is consistent with a more specific role for *SND1* as a regulator of secondary cell wall formation in fibres (Zhong *et al*., 2006, 2010*b*; Ohashi-Ito *et al*., 2010).

The proposed role of the cysteine proteases in executing PCD was confirmed by the examination of *xcp1* single mutants and *xcp1xcp2* double mutants, which displayed incompletely

degraded cellular contents within tracheary elements (Avci *et al*., 2008). The localization of *XCP1* expression to tracheary elements is mediated by SNBE regulatory regions in the *XCP1* promoter, but other unidentified factors or interactions must be involved to restrict the expression of SNBE-regulated genes effectively (e.g. *XCP1*) to specific cell types such as the tracheary elements (Zhong *et al*., 2010*b*).

Gene transcript profiling in the *Zinnia* tracheary element differentiation cell culture system has helped identify which genes are specifically up-regulated prior to the onset of PCD in these cells (Groover *et al*., 1997; Fukuda, 2000). These data, together with several whole plant studies, suggest that PCD during tracheary element differentiation is an orderly and actively regulated cell-autonomous process (Fukuda and Komamine, 1980; Groover *et al*., 1997). Unlike many other types of PCD in plants, the large central vacuole of the nascent tracheary element cell plays a critical role during this process (Roberts and McCann, 2000). Modifications or disruptions of the tonoplast (vacuolar membrane), and accompanying changes in the vacuolar contents, define the initial stage of PCD. The subsequent rupture of the vacuole and release of digestive enzymes such as nucleases and proteases results in digestion of all the cell contents, leaving only the cell wall intact (Fukuda, 2000). XCP1 and XCP2 proteins appear to be localized within tracheary elements prior to this vacuolar implosion, and can still be detected, post-implosion, in the space formerly occupied by the vacuole (Avci *et al*., 2008). Transcript profiling in the *Zinnia* cell culture system has also identified other nucleases, proteases, and lytic enzymes putatively stored in the vacuole, which are likely to be involved in the vacuole-mediated tracheary element PCD (Groover *et al*., 1997; Fukuda, 2000).

A study in poplar (Courtois-Moreau *et al*., 2009) has confirmed that tracheary elements undergo PCD and associated vacuolar collapse much earlier than do fibres. In contrast to the rapid vacuolar collapse that marks tracheary element PCD, PCD in fibres starts with DNA degradation and cellular dismantling in advance of vacuole collapse (Courtois-Moreau *et al*., 2009). The difference in cell death programming between these developmentally distinct cell types is reflected not only in the specific events leading to the 'dead cell' end-point, but also in the speed of the autolysis. Tracheary element PCD occurs rapidly, with the vacuolar implosion requiring only a few minutes, and the clearance of the remainder of the cell contents is completed within a few hours (Groover *et al*., 1997), whereas fibre PCD takes much longer. The timing of fibre PCD has not been extensively studied, but a study performed in poplar (Courtois-Moreau *et al*., 2009) showed that xylem tracheary elements positioned within 400 µm of the cambium undergo PCD, i.e. at an early stage of xylem development, while xylem fibre PCD occurs 650–1000 µm from the cambium.

While PCD appears to operate as a cell-autonomous process, it has been hypothesized that lignification of the cell walls in tracheary elements may be non-cell autonomous (Pickett-Heaps, 1968). The non-cell-autonomous lignification model suggests that non-lignifying cells such as xylary parenchyma cells, positioned adjacent to lignifying cells such as tracheary elements, are capable of synthesizing monolignols and exporting them to the cell wall of the neighbouring lignifying cells. Studies in the *Zinnia* cell culture system have demonstrated that it is possible for lignification of tracheary element-like cells to proceed even after PCD. Thus, when dead tracheary elements were moved from the tracheary element induction medium to another medium containing added monolignols, the tracheary elements were able to use monolignols from the extracellular solution to continue lignification post-mortem (Hosokawa *et al*., 2001; Tokunaga *et al*., 2005). Because *Zinnia* cell cultures, in which mesophyll cells are induced to transdifferentiate to form tracheary element-like cells, contain many cells that remain in a state similar to xylem parenchyma cells (McCann *et al*., 2001), it has been hypothesized that these parenchyma-like cells may act as 'good neighbours' for the differentiating tracheary elements and provide them with an exogenous supply of monolignols.

Further support for such a model (Fig. 4) comes from the observation that some lignin biosynthesis genes are expressed not only in lignifying tracheary elements and fibres of *Arabidopsis*, tobacco, and poplar, but also within xylem parenchyma cells and poplar ray cells situated adjacent to dead tracheary elements (Bevan *et al*., 1989; Hauffe *et al*., 1991; Feuillet *et al*., 1995; Chen *et al*., 2000; Baghdady *et al*., 2006). There is no evidence that a similar cooperative lignification process occurs in fibres (Baghdady *et al*., 2006), and the longer development time for fibres is likely to be sufficient to allow for complete cell-autonomous lignification. In contrast, the rapidity with which tracheary elements undergo PCD may leave limited time for cell-autonomous lignification, in which case the xylem parenchyma neighbours might continue to export monolignols to the cell wall after tracheary element PCD, and thereby further strengthen the wall.

It has also been suggested that, in addition to the degradative enzymes released by the vacuole during tracheary element vacuole collapse, monolignols stored in that compartment could be released, and that lignification is therefore primarily a post-mortem process (Pesquet *et al*., 2010). In this model, monolignols would be synthesized prior to cell death, and small amounts might be deposited and polymerized in the cell wall. However, the bulk of the monolignol pool would be stored in the vacuole (Pesquet *et al*., 2010), and only when the vacuole collapses would the monolignols diffuse rapidly into the cell wall to be polymerized (Pesquet *et al*., 2010). Most lignin polymerization would therefore occur after PCD of the tracheary element. However, as with the 'good neighbour' hypothesis described above, there is little direct *in planta* evidence for such a model. Monolignol localization using microautoradiography suggests that tracheary elements in *Arabidopsis* are still living while the cell wall is being lignified (Kaneda *et al*., 2008; R. Smith, unpublished data), but this observation does not preclude lignification continuing to proceed following PCD, either through vacuolar release of monolignols or their acquisition by donation from neighbouring, non-lignifying cells.

## Acknowledgements

This work was supported by the Working on Walls (WOW) Collaborative Research and Training Experience Program funded by NSERC. We thank Etienne Grienenberger, Teagen Quilichini and Yoshi Watanabe for helpful suggestions to improve the manuscript, and Vicky Earle (UBC IT creative media) for assistance with figure preparation.

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