Genetic Networks in Plant Vascular Development

Raili Ruonala $^{1,2,*}$, Donghwi Ko $^{2,*}$, and Ykä Helariutta $^{1,2}$

$^1$Institute of Biotechnology and Department of Biosciences, University of Helsinki, 00014 Helsinki, Finland
$^2$Sainsbury Laboratory, University of Cambridge, Bateman Street, Cambridge CB2 1LR, UK

*These authors contributed equally to this work.

raili.ruonala@slcu.cam.ac.uk
donghwi.ko@slcu.cam.ac.uk
yrjo.helariutta@slcu.cam.ac.uk

Author for correspondence: Ykä Helariutta (yrjo.helariutta@slcu.cam.ac.uk)

Keywords:
Plant vascular tissue patterning, cambial development, xylem and phloem differentiation, phytohormone signalling, transcriptional network, genetic approach

ABSTRACT

Understanding the development of vascular tissues in plants is crucial since the evolution of vasculature enabled land plants to thrive on land. Various systems and approaches have been used to advance our knowledge about the genetic regulation of vasculature development from the scale of single genes to networks. In this review, we provide a perspective on the major approaches used in studying plant vascular development, and we cover the mechanisms and genetic networks underlying vascular tissue specification, patterning and differentiation.
INTRODUCTION

Searches for information about vascular development typically yield an impressive number of results from medical research. A cardiovascular network consisting of a heart and blood vessels is formed very early during organogenesis and is essential in supporting vertebrate life. In plants, an equally crucial vascular system of connective tubing is formed during early embryogenesis; at the early globular stage, cells that will develop as vascular initials can already be identified (130). From an evolutionary perspective, the invention of vasculature has been crucial to enable plants thrive on land (79; 151) since these tissues provide rapid, controlled delivery paths throughout the plant, as well as mechanical support. The plant vascular system is complex, with a vascular cylinder that consists of three cell types arranged in a highly organized manner, each with a specialized function (Figure 1). The middle layer, the (pro)cambium, is composed of cells with meristematic capacity, which remain inactive at the early procambial stage. These pluripotent cells give rise to cells that differentiate into xylem towards the inner side of the vascular cylinder and phloem towards the outer side. Xylem and phloem cells are specialized for water and nutrient transport, respectively, and also facilitate the transport of various signalling molecules. Later in development, the plant may also expand laterally when the cambial cells are activated and divide periclinally; this is typically referred to as secondary vascular development. In this review, we discuss the processes of vascular specification and differentiation and touch briefly on secondary vascular development and lateral growth. We aim to emphasize the various genetic approaches and plant systems that have improved our understanding of plant vascular development and to provide an overview of signalling mechanisms that have been recently reviewed in detail (16; 25; 75; 105). We therefore start with a historical overview of the major approaches that have been valuable in this important research field.

Diversity of plant vascular developmental systems and genetic approaches

Classical descriptive and experimental studies on plant vascular development established the anatomical and physiological foundations of the research field (133). For instance, the reticulated vein networks in the foliage leaves of various angiosperms and the well-organized vascular cylinder of roots were described between the 1850s and early 1900s (41; 140). The importance of plant hormones in the regulation of vascular patterning was highlighted during the same period. In the 1930s, treatment with auxin was shown to induce cambial growth in various plant species, including Coleus, Vicia faba, and Tradescantia (134). Young and coworkers provided experimental evidence that the young leaves of Lupinus albus are a source of auxin for the stem, inducing procambial development as well as xylem differentiation, implying that auxin transport is crucial for vascular patterning (155). Later, cytokinins were suggested to also be important signalling molecules in vascular patterning based on phenotypic changes observed in the vascular tissue of pea epicotyls upon cytokinin treatment (135).

Before 1980, most studies of xylem formation used entire multicellular organisms, which did not allow investigation of the stages of xylem cell differentiation. In order to overcome this problem, Fukuda and coworkers established a method to isolate single mesophyll cells from Zinnia leaves and initiate differentiation of the mesophyll cells into xylem vessels (tracheary elements, TEs) (43). Since then, the Zinnia cell culture system has been used to investigate the morphogenesis of xylem cells in detail, including secondary cell wall formation, programmed cell death, and genome-wide changes in expression during xylogenesis (29; 44; 108). In addition, the Zinnia cell culture system was crucial in identifying signalling peptides important for cambial proliferation, known as Tracheary Differentiation Inhibiting Factor (TDIF)
In 2005, microarray analysis of xylem vessel formation using a newly established in vitro Arabidopsis system identified key transcription factors involved in Arabidopsis xylem differentiation, VASCULAR-RELATED NAC DOMAIN 6 (VND6) and VND7. The in vivo physiological significance of these transcription factors was validated by reverse genetics approaches in Arabidopsis and Populus (74). The Vascular cell Induction culture System Using Arabidopsis Leaves (VISUAL) was established more recently and has been used to investigate genome-wide expression pattern of genes during phloem differentiation (73).

Since the 1980s, Arabidopsis thaliana has served as a prominent model plant not only for investigating vascular development but also for other research fields due to its relatively short life span, small genome size, ease of crossing, and amenability to saturation mutagenesis screens in the laboratory (116). Indeed, in the early 1990s, various mutants were isolated from forward genetic screens of Arabidopsis mutant pools produced by a variety of methods, including ethyl methanesulfonate (EMS) treatment, gene traps, T-DNA insertions, and transposon insertions, accelerating studies of the genetic networks governing vascular development in plants (8; 46; 90). For instance, MONOPTEROS (MP), an auxin-inducible transcription factor which plays a crucial role in vascular patterning in both embryonic and post-embryonic organs was identified from an EMS-mutagenized mutant pool (90). In order to elaborate the genetic networks involved in the MP pathway, genome-wide transcriptional changes in the mp mutant and related transgenic plants were analysed, revealing several TARGET OF MONOPTEROS (TMO) genes (132). In addition, the MP pathway was scrutinized further using multiple biochemical approaches, including chromatin immunoprecipitation and co-immunoprecipitation followed by liquid chromatography-tandem mass spectrometry (26; 66; 146). In addition to the forward genetic approach, a study of natural genetic variation in root growth identified BREVIS RADIX (BRX) as an important factor in protophloem differentiation in Arabidopsis. Of the tree species, Populus is currently the best understood, thanks to the availability of its full genome sequence and organ/tissue-specific gene expression databases created by whole genome transcriptome profiling, as well as the ability to routinely generate transgenic trees for the functional characterization of genes (56; 153). In 1998, expressed sequence tags (ESTs) were constructed from the wood-forming tissues of poplar to help identify genes involved in controlling the development of vascular tissues in this tree (137).

During recent decades, a comprehensive picture of the gene regulatory machinery involved in vascular development has started to emerge from genome-wide approaches. In the context of the Arabidopsis primary root, global transcriptional profiling in a spatiotemporal manner, facilitated by fluorescence-activated cell sorting and microarray techniques, has provided the first atlases of gene expression, covering many root cell types at different development stages (11; 14). These atlases have laid the foundations for network analyses and systems biology approaches which have been used to study secondary cell wall formation, for example. In addition to cell sorting, microdissection was also used to collect specific vascular tissues for transcriptome analysis (3; 27; 48). Genes identified via transcriptomics were investigated further by reverse genetics approaches (74), which have been facilitated by the availability of a genome-wide T-DNA insertional mutant library (116). More recently, a yeast one-hybrid network was used to establish the presence of a feedback loop in the transcriptional regulation of secondary cell wall formation (139). The genome-wide approaches have accumulated massive amounts of data and, together with the emergence of mathematical modelling (ideally with the capacity to predict gene regulatory networks), have begun to expand our understanding of these developmental processes (23; 33; 93; 102). The major approaches described here are illustrated in Figure 2 in consecutive order.
GENETIC NETWORKS IN PLANT VASCULAR DEVELOPMENT

Patterning of the vascular tissues in diverse vascular meristems

**Provascular development during embryogenesis.** The Arabidopsis zygote undergoes a series of cell divisions to give rise to an embryo comprised of cells with designated, specific fates. The establishment of the provascular tissue in the root, which is the origin of the post-embryonic vascular cylinder, is initiated by periclinal division of the four central cells in the lower-tier domain of the early globular stage embryo (24; 130). The *monopteros* (*mp*) mutant, which displays severe defects in root-pole formation and vascular development, was first isolated by a forward genetic screen of an EMS-generated mutant pool (90). *MONOPTEROS/AUXIN RESPONSE FACTOR 5 (MP/ARF5)* is an auxin-dependent transcription factor which plays an important role in the specification of the provascular tissue in the embryo, as well as in post-embryonic vascular patterning (9; 53; 148). In the early stages of embryogenesis, *MP/ARF5* is broadly expressed, but its expression is gradually confined to the provascular tissue (53), which coincides with the auxin response maximum produced by the concentration of auxin in those tissues by the PIN proteins, which export auxin from cells (42; 136). Examination of expression profiles revealed that *MP/ARF5* act as a positive regulator of *PIN1* expression, thereby forming a positive feedback loop to generate an auxin maximum in the provascular tissue during embryogenesis (Figure 3a) (110; 132; 147; 148). Consistent with this, mutation of either *MP/ARF5* or *PIN1* abolishes the auxin maximum, as well as provascular patterning in embryo, indicating that *MP/ARF5*-mediated auxin maximum formation and signalling is essential for provascular establishment (9; 42; 53).

Auxin signalling initiates upon the binding of auxin to TRANSPORT INHIBITOR RESPONSE 1 (TIR1) in the SKP1-CUL1-F-box (SCF)*TIR1/AFB* ubiquitin ligase complex, which triggers the 26S proteasome-dependent degradation of the AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) transcriptional repressors, releasing the ARFs to act (125). Another EMS-generated mutant screen isolated the *bodenlos* (*bdl*) mutant, which shows phenotypes similar with those of the *mp* mutant and harbours a point mutation in the conserved ubiquitination domain of the *AUX/IAA12* transcriptional repressor (50; 51). The *mp*-like phenotypes of *bdl* are attributed to this gain-of-function mutation of *AUX/IAA12*, which causes it to constitutively inhibit MP activity, thus mimicking the *mp* mutation (50) (Figure 3a). Transcriptomic analysis of the *mp* mutant and seedlings expressing dexamethasone-inducible *bdl* revealed that *MP/ARF5* directly upregulates the transcription of TARGET OF MONOPEROS (*TMO*) 3, 5, 6 and 7 (132). *TMO5* encodes a basic helix-loop-helix (bHLH) transcription factor which is sufficient to rescue the provascular initiation defects in *mp* (26). Furthermore, co-immunoprecipitation followed by liquid chromatography tandem mass spectrometry analysis revealed that TMO5 forms a heterodimer with LONESOME HIGWAY (LHW), an atypical bHLH transcription factor, to promote the periclinal division of provascular and procambial cells (Figure 3a,b) (26; 111; 112). High-order knockout mutants of either *TMO5* or *LHW* with their corresponding parologue, *TMO5-LIKE1* (*T5L1*) and *LHW-LIKE1* (*LL1*), exhibit a severe reduction in the number of periclinal divisions of the provascular and procambial cells in the embryo and root, respectively, indicating that these bHLH transcription factors are crucial components in *MP/ARF5*-mediated provascular and procambial patterning (26; 111). *TMO3* encodes an AP2 transcription factor which is also known as CYTOKININ RESPONSE FACTOR 2 (*CRF2*), a cytokinin-inducible gene which is crucial for embryonic development (119), indicating that auxin and cytokinin signalling converge in provascular patterning.

Cytokinins, like auxin, are key signalling molecules implicated in vascular development. The strong expression of the cytokinin receptor *WOODENLEG* and the synthetic cytokinin reporter
Two Component signalling Sensor new (TCSn)::GFP in embryo provascular tissue suggests that cytokinin functions in provascular patterning (81; 160). In addition, impaired embryonic root pole formation results from the overexpression of PURINE PERMEASE 14 (PUP14), which takes up bioactive cytokinins from the apoplast and inhibits cytokinin signalling mediated by plasma-membrane localized cytokinin receptors (160). Local biosynthesis of active cytokinins by the LONELY GUY (LOG) genes induced by TMO5/LHW has also been revealed to promote periclinal divisions to establish the provascular and procambial tissues (Figure 3) (23). The crosstalk between auxin and cytokinin regulating vascular development will be further discussed below.

Hormonal signalling governing procambial patterning of the root. The root vascular cylinder is a uniform structure composed of a central xylem axis flanked by phloem and the lateral meristem cambium; during secondary growth, the procambial cells give rise to xylem, phloem and the lateral meristem cambium (Figure 3). WOODENLEG (WOL), SHORTROOT (SHR), and SCARECROW (SCR), key regulators involved in patterning the root vascular cylinder, were isolated from mutant pools generated by EMS-mediated mutagenesis in the early 1990s (8; 131). The wol mutant exhibits a significant reduction in the number of vascular cell files in the root which is attributed to defects in periclinal cell division in the procambium and the abnormal differentiation of all of the vascular cell files as protoxylem (81; 129). WOL encodes a cytokinin receptor, a two-component histidine kinase also known as CYTOKININ RESPONSE1 (CRE1) and ARABIDOPSIS HISTIDINE KINASE4 (AHK4) (62; 81; 129; 143). Cytokinins are perceived by three homologous receptors, AHK2, 3, and 4, which initiate a phosphorelay involving the ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEINs (AHPs) and ARABIDOPSIS RESPONSE REGULATORs (ARRs) to regulate target genes (60).

Interestingly, the wol mutant harbours an amino acid substitution (T278I) in the cytokinin-binding domain of AHK4, locking the receptor in a form with reduced kinase activity but constitutive phosphatase activity, resulting in an impaired cytokinin response and changes in vascular patterning similar to the triple cytokinin receptor mutant (82). The phenotypes of wol and the ahk2ahk3ahk4 triple mutant, together with the procambium-specific expression of cytokinin-inducible ARR5, indicate that cytokinin signalling is restricted to procambial cells and mediates procambial proliferation and maintenance (12; 62; 81). Consistent with this, treatment with exogenous cytokinin suppresses the differentiation of protoxylem, while overexpression of the cytokinin oxidases, which degrade cytokinins, phenocopies the wol mutant (80; 149). A screen for suppressors of wol isolated AHP6, a negative regulator of cytokinin signalling which is primarily expressed in the protoxylem. AHP6 inhibits cytokinin signalling in the developing protoxylem and stimulates protoxylem differentiation at the marginal position of the central xylem axis in roots (Figure 3b) (80).

In contrast to cytokinins, auxin promotes the differentiation of xylem cells, as evinced by the repression of protoxylem formation following treatment with the auxin transport inhibitor 1-Naphthalylphthalamic (NPA) and the absence of xylem cells in the auxin-signalling-resistant mutant auxin-resistant1 (axr1) (12). Auxin response reporters, such as DR5 and IAA2, are primarily expressed in the xylem axis, but their expression expands into the procambial cells following changes in cytokinin signalling, suggesting that cytokinin confines the auxin maximum to the xylem axis of the root (12). On the other hand, auxin-induced LHW induces expression of AHP6 in the protoxylem and adjacent pericycle cells (112), where it inhibits cytokinin signalling, demonstrating that there is a mutual negative feedback loop between auxin and cytokinin signalling which generates the distinct domains of the hormones in the procambium and xylem axis to pattern the vascular cylinder (12). Indeed, detailed analysis of the expression and subcellular localization of the PIN proteins in the wol mutant or upon cytokinin treatment revealed that cytokinin is required not only for the proper expression of
PIN1, 3 and 7 but also for the appropriate subcellular localization of PIN1 in procambial cells (12; 83; 84). Likewise, the pin1pin3 mutant displays distorted development of protoxylem (12), indicating that cytokinin signalling regulates the PINs in order to steer auxin flow toward the xylem axis. Recently, it has been established that local cytokinin biosynthesis by LOG3 and 4, which are directly upregulated by auxin-induced TM05/LHW in the xylem axis, plays an important role in forming the asymmetric hormone response domain in the root vascular cylinder (23). Using the key components described above, three distinct mathematical models have examined the mechanisms underlying the formation and maintenance of these asymmetric hormone signalling domains (23; 33; 93; 102).

Radial patterning of xylem during root development. As discussed above, the role of plant hormones such as cytokinin and auxin in vascular cell specification is evident and is closely linked to root patterning and cell differentiation. These processes are often mediated by transcriptional regulation; for example, several members of the MYB, NAC and HD-ZIP III transcription factor families are well-known players in vascular differentiation. In the Arabidopsis root, xylem cell types can be distinguished morphologically by the pattern of their secondary cell wall thickening (pitted in metaxylem vessels vs. spiral in protoxylem) or molecularly by cell-type specific markers, such as ACAULISS for metaxylem (101) and AHP6 for protoxylem (80). Combined with the consistent organization of the xylem axis, with metaxylem cells in the centre and protoxylem in the periphery, this has allowed the detailed tracing of xylem identity determination by traditional phenotype-to-genotype mutant analyses.

Following the independent identification of a set of key transcription factors, a fascinating mechanism that balances metaxylem-versus-protoxylem identity in the Arabidopsis primary root was discovered. In the primary root, the identity of the two types of xylem vessels is determined by the SHR–SCR–microRNA 165/166–HD-ZIP III pathway in a reciprocal manner; while a high dose of miR165/166 and the consequent low level of the HD-ZIP III genes promote protoxylem identity, metaxylem is specified by high levels of the HD-ZIP III genes (Figure 3b) (20). This finely tuned transcriptional regulation involves non-cell-autonomous activity of the mobile transcription factor SHR, together with SCR (55; 104), to activate miR165/166 outside the stele (20). Upon moving back into the stele, miR165/166 degrades HD-ZIP III transcripts, such as PHABULOSA (PHB), producing a gradient that determines the vessel type. Recently, PHB has been reported to upregulate IAA20/30 and MP, stabilizing the auxin response in the xylem axis, which suggests that there may also be a feedback loop between hormone signalling and the SHR–SCR–miRNA165/6–HD-ZIP III pathway (Figure 3b) (100). Complementing the experimental evidence, a computational model suggests that degradation of miRNA165/166 itself, in addition to its degradation of the HD-ZIP III transcripts, is necessary for correct vascular patterning (102).

The players in this dynamic pathway were identified in mutant screens for altered vascular development in the Arabidopsis primary root (8; 20; 55; 131). Phenotypes of the short-rooted shr and phb mutants share similarities in many aspects, including the formation of ectopic metaxylem in the protoxylem position and delayed phloem formation (20). Loss-of-function of all five HD-ZIP III genes completely abolishes the differentiation of procambial cells into xylem. SHR, together with SCR, is also involved in controlling cell division to form the cortex and endodermis cell layers in the ground tissue of the primary root (55), while the HD-ZIP III genes redundantly restrict procambium proliferation (20).

In addition to their activity in the root, the HD-ZIP III transcription factors play crucial roles in radial vascular patterning in the shoot (34; 92). Antagonistic regulation between the HD-ZIP IIIs and the KANADI (KAN) genes, a family of GARP transcription factors primarily expressed in developing phloem, has been reported to regulate radial vascular patterning in the shoot (34;
The phv phb rev mutant displays abaxialized cotyledons and a distorted radial vascular pattern in which the xylem is surrounded by phloem (34). By contrast, mutation of the KAN genes in the kan1kan2kan3 mutant results in a phloem-surrounded-by-xylem pattern in the shoot similar to the gain-of-function HD-ZIP III mutants (34; 36). However, kan1kan2kan3 does not exhibit an apparent phenotype in root vascular patterning, suggesting that the KAN genes are not required for radial pattern formation in the root vascular cylinder (54). In Populus, transgenic trees misexpressing the HD-ZIP III transcription factor REVOLUTA showed ectopic formation of cambium and severe patterning defects with reversed polarity; secondary xylem was produced towards the outside and phloem towards the inside of the stem (120). Other HD-ZIP III genes have been shown to affect the rate of secondary xylem and phloem differentiation in Populus (32; 159), further indicating that cambial expression of the HD-ZIP III is important in vascular patterning as well as differentiation.

**Phloem specification in the root.** As for early phloem specification and differentiation in the Arabidopsis root, a few important genes have been identified using different approaches. OCTOPUS (OPS) (141) was discovered in a systematic survey for genes expressed during early procambial (103) and protophloem (7) development. OPS thus represents an example of reverse genetics in identifying novel genes involved in vascular development. The initial expression domain of OPS in the embryonic provascular cells narrows later in development to only the phloem lineage (7; 141). The ops-1 mutant, which originated from a promoter-trap collection, is characterized by a short primary root and adventitious roots growing out from the root-hypocotyl junction at an early age, as well as reduced venation in the cotyledons. Phloem development is discontinuous in the mutant root; so-called gap cells that do not differentiate into protophloem interrupt the cell file (141). OPS is a plant-specific plasma membrane protein that was recently shown to regulate brassinosteroid signalling via direct repression of the GLYCOGEN SYNTHASE KINASE 3 (GSK3) family member BRASSINOSTEROID-INSENSITIVE 2 (BIN2) (2) (Figure 4a). Further work has confirmed that brassinosteroid signalling, which was initially described in vascular differentiation of the inflorescence stem (17), also plays a role in Arabidopsis root protophloem differentiation (65; 124).

An interesting study on natural variation in root growth among 44 Arabidopsis accessions prompted the discovery of a natural loss-of-function allele brevis radix (brx) in the accession Umkirch-1 (98); later, BRX was linked to early protophloem development in a manner similar to OPS (121). In the short-rooted brx mutant, the levels of brassinosteroids are down and auxin responsiveness is reduced, indicating feedback regulation between these two hormones at an early stage in Arabidopsis root growth (98; 99). A second-site suppressor screen in the brx mutant background added more dimensions to the regulatory network related to phloem differentiation (Figure 4a). Depuydt and coworkers (30) reported that BRX restricts the expression of BARELY ANY MERISTEM 3 (BAM3), the receptor of a small peptide ligand, CLAVATA3/ENDOSPERM SURROUNDING REGION 45 (CLE45). CLE45 suppresses protophloem differentiation (30); this signal is amplified by MEMBRANE-ASSOCIATED KINASE REGULATOR 5, which acts downstream of BAM3 (65). Given that phloem files, though discontinuous, form in the ops and brx mutants, these genes are not required for phloem differentiation but rather seem to be involved in regulating the rate of this process (30; 121; 141). COTYLEDON VASCULAR PATTERN 2 (CVP2) was also isolated from a brx suppressor screen (122); a double mutant with its close homolog CVP2-like 1 has discontinuous cell files in the root protophloem and abnormal level of phosphoinositides. These genes had also been characterized earlier from a mutant screen for discontinuous vein patterns in the cotyledons, suggesting that phosphoinositides may act as signalling molecules for vascular strand propagation (18; 19).
Leaf vein patterning. Leaf primordia develop preprocambial tissue composed of subepidermal cells which are morphologically inconspicuous but are marked by auxin response genes (41; 145). The preprocambial cells give rise to procambium which consist of narrow cells arranged in continuous strands and which differentiate into the vascular cylinder of leaves (5; 41; 127; 128). Since an early 1950s study which showed that exogenous auxin treatment mimics the endogenous signal from young leaf primordia in inducing vascular development (64), numerous studies have supported the role of auxin as a positional cue in the formation of the vascular system in leaves (87; 123; 128; 145). The expression profile of the synthetic auxin reporter DR5 shows an auxin maximum at the site where veins will form (87). In addition, treatment with the auxin transport inhibitor NPA restricted the auxin maximum and vascular formation to the primordium margin, suggesting that appropriate endogenous auxin transport toward the site of the preprocambium is a prerequisite for proper leaf venation (87). Indeed, analysis of the expression profiles and subcellular polarity of the PIN proteins demonstrated that PIN1 is expressed prior to preprocambial cell markers and localized on the plasma membrane in order to steer auxin flow toward the site of preprocambium (128). Accordingly, the pin1 mutant exhibits reduced auxin transport and a distorted vascular pattern which can be reproduced by NPA treatment, indicating that PIN1 is the major auxin exporter involved in forming the auxin maximum necessary for correct leaf venation (87; 88; 113). The defects in leaf venation of the pin1 mutant are exacerbated in higher order mutants that include pin6 or pin6pin8, the endoplasmic reticulum-localized PINs, indicating that intracellular auxin transport is also important for leaf vein patterning (126). Distorted leaf vein formation in the cotyledons of mutants of the auxin importer LIKE AUXIN RESISTANT 2 (LAX2) suggests that auxin import is also necessary for formation of the maximum and veins (114). In addition, defective auxin signalling in the mp mutant results in impaired vascular patterning in leaves, as well as in embryos (9; 117). The spatiotemporal expression pattern profiles of MP and PIN1 in developing leaf primordia revealed that auxin up regulates the expression of MP which, in turn, induces the expression of PIN1 to form a positive feedback loop generating auxin maxima at the preprocambial sites of leaf primordia, similar to the feedback loop observed in the embryo (Figure 3c) (148).

In 1995, studies of Arabidopsis homeobox genes containing a leucine-zipper motif (HD-ZIP) identified the class III HD-ZIP gene ARABIDOPSIS THALIANA HOMEBOX 8 (ATHB8) as an auxin-inducible preprocambial and procambial marker specifically expressed in the vascular tissues of the embryo and post-embryonic organs, as well as during the regeneration of vascular strands (5). Expression profiling of ATHB8 promoter fragments revealed that the sequence TGTCTG in the promoter is a functional auxin response element and is essential for the gene’s specific expression in the vascular tissues (31; 132). Expression of ATHB8 is dramatically diminished in the mp mutant and increased by overexpression of MP, suggesting that MP is a direct transcriptional activator of ATHB8 in the preprocambial and procambial cells (Figure 3c) (31). Recently, Baima and coworkers identified ACAULIS5 (ACL5) and BUSHY AND DWARF2 (BUD2) as downstream targets of ATHB8 (4). The acl5 mutant was originally identified from an EMS mutant screen because of its dwarf phenotype and distorted xylem development (4; 52; 101). An in vitro enzymatic activity analysis revealed that ACL5 encodes thermospermine synthase, which produces thermospermine, a polyamine which is important in various plant developmental processes (68). The bud2 mutant was isolated from a mutant pool produced by a sense/antisense RNA expression system (97); it shows retarded shoot growth and impaired vascular patterning similar to the acl5 mutant (47). BUD2 encodes S-adenosylmethionine decarboxylase, an enzyme necessary for polyamine synthesis. The mutation decreases the production of polyamines, including spermine; together, these phenotypes suggest that the polyamine produced by ACL5 and BUD2 plays an important role in vascular patterning (47). Treatment with exogenous polyamines inhibits auxin-induced
xylem differentiation in leaves and prolongs xylem differentiation in Zinnia cell cultures (101; 154). Interestingly, overexpression of POPACAULIS5, the orthologue of ACL5 in poplar, suppresses biosynthesis of auxin, while PttHB8, the poplar orthologue of ATHB8, increases expression of POPACAULIS5, suggesting that there is a negative feedback loop between thermospermine biosynthesis and auxin signalling (94). Indeed, chromatin immunoprecipitation (ChIP) of ATHB8 showed that it directly binds to the promoters of ACL5 and BUD2 to upregulate their expression in Arabidopsis; in turn, these genes suppress premature xylem differentiation from the procambium by inhibiting the expression of HD-ZIP III and auxin signalling genes (Figure 3c) (4; 101). More recently, two studies have established the existence of a negative feedback network involving ACL5, four bHLH transcription factors belonging to the SUPPRESSOR OF ACACULIS51 LIKE (SACL) clade, and TMO5/LHW, all of which are specifically expressed in the vascular cylinder of the leaf, root and stem (66; 146). The LHW heterodimer with either TMO5 or T5L1 increases the activity of the SACLs either by directly increasing transcription or via ACL5-thermospermine-mediated translational activation, which, in turn, represses the TMO5-LHW interaction because the SACLs bind to LHW (Figure 3c) (66; 146).

Vascular cambium: establishment and patterning of the secondary meristem. Vascular cambium, a layer of meristematic cells, typically separates the differentiated tissues xylem and phloem in the vasculature; such an organized pattern requires coordinated and properly oriented division of the cambial cells, as well as timely differentiation processes of the specialized cell types. In a mutant screen for irregular vascular organization in the Arabidopsis inflorescence stem, Fisher and Turner (40) identified an LRR kinase which they named PHLOEM INTERCALATED WITH XYLEM (PXY). In the vascular bundles of the pxy mutant, phloem and xylem were not separated by a distinct cambial layer since the cambial cell division plane was abnormal; in addition, the ratio of vessels compared to other xylem cell types was reduced. PXY was also discovered in an independent set of experiments because of the mutant’s insensitivity to TDIF, a CLE peptide that was originally purified from a Zinnia cell culture medium because of its capacity to suppress TE differentiation (43; 58). In Arabidopsis, two genes, CLE41 and CLE44, encode the TDIF peptide (63), which is found in phloem, while PXY is expressed in dividing cambial cells (40; 58). This implies that PXY-CLE41/44 signalling regulates cell fate in a non-cell-autonomous manner (Figure 3d). In the Arabidopsis hypocotyl, PXY-CLE41/44 was reported to suppress TE differentiation through CLE41/44-dependent interaction of PXY with BIN2, a GSK3 protein that transcriptionally downregulates the transcription factor BES1 in brassinosteroid signalling (Figure 3d) (72). PXY-CLE41/44 promotes cambial proliferation during secondary growth of the Arabidopsis hypocotyl and inflorescence stem by activating WUSCHEL-related HOMEOBOX 4 (WOX4) and WOX14 (Figure 3d) (38; 57). Interestingly, PXY-CLE41/44 also controls secondary growth in the model tree species Populus; transgenic trees overexpressing the Populus orthologues of PXY and CLE41 specifically in the cambium and phloem, respectively, showed an increased rate of cambial cell division, leading to a dramatic increase in biomass (37). Thus, the PXY-CLE41/44 signalling pathway may affect vascular organization in many ways, and manipulation of this conserved pathway might even be considered as a means for improved production of sustainable energy.

In contrast to the pxy mutant, which displays defects in interfascicular cambium formation, the rul1 and mol1 mutants do not exhibit impaired interfascicular cambium formation. However,
$rul1$ and $mol1$ show a decrease and increase in interfascicular cambium-derived tissue, respectively, suggesting that they mediate opposing signals regulating cambium activity (1). More recently, Gursanscky and coworkers suggested that $MOL1$ negatively regulates ethylene and jasmonic acid signalling, which induce lateral growth (49).

Alongside $PXY$-$CLE41/44$, several other factors relevant for vascular development have emerged from Zinnia and Arabidopsis cell culture studies. For example, xylogen was purified from Zinnia cultures and described as a polar-localized proteoglycan-like factor essential to direct continuous xylem differentiation by promoting cell-cell interactions in Zinnia and Arabidopsis (96). The involvement of various hormones in xylem vessel differentiation, such as auxin, cytokinins, brassinosteroids and ethylene, has also been convincingly demonstrated in cell culture studies (43; 74; 109; 115; 152); these hormones may also act as important regulators of cambial activity.

**Hormone signalling and networks regulating cambial activity.** Much of our current understanding of secondary growth arises from research on the herbaceous species Arabidopsis, which can be regarded as a valid model since there is evidence of conserved mechanisms controlling cambial activity across species. However, secondary development is most evident in the secondary xylem of a tree trunk (also known as wood), and Populus has emerged as a popular forest tree model. An increasing amount of data indicates that cambial activity is regulated by several plant hormones, such as auxin, cytokinin, ethylene, gibberellic acid, strigolactone, and jasmonic acid. The role of auxin in the cambium was highlighted in the early 1930s, as treatment with exogenous auxin stimulated cambial growth in the petiole and hypocotyl (134); since then, numerous studies have supported the role of basipetally transported auxin as one of the major regulators of cambial activity (70; 77). For instance, the auxin-signalling-resistant mutant $axr1$ displays reduced interfascicular cambium activity in the Arabidopsis stem, and auxin concentration peaks in the cambium of Populus and Scots pine (10; 144). In the Arabidopsis stem, expression of the auxin response reporter $DR5$ is upregulated in parenchyma cells differentiating into interfascicular cambium (91). Furthermore, auxin activates expression of $WOX4$, a transcription factor downstream of $PXY$ which is involved in cambial cell proliferation (Figure 3d) (138). Accumulation of auxin in the Arabidopsis stem after a 1-day treatment with NPA induces the expression of $WOX4$ in wild-type plants and in the $pxy$ mutant. However, prolonged auxin accumulation following 7 days of NPA treatment results in high expression of $WOX4$ in wild-type but not in $pxy$, indicating that the induction of $WOX4$ by auxin is independent of $PXY$, but stable auxin-mediated $WOX4$ induction requires $PXY$ (Figure 3d) (138). In Populus, functional studies have shown that reduced auxin responsiveness reduces cambial activity (107).

Cytokinins are also well-known to stimulate cell division in meristems. The high-order Arabidopsis mutant of the ISOPENTENYLADENINE TRANSFERASE ($IPT$) genes, which are rate-limiting enzymes in cytokinin biosynthesis, has a severe reduction in the size of the cambium (86). Consistent with this, cytokinin signalling genes are primarily expressed in cambial cells, and overexpression of the cytokinin-degrading enzyme $CYTOKININ OXIDASE 2$ ($CKX2$) results in a reduction of cambial size in poplar (106), while suppression of $CKX$ genes in Arabidopsis generates a thicker inflorescence stem (6). In Populus, overexpression of $IPT3$ increased auxin levels and greatly enhanced radial growth. High-resolution profiling of the cambial region by RNA sequencing revealed distinct profiles for auxin and cytokinin response, indicating a hormonal gradient in the cambium (61) and further supporting the role of these two hormones as critical regulators of secondary growth. Just as basipetal auxin transport is important for cambial function, cytokinin transport is also crucial for cambial activity, as evinced by the severe growth retardation in the stem of the $abcg14$ mutant, which is defective in cytokinin transport (69; 156). Recently, it has also been demonstrated that
cytokinin induces the expression of the APETALA2-like transcription factor AINTEGUMENTA (ANT) and the cell cycle factor CYCLIN D3;1 (CYCD3;1) to promote cell proliferation in the cambium (21, 118). Accordingly, mutants of ant and cycd3;1 exhibit impaired cambial activity similar to that of cytokinin biosynthesis and signalling mutants. Compared with the single mutant phenotypes, the additive phenotype of the ant cycd3 double mutant indicates that they are both regulated by cytokinins but contribute independently to cell proliferation (Figure 3d) (118).

Another example of the hormonal regulation of cambial activity comes from Populus, where gibberellin levels are positively correlated with biomass accumulation (35) and gibberellin signalling appears to stimulate secondary xylem differentiation, as well as biomass accumulation in terms of plant height and fibre length (89). Furthermore, exogenously applied ethylene or overexpression of an ethylene biosynthesis gene stimulates cambial growth and wood formation in Populus (78). Ethylene appears to function in parallel with the PXY pathway to induce cambial proliferation (39, 78). The mild reduction in vascular cell number by the mutation of PXY is attributed to compensatory upregulation of ETHYLENE RESPONSE FACTORS (ERFs), such as ERF018 and ERF109, in Arabidopsis. The expression of ERF018/109 is increased in both the pxy and wox4 mutants, and the phenotype of pxy is exaggerated by mutation of ERF018/109, suggesting that both the PXY pathway and ethylene signalling positively regulate cambial activity, while the PXY pathway also represses ethylene signalling (Figure 3d) (39).

Differentiation of phloem and xylem
In the previous sections, we focused on the establishment and patterning of various meristems in plant vascular development. Meristematic cambial tissues in the stem, as well as in the hypocotyl and root, can be activated to produce secondary phloem and xylem by periclinal cell divisions and subsequent cell differentiation processes. Specialization of xylem and phloem cell fate culminates in the development of secondary cell wall and programmed cell death once the final size of the cell has been reached (Figure 4). All of these processes are important to generate tissues in organs such as the Arabidopsis inflorescence stem or tree trunks that can support upright growth and facilitate the flow of essential fluids and compounds throughout the growing plant. Since much of plant biomass derives from the development of the secondary cell walls of xylem vessels and fibres, these major components of wood are also important natural sources of support for human life.

Transcriptional regulation in xylem secondary cell wall development. The NAC family of transcription factors appears to be very important in the activation of the secondary cell wall regulatory network. Various approaches have identified five NACs that are sufficient to regulate xylem vessel formation and are therefore regarded as master regulators. Ectopic overexpression of VASCULAR-RELATED NAC-DOMAIN 6 (VND6), VND7, NAC SECONDARY WALL THICKENING PROMOTING FACTOR 1 (NST1), NST2, or SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN (SND1) results in ectopic secondary cell wall formation in various cell types (74; 95; 157). VND6 and VND7 emerged from comprehensive transcriptomics analysis of in vitro Arabidopsis xylogenesis cell suspension cultures, and overexpression studies subsequently linked them to transdifferentiation into metaxylem and protoxylem-like cells, respectively, in Arabidopsis roots and Populus leaves (74). In the Arabidopsis inflorescence stem, VND6 expression is restricted to the metaxylem vessels, while the VND7 expression domain includes both meta- and protoxylem vessels (158). NST1 and NST2 were discovered because of their role in another dehiscence in Arabidopsis by dominant EAR-motif-mediated repression analysis of the NAC
genes (95). NST1 and NST2 function redundantly; in the double knockout nst1 nst2, secondary walls are not formed in the endothecium, which results in anther indehiscence. Promoter analysis of NST1 and NST2 indicated strong expression in tissues other than the anthers; in particular, NST1 is strongly expressed in the xylem vessels and fibres of the Arabidopsis inflorescence stem (95). Finally, the fifth master regulator, SND1 was discovered as a highly expressed NAC transcription factor in xylem fibre cells that were isolated from Arabidopsis inflorescence stems by laser microdissection (157).

Transcriptional regulatory networks down- and upstream of the five master regulator NACs in secondary cell wall formation have been extensively studied, and a comprehensive view of the gene regulatory machinery has started to emerge from genome-wide approaches (59; 75; 139). Recently, interesting new connections were made in a large-scale yeast one-hybrid (Y1H) screen and subsequent network analysis to study protein interactions with transcription factors expressed in the root xylem (Figure 4b) (14; 15; 139; 158). For example, the E2Fc transcription factor, which is a negative regulator of endoreduplication (28; 85), was placed upstream of VND7 by the network analysis, with either a positive or negative effect, depending on dosage (139). This interaction was confirmed by monitoring luciferase activity under the VND7 promoter in tobacco leaves co-infiltrated with transiently overexpressed E2Fc at different ratios. Furthermore, VND7 was found to act as an upstream regulator of the HD-ZIP III genes REV and PHB, adding a third major transcription family to the secondary cell wall transcriptional regulatory network alongside the NACs and MYBs. Several other feed-forward loops that contribute to xylem differentiation were also highlighted in the transcriptional network (139). Thus, a systems biology approach conducted in a heterologous yeast system appears to have greatly enhanced our understanding of xylem development, pending validation in planta.

In the case of xylem fibre differentiation, perhaps surprisingly, Liebsch and coworkers (76) discovered a redundant function for the class I KNOX transcription factors SHOOT MERISTEMLESS (STM) and KNAT 1, via transcriptional repression of the meristem boundary genes BLADE-ON-PETIOLE 1 (BOP1) and BOP2 in the Arabidopsis hypocotyl. In the weak mutant alleles stm and knat1, SND1 and NST1 expression levels were significantly reduced, correlating with the reduced formation of fibres in the xylem. This is an interesting finding of important regulators in shoot meristem maintenance having an opposite role in secondary growth by promoting cell differentiation.

**Phloem sieve element differentiation in the Arabidopsis primary root.** While many pathways leading to xylem differentiation have been established, less is known about phloem differentiation. The first identified gene to mark phloem identity emerged from a mutant screen for compromised root growth: ALTERED PHLOEM DEVELOPMENT (APL), a MYB transcription factor that is required for the formation of phloem poles (13). APL also represses xylem formation; in the apl/loss-of-function mutant, the cells positioned in the expected phloem poles are characterized by lignified cell walls characteristic of tracheary elements rather than sieve elements (SEs). The apl mutant was selected from a transposon-tagged pool of Arabidopsis (150) because of its wol-like phenotype: a short, determinate primary root and arrested shoot development. In contrast to wol, the severe developmental effects of the recessive, seedling-lethal apl mutant could not be rescued, suggesting that APL function is required for phloem identity throughout the plant. Since protophloem can develop in a normal manner in apl embryos (142), APL appears necessary for the later stages of phloem development. More recently, the molecular regulatory pathway downstream of APL has been studied, providing a detailed view of SE maturation in the Arabidopsis root and highlighting the involvement of NAC transcription factors and their targets, the NAC-DEPENDENT EXONUCLEASEs (NENs), in this process (45) (Figure 4a). NAC45/86 were identified by
comparing transcript profiles of the apl mutant and wild-type plants, while the downstream NENs were found by comparison of cell-type specific transcript profiles from the nac45/86 double mutant, wild-type plants, and NAC45 overexpressing plants. Analysis of the NAC double mutant plants revealed that these genes were involved in the breakdown of the nucleus during SE differentiation, which was imaged at high resolution using serial block-face scanning electron microscopy (45).

An upstream regulator of APL was recently identified through an interesting in vitro system. Ectopic differentiation of xylem and phloem was induced in Arabidopsis leaf discs cultured in a medium containing auxin, cytokinin and the GSK3 inhibitor bikinin; mesophyll cells were converted into procambial cells prior to differentiation in a highly synchronized manner (71; 73). In this system, named VISUAL (Vascular cell Induction culture System Using Arabidopsis Leaves), an increased number of phloem-marker-expressing cells was recorded in the bikinin-induced leaf discs, as quantified by flow cytometry. Transcriptomics analysis coupled with cell sorting of the induced SE-like cells identified NAC20 as an upstream negative regulator of APL (73). Although not all of the cellular events related to SE morphogenesis were observed, VISUAL appears to have significant potential for identifying novel regulatory elements in TE and SE differentiation.

**SUMMARY POINTS**

1. The plant vascular system is highly organized at the whole-plant level, and it supports the coordinated growth of organs in response to environmental changes by providing a pipeline for the delivery of nutrients and signalling molecules.

2. Various systems and approaches have been invented and used to study the mechanisms governing plant vascular patterning and identify multiple genetic components and networks important for vascular patterning and development.

3. The development of the vascular system starts with cell specification, which involves numerous positional cues based on hormone signalling and transcriptional regulation. The interaction between auxin and cytokinin plays crucial roles in determining cell fates in the vascular cylinder. In addition, mobile transcription factors and miRNAs generate gradients of HD-ZIP IIIs along the vascular tissues to pattern the vasculature. Furthermore, antagonistic regulation between two groups of transcription factors, HD-ZIP IIIs and KAN, is crucial for adaxial and abaxial patterning.

4. Signalling mediated by LRR-RLKs and their ligands interacts with hormonal signalling to regulate cell specification and proliferation during vascular development.

5. Several members of the MYB and NAC transcription factor families play important roles in vascular tissue differentiation processes during vasculature maturation.

**FUTURE ISSUES**

1. Cell fate is determined by specific proteins, which can be regulated transcriptionally, post-transcriptionally, translationally, post-translationally and epigenetically. Studies during recent decades have characterized not only (post-)transcriptional regulation by transcription factors and miRNAs but also protein-protein interactions involved in vascular patterning. Recently, it has been reported that the expression of SACL genes is positively regulated in translation through thermospermine generated by ACL5, which is important for appropriate vascular
patterning. This suggests that translational regulation is indeed a crucial step controlling the specific expression of genes involved in vascular patterning. Furthermore, the importance of epigenetic regulation in vascular development has been suggested by a recent study which reported that trimethylation of histone by POLYCOMB REPRESSIVE COMPLEX 2 has a role in vascular cell proliferation and differentiation (22). However, the detailed mechanisms underlying translational and epigenetic regulation of vascular-specific genes remain elusive. Research on translational and epigenetic regulation may identify novel factors and further elaborate the processes governing vascular patterning.

2. Network construction and analysis based on bioinformatics and statistics, combined with dynamic and predictive modelling, may become even more powerful tools once data about the translatome and protein interactions are incorporated. This may also be extended to explore evolutionary aspects of plant vascular development and connect the disparate genetic networks.

3. Understanding the mechanisms of plant vascular development is important not only for the sake of advancing basic knowledge but also to improve agriculture and forestry in order to support the ever-growing human population. After all, in crop species, the storage organs we eat consist mostly of vascular tissues, and wood is composed of secondary xylem, the water-conducting tissue of the vasculature.

ACKNOWLEDGEMENTS
We sincerely thank Bonghee Heo for assistance with graphical illustrations, Sedeer el-Showk for helpful comments while proof-reading the manuscript and Ari Pekka Mähönen for discussions. The Y.H. lab is generously supported by the Gatsby Foundation, the European Research Council Advanced Investigator Grant Symdev (No. 323052), the Academy of Finland Centre of Excellence programme, the University of Helsinki, Tekes (the Finnish Funding Agency for Technology and Innovation) and the Biotechnology and Biological Sciences Research Council.

LITERATURE CITED


65. Kang YH, Hardtke CS. 2016. *Arabidopsis* MAKR5 is a positive effector of BAM3-dependent CLE45 signaling. *EMBO reports* 17:1145-54


**TERMS AND DEFINITIONS**

**Forward genetics:** gene discovery by untargeted mutation (or overexpression), followed by screening of mutants (or transgenics) for a phenotype of interest

**Reverse genetics:** targeted mutation, deletion, inhibition, or expression of a gene thought to function in a given process, followed by phenotypic analysis

**T-DNA:** a portion of Agrobacterium derived Ti plasmid that is inserted into the host genome and used as an insertional mutagen

**Provascular tissue:** Embryonic tissue which originates the future vascular cylinder

**Vascular bundle (cylinder):** Assembly of xylem, phloem and (pro)cambial cell files
Preprocambium: Undifferentiated leaf tissue consisting of cells that are morphologically inconspicuous but genetically distinct from mesophyll cells, and develop to procambium.

Procambium: Undifferentiated tissue with narrow and cytoplasm-dense cells which differentiate to primary xylem and primary phloem, and later develop to cambium.

Cambium: Lateral meristem which generates secondary xylem and secondary phloem during secondary growth in stem and root

Secondary growth: Lateral growth of stem and root by division of cambial cells

Interfascicular cambium (IFC) and fascicular cambium (FC): Cambial cells arising between (IFC) and within (FC) vascular bundles of stem; IFC connects FC in a ring structure.

Protoxylem: Primary xylem that develops from procambium earlier than metaxylem, characterized by spiral cell wall thickenings

Metaxylem: Primary xylem that develops from procambium, characterized by shorter and broader cells than protoxylem, and pitted cell wall thickenings

Tracheary element (TE): Xylem conductive vessel element characterized by secondary cell walls, perforation plates that connect TEs into a continuous file, and autolysis.

Protophloem (PP) and metaphloem (MP): Phloem tissues arising from procambium. Cell divisions first give rise to PP while MP develops later; both differentiate into SEs.

Sieve element (SE): Phloem conductive cell characterized by thick cell walls, sieve plates with pores connecting SEs into a continuous file, and enucleation.

Periclinal division: Cell divisions that occur parallel to the surface of the plant body, resulting in radial growth.

Secondary cell wall: Multi-layered polymer structure formed inside the primary cell wall of differentiated cells in some tissues such as xylem and fibres

Hypocotyl: The embryonic stem connecting the cotyledons with the embryonic root

Cotyledons: The embryonic leaves of the plant; post-embryonically, the first true leaves are formed from the shoot apical meristem.

Abaxial and adaxial: Refers to the under and upper sides of leaves, respectively
Figure 1. Vascular development in plants. Vascular development begins with the periclinal division of the lower tier of cells (marked in yellow) in globular stage embryos (top left). Controlled division of these cells forms the well-organized provascular tissue in the centre of the developing embryo throughout embryogenesis, which can be divided into 5 stages: zygote, globular, heart, torpedo and mature embryo. The vascular cylinder in newly developing post-embryonic organs originates from the shoot apical meristem or the root apical meristem. Intriguingly, despite the structural differences between vascular cylinders in different organs and growth stages, the vascular system tightly connects all of the organs together, indicating that vascular patterning is highly organized at the whole-plant level. Newly emerging leaves develop a reticulate vein structure (leaf venation) starting from the mid-vein, which is connected to the rest of the plant body; phloem and xylem develop on the abaxial and adaxial side, respectively. A uniform structure is seen not only in the leaf vascular cylinder but also in other plant organs. In the young root, the central xylem axis is sandwiched by intervening procambial cells and phloem; it will later develop to have a central core of xylem tissue surrounded by cambium and phloem tissue. In the young stem, individual vascular cylinders composed of procambial cells between (inner) xylem and (outer) phloem are disconnected, but they become connected in older stems and give rise to continuous xylem tissue on the inside surrounded by cambium and then phloem tissue on the outside.
Figure 2. Major approaches used in the study of plant vascular development. Approaches are shown in consecutive order based on the time when each technique started to be used for research on vascular development.
Figure 3. Genetic networks underlying vascular tissue specification. Vascular patterning in plants is controlled by complex genetic networks involving multiple transcription factors and phytohormones. (a) Provascular tissue specification. Auxin-inducible MP positively regulates PIN1 expression, which, in turn, forms a positive feedback loop resulting in an auxin maximum in the provascular tissue. BDL encodes AUX/IAA12 which represses MP-mediated transcriptional activation of target genes. MP upregulates the expression of HD-ZIP IIIIs, including ATHB8, which play crucial roles in vascular patterning. Another direct target of MP is TMOS5, which forms a heterodimer with LHW and induces expression of the LOGs to produce bioactive cytokinins that stimulate periclinal cell division. The expression pattern of MP in heart stage embryo is marked in yellow. (b) Cell specification in root procambial cells. SHR, a transcription factor expressed in the stele, moves to the endodermis and activates the transcription factor SCR. Together, SHR and SCR induce expression of miRNA165/6 in the endodermis, which diffuse toward the centre of stele to form a gradient. miRNA165/6 degrade the HD-ZIP IIIIs, resulting in an HD-ZIP III gradient toward the centre of the xylem which specifies metaxylem versus protoxylem. In addition, the HD-ZIP III PHABULOSA upregulates MP and IAA20/30 to stabilize the auxin response in the xylem axis of the root. TMOS5 induces expression of ACL5, a thermospermine synthase, as well as the SACL genes. Thermospermine generated by ACL5 induces translation of the SACLs. Furthermore, ATHB8 directly upregulates the expression of the SACLs, which inhibit TMOS5 and LHW heterodimerization by binding to LHW. ACL5 is likely to inhibit HD-ZIP III expression in roots based on the enhanced protoxylem cell differentiation in acl5 roots, but this has not been experimentally demonstrated in roots. (c) Leaf (pre)procambial tissue specification. The auxin-MP pathway also plays key roles in leaf (pre)procambial development. BUD2 encodes S-adenosylmethionine decarboxylase, which is involved in the synthesis of polyamines such as thermospermine. BUD2 and ACL5 are directly induced by ATHB8, but they repress expression of ATHB8, forming a negative feedback loop. (d) Cambial specification. PXY, a leucine-rich repeat receptor-like kinase, is activated by its ligands, CLE41/44, and upregulates the expression of WOX4/14, resulting in cell proliferation in the cambium. In addition, the PXY pathway activates BIN2, a GSK3 protein that inhibits the expression of the transcription factor BES1, stimulating xylem differentiation. Ethylene-induced ERFs and cytokinin-induced ANT and CYCD3;1 are also involved in cambial cell proliferation.
Figure 4. Morphogenetic events and transcriptional networks involved in vascular tissue differentiation. (a) Over the course of phloem differentiation from protophloem to mature sieve elements (SE), cell walls thicken and the cells undergo cellular re-arrangements during which organelles are modified and re-aligned. The process culminates in the breakdown of the nucleus and the formation of sieve plate pores that connect SEs in a cell file. Via phloem pores, the mature SE is associated with a companion cell that supports the enucleated SE. *OPS*, *BRX* and *CVP2* are positive regulators of protophloem specification and early SE differentiation. *OPS* represses *BIN2*, a suppressor of *BES1* in the brassinosteroid signalling pathway that promotes xylem differentiation. *BRX* represses *BAM3*, which is a receptor of *CLE45* that inhibits differentiation. *CVP2* is involved in balancing phosphoinositide levels, which also affect differentiation. The *ops* and *brx* mutants (as well as combinations with *cvp2*) are characterized by discontinuous protophloem files, suggesting that these genes have a role in regulating the protophloem differentiation rate. *APL* is required in the later stages of SE differentiation. It regulates *NAC45/86* expression and is downregulated by *NAC20*. *NEN*1,2, and 4 are key regulators of SE nuclear degradation that act downstream of *NAC45/86*. (b) Xylem vessel elements undergo programmed cell death, and their secondary cell walls develop pitted or spiral deposition patterns. The nuclear contents are degraded, and perforation plates that connect the cells are formed. Secondary cell wall development is controlled by a complex transcriptional network involving multiple steps, presented here schematically by arrows. The arrows also represent a multitude of feed-forward loops that are important in this process. The NAC transcription factors *VND6*, *VND7*, *SND1*, *NST1* and *NST2* are sufficient to regulate xylem formation and are therefore considered master regulators. Two recently identified novel pathways are also indicated; upstream of *VND7*, *E2Fc* may have either a positive or negative effect, while the HD-ZIP III transcription factors *REV* and *PHB* are suppressed by *VND7*. In a separate pathway, *KNAT1* and *STM*, which are typically associated with shoot meristem maintenance, were found to repress *BOP1* and 2, which suppress xylem fibre differentiation.