Connections in the cambium, receptors in the ring

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Highlights

In plants, pluripotent cells in meristems divide to provide cells for the formation of postembryonic tissues. The cambium is the meristem from which the vascular tissue is derived and is the main driver for secondary (radial) growth in dicots. Xylem and phloem are specified on opposing sides of the cambium, and tightly regulated cell divisions ensure their spatial separation. Peptide ligands, phytohormones, and their receptors are central to maintaining this patterning and regulating proliferation. Here, we describe recent advances in our understanding of how these signals are integrated to control vascular development and secondary growth.

Introduction

A prerequisite for size is structural support, and the ability to distribute water and nutrients. The vascular tissue performs these functions, with xylem providing mechanical strength and movement of water and solutes from roots, and phloem distributing photosynthates and bulk flow of phytohormones (reviewed in [1, 2]) including auxin [3], cytokinin [4-7], gibberellin [8-10] and abscisic acid (ABA) [11-14], to facilitate physiological responses and regulate plant development. Specification of xylem and phloem cell-type-identity and function has been summarised in recent reviews [15-22]. Here, we review signalling mechanisms that regulate the homeostasis of the cambium, the meristematic tissue from which the xylem and phloem are derived.

Hormone harmonies

Most plant hormones play a role in the regulation of cambial activity [10, 23-25], but the most prominent and well-studied are auxin and cytokinin. Auxin is involved in numerous developmental processes, operating through a signalling pathway that includes auxin/indole-3-acetic acid inducible Aux/IAA proteins, TRANSPORT INHIBITOR RESPONSE 1 (TIR1) receptor, and the AUXIN RESPONSE FACTOR (ARF) family of genes [26-31]. ARFs act as transcriptional regulators [30, 31]. Of these, ARF5/MONOPTEROS (MP) regulates proliferation in the vascular stem cell niche, as well as performing distinct roles in early and late stages of vascular development. During embryogenesis, *mp* mutants fail to establish a central axis in the provascular cylinder [32, 33]. Weak *mp* alleles also demonstrate disrupted auxin transport [32, 34, 35] due to MP directly activating transcription of several PIN-FORMED (PIN) auxin efflux transporters [36, 37]. Thus, in early development, MP promotes vascular proliferation. Late in development, during secondary growth, *mp* mutants demonstrate increased cambial divisions, suggesting that in this context MP suppresses vascular expansion [38, 39]. Conversely, other auxin response factors, ARF3 and ARF4, have been shown to operate in concert to upregulate cambium activity [39].

Cytokinin also contributes to cambium development, with loss of cytokinin-synthesizing genes deterring cambium formation and thus radial vascular expansion [40, 41]. Cytokinin signalling occurs via a phosphorelay, which begins with cytokinin perception by its family of receptors CYTOKININ RESPONSE 1 (CRE1)/WOODEN LEG (WOL)/*Arabidopsis* HISTIDINE KINASE4 (AHK4), AHK2 and AHK3 [41-45]. Following perception, *ARABIDOPSIS* PHOSPHOTRANSFER PROTEINS (AHPs) AHP1-AHP6 are activated [46-48], with AHP1-AHP5 promoting cytokinin signalling, and AHP6, acting as a pseudo-AHP and thus as a negative regulator of the signal [46-49]. In the final steps of the signalling cascade, AHPs 1-5 phosphorylate type-B *ARABIDOPSIS THALIANA RESPONSE REGULATORS* (*ARRs*), transcription

factors that promote cytokinin responses including vascular proliferation. AHPs also trigger the transcription of type-A ARRs, which in turn suppress cytokinin responses, thus buffering the system [50-55].

Auxin and cytokinin ratios influence the balance between cell division and differentiation during plant development [56-61]. Their concentration gradients span the vascular tissue with a cytokinin maxima in the phloem, and an auxin maxima on the xylem side of the cambium (Figure 1)[38, 39, 62]. Cross-talk between these hormones is likely important in establishing the auxin/cytokinin ratios. Auxin stimulates the expression of cytokinin oxidase (CKX), a major cytokinin deactivating enzyme [63], and suppresses the transcription of *isopentenyl transferase* (*IPT*) genes that encode cytokinin-promoting enzymes [64, 65]. Auxin also increases expression of *AHP6* which as described above dampens cytokinin signalling [5, 47, 49]. In the root xylem axis, MP/ARF5 promotes the transcription of TARGET OF MONOPTEROS 5 (TMO5), a bHLH transcription factor that forms a heterodimer with LONESOME HIGHWAY (LHW). In turn, the TMO5-LHW heterodimer upregulates cytokinin biosynthesis genes *LONELY GUY3/4 (LOG3/4)* [66, 67]. Cytokinin notoriously acts on auxin by controlling distribution and levels of auxin transport's main conductors, the PIN-FORMED (PIN) proteins [5, 58, 60, 68, 69]. Cytokinin application strongly affects PIN transcription levels, downregulating PIN1-PIN4 and upregulating PIN7 [60]. In developing roots and shoots, transcription levels of auxin biosynthesis genes were stimulated by cytokinin, thus promoting auxin production [70]. Cytokinin also induces expression of group of related DOF-family transcription factors, DOF2.1, DOF6, TMO6, PHLOEM EARLY DOF 1 (PEAR1), PEAR2, OBF BINDING PROTEIN 2 (OBP2) and HIGH CAMBIAL ACTIVITY 2 (HCA2) which promote procambial cell divisions [71-73].

Peptides and proliferation

Peptide ligands and their cognate receptors contribute substantially to secondary growth and patterning. The cambium-expressed leucine-rich repeat receptor-like protein kinase (LRR-RLK) PHLOEM INTERCALATED WITH XYLEM (PXY), also known as TDIF-RECEPTOR (TDR) [74, 75] and its phloem-expressed ligand TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF) are essential for cell proliferation and division plane specification (Figure 1; Figure 2a) [75-79]. TDIF, encoded by *CLAVATA3/ENDOSPERM SURROUNDING REGION 41* (*CLE41*), *CLE42* and *CLE44*, was identified as a repressor of xylem differentiation and is structurally similar to CLAVATA3 (CLV3) [76], a peptide ligand that regulates meristem maintenance in shoots and signals to receptor CLV1 [80, 81]. *pxy* mutants were first described as lacking separation between cambium-derived phloem and xylem tissues and as having disrupted orientation of cambial cell divisions [74]. Hirakawa et al. (2008) independently identified PXY by testing loss-of-function mutants in relatives of CLV1, for TDIF insensitivity [75].

Since CLV signalling acts to repress expression of homeodomain transcription factor WUSCHEL (WUS) [82, 83], potential transcript targets of TDIF/PXY signalling were hypothesised to be members of the WUSCHEL-RELATED HOMEOBOX (WOX) family [84, 85]. WOX4 exhibited a rise in expression levels following TDIF treatment, and WOX14 was identified as being down-regulated in *pxy* mutants. Both WOX4 and WOX14 were seen to stimulate cambial cell proliferation [78, 79], with WOX14 cooperatively controlling expression of LOB DOMAIN-CONTAINING PROTEIN (LBD4) transcription factor with a DOF transcription factor, TMO6 (Figure 1, 2a) [86].

The PXY/TDIF signalling module influences outputs of auxin signalling. For instance, PXY acts to represses one glycogen synthase kinase-3 (GSK3), BIN2-LIKE 1 (BIL1). In the absence of PXY, BIL1 phosphorylates MP (Figure 2A), which is thought to loosen MP's interaction with an IAA suppressor, thus releasing it to control gene expression [38]. Recently, Smetana et al (2019) have reported a positive influence of auxin/MP on PXY expression in the initial stages of cambium formation in roots [87]. Since the PXY-BIL1-MP negative interactions were shown to function in the stem [38], an

interesting question is whether a negative feedback loop might exist between MP and PXY, wherein MP attenuates its own activity by boosting PXY expression – or whether the regulation is organ-specific. While PXY represses BIL1, it activates other GSK3s and most notably, BRASSINOSTEROID INSENSITIVE 2 (BIN2) in the presence of TDIF. Active BIN2, in turn, phosphorylates a transcription factor BRI1 EMS SUPPRESSOR 1 (BES1), marking it for degradation. BES1 promotes xylem differentiation (Figure 1, 2A), thus its removal protects the cambium from differentiation [88].

A ring of receptors

LRR-RLKs of the SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (SERK) family, including BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1), are thought to form complexes with PXY at the plasma membrane in the presence of TDIF (Figure 2A) [89]. BAK1 also functions as a co-receptor for brassinolide with BRASSINOSTEROID INSENSITIVE 1 (BRI1), and as a co-receptor for bacterial flagellin peptide (flg22) with FLAGELLIN SENSING 2 (FLS2), and in these interactions the ligands act as molecular glue for the BAK1-BRI1 and BAK1-FLS2 interaction [90-92]. The PXY-SERK interactions likely differ from those described for other receptors. PXY LRR domains are shorter, and the receptor domain lacks the curvature of BRI1 and FLS2. TDIF binds PXY further from the membrane, clear of the BAK1-PXY interaction site and is thus its function in this respect is distinct in that it is unlikely to mediate a SERK-RLK interaction [93].

In pursuit of other vascular regulators, Yang et al. (2019) analysed gain-of-function activation-tagging lines, one of which, *xvp-d*, demonstrated *pxy*-like morphology [94] (Figure 2). *XVP* encodes a cambium-expressed transcription factor of the NAC family which surprisingly localised to the plasma membrane. Bimolecular fluorescence complementation (BiFC), a split ubiquitin yeast-two-hybrid system (mbSUS) and a Fluorescence Resonance Energy Transfer (FRET) assay support the notion that XVP binds to the PXY-BAK1 complex (Figure 1; Figure 2A, D, E). Removal of *XVP* enhanced TDIF activity, suggesting that XVP represses vascular proliferation by allowing xylem differentiation to occur. *xvp-d* gain-of-function lines demonstrated increased *CLE44* expression, while *CLE41* and *CLE44* overexpression lines demonstrated reduced XVP expression. Thus, XVP promotes the expression of TDIF-encoding genes, but suppresses the TDIF signal and is itself repressed by TDIF (Figure 2A) [94].

In the hypocotyl, *ERECTA (ER)* and its paralogues *ERECTA-LIKE (ERL1)* and *ERECTA-LIKE (ERL2)* have been reported to promote auxin biosynthesis [95]. Of these, *ER* and *ERL1*, have been shown to prevent premature xylem fibre formation, as *er erl1* lines exhibited precocious fibre differentiation [96]. *er* enhances the loss-of-function phenotype for another LRR-RLK, SUPPRESSOR OF BIR-1 (SOBIR1)/EVERSHED (EVR) [97], which is also responsible for preventing early xylem fibre formation in *Arabidopsis* hypocotyls. ER and SOBIR1 physically interact at the plasma membrane to perform this function. ER family members regulate not only the xylem, but also the cambium. ER and ERL1 are thought to restrict radial expansion of hypocotyls as *er erl1* lines exhibit increases in xylem area (Figure 1; Figure 2A) [96]. By contrast, the *er erl1 erl2* triple mutants demonstrate a reduction in secondary growth [98], thus interplay between these three receptors in the context of cambium regulation requires further investigation.

ER family regulation of vascular development occurs via a genetic interaction with members of the *PXY* gene family. In the absence of the *PXY* gene family (*PXY*, *PXY-LIKE 1(PXL1)* and *PXL2*), vascular cells are larger, however this increase is dependent upon *ER* and *ERL2*, as *pxy pxl1 pxl2 er erl2* lines have cell sizes similar to those of wild type. Removing all members of both families prevented the transition to true secondary growth, as cell division was vastly reduced and phloem was present in poles rather than a continuous ring as is the case in wild type. Thus, interacting PXY and ER families regulate cell division, cell size, and organisation in the vascular tissue (Figure 1; Figure 2B, C) [98].

Like *ER* and *ERL1,* a LRR-RLK, MORE LATERAL GROWTH (MOL1), also suppresses cambial activity as *mol1* mutants demonstrated larger cambium-derived domains compared to wild type [99, 100]. MOL1 was identified in a set of experiments where *Arabidopsis* inflorescence stem explants were subjected to auxin (NAA) treatments. These treatments initiated cambium formation in the explants which were then subjected to transcriptome analysis. REDUCED IN LATERAL GROWTH (RUL1), a receptor with a positive effect on cambium activity was additionally identified in these experiments [99]. While ER's signal peptides have been determined to belong to the *EPIDERMAL PATTERNING FACTOR LIKE* (*EPFL)* family [101-108], exactly which of them control cell division in the cambium is yet to be determined. Ligands for MOL1, RUL1 and SOBIR1 are also to be discovered.

Ontogeny of the Organiser

The cambium represents a group of mostly periclinally dividing cells with the ability to generate xylem and phloem, on its two opposite sides [87, 109, 110]. A vascular organizer in xylem cells adjacent to the initiating cambium that is characterized by high auxin levels, imposes stem-cell function on its neighbour to initiate cambial divisions [87]. Since at the secondary growth stage xylem cells have already undergone programmed cell death thus stripping them from signalling ability, Smetana et al. (2019) proposed that cell identity information must be passed on earlier, during xylem formation [87]. Auxin, acting through MP, ARF7 and ARF19, promotes the expression of HD-ZIP III genes, which have been previously reported as regulators of xylem identity [111-114] downstream of auxin [115, 116]. Here, they were linked to the correct establishment of the vascular organizer [87]. *WOX4* and *PXY*, which are required for auxin responses in the cambium [117], were also required in the stem-cell organizer [87].

While Smetana et al. (2019) characterised the ability of the xylem to specify the position of the initiating cambium in adjacent cells in the *Arabidopsis* root [87], Shi et al. (2019) aimed to explore pattern in the established hypocotyl vasculature [109]. The cambium was found to be separated into three distinct sub-domains in each cell file along the radial axis – proximal, central, and distal. Independently, both Smetana et al. (2019) and Shi et al. (2019) defined PXY and WOX4 as part of the xylem-facing side of the cambium, i.e. the organizer side of the cambium, verifying the importance of these components for the cambium activity [87, 109]. They also confirmed a long-standing hypothesis in which the cambium stem cells (central) are flanked by mother cells of the xylem (proximal) and phloem (distal) within each vascular cell file [118].

Sapling similarities

A significant proportion of the molecular mechanisms controlling cambium growth and development comes from studies of Arabidopsis, but evidence suggests that much is conserved in forest trees. One such example is ethylene, which promotes cell division in the cambium of both *Arabidopsis* and poplar [23, 119]. In poplar, ethylene-induced *ETHYLENE RESPONSE FACTORS (ERFs)* that were overexpressed altered wood formation and stem diameter [120]. Our understanding of the distribution of auxin and cytokinin in the dividing cambia and phloem arose from experiments in poplar. These patterns are supported by transcript profiling showing that cytokinin and auxin responses coincide with tissue-specific hormonal gradients. Transgenic *Populus* with elevated cytokinin biosynthesis displayed increases in cambial auxin concentration and a dramatic increase in secondary growth, confirming the auxin-cytokinin connection [62, 121].

Alongside the TDIF-PXY-WOX4 signalling module [122, 123], further members of the CLE family also regulate the cambium in poplar. *PttCLE47* positively regulates cell division in the vascular cambium, as its repression led to reduced secondary xylem formation. PttCLE47 appears to act in a cell-autonomous fashion in the vascular cambium [124]. By contrast, *PtrCLE20*, expressed specifically in developing xylem, was found to reduce cambial divisions in part by reducing *PtrWOX4* expression [125]. Thus, CLE peptides influence the cambium from opposing sides; *PttCLE41* (from which TDIF is

derived) acting from phloem, *PtrCLE20* from the xylem, and *PttCLE47* operating from within the cambium [122-125].

Conclusions

Interactions between LRR-RLKs, their ligands, cytoplasmic signalling intermediates, and their targets are increasingly well-defined in our understanding of cambium regulation [126, 127]. A recent study has proposed a transcriptional network that may explain many of the relationships between these components [86]. Identification of further signalling elements, such as ligands for MOL1 and RUL1 will help refine this picture. Remaining challenges surround hormones such as gibberellic acid and jasmonic acid, known to contribute to radial growth [24, 128], but whose role in the existing networks is largely unexplored. Much of what we know has also been characterised in a single tissue type, but differences in cambium regulation occur along the apical-basal axis of the plant [98] and how those differences underpin variations in morphology remains unclear. Finally, this review has focussed mostly on Arabidopsis, and entirely on dicot species. A recent analysis of cambium-regulating genes identified a small number of genes that were absent in the monocot clade [129]. Thus, an important question concerns how these networks may have been modified to give rise to the significantly different scattered vascular morphology of grass species.

Conflict of interest statement

Nothing declared.

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Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

Of outstanding interest

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Intergration of multiple signalling components into a single network.

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Figures and legends

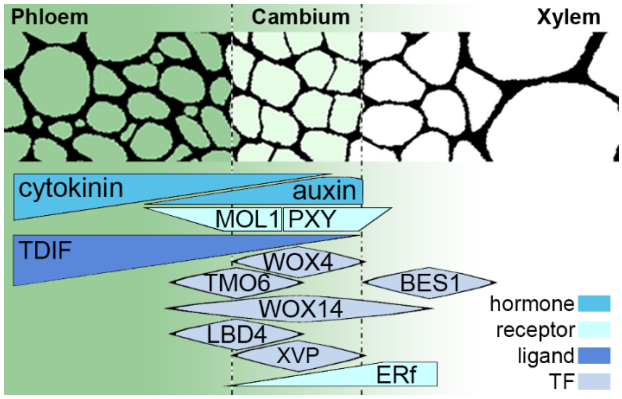

Figure 1. Stylised depiction of protein distribution and auxin and cytokinin accumulation across the vascular cambium in wild type plants. Cytokinin has a concentration maxima in the phloem; auxin on xylem–adjacent cambium. RLK's MOL1 and PXY are expressed on phloem facing and xylem facing cambium, respectively; ERf receptor expression spans the cambium. TDIF ligand is expressed in the phloem and perceived by PXY. Transcription factors WOX4, WOX14, and XVP exhibit maxima in the cambium. BES1 is present in the xylem; TMO6 and LBD4 expression as at the edge of the cambium on the phloem side.

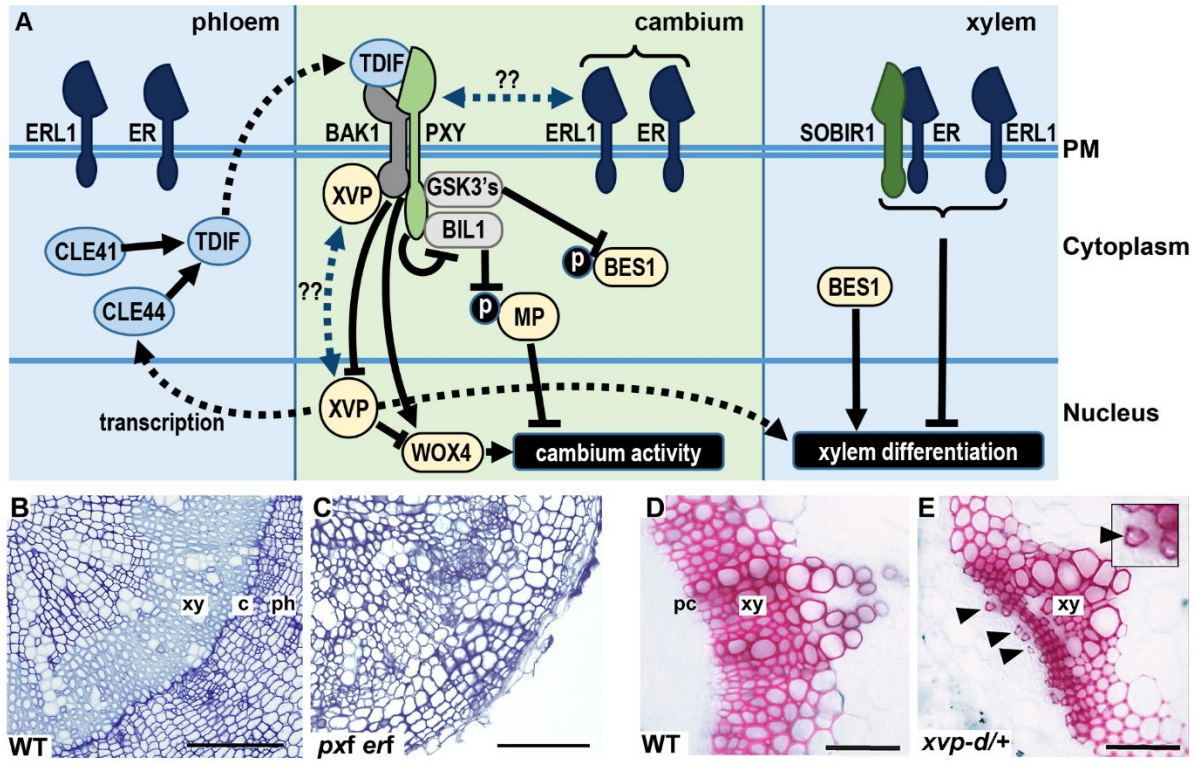

Figure 2. Signals that regulate cambium activity in *Arabidopsis*. (A) Schematic representation of phloem, cambium and xylem with signal components shown in the plasma membrane (PM),

cytoplasm, and nucleus. RLK's are shown in the PM, PXY ligand components are blue, transcription factors are yellow, and GSK3's are grey. '??' on blue dashed arrows indicates limited understanding (signals promoting XCP translocation to the nucleus are not known; partial evidence for a physical interaction between PXY and ER family receptors has been reported). 'P' indicates phosphorylation. (B-C) Hypocotyl transverse sections, with wild type (WT; B) showing distinct phloem (ph), xylem (xy) and cambial (ca) domains. (C) Loss of both *PXY* and *ER* family of genes results in loss of distinct tissue domains. Plants also fail to make the transition to true secondary growth. (D-E) Stem sections stained for lignin, adapted from Yang et al. (2019), with the permission from the publisher. (D) WT shows lignin deposition and thus xylem differentiation in a single arc (D). *xvp-d/+* lines demonstrate premature xylem differentiation in the regions marked by arrowheads.

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