Plant Vascular Development: from early specification to differentiation

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Preface text

Vascular tissues are crucial to provide physical support and transport water, sugars, hormones and other small signalling molecules throughout the plant. Recent genetic and molecular studies have identified and interconnected some of the major signalling networks that regulate vascular development. Using Arabidopsis as a model system, this now allows describing this developmental process from the earliest specification during embryogenesis to the differentiation events of phloem and xylem tissues, as well as to reassess how oriented cell divisions are able to produce a three-dimensional vascular bundle within the root meristem.

Introduction

The development of vascular tissues is one of the most important evolutionary adaptations that allowed plants to grow in environments other than water and populate the land [1]. Vascular tissues provide mechanical support and facilitate transport of water, nutrients, hormones and other signalling molecules throughout the plant. These functions have also enabled land plants to grow beyond the size of mosses. Early land plants adopted a tissue organisation comprising three major tissue types, which can be found in about all organs: the outer epidermis, ground tissues and centrally localised vascular tissues. This organisation proved to be evolutionary very successful as it is still found in leaves, stems and roots of most modern land plants (**Figure 1**).

Our current understanding of the molecular pathways that regulate vascular development is mostly based on studies in *Arabidopsis thaliana*. Vascular development in Arabidopsis occurs in four main processes: specification, during which cells obtain their specific vascular cell identity from naïve precursor cells; establishment, which combines growth and patterning; maintenance and differentiation. Specification of four provascular initial cells occurs early in embryogenesis [2-4]. Highly regulated cell divisions with defined stereotypical orientations and simultaneous patterning events next establish the vascular tissue by the end of embryogenesis, leading to fully functional tissues with an adequate number of cells with correct identities (**Figure 1**). These embryonic provascular cells will generate the vascular tissues of the root and hypocotyl, while those of the shoot originate from the shoot apical meristem (**Figure 1**). Moreover, post-embryonically, the growth and maintenance of patterned vascular tissues occurs through cell divisions

in zones of the plant with high mitotic activity called meristems. Vascular tissue comprises two functionally distinct domains: phloem and xylem, which transport respectively solutes and water through the plant. Once the cells with xylem and phloem fate exit the meristematic regions, differentiation events will create the conducting cell types, tracheary and sieve elements, respectively, with their characteristic secondary cell wall and other cellular modifications [1] (**Figure 1**). The organization of vascular systems is very different depending on the plant organ. For example, the young root has a central xylem axis flanked by two phloem poles (diarch pattern), whereas stems contain several vascular bundles consisting of phloem on the outside and xylem on the inside (collateral pattern) (see **Figure 1**). It is important to note that the vascular organisation found in Arabidopsis is just one of the vast amount of different vascular topologies found throughout the plant kingdom. These topics have recently been discussed extensively [1, 5, 6] and we refer to these reviews for more detailed information.

The field of vascular biology has seen major advances in the past few years, substantially increasing our understanding of vascular development from early embryonic development to late differentiation steps. Known molecular pathways have been extended and previously unknown links between these pathways have been recently uncovered. In this Review, we discuss the most recent progress in identifying the regulatory networks that control vascular development during Arabidopsis embryonic root formation and its post-embryonic maintenance. We refer to several excellent reviews for discussions on cambial secondary growth [1, 5, 6], leaf venation [7] and other topics throughout the text when relevant. We will also highlight current open questions and discuss how general concepts regarding stem cell functions in the root meristem can be extrapolated to vascular tissues.

Root Vascular Ontogeny and Specification

Vascular tissues are first formed during the early globular stage of embryogenesis from four inner procambium precursor cells that each generate a ground tissue and a vascular tissue cell through a periclinal division [2, 4] (**Figure 1**). From the latter, in a next round of periclinal cell divisions, the outer pericycle cell layer is generated [2-4]. The resulting four inner provascular cells will generate all the cells of the xylem, phloem and procambium in the root and hypocotyl. In contrast, all vascular tissues in the aboveground tissues originate from the shoot apical meristem [1]

Although the ontogeny of these cell types in the root was first described over 20 years ago [2, 8] and in 3D more recently [4]; molecular markers for the earliest cellular identities remain scarce. Thus, it is at present unclear if vascular and ground tissue lineages are both established *de novo* from an uncommitted precursor, or whether one of the identities derives from the other. This also implies that it is still unknown how the actual vascular identity itself is determined or controlled. Despite this clear gap in our understanding of vascular development, it is possible to speculate on when the different cell types are specified within the tissue once a generic vascular identity has been established. Based on cell type specific reporters and hormone response marker analysis [9, 10], xylem identity is established first in two of the four initial cells around globular stage (**Figure 2a**). Although existing phloem markers have not been clearly described during embryogenesis, no early phloem markers have been identified so far [11, 12]. It thus seems possible that phloem identity is established later than xylem, towards the end of embryogenesis (after heart stage) when many more cell files are present.

Although final differentiation only occurs post-embryonically, all cell identities of the root vasculature; including xylem (which comprises protoxylem and metaxylem), phloem (which comprises sieve elements, companion cells and protophloem) and procambium; are present at the end of embryogenesis based on morphology and marker analysis (**Figure 2a**; [11, 13]). Post-embryonic primary root development is thus marked by patterning and cell specification events based on a previously established template (**Figure 2a, b**). In most other plant organs, such as stems, leafs, flowers and lateral organs, vascular tissues are formed from non-vascular precursor cells derived from the shoot apical meristem (**Figure 1**). It is very likely that the early processes of specification, growth and patterning are reiterated in these different tissue contexts.

Self-organization versus determinism

Although the stereotypic diarch vascular pattern of the Arabidopsis root **(Figure 2)** is laid down during embryogenesis, the regulatory mechanisms that establish this pattern are also able to generate different architectures when fewer or more cells are present. For example, mutants with half the number of vascular cell files can generate a monarch symmetry with opposing xylem and phloem poles [3, 14], while in other dicot species with larger vascular bundles, the number of xylem and phloem poles generally positively correlates with the size of the bundle [1]. Also, when new organs are formed or the vascular continuity is physically damaged, new strands will quickly be formed to restore connectivity of this elaborate network [14-16]. This intrinsic flexibility suggests a high degree of self-organisation underlying the arrangement of cell types in vascular tissues. In all cases, there is a link between the size of the vascular bundle and the

number of xylem and phloem poles. Moreover, some mutants have revealed a possible correlation between number of xylem poles and the number of embryonic leaves (cotyledons) [17].

In contrast to potential self-organizing properties underlying vascular pattern formation, recent observations have shown that the bisymmetry of the embryo, and thus also of the post-embryonic plant, is determined early after fertilisation of the egg cell. Intriguingly, the orientation of the first divisions of the proembryo is constrained relative to the axis of the surrounding developing seed (**Figure 1**) [4]. Because four-way junctions of adjoining cell walls are rare, if not actively prevented in plant development [18], a small connection between two of the four cells in the four-cell stage embryo is formed (**Figure 2a**) [9]. This connection between two cells at the centre of the embryo is maintained throughout embryogenesis and may later contribute to xylem axis formation [9]. Although there is no molecular evidence to support these observations, they suggest some degree of early determinism in plant development. It is plausible that during early stages, when the number of cells participating in tissue establishment is limited, and seeds provide external constraints, a deterministic mode of development ensures formation of a minimal but correct pattern. Yet during later, post-embryonic development, vascular development becomes plastic and acquires self-organizing properties, to allow maximal adaptability to the environment. An important future question is whether the same regulatory network can have both deterministic and plastic properties depending on the number of cells available.

Early establishment of root vascular tissues

Early establishment of the root vascular tissue is tightly linked to growth, patterning and hormone signalling pathways. Although the plant hormones auxin and cytokinin (CK) have long been known to be crucially involved, we have only recently begun to understand how these signalling pathways interact to control vascular development on a molecular level.

Mobile signals control vascular patterning

When the two cotyledons initiate early in embryogenesis, auxin produced at these sites is transported towards the embryonic root through auxin transporters of the PIN family [19, 20]. Because of their position relative to the incipient cotyledons, the two connected provascular initial cells receive more auxin that the other two cells (**Figure 2a**) [20]. The auxin response transcription factor MONOPTEROS (MP) is crucial for proper auxin signalling and vascular tissue formation, as mutations lead to very early division defects in the provascular initial cells [21]. An MP transcriptional target gene, the basic Helix-Loop-Helix (bHLH) transcription factor *TARGET OF MONOPTEROS5* (*TMO5*) is first expressed in the two provascular cells receiving more auxin [9, 22]. TMO5 and its homologs form heterodimeric complexes *in vivo* with another bHLH subclade including LONESOME HIGHWAY (LHW) and its close homologs [3, 23]. Loss-of-function of either TMO5 or LHW family members lead to a reduced number of periclinal cell divisions in the vasculature [3, 23, 24]. Thus, TMO5/LHW complexes mediate MP-dependent cell division activity in vascular tissues during embryogenesis.

Procambial cells undergo characteristic periclinal cell division both during and after embryogenesis; increasing the number of vascular cell files from the four provascular

initial cell files up to 30 in a mature root (**Figure 2b**) [8, 25]. These periclinal divisions are reduced in the *wooden leg* (*wol*) mutant, which is mutated in a cytokinin receptor *ARABIDOPSIS HISTIDINE KINASE 4* / *CYTOKININ RESPONSE 1* (*AHK4*/*CRE1*) [25, 26]. Additionally, in the *wol* mutant, all cell files within the vasculature differentiate as protoxylem, and conversely, cytokinin treatments inhibit protoxylem differentiation [8, 25-27]. These results show that cytokinins have a dual role in vascular development as inhibitors of protoxylem formation and as promoters of periclinal divisions. Protoxylem differentiation is facilitated in part by protoxylem-specific expression of cytokinin signalling inhibitor *ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6* (*AHP6*) [26]; which is also a *MP* target gene [28]. *AHP6* expression is thus auxin-dependent, and protoxylem positions are therefore not only characterized by high auxin, but also by low cytokinin responses [26, 28]. In the adjacent procambial cells, cytokinins promote a cell identity associated with the expression and/or lateral localization of PINFORMED (PIN) auxin efflux carriers, thus leading to accumulation of auxin in xylem cell files. Therefore, a mutually inhibitory feedback between cytokinins and auxin establishes the bisymmetric vascular pattern [28].

Recently, a connection between the auxin-MP-TMO5 and cytokinin-AHP6 pathways was identified. TMO5/LHW heterodimers were found to activate local CK biosynthesis through direct transcriptional activation of the *LONELY GUY4* (*LOG4*) gene and its closest homolog *LOG3* [9, 29] (**Figure 2c**). Mathematical modelling efforts using both growing embryonic [9] and static post-embryonic root [30] templates have shown that this regulatory network is able to create and maintain a zone of high auxin signalling along the xylem axis with flanking zones of high CK signalling in procambium cells within the growing vascular tissue as described above. Although the dynamic embryonic

model and the static post-embryonic model used different PIN regulation dynamics (**Figure 2d**), each highlights the intricate complexity in space and time of the auxin-CK interactions at play during vascular development. These modeling studies have predicted the existence of a novel inhibitor of CK signalling acting in the metaxylem [30], and have suggested that differential mobility of the key intermediates in this pathway as well as tissue geometry are essential for tissue patterning by this hormonal interaction network [9].

The major molecular hubs in this pathway; e.g. MP, PIN1, TMO5-LHW and cytokinin signalling components; and their interactions have been best documented in embryo and root tissues. All of these are however expressed in vascular tissues throughout the plant [19, 23, 26]. Therefore, it is plausible that the activity of this interaction network mediates vascular development in other plant tissues. However, not all vascular periclinal cell divisions (e.g. those at the phloem poles) can be explained by this regulatory network. Thus, other parallel pathways may act in the phloem (see below) and elsewhere.

A second pathway involving mobile signals is next required to maintain a sharp boundary between the xylem axis and the neighbouring procambial cells. The *AT-HOOK MOTIF NUCLEAR LOCALIZED PROTEIN3* (*AHL3*) and its interacting homolog *AHL4* are expressed in procambium cells neighbouring the xylem axis (**Figure 2e**). The AHL3 and AHL4 proteins move towards the xylem where they are required to regulate tissue boundaries between xylem and procambium [31]. Consistent with their expression in the zone with high CK-signalling, they were shown to be CK

inducible; suggesting that these AT-HOOK factors could function downstream of the TMO5-LHW pathway.

Patterning of the xylem axis into proto- and metaxylem cells is controlled by a third pathway, which has so far not mechanistically been linked to the previous two. The SHORTROOT (SHR) transcription factor, involved in ground tissue specification [32, 33] also plays an important role in vascular tissue patterning; exemplified by metaxylem formation at the protoxylem position in the *shr* mutant [34]. *SHR* is expressed in the stele and the SHR protein moves towards the endodermis [35], where it sequestered into the nucleus by SCARECROW (SCR) and induces *miRNA165/6* expression [34, 36] (**Figure 2f**). These mobile RNAs diffuse to create a gradient of *miRNA165/6* with highest levels at the periphery of the vascular bundle and lowest levels in the inner domain of the stele. The class III HOMEODOMAIN LEUCINE-ZIPPER (HD-ZIPIII) family proteins PHABULOSA (PHB), PHAVOLUTA (PHV), REVOLUTA (REV), ATHB8 and ATHB15/CORONA (CNA) are all present in the stele [37-41], require auxin biosynthesis for their proper expression [42] and are targeted by miRNA165/6 [34, 43]. As a result, high miRNA and resulting low HD-ZIPIII levels control protoxylem identity, while metaxylem is characterized by the inverse gradient. This is supported by only protoxylem identity in the quadruple loss-of-function *athb8 phb phv rev* mutant; while a miRNA165/166 insensitive dominant *phb-7* mutant shows ectopic metaxylem at the protoxylem position, just like the *shr* mutant [34]. Moreover, a modelling study has indicated that miRNA165/6-PHB mRNA interactions probably contribute to sharp boundaries of gene expression in the vascular bundle [30].

Although this pathway has only been shown to act post-embryonically in the root, it is likely also active during embryogenesis to establish proto- and metaxylem identity.

Early embryonic expression has been shown for least five miRNA165/6 members [44] in the lower tier of the embryo. These miRNAs restrict PHB expression to the upper tier during this stage of development. Nevertheless, this very early expression is most likely linked to its function in ab- and adaxial polarity, because other HD-ZIPIII members that are involved like *PHB*, *PHV* and *REV* are only expressed around late heart to torpedo stage embryos [44-47].

From these examples, it is clear that diverse mobile signals play a crucial role controlling xylem patterning. These signals move between the cells through various mechanisms depending on their nature. Polar auxin transport is based on the PIN protein efflux transport [48]. The SHR and AHL proteins as well as the miR165/6 species move through the plasmodesmata [31, 49]. The role of protein and cellular factors of SHR movement has been further investigated [50, 51]. The movement mechanism for cytokinins is however less clear. It appears possible that they might move through the plasmodesmata, but also various transporters have been implicated in CK transport in contexts other than xylem development [52, 53].

Unravelling early phloem development

The first identified factor controlling phloem development was the MYB type transcriptional factor ALTERED PHLOEM DEVELOPMENT (APL) [11]. The *apl* mutant shows xylem like cells at the phloem positions, while ectopic expression represses xylem development. Moreover, because the *apl* mutants lack sieve elements and companion cells, APL is most likely both a negative regulator of xylem differentiation and a positive regulator of phloem differentiation. In addition to APL functioning as master regulator of phloem identity, several counteracting pathways

specify the individual cell types within the phloem lineage (protophloem, metaphloem and companion cells; **Figure 3**).

The membrane protein OCTOPUS (OPS) was identified through screening gene-trap lines [13] for phloem specific genes. In *ops* mutants, individual protophloem cells fail to differentiate and thus interrupt the phloem strand integrity [12]. Very similar phloem defects have been described in another of these gene-trap lines, previously identified as *BREVIS RADIX* (*BRX*), which is itself a MP target gene [54, 55] and shows low penetrance *mp*-like embryo phenotypes [56]. Both OPS and BRX are polar membrane associated proteins, although BRX also seems to be nuclear [12, 55]. These factors promote the transition to sieve element identity and to maintain it; while CLAVATA3/EMBRYO SURROUNDING REGION45 (CLE45) peptide treatments suppress protophloem differentiation [57]. CLE45 response requires the BARELY ANY MERISTEM3 (BAM3) receptor like kinase [57]. All these factors are expressed at the precursor cells of protophloem sieve element, and it appears that the balanced interplay between this CLE45-BAM3 pathway on one hand and the BRX and OPS regulators on the other hand would regulate the timing of protophloem specification. Furthermore, auxin appears to have a role in regulating the timing of the asymmetric periclinal division resulting in the specification of the protophloem and metaphloem cell lineages [58].

Xylem and Phloem Differentiation

Once specification, growth and patterning events are completed, all cell types are present in the vascular bundle. In order to create functional conductive tissues, cells with xylem and phloem identities will differentiate into tracheary and sieve elements respectively (**Figure 4**). These processes involve drastic cytological changes in these

cells and will result in tissue-specific secondary cell walls. Because the differentiation of xylem and phloem cell types was recently reviewed in detail [59-61], we will only discuss the molecular mechanisms that control these processes briefly in this section.

Tracheary element formation

Using transcript profiling of xylem vessel element differentiation in *in vitro Zinnia* cell cultures, the NAC transcription factors VASCULAR-RELATED NAC-DOMAIN6 (VND6) and VND7 were identified as transcriptional switches controlling differentiation into meta- and protoxylem cells respectively [62]. However, it remains unclear if besides their role in differentiation, they also control cell identity determination of these xylem cell types. Although fusions to a dominant transcriptional repressor domain inhibited differentiation into the respective vessel elements, loss-of-function mutants did not show phenotypes, suggesting redundancy within the VND family [62]. Both VND6 and VND7 directly up-regulate genes involved in programmed cell death and cell-wall thickening, leading to tracheary element differentiation [63, 64]. Within this pathway (**Figure 4a**), VND INTERACTING 2 (VNI2) was identified to interact with VND7 and negatively regulate xylem differentiation [65]. A systems-biological approach was recently applied to determine the intricate transcriptional networks that act during xylem differentiation and has shown that a multitude of feed-forward loops in this network ensures robust regulation of this process [66]. Intriguingly, the orthologs of the same NAC type transcription factors in the moss *Physcomitrella patens* control the differentiation of their water-conducting hydroid cells. The functional conservation in moss and vascular plants thus suggests that these transcription factors played a major role in the evolutionary adaptations of plants to life

on land [67]. Differentiation of xylem is on the other hand repressed by two members of the *CLE* gene family, CLE41-CLE44/ TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF), which are produced in the phloem [68, 69]. These peptides then move to the procambium where they are perceived by the leucine-rich repeat receptor like kinase PHLOEM INTERCALATED WITH XYLEM/TDIF RECEPTOR (PXY/TDR) [68-71]. This peptide-receptor complex activates GLYCOGEN SYNTHASE KINASE 3 PROTEINS (GSK3), leading to a repression of the BRI1-EMS SUPPRESSOR 1 (BES1) transcription factor and thereby preventing xylem differentiation [72].

First parts of the phloem differentiation puzzle

In contrast to tracheary elements that undergo programmed cell death, phloem cells interconnect via sieve plates, generate secondary cell walls and loose most of the organelles and the nucleus. They manage to stay alive by establishing numerous cytoplasmatic connections through plasmodesmata with the neighbouring companion cells (CC). Recent work has shown that sieve plate biogenesis requires the CHOLINE TRANSPORTER-LIKE1 (CHER1/AtCTL1), indicating that the regulation of choline levels is crucial for phloem development and long-range transport in plants [73]. Sieve element formation is further controlled by two redundant APL target genes, *NAC45* and *NAC86* [74]. Among the target genes of these NAC transcription factors, a family of nuclease domain containing proteins NAC45/86-DEPENDENT EXONUCLEASE-DOMAIN PROTEIN1 to 4 (NEN1-4) control the enucleation process [74] (**Figure 4b**). Despite these recent advances, most of the molecular mechanisms that regulate the vast array of cellular changes during phloem differentiation remain elusive to date.

A meristem within the root meristem

To generate a growing three-dimensional structure, the root meristem undergoes a series of ordered cell divisions. Those divisions underlying the longitudinal growth of the root are called anticlinal divisions (AD), while radial growth is controlled by periclinal divisions (PD) (**Figure 5**). Over 60 years ago, Clowes described a group of cells at the centre of the meristem with very low division rates [75, 76], which we now call the quiescent centre (QC). These cells are also characterized by specifically high auxin signalling as shown by auxin responsive reporters [77-79]. Although QC cells hardly divide, the cells immediately surrounding the QC are actively dividing anticlinally and are commonly called the stem cells. A series of elegant laser ablation studies and genetic experiments [80-84] has shown that stem cells continuously undergo asymmetric, anticlinal divisions generating a new stem cells, while the daughter cell (which is no longer in contact with the QC) undergoes several more rounds of AD and finally differentiates when exiting the meristem. The *PLETHORA* (*PLT*) transcription factors sit at the basis of the transcriptional regulation of the stem cell niche [85-87]. The stem cell model has been well established for the distal (root cap) stem cell niche and paved the way for our understanding of stem cell niches in plants.

This concept however does not appear to seamlessly apply to the high number of PD that occur in the vascular tissues [25], giving the root tip its typical conical shape (**Figure 2a**) by increasing the width. Radial growth through PD has been recently shown to depend on cytokinin and the TMO5/LHW transcription factors (see above). TMO5/LHW dimer is sufficient to specifically trigger PD in any cell type of the root

when ectopically expressed, while the number of AD is not significantly altered. Also, in *tmo5tmo5like1* and *lhw* loss of function mutants, longitudinal growth is only moderately reduced compared to wild type, while radial growth in the vasculature is strongly reduced [3]; suggesting that control of PD and AD can be genetically separated. Moreover, the TMO5/LHW dimer controls PD in the neighbouring procambium cells through local cytokinin (CK) production [9]. Although CK induces radial growth through PD in root meristem and also in vascular cambium (**Box 1)** [25, 26, 88-91], it has a negative effect on meristem length by repressing AD [92, 93]. Thus both genetic and hormone response networks support that radial and longitudinal growth are controlled by distinct networks.

Intriguingly, the TMO5/LHW dimer expressing cells with xylem identity themselves only very rarely divide along the radial axis and contain high levels of auxin signalling; two characteristics that are shared with the QC. Indeed, root vascular bundles containing only xylem cell identities, such as in the *log* heptuple or *wol* mutants [9, 25, 90], show hardly any PD. Radial growth thus appears to be controlled by a 'radial meristem' in which the xylem axis acts as an organising centre driving radial growth through PD in the neighbouring procambium cells. However, it seems unlikely that the xylem located *TMO5/LHW* network, would be to be sufficient to explain all vascular formative divisions (e.g. those that generate the distinct phloem cell types: companion cell, protophloem, metaphloem), suggesting the existence of yet unknown factors controlling for example phloem cell fates.

In conclusion, a growing root tip contains a longitudinal meristem that generates more cells in the existing cell files through AD under control of the well-described *PLT*

transcriptional network [85-87]. On top of this, we propose that a radial meristem produces more cell files within the vascular bundle through PD at least partly through the independently acting *TMO5/LHW* network. A combination of both meristem activities thus generates the ordered three-dimensional structure of the root apical meristem.

Box 1 | **Vascular cambium and its similarity to the radial meristem**

While a fraction of procambial cells differentiate into various xylem and phloem cell types, the remaining fraction of the cells persists undifferentiated as the tissue matures. Later in the development these intervening procambial cells form the vascular cambium, a secondary meristem that undergoes periclinal cell divisions to produce xylem cells inside and phloem outside the meristem, thus resulting in a radial (secondary) growth in number of plant organs. Cambial activity therefore resembles the activity of the radial meristem in the root tip. Similar to the radial meristem, cytokinins are critical for the periclinal divisions in cambium [25, 26, 88, 91], however there are also differences: the key cambial regulators WUSCHEL-RELATED HOMEOBOX4 (WOX4), TDIF and PXY/TDR are present in cambium but absent from the root tip including the procambium [68-71, 94]. TDIF peptide is produced in secondary phloem and diffused into the cambium where it binds to its receptor, PXY/TDR to regulate rate and orientation of cell division, in part through promoting the expression of a transcription factor, WOX4 [69, 70, 95]. Another striking dissimilarity is that phloem-related cells show a peak in auxin signalling during secondary growth [96]. More cambial regulators and their signalling pathways have been reviewed recently [5, 6].

Perspectives

In recent years, several important studies have helped to generate a more comprehensive understanding of the development and regulation of vascular tissues. Early steps in tissue formation can now be connected to differentiation though connections identified between individual components of regulatory networks. Thus, vascular tissue initiation, formation, patterning, growth and differentiation can now be seen as parts of a continuum. However, with this view, it also becomes clear where knowledge is still lacking. For example, while many genes are activated concurrently at the time of vascular tissue initiation, it is entirely unknown how the tissue is first specified by identity determinants. Secondly, while several regulatory networks have been identified in a specific developmental context, it remains to be seen whether these are universal principles that also mediate patterning in other organs. For example, the specific vascular architecture of the meristematic root is changed completely during root secondary growth and vascular organisations similar to those of the root apical meristem can be obtained in other context, raising the question if the same pathways are reused at this point or whether completely parallel pathways exist. It will also be crucial to evaluate if the well-studied pathways in Arabidopsis can be transferable to other plant species and if these mechanisms are conserved throughout evolution. Finally, it appears that oriented cell division is an important factor in vascular tissue development, and a key question is how division orientation is controlled in space and time to create and maintain the three-dimensional shape of vascular tissue. We propose a model in which two distinctly regulated orthogonal meristems each controls a separate axis of growth to sustain tissue development as a whole. It will be interesting to see whether the downstream networks overlap or whether distinct regulatory modules control radial and longitudinal growth separately.

Figure 1 | Vascular development in the *Arabidopsis thaliana* lifecycle.

After fertilization of the egg cell, provascular tissues are established around the early globular stage of embryogenesis. Highly controlled oriented divisions next generate the entire vascular system throughout the plant. The vascular tissue in the post-embryonic root and hypocotyl derives from the embryonic vasculature (red cells), while the vascular tissue in all newly initiated post-embryonic organs and tissues (leaf, stem, lateral root; orange cells) is established *de novo* from the apical meristems (yellow cells). Note that the exact architecture of vascular tissues differs between the individual organs of the plant. For example: in young roots, a central xylem axis is separated from the phloem poles by procambium cells. In older roots that have undergone secondary growth, concentric rings of xylem (inner), cambium and phloem (outer) are formed. Leaves show xylem on the adaxial side and phloem on the abaxial side. In young stems, vascular tissues are first organised in bundles with xylem on the inside and phloem on the outside. Later in development, the procambium cells of the different bundles connect forming a ring. Finally, in the old stem, a similar structure as in the mature root is formed with concentric rings of xylem (inner), procambium and phloem (outer).

Figure 2 | Regulatory networks controlling early vascular development.

a | Ontogeny of the xylem tissues during embryogenesis. Two provascular initial cells share a cellular connection and receive more auxin than the other two through the incipient cotyledons forming above (indicated as asterisk next to early xylem in the early globular stage embryo). These cells will form the xylem axis of the root and are marked by high auxin signalling. The other provascular cells will form procambium and phloem cell lineages, with the procambium marked by high cytokinin (CK) signalling. **b** | Schematic longitudinal (lower panel) and radial (upper panel) cross-

section through the vascular bundle of the root apical meristem. Different colours indicate the various cell types. Note that all distinct cell identities are present in the mature embryo. Longitudinal zones in the root are not to drawn scale. **c** | The Auxin-TMO5/LHW-LOG4-CK pathway controls growth and patterning of the vascular bundle through local production of CK along the xylem axis, which triggers periclinal cell divisions (PD) in the neighbouring procambium cells. **d** | Summary of regulatory connections included in computational models describing vascular patterning in the root meristem as described in **c**, including AHP6 as negative regulator of CK signalling. Connections included in Ref. 30 are in red, those included in Ref. 9 in blue, and those common to both models in black. **e** | The AHL proteins are expressed in procambium cells and migrate towards the xylem axis, thereby controlling strict boundaries between xylem and procambium through an unknown mechanism. **f** | Control of metaxylem versus protoxylem identity by the SHR-miR165/166-HD-ZIPIII pathway. SHR expressed in the stele travels to the endodermis, where it is sequestered in the nucleus by SCR. There, miRNA165-166 is induced, which moves back inwards inhibiting members of the HD-ZIPIII family of transcription factors. These levels control the metaxylem vs. protoxylem identity.

Figure 3 | Key factors regulating early phloem development.

a | Schematic representation of phloem development in the root meristem showing the longitudinal ontogeny and organization of phloem cell types. **b** | After one or more anticlinal cell divisions, one procambium cell (expressing *OPS*) next to the pericycle on each side of the xylem axis undergoes a periclinal cell division, generating another procambium cell and a sieve element precursor cell. This cell (containing the CLE45 peptide, its putative receptor like kinase BAM3 and the transcription factor BRX)

undergoes another periclinal cell division generating proto- and metaphloem sieve elements. The companion cells (CC) are formed through yet another periclinal division from two flanking procambium cells. Protophloem specification is controlled in parallel by the dose-dependent CLE45-BAM3 factors, and the opposing activity of OPS and BRX proteins.

Figure 4 | Differentiation events during xylem and phloem development.

a | A network of transcription factors including VNDs under control of LBD genes, VNI2 and SND1 activate MYB transcription factors that regulate the expression of genes required for secondary cell wall synthesis and programmed cell death during xylem differentiation (left panel). In a first step, the secondary cell wall pattern is established and hydrolytic enzymes accumulate. Next the vacuoles rupture and programmed cell death occurs; together with perforation of the cell wall, generating a hollow tracheary tube (right panel). **b** | Phloem differentiation involves a number of distinct cellular modifications, including nuclear breakdown and sieve plate formation (left panel). These cellular events are controlled as different outputs of a regulatory network (right panel). So far it was shown that APL induces nuclear breakdown via NEN1-4 nucleases through NAC45/86 transcription factors.

Figure 5 | Orthogonal meristems control three-dimensional vascular growth in the root meristem.

Model for control of vascular tissue growth in the post-embryonic root: Two orthogonal meristems operate to control either extension of the tissue in the longitudinal dimension (left panel), or in the radial dimension (middle panel). Arrows indicate the axis of growth. These two meristems are differentially regulated, as the longitudinal meristem

depends on the PLT pathway, while the radial meristem is under control of the auxin-TMO5/LHW-cytokinin pathway. Note that these two meristems rely on distinctly oriented cell division planes: anticlinal for the longitudinal meristem, and periclinal for the radial meristem. In both cases, dividing cells are neighbouring cells that do not divide along the axis of the respective meristem (QC; xylem identity). The combination of these two orthogonal meristems can explain three-dimensional growth of the vascular tissue within the root meristem.

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Bert De Rybel recently became project leader in the Department of Plant Systems Biology (PSB) at VIB-Ghent University (Belgium). He also performed his PhD work here in the group of Tom Beeckman, but then moved to the lab of Dolf Weijers at the department of Biochemistry in Wageningen University (the Netherlands) where he studied early vascular growth and development. He now focuses on the hormonal control of cell division orientation during early vascular development.

Ari Pekka Mähönen has been a research group leader at the Institute of Biotechnology, University of Helsinki, Finland, since 2011. He carried out his doctoral studies with Ykä Helariutta in the University of Helsinki, and then did his postdoctoral studies with Ben Scheres at Utrecht University, the Netherlands. His lab is studying cambium development using Arabidopsis root as a model

Ykä Helariutta recently joined the Sainsbury Laboratory Cambridge University, UK, after leading the Wood Development Group at the University of Helsinki, Finland for 16 years. He is a Professor in Plant Developmental Biology. He carried out his PhD thesis work with Teemu Teeri (University of Helsinki) and conducted the postdoctoral period with Philip Benfey (NYU), New York City, USA. His laboratory focuses on procambial development with the emphasis on phloem specification and differentiation.

Dolf Weijers is professor in Biochemistry of Plant Development at Wageningen University (the Netherlands), where he started as a group leader in 2006. He did his PhD work at Leiden University (the Netherlands) with Remko Offringa, and then did post-doctoral work with Gerd Jürgens at Tübingen University (Germany). His group studies

early plant embryos as a model for multicellular plant development, and in addition focuses on transcriptional regulation of cell type-specific gene expression.

Key points

- Due to recent advances, we can now describe plant vascular developmental from early specification during embryogenesis up to late differentiation events.
- Plant vascular tissues display both deterministic and plastic properties during development.
- Most of the molecular pathways controlling vascular tissue patterning involve mobile signals.
- Although a rather elaborate transcriptional network has been established for xylem differentiation processes, very little is known so far about phloem differentiation.
- We propose a model in which two distinctly regulated orthogonal meristems each controls a separate axis of growth to sustain tissue development as a whole.

Table of contents summary

Recent advances in the field of plant vascular development have identified and interconnected some of the major signalling networks, allowing us to describe this developmental process from the earliest specification during embryogenesis up to the late differentiation events.

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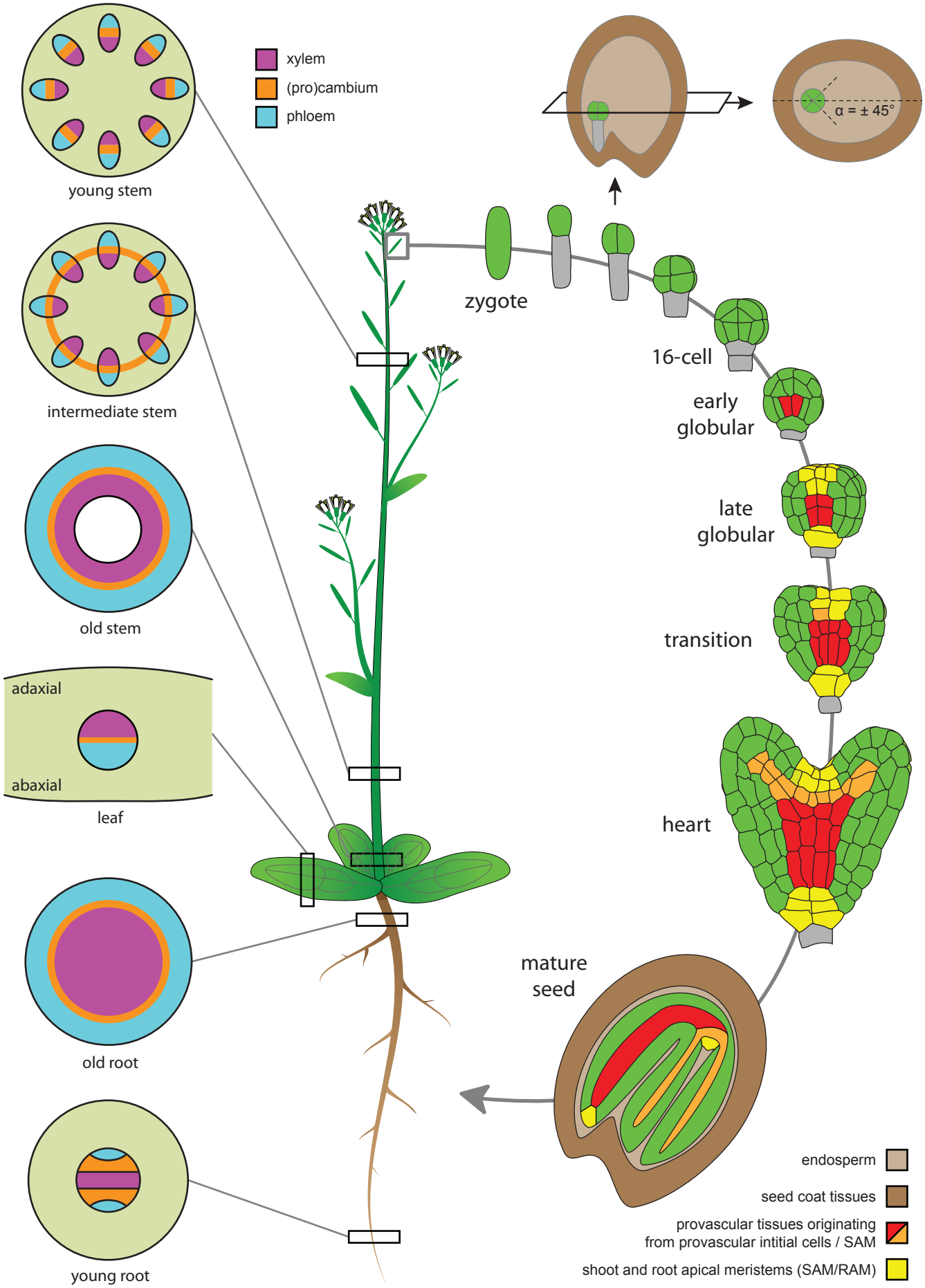

xylem
(pro)cambium
phloem
young stem
intermediate stem
old stem
adaxial
abaxial
leaf
old root
young root
α = ± 45°
zygote
16-cell
early globular
late globular
transition
heart
mature seed
endosperm
seed coat tissues
provascular tissues originating from provascular intitial cells / SAM
shoot and root apical meristems (SAM/RAM)

Figure 1

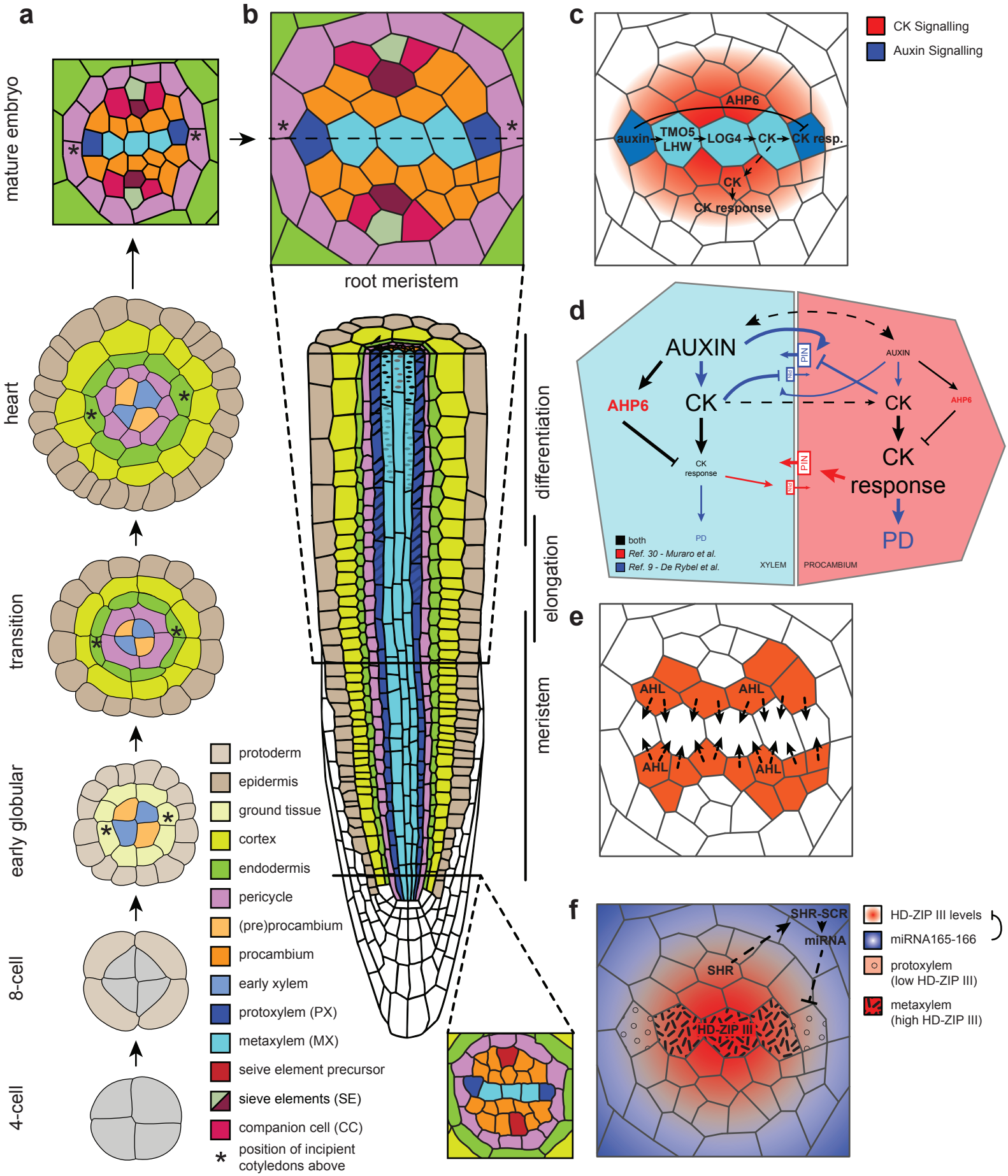

a
b
c
d
e
f
mature embryo
heart
transition
early globular
8-cell
4-cell
root meristem
differentiation
elongation
meristem
protoderm
epidermis
ground tissue
cortex
endodermis
pericycle
(pre)procambium
procambium
early xylem
protoxylem (PX)
metaxylem (MX)
seive element precursor
sieve elements (SE)
companion cell (CC)
position of incipient cotyledons above
CK Signalling
Auxin Signalling
AHP6
auxin
TMO5 LHW
LOG4
CK
CK resp.
CK response
AUXIN
PIN
CK response
PD
both
Ref. 30 - Muraro et al.
Ref. 9 - De Rybel et al.
XYLEM
PROCAMBIUM
AHL
SHR-SCR
miRNA
SHR
HD-ZIP III
HD-ZIP III levels
miRNA165-166
protoxylem (low HD-ZIP III)
metaxylem (high HD-ZIP III)

Figure 2

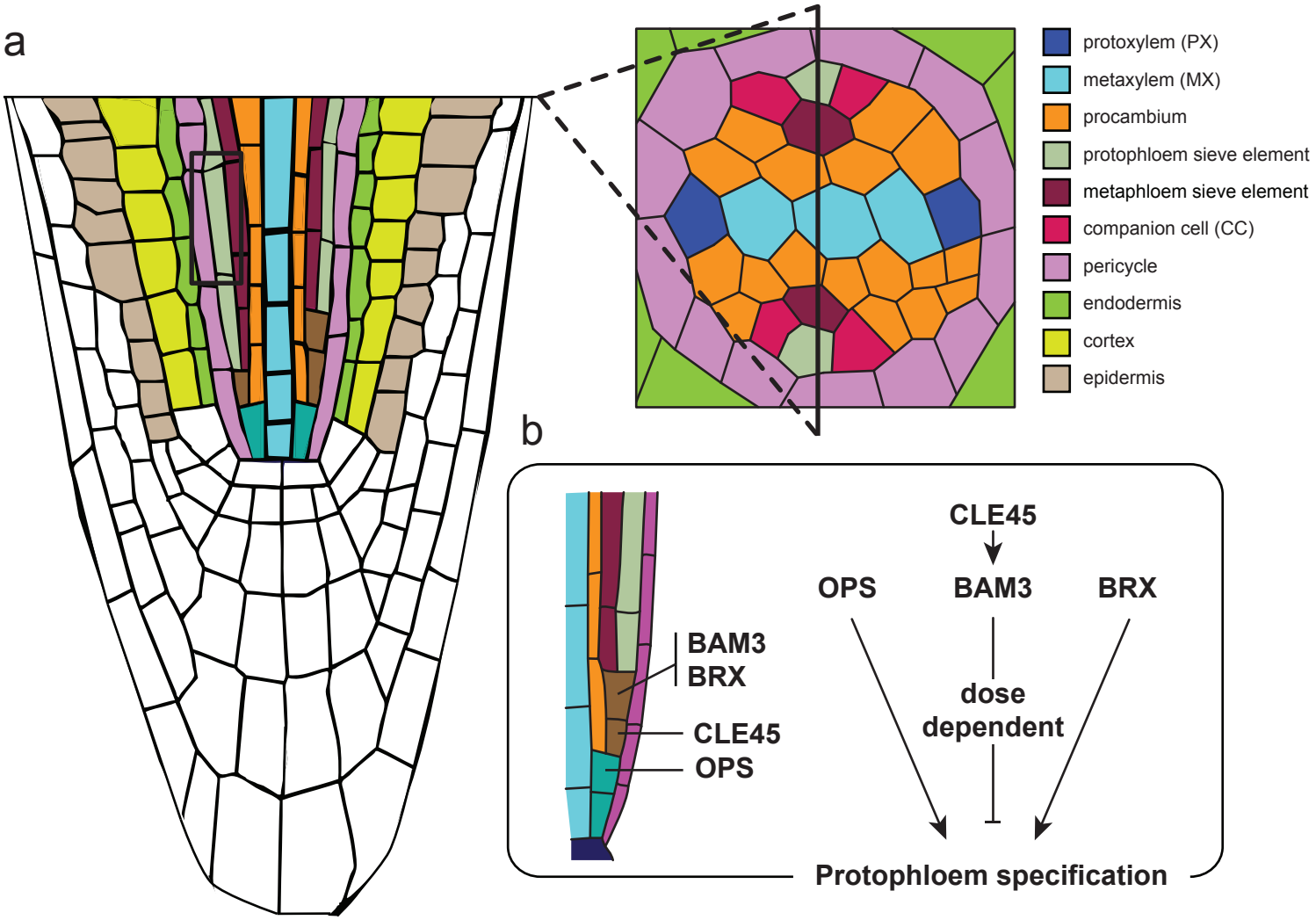

a
protoxylem (PX)
metaxylem (MX)
procambium
protophloem sieve element
metaphloem sieve element
companion cell (CC)
pericycle
endodermis
cortex
epidermis
b
BAM3
BRX
CLE45
OPS
CLE45
OPS
BAM3
BRX
dose
dependent
Protophloem specification

Figure 3

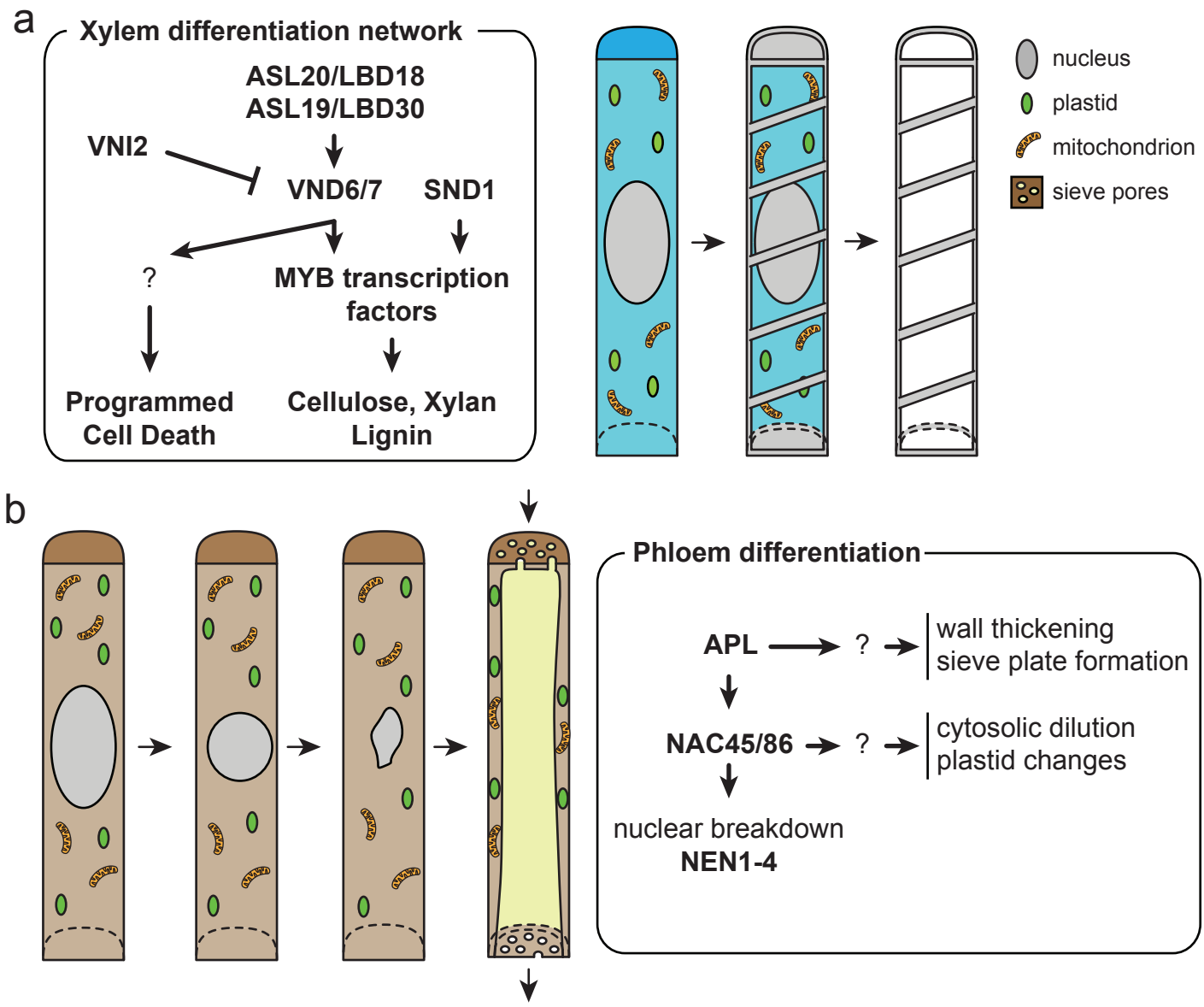
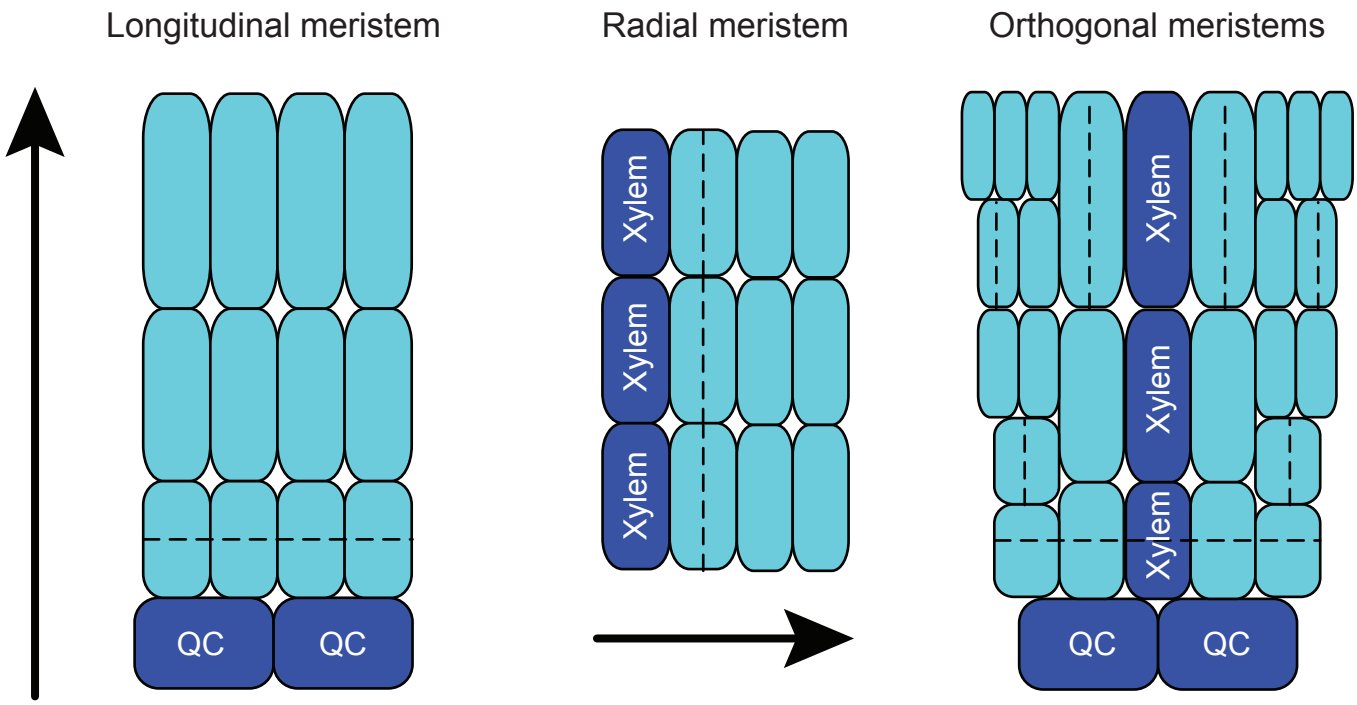

a
Xylem differentiation network
ASL20/LBD18
ASL19/LBD30
VNI2
VND6/7
SND1
?
MYB transcription factors
Programmed Cell Death
Cellulose, Xylan Lignin
nucleus
plastid
mitochondrion
sieve pores
b
Phloem differentiation
APL
?
wall thickening
sieve plate formation
NAC45/86
?
cytosolic dilution
plastid changes
nuclear breakdown
NEN1-4

Figure 4



anticlinal divisions (AD)
adding cells to file

PLT network

periclinal divisions (PD)
generating more cell files

TMO5-LHW network

both AD and PD
contribute to a
3D meristem

Figure 5