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(Pro)cambium formation and proliferation: two sides of the same coin?

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The body of higher plants is usually pervaded by the (pro)cambium, a reticulate system of meristematic cells harboring the potential for producing vascular tissues at critical times and places. The (pro)cambium thereby provides the basis for the differential modulation of long-distance transport capacities and plant body stability. Distinct regulatory networks responsible for the initiation and proliferation of (pro)cambium cells have been identified. However, although a tight interaction between these networks can be expected, connections have been established only sporadically. Here we highlight recent discoveries of how (pro)cambium development is regulated and discuss possible interfaces between networks regulating two processes: (pro)cambium formation and cambium proliferation.

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Introduction

Maintaining the potential for regenerating and modulating body structures after embryogenesis is a critical aspect during the development of multicellular organisms. In this context, plants have developed an unparalleled ability to continuously adapt their growth to changing environmental conditions and to renew themselves after major damages. This developmental plasticity is based on the activity of stem cells present in meristematic tissues, which are located at key positions within the plant body. Among these meristems, the procambium and — in mature organs — the vascular cambium pervade the whole body of most vascular plants, giving rise to vascular tissues. In many species, including *Arabidopsis thaliana*, the xylem (wood) is delivered toward the center of the growth axis (adaxially) and the phloem (bast) toward the periphery (abaxially). These tissues are not only essential for the long-distance transport of water, assimilates, signaling molecules and nutrients but also to provide mechanical support for the growing plant body. Thus, (pro)cambium formation and activity is a major determinant of postembryonic plant growth, since it represents a connected and omnipresent system of stem cells, which, when required, generates tissues important for the modulation of body structures [1–4]. Strikingly, the establishment and maintenance of this essential network of stem cells in a differentiated cellular environment at the level of individual cell types is hardly understood, and only recently have studies started to generate insight into this fundamental aspect of plant growth and development.

Here, we review investigations on procambium formation and the control of cambium activity by focusing on latest insights obtained using the reference plant *Arabidopsis thaliana* and translate these into a broader context. For a more comprehensive overview of the topics, we refer the reader to excellent summaries published elsewhere [5–8].

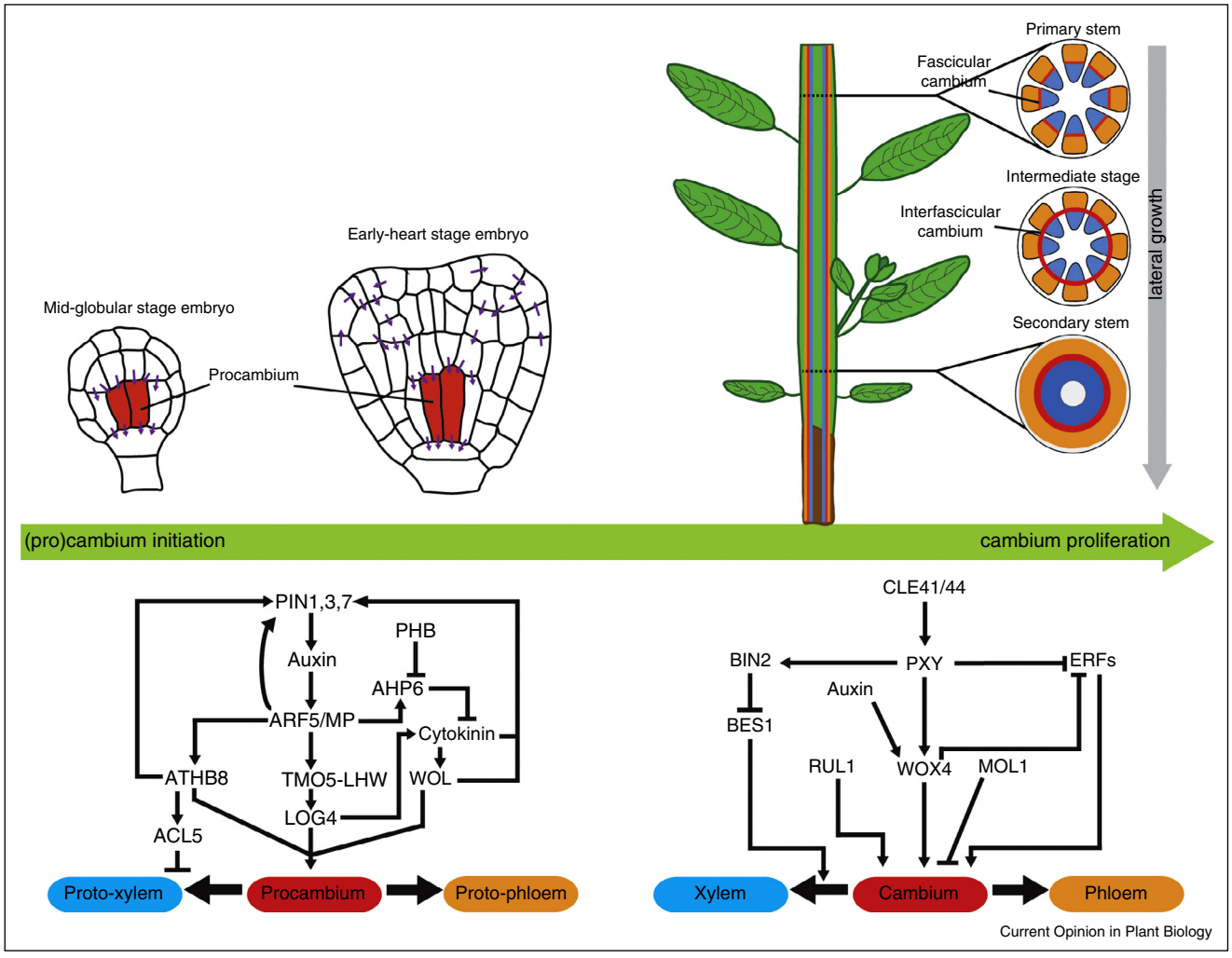
Procambium initiation and development

The first procambium appearance in the embryo

During seedling germination, vascular tissues of the root, hypocotyl and cotyledons differentiate from a predetermined tissue, the procambium, located in the innermost domain of these organs [9]. In *Arabidopsis*, the procambium goes back to four initial cells established as early as the globular embryo stage [10] (Figure 1). After their specification, these cells elongate and undergo oriented and coordinated cell divisions, thereby increasing the number of procambial cells and establishing their typical strand-like anatomy up to the mature embryo stage [11] (Figure 1). At this point, a subset of procambial cells undergo asymmetric divisions, generating precursors of phloem and xylem cells while maintaining a pool of procambium cells between these tissues [12].

The plant hormone auxin is strongly associated with procambium formation, as the establishment of local auxin maxima precedes procambium formation in all cases investigated so far. Among auxin signaling factors, the auxin-dependent transcription factor MONOPTEROS/AUXIN RESPONSE FACTOR 5 (MP/ARF5) plays a major role in translating auxin accumulation into the establishment of procambium identity [13,14]. Although expressed before the globular stage, *MP/ARF5* expression soon becomes strongly associated with procambial tissues (Figure 2)

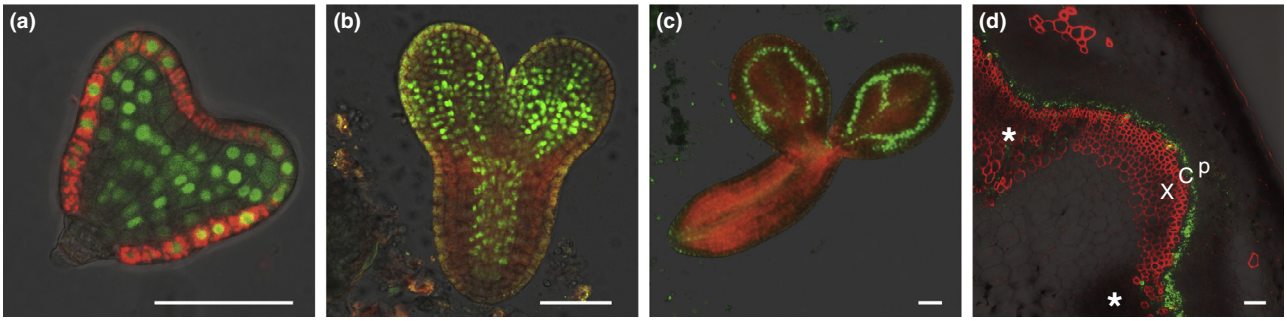
Figure 1



Initiation and proliferation of the (pro)cambium along plant life. The two diagrams render the pathways implicated in the initiation of the procambium (lower part left) and in cambium proliferation (lower part right). A schematic view of two embryo stages is depicted in the upper left part, with procambium initial cells in red. A schematic view of vascular tissue organization in stems is shown in the upper part right. The xylem is represented in blue, the phloem in orange and the cambium in red. Auxin flow in the embryo is indicated by purple arrows. Spatial arrangements of signaling components are not represented in the bottom diagrams.

and strong *mp/arf5* mutants display severe defects in procambium formation [13,14,15••]. How MP/ARF5 fulfills its role in procambium formation was elucidated very recently by identifying candidates for direct and procambium-specific MP/ARF5 targets [15••,16,17]. Among these, the basic helix-loop-helix (bHLH) transcription factor *TARGET OF MONOPTEROS5* (*TMO5*) is first expressed in all four procambium initials at the globular stage. Later, *TMO5* expression is restricted to xylem precursor cells in the root apical meristem (RAM) and presumably along the whole vasculature [15••,18••]. In the embryo and the RAM, periclinal (i.e., in parallel to the organ surface) divisions of procambium cells depend on a protein dimer formed by TMO5 and LONESOME HIGHWAY (LHW) (Figure 1), another bHLH transcription factor [18••,19]. Redundantly acting protein dimers are also formed by close homologs of TMO5 and LHW, mainly T5L1 and LL1, respectively [18••,20]. LHW and LL1 are expressed in the basal domain of the globular embryo and, later, in the RAM, thereby contributing to the spatial specificity of respective dimer activity [18••,19,20]. Importantly, ectopic expression of *TMO5* and *LHW* is sufficient for inducing periclinal cell divisions in other tissues, suggesting that the TMO5/LHW dimer mediates this fundamental procambium attribute independently of cell identity [18••]. Furthermore, ectopic *LHW* expression is sufficient to induce auxin responses like *PIN1* and *MP/ARF5* expression, suggesting that *LHW* is not only downstream but also upstream of auxin signaling [19].

Figure 2



MP/ARF5 promoter activity at different developmental stages. **(a)–(c)** *MP/ARF5* (TAIR: AT1G19850) promoter activity in the *Arabidopsis* embryo (heart (a), early torpedo (b), and mature (c) stage) visualized by a stably transformed *pARF5:SV40-3xGFP* reporter [60] (green). Cell walls are counterstained by FM4-64 (red). **(d)** *MP/ARF5* promoter activity in the mature *Arabidopsis* stem visualized by a *pARF5:ER-EYFP* reporter (green). Cell walls are counterstained by propidium iodide (red). c, cambium; p, phloem; x, xylem. Scale bars: 50 μm. Asterisks in (d) label the position of primary vascular bundles.

It is worth mentioning that MP/ARF5 requires the PHD-finger proteins OBERON1 (OBE1) and OBE2 for *TMO5* activation in the basal part of the embryo [21]. However, whether OBE-like proteins are auxin-specific or more general transcriptional regulators remains as yet obscure. Similarly, although MP/ARF5 activity is maintained in the postembryonic (pro)cambium (Figure 2), the role for the MP-TMO5/LHW module in later developmental stages is unclear.

Mutual interaction between vascular tissues establishes procambium cell identity

A mutually inhibitory interaction between auxin and cytokinin (CK) signaling is an important aspect of procambium formation [22•,23]. In the growing root, perturbation of CK signaling, by mutating *ARABIDOPSIS HISTIDINE KINASE* genes (*AHK2*, *AHK3*, *AHK4/WOL/CRE1*) encoding two-component signal transducers of CK signaling, results in the reduction of periclinal cell divisions in the procambium and in the differentiation of all procambium cells into protoxylem, reflecting a cell-autonomous CK-dependent promotion of procambium identity [12,24••]. Reduced CK-signaling leads also to altered subcellular polarity of the auxin efflux carriers PIN-FORMED 1 (PIN1), PIN3 and PIN7, and a loss of *PIN7* expression in the procambium and the phloem along which CK is symplastically transported [22•,23] (Figure 1). PIN-mediated lateral transport of auxin, in turn, generates an auxin maximum in the protoxylem pole [22•] whose orientation is initially aligned with the position of the two cotyledons [25,26••]. Here auxin signaling induces the expression of the histidine pseudophosphotransfer protein *AHP6*, potentially via a direct regulation by MP/ARF5 [17] or by the LHW-T5L1 dimer [27] (Figure 1). Thereby, CK signaling is inhibited allowing protoxylem formation [22•,23,24••]. In the procambium and the phloem, CK signaling negatively regulates *AHP6* expression in an auxin-dependent manner [23,24••,28] (Figure 1). Modeling of the interactions of these and other factors, including the *CLASS III HOMEODOMAIN-LEUCINE ZIPPER* (*HD-ZipIII*) gene *PHABULOSA* (*PHB*) which represses *AHP6* expression [29,30•] (Figure 1), was sufficient for recapitulating patterning events in the early *Arabidopsis* root in a computational approach [31]. This highlights the importance of a complex and mutual intercellular crosstalk for procambium development. Very recently, a connection between the MP-TMO5/LHW module and CK signaling was established by the discovery that the TMO5/LHW dimer activates directly *LOG4*, a gene encoding for a rate-limiting enzyme in CK biosynthesis [26••,27]. This finding identifies CK as an — until then missing — non-cell autonomous signal inducing periclinal cell divisions in cells outside of the TMO5/LHW-expressing xylem pole. Again, cell-based computational modeling of the inhibitory interactions between auxin and CK signaling taking the new findings into account generated sharp boundaries between signaling domains. Starting with the globular embryo, the model was able to simulate vascular pattern formation in wild type and various auxin and CK signaling mutants [26••]. Of note, CK depletion or impaired CK signaling leads also to vascular cambium deficiency later in development [32,33] arguing for a similar role of CK in promoting cambium activity.

Laying the roads: establishing auxin transport routes for the connectivity of vascular strands

In leaf primordia, procambial strands emerge de novo from naïve ground tissue cells. Taking advantage of this easily accessible system, formation of focused auxin

transport routes according to the principles of auxin canalization and important for the continuity of vascular bundles has been extensively investigated in *Arabidopsis* (reviewed in [7]). As in the embryo, *MP/ARF5* is activated in pre-procambial strands in response to auxin accumulation established by polar auxin transport, leading to the acquisition of procambial cell identity [34•,35•,36]. *MP/ARF5* positively regulates *PIN* genes but also *ATHB8* (Figure 1), another member of the *HD-ZipIII* gene family [15••,16,37]. *ATHB8* is required to stabilize *PIN1* expression against auxin transport perturbation, to limit pre-procambial cell fate acquisition to narrow zones and to synchronize procambial cell identity assignment within and between veins [16]. *ATHB8* activates *ACAULIS5* (*ACL5*) (Figure 1), a gene important for thermospermine production which, in turn, attenuates xylem differentiation through a negative feedback loop involving other *HD-ZipIII* genes [38,39]. Surprisingly, while the mild venation defects found in *pin1* mutants are not enhanced by removing other plasma membrane-localized PIN proteins, they are particularly enhanced by removing PINs localized in the endoplasmic reticulum (ER) [40]; this underlines the importance of a fine-tuned system of auxin flow between, but also within, cells. Overall, these observations suggest the existence of an integrated and essential feedback loop involving PIN proteins, auxin, *MP/ARF5* and *HD-ZipIII* genes during procambium formation.

The initiation and activity of the vascular cambium

Later in the development of most dicotyledonous plants and conifers, (pro)cambium cells maintained between primary xylem and phloem resume periclinal division, producing secondary vascular tissues that lead to lateral growth of roots, hypocotyls and stems. The transition to lateral growth includes major anatomical transformations of these organs, resulting in the formation of a cylindrical meristematic domain designated as the vascular cambium [6] (Figure 1). Whether undifferentiated stem cells within the vascular cambium are comparable to procambium cells established during earlier stages has been a matter of debate [41]. However, considering their elongated anatomy, their predetermination for vascular development and the shared expression of major regulators like *MP/ARF5* (Figure 2), a common developmental constitution seems likely. Interestingly, the investigation of procambium formation and of the regulation of vascular cambium activity hardly overlapped in the past, which resulted in the establishment of distinct regulatory networks for the two processes (Figure 1). It is possible, however, that the apparent isolation of the two networks does not reflect fully independent processes but is at least partly based on different experimental readouts and genetic accessibility when targeting both processes. Analyzing molecular events during transdifferentiation of cells during vascular cambium formation in root pericycle cells [42] and in interfascicular regions in stems (Figure 1) [41,43,44] may provide means for comparing procambium and cambium formation.

The CLE-PXY-WOX module controls cambium proliferation

In *Arabidopsis*, a CLAVATA3/ESR-RELATED (CLE)-like peptide encoded by two members of the *CLE* gene family, *CLE41* and *CLE44*, stimulates cambium activity and represses xylem differentiation [45,46]. The peptide is synthesized in the phloem and travels to the cambium where it binds and activates the leucin-rich repeat receptor-like kinase PHLOEM INTERCALATED WITH XYLEM (PXY, also known as TDIF RECEPTOR, TDR) [45–48] (Figure 1). Due to disturbed cambium activity, *pxy* mutants exhibit perturbation in vascular bundle organization [45,49] and a dramatic reduction of lateral growth [43]. The CLE41/44-PXY signaling cascade regulates (pro)cambium proliferation through its positive effect on the *WUSCHEL-RELATED HOMEOBOX4* (*WOX4*) transcription factor gene [46,47••,50] (Figure 1). Although *PXY* and *WOX4* are already expressed in the procambium [43,51,52], several lines of evidence point to roles for *PXY*, *WOX4* and its redundantly acting homolog *WOX14* [50] in promoting cambium activity rather than in initiating procambium identity. First, in *pxy* and *wox4* mutants cambium activity is not entirely abolished and vascular bundle organization in *wox4* single or in *wox4 wox14* double mutants is not altered [47••,50,52]. Second, ectopic expression of *PXY* or *WOX4* does not induce cambium formation [47••,49,52]. Third, transcriptional profiling and expression analyses have shown that cambium marker activities are barely reduced in *wox4* mutant plants [47••,52]. Fourth, defects in procambium strand formation have not been reported for *pxy* single and *wox4 wox14* double mutants [45,46,50]. Interestingly, *WOX4* is auxin-responsive in a *PXY*-independent manner [52] (Figure 1), providing a possible link to the auxin signaling machinery involved in procambium formation described above.

Cambium activity is promoted by several parallel pathways

In the *Arabidopsis* stem and hypocotyl, twelve *ETHYLENE RESPONSE FACTOR* (*ERF*) genes, encoding members of the ERF/AP2 transcription factor family, have been shown to be up-regulated in *pxy* and *wox4* mutants [53]. Furthermore, because defects in vascular bundle patterning typical for *pxy* mutants are enhanced in the triple *pxy erf109 erf018* mutant [53], it is likely that the ERF transcription factors promote cambium activity in the absence of PXY (Figure 1).

ERECTA (*ER*) encodes a receptor-like kinase which likewise supports *PXY* function in vascular development: in the *er pxy* double mutant the hypocotyl diameter is more reduced and primary bundle organization in stems is

more severely affected than in the *pxy* single mutant [50]. However, because *ER* and *PXY* expression does not seem to overlap in stems [49,54], the interaction between *ER* and *PXY* might be indirect [50,55]. Two peptides belonging to the secreted cysteine-riche peptide family, EPIDERMAL PATTERNING FACTOR LIKE 4 (EPFL4) and EPFL6, bind directly to ER [55] and mediate the ER-dependent effect on vascular bundle organization [55]. Interestingly, *EPFL4* and *EPFL6* genes are expressed specifically in the starch sheath external to the vascular bundles [54], again highlighting the importance of intercellular communication for vascular development.

Beside a WOX4-dependent promotion of cambial cell proliferation, the CLE-PXY signaling module inhibits xylem differentiation by the direct activation of members of the GLYCOGEN SYNTHASE KINASE 3 (GSK3) protein family, including BRASSINOSTEROID-INSENSITIVE 2 (BIN2) [47••,56•,57] (Figure 1). Applying bikinin, a specific inhibitor of GSK3 activity, results in the depletion of cambial cells in favor of xylem cells in the hypocotyl, a phenotype similar to what is observed in a *gsk3* sextuple mutant [56•]. In the brassinosteroid (BR) signaling pathway, BIN2-dependent phosphorylation inhibits the activity of the BRASSINAZOLE-RESISTANT 1 (BZR1) and BZR2/BRI1-EMS SUPPRESSOR 1 (BES1) transcription factors [58] (Figure 1), suggesting that BR signaling is suppressed in early xylem cells, thereby counteracting differentiation. The role of BR signaling in promoting xylem differentiation has also been demonstrated by genetic studies: gain-of-function *bes1* mutants display an increased number of xylem cells and fewer cambium cells [56•] while mutations in BR receptor genes, in particular *BRASSINOSTEROID INSENSITIVE 1* (*BRI1*) and *BRI1-LIKE 1* (*BRL1*), result in reduced xylem differentiation [59]. The interaction between PXY and BIN2 has also been shown in the context of lateral root (LR) formation. BIN2 promotes lateral root initiation by phosphorylating ARF7 and ARF19 in a PXY-dependent manner [57]. BIN2 thereby suppresses their interaction with AUXIN/INDOLE-3-ACETIC ACID (AUX/IAAs) proteins and enhances their positive effect on gene transcription. Interestingly, exogenous application of CLE41/44 peptides enhances phosphorylation of ARF7, but this effect is not observed in *pxy* mutants or when GSK3 activity is blocked by bikinin application [57]. This indicates that a CLE-PXY-dependent phosphorylation of ARF transcription factors is required to mediate the auxin response during LR initiation, suggesting another molecular link between auxin signaling and cambium regulation.

In the *Arabidopsis* stem, identification of genes induced during cambium formation has led to the characterization of two novel receptor-like kinases *MORE LATERAL GROWTH1* (*MOL1*) and *REDUCED IN LATERAL GROWTH1* (*RUL1*), which regulate secondary vascular tissues formation in an opposite manner [43] (Figure 1). Due to the absence of obvious defects in procambium formation in corresponding mutants and their delayed or absent expression in procambium cells, respectively [43], it is tempting to speculate that both factors, together with the CLE/PXY/WOX4 signaling module, belong to a developmental program specific for regulating cambium activity and superimposed on the program described above for regulating procambium development.

Conclusion

Due to the diversity of tissues and organs investigated in the context of research of vascular development, the establishment of an integrated regulatory network influencing (pro)cambium attributes is challenging. Indeed, procambium initiation has been studied in the embryo, early root tips and leaf primordia, but the role of participating factors later in development is unknown even if several factors have been shown to be expressed in vascular tissues during the plant's entire life. Conversely, factors implicated in the regulation of cambium activity have not yet been related to the regulation of procambium dynamics, despite some of them being expressed in this tissue. This raises the question whether the initiation of procambium identity and the regulation of cambium activity are two genetically distinct processes, or whether the network regulating cambium activity is superimposed on the procambium developmental program and converges on similar key regulators. Investigations of (pro)-cambium-specific genes in different tissues and at different developmental stages, as well as their functional relation, should help answer this central question. Moreover, this should help identify a core mechanism conserved by the different plant species for the formation of vascular tissues, whose acquisition was a major invention during the evolution of land plants.

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