Tansley review

Phloem development

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Summary

The evolution of the plant vascular system is a key process in Earth history because it enabled plants to conquer land and transform the terrestrial surface. Among the vascular tissues, the phloem is particularly intriguing because of its complex functionality. In angiosperms, its principal components are the sieve elements, which transport phloem sap, and their neighboring companion cells. Together, they form a functional unit that sustains sap loading, transport, and unloading. The developmental trajectory of sieve elements is unique among plant cell types because it entails selective organelle degradation including enucleation. Meticulous analyses of primary, so-called protophloem in the *Arabidopsis thaliana* root meristem have revealed key steps in protophloem sieve element formation at single-cell resolution. A transcription factor cascade connects specification with differentiation and also orchestrates phloem pole patterning via noncell-autonomous action of sieve element-derived effectors. Reminiscent of vascular tissue patterning in secondary growth, these involve receptor kinase pathways, whose antagonists guide the progression of sieve element differentiation. Receptor kinase pathways may also safeguard phloem formation by maintaining the developmental plasticity of neighboring cell files. Our current understanding of protophloem development in the *A. thaliana* root has reached sufficient detail to instruct molecular-level investigation of phloem formation in other organs.

I. The plant vascular system

The evolution of the vascular system enabled plants to effectively colonize land and thus had a major impact in shaping the extant terrestrial biosphere (Raven, 2003; Beerling, 2007). Vascular tissues (see Box 1 for a glossary) permitted the long-distance separation of the sites of water and nutrient acquisition from the sites of photosynthesis and thereby also body plan expansion. Generally, xylem transports water and inorganic nutrients absorbed by the root system to shoot organs, whereas phloem distributes photosynthates and organic metabolites throughout the plant from source to sink organs. Xylem sap transport is mainly driven by the water potential differential between the soil and the atmosphere (Pratt & Jacobsen, 2017; Endo *et al.*, 2019). By contrast, transport of the viscous phloem sap is driven by an osmotic pressure differential, which builds up through the controlled loading of

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Box 1 Plant vasculature glossary

- **Plant vasculature** – The network of xylem and phloem strands that distributes water, nutrients, metabolites, and signaling molecules throughout the plant body.
- **Xylem** – The plant vascular tissue that transports xylem sap, generally from the root system to the shoot organs.
- **Phloem** – The plant vascular tissue that transports phloem sap, generally from source to sink organs.
- **Protoxylem** – The early xylem formed by stem cells in the growth apices of plants, the meristems.
- **Metaxylem** – The later primary xylem that persists throughout a plant organ's life cycle.
- **Secondary xylem** – Xylem that is formed by the cambium during radial thickening of stem(-like) organs and roots.
- **Protophloem** –The early phloem formed by stem cells in the growth apices of plants, the meristems.
- **Metaphloem** – The later, durable primary phloem of mature plant organs.
- **Secondary phloem** – Phloem that is formed by the cambium during radial thickening of stem(-like) organs and roots.
- **Cambium** – The cylindrical ring of vascular stem cells that is established as stem(-like) organs and roots mature, and which produces xylem to one, and phloem to the other side.
- **Xylem sap** – The aqueous solution of dissolved minerals as well as signaling molecules transported by the xylem vessels.
- **Phloem sap** – The viscous solution of photosynthates and metabolites as well as signaling molecules transported by the sieve elements.
- **Vessels** – Hollow, dead cells reinforced by secondary cell walls that align to form continuous xylem strands and transport xylem sap.
- **Sieve elements** – Enucleated cells with reduced organelle content that align to form continuous sieve tubes and are adapted to transport phloem sap.
- **Companion cells** – Specialized cells that provide metabolic support to directly neighboring sieve elements and that are also involved in phloem sap loading and unloading.
- **Fibers** – Specialized vascular cells with strong, lignified secondary cell walls in the xylem or phloem that provide structural support.
- **Sieve plate** – The highly perforated cell wall between two interconnected sieve elements.
- **Phloem pole** – The functional unit formed by sieve elements and their associated companion cells. Sometimes also meant to include other, directly neighboring cell files such as incipient metaphloem sieve elements or pericycle cells.

osmotic sugars in the source tissue and their unloading in the sink tissue (Riesmeier *et al.*, 1993; Chen *et al.*, 2012; Knoblauch *et al.*, 2016; Ross-Elliott *et al.*, 2017; Zhang & Turgeon, 2018). Because water exchange between xylem and phloem is necessary for the bulk transport of phloem sap, the two tissues always develop near one another. For example, in secondary growth, the radial expansion of plant stems in dicotyledons, cambial stem cells produce xylem to the inside and phloem to the outside in a likely synchronized manner (Sankar *et al.*, 2014; Lehmann & Hardtke, 2016; Smetana *et al.*, 2019; Bagdassarian *et al.*, 2020; Fig. 1a).

Although simple conducting cells can already be found in bryophytes and even macroalgae (Raven, 2003; Brodribb *et al.*, 2020), conspicuous xylem and phloem first emerge in the lycophytes (Spencer *et al.*, 2021). In the dominant group of land plants, the angiosperms, the vascular tissues show the highest complexity with respect to cell types and specialization. In the xylem, end-to-end joined vessel elements form open pipes for unimpeded xylem sap transport. They are surrounded by xylem parenchyma cells that perform metabolic support functions such as water storage, and fiber cells that add structural support (Esau, 1977; Spicer & Groover, 2010; Slupianek *et al.*, 2021). In the phloem, sieve elements join end-to-end via their perforated sieve plates to form sieve tubes that transport phloem sap. Laterally, the sieve elements are also intimately connected to their neighboring companion cells via plasmodesmata. Together, sieve elements and companion cells form a functional unit since the former are maintained by the latter. They can also be surrounded by parenchymatic and fiber cells (Esau, 1977; Sankar *et al.*, 2014; Heo *et al.*, 2017). Notably, unlike xylem vessels (which undergo programmed cell death and lose their protoplasmic content (Esau, 1977; Fukuda, 1997, 2000)), sieve elements are not dead. During their differentiation, they undergo a selective autolysis which drastically alters their cellular makeup (Esau, 1977; Heo *et al.*, 2017). That is, to optimize phloem sap flow, mature sieve elements have lost their vacuole, cytoskeleton, Golgi apparatus, and ribosomes, and only retain reduced mitochondria, plastids, and endoplasmic reticulum (Knoblauch & van Bel, 1998; Knoblauch & Oparka, 2012). Moreover, they have also degraded their nucleus and therefore depend on the neighboring companion cells for the maintenance of their transport functions. The focus of this review are molecular mechanisms that participate in the initiation, proliferation, and differentiation of phloem sieve elements, and their role in patterning the phloem pole in *Arabidopsis thaliana* (Arabidopsis).

II. Phloem pole patterning in secondary growth

Vascular tissues are produced by the root and shoot apical meristems during primary growth, as well as by a lateral meristem, the vascular cambium, during secondary growth. Molecular-genetic analyses of secondary growth dynamics in Arabidopsis roots, hypocotyls, and inflorescence stems have revealed a central role of receptor kinase pathways in vascular tissue patterning.

1. Secondary growth is a massive yet patterned growth process

Besides in some early diverging angiosperm lineages, the phenomenon of secondary growth is almost exclusively observed in dicotyledons and gymnosperms (Spicer & Groover, 2010). Although it is most prominent in perennial plants, notably trees, it also occurs in annual, herbaceous plants such as Arabidopsis (Chaffey *et al.*, 2002; Sibout *et al.*, 2008; Ragni *et al.*, 2011). The Arabidopsis hypocotyl is particularly well suited to investigate the dynamics of secondary growth, because secondary thickening in hypocotyls only starts once elongation growth has ceased and is moreover not obscured by parallel primary meristem activity (Chaffey *et al.*, 2002; Sibout *et al.*, 2008; Ragni *et al.*, 2011). Hypocotyl secondary growth accelerates upon flowering (Sibout *et al.*, 2008; Ragni *et al.*, 2011), which triggers a massive proliferation from a few hundred to several thousand longitudinal

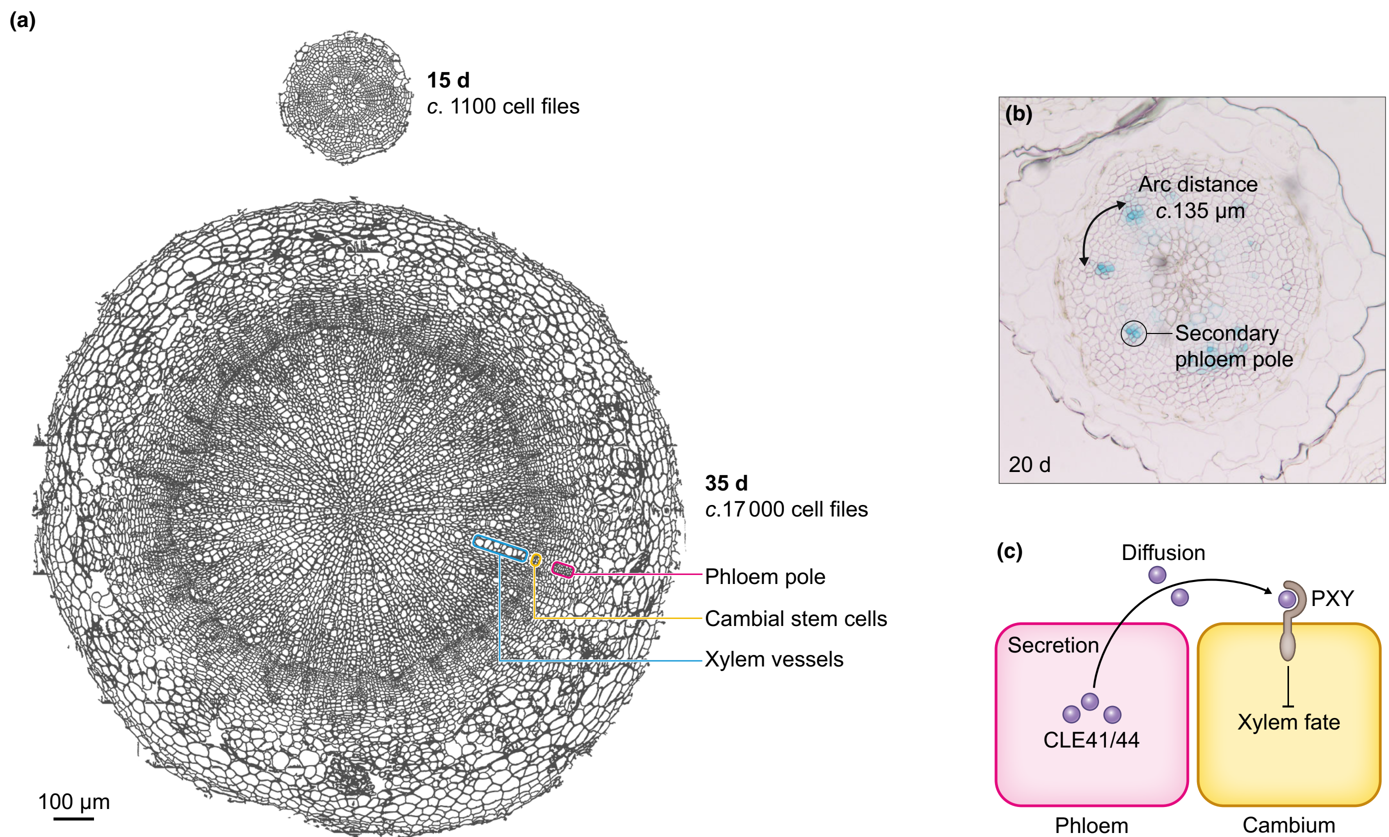

Fig. 1 Secondary growth of Arabidopsis hypocotyls. (a) Cell outlines of Col-0 wild-type hypocotyl cross-sections obtained from computational segmentation of tiled images (Sankar *et al*., 2014). The two sections demonstrate the progression of radial growth that occurs within 3 wk. The xylem area on the inside and phloem area on the outside are separated by the clearly visible ring of small cambial stem cells. The large arrays of xylem vessels are typically found opposite of phloem poles. (b) Cross-section of a (transgenic) hypocotyl after transition from early diarch to radial symmetry, stained for expression of *CVP2*, a marker of emerging phloem sieve elements (blue, β-glucuronidase staining). Computational analyses suggest that the cambium produces phloem poles at a constant arc distance. (c) Schematic presentation of noncell-autonomous control of receptor kinase pathway activity by their ligands, exemplified by the CLE41/44-PXY ligand-receptor pair. Developing phloem produces the CLE41/44 peptide ligands, which are secreted and can diffuse in the apoplast to prevent commitment of cambial stem cells to xylem fate by stimulating PXY activity. See Bagdassarian *et al*. (2020) for a recent detailed review of receptor kinase pathway action in secondary growth patterning.

cell files within a few weeks (Sankar *et al*., 2014; Fig. 1a). During this radial expansion, the relative cross-section xylem area increases and pushes the phloem and periderm further to the outside (Sankar *et al*., 2014; Serra *et al*., 2022). Due to this mechanical strain, the secondary phloem poles are shifting position, especially in older tissue as it is displaced further and further to the periphery. However, image segmentation of tiled high-resolution cross-sections, combined with a machine learning approach for automated cell type identification, revealed that the cambium periodically produces phloem poles at a constant arc distance (Sankar *et al*., 2014; Fig. 1b). This suggests that despite the slightly chaotic visual appearance, secondary phloem pole formation is a highly controlled and patterned process.

2. Receptor kinase pathways are prominent in vascular tissue patterning

Secondary growth dynamics displays substantial variation across Arabidopsis accessions (Sibout *et al*., 2008; Ragni *et al*., 2011). For instance, the cellular-level quantitative analysis revealed a relatively lower phloem cell proliferation in the Landsberg *erecta* (Ler) accession as compared to the common Columbia-0 (Col-0) laboratory strain (Sankar *et al*., 2014). This could be attributed to a quantitative trait locus that is either identical or closely linked to the *ERECTA* (*ER*) locus (Ragni *et al*., 2011; Ikematsu *et al*., 2017). A closely linked locus may appear more likely because *ER* gene family knock-out in Col-0 reportedly has the opposite morphological effect on secondary growth (Ikematsu *et al*., 2017). However, it is conceivable that accession-specific genetic modifiers condition *ER* gene family impact. Indeed, although single knock-out of *ER* in Col-0 does not lead to conspicuous secondary growth defects (Etchells *et al*., 2013; Ikematsu *et al*., 2017), as a second-site mutation, it specifically enhances the scrambled secondary growth patterning observed in mutants of the *PHLOEM INTERCALATED WITH XYLEM* (*PXY*) gene (a.k.a. *TDIF-RECEPTOR*; Fisher & Turner, 2007; Etchells *et al*., 2013; Uchida & Tasaka, 2013). Moreover, *ER* family genes redundantly maintain procambial cell divisions in stems, and for this activity, their phloem-specific expression is sufficient (Uchida & Tasaka, 2013). The phenotype of higher order mutants in which both the *ER* and

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PXY gene families are completely knocked out suggests that together, they control vascular tissue organization and its maintenance, cambial cell divisions, and the rate of vascular tissue differentiation in an organ-specific manner, but also that they are not absolutely necessary for either phloem sieve element or xylem vessel formation (Wang *et al.*, 2019).

Both *ER* and *PXY* encode leucine-rich-repeat (LRR) transmembrane receptor kinases (RKs) that transmit secreted peptide signals (Hirakawa *et al.*, 2008, 2010; Abrash *et al.*, 2011; Uchida *et al.*, 2012; Zhang *et al.*, 2016). Cambial activity is also modulated in antagonistic manner by the MORE LATERAL GROWTH 1 (MOL1) and REDUCED IN LATERAL GROWTH 1 (RUL1) LRR-RKs (Agusti *et al.*, 2011; Gursanscky *et al.*, 2016). Moreover, vascular tissue organization also involves the brassinosteroid receptor BRASSINOSTEROID INSENSITIVE 1 (BRI1) and its BRI1-LIKE (BRL) homologs BRL1, BRL2 (a.k.a. VASCULAR HIGHWAY 1), and BRL3 (Clay & Nelson, 2002; Ibanes *et al.*, 2009). Finally, the receptors and their ligands, as far as they are known, are typically not expressed in the same cell types (Fig. 1c), suggesting that they mediate important inter-cellular communication in vascular tissue patterning (Bagdassarian *et al.*, 2020). Thus, molecular-genetic analyses of secondary growth revealed a preponderance of LRR-RK signaling pathways in vascular tissue organization, which is also a recurring theme in phloem differentiation (to be described later).

III. Seeing clear through VISUAL

Developmental plasticity is a hallmark of plant ontogeny, as demonstrated by the capacity of already specified plant cells to adopt a new fate depending on their relative position in the organ (van den Berg *et al.*, 1995). A drastic example of this plasticity is the reprogramming of leaf mesophyll cells into vascular cells in tissue culture, which has yielded major insights into the transcriptomics of vascular tissue formation.

1. Inducible trans-differentiation of mesophyll cells into conductive vascular tissues

A key intermediate in the signaling cascade downstream of BRI1 is BRASSINOSTEROID INSENSITIVE 2 (BIN2), a cytoplasmic kinase of the Glycogen Synthase Kinase 3 (GSK3)/SHAGGY family. BIN2-LIKE (BIL) proteins are also targeted by PXY (Cho *et al.*, 2014; Kondo *et al.*, 2014; Han *et al.*, 2018). Stimulation of PXY signaling by its CLAVATA3/EMBRYO SURROUNDING REGION-RELATED 41 (CLE41) and the sequence-identical CLE44 dodeca-peptide ligands (a.k.a. TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR) is important for precise cambium positioning in secondary growth (Fig. 1c) and activates GSK3s to suppress xylem differentiation (Kondo *et al.*, 2014). The discovery of a pharmacological small-molecule inhibitor of BIN2 and GSK3s/BILs, called bikinin (De Rybel *et al.*, 2009), could thus be exploited to establish an *in vitro* system for xylem tissue differentiation. Bikinin treatment of leaf disks or excised cotyledons combined with simultaneous auxin and cytokinin application was initially found to promote trans-differentiation of mesophyll cells into xylem vessels via an intermediate procambial stage (Kondo *et al.*, 2015). Subsequently, it was discovered that in this same assay, which was named *Vascular Cell Induction Culture System Using Arabidopsis Leaves* (VISUAL), a roughly equal portion of mesophyll cells transdifferentiated into phloem sieve elements (Kondo *et al.*, 2016; Fig. 2a). This highlights once more the strong coupling of xylem and phloem formation, which has also been confirmed by high-resolution clonal sector analyses of secondary growth (Shi *et al.*, 2019; Smetana *et al.*, 2019). These studies found that cambial stem cells are bifacial, that is they possess the capacity to produce either xylem or phloem (Fig. 1a).

2. Auxin levels determine the abundance of VISUAL phloem cell types

In the organ context, the bifacial activity of the cambial stem cells is usually firmly polarized, that is they exclusively produce future xylem to one side and future phloem to the other (Fig. 1a). The fate of their daughter cells is at least in part determined by the local phytohormone status, notably the level of auxin, which is maintained in a gradient across the phloem-xylem border and therefore is likely responsible for the organ-level polarity (Suer *et al.*, 2011; Liebsch *et al.*, 2014; El-Showk *et al.*, 2015; Han *et al.*, 2018; Smetana *et al.*, 2019; Smit *et al.*, 2020; Makila *et al.*, 2023). The importance of the relative auxin level is also reflected in a variation of the VISUAL assay, VISUAL-CC, which increases the low abundance of companion cells observed in VISUAL (Tamaki *et al.*, 2020). Compared with VISUAL, the higher ratio of companion cells to sieve elements was achieved by combining a lower auxin level with higher GSK3 activity (Fig. 2a). Bulk transcriptomic analyses of VISUAL and VISUAL-CC time courses revealed the temporal dynamics of the trans-differentiation process, which takes 72–96 h to complete (Kondo *et al.*, 2016; Tamaki *et al.*, 2020; Fig. 2b). These time courses not only identified the NAC DOMAIN-CONTAINING PROTEIN 20 (NAC020) transcription factor as a likely regulator of sieve element differentiation (Kondo *et al.*, 2016), but also found that known regulators of phloem development in the root context are differentially expressed in VISUAL(-CC) over time (Fig. 2c). Their sequence roughly matches their spatio-temporal expression in the root protophloem (to be described later).

IV. Protophloem development in the Arabidopsis root tip

Historically, phloem observation is challenging because sieve elements are thin, highly anisotropic, and buried rather deep inside plant organs. However, during organ formation in primary growth, xylem and phloem are continuously formed from the stem cell niches in meristems. The meristems are terminal sinks, whose growth is sustained by phloem sap delivery through the early, so-called protophloem (Ross-Elliott *et al.*, 2017). Compared with the later metaphloem or with secondary phloem, protophloem thus develops relatively close to the meristem surface in a predictable location and orientation. Especially, the

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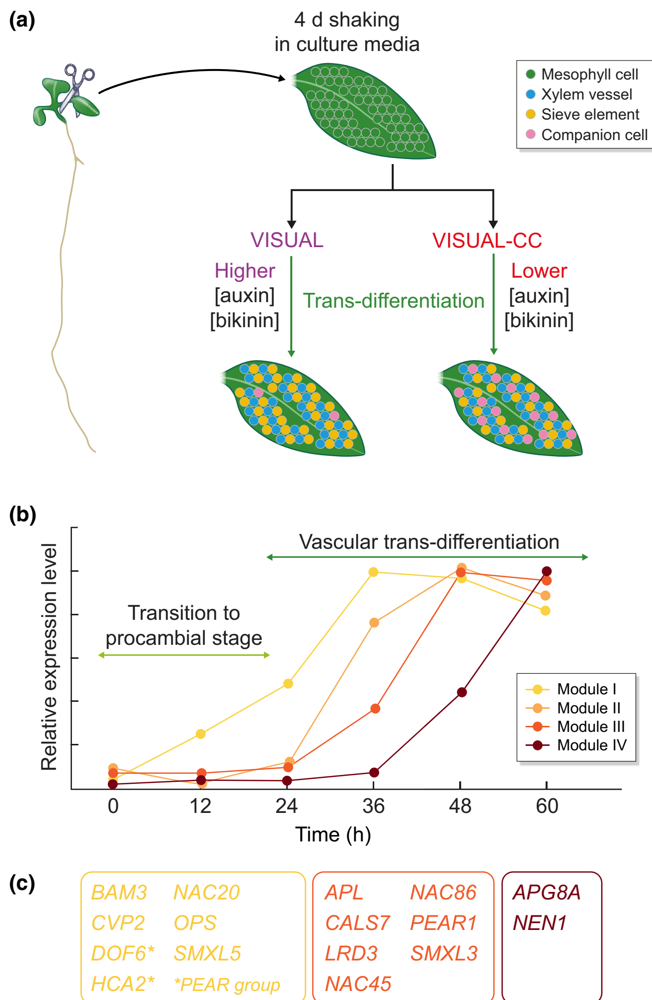

Fig. 2 Trans-differentiation in the VISUAL assay. (a) Schematic presentation of VISUAL assays. Cut cotyledons (or leaf disks) are transferred into VISUAL culture media and gently shaken in the dark to induce re-specification of mesophyll cells toward xylem vessel or phloem sieve element fate, via an intermediate procambial stage. Lower auxin and bikinin concentrations in the media promote the concomitant formation of companion cells (VISUAL-CC). (b) Timeline of the VISUAL trans-differentiation process (see Kondo *et al*., 2016), as defined by temporal gene expression profiles. Bulk transcriptomics identified four distinct temporal modules comprising a total of *c*. 200 genes that are associated with phloem sieve element formation after peak expression of procambial markers. (c) They include several genes that have been described in the context of root protophloem development, suggesting that sieve element development in different organ contexts follows a common genetic program.

protophloem in the Arabidopsis root tip has therefore become the tissue of choice for (noninvasive) molecular-genetic and cell biological investigation of phloem development. Moreover, progress in the performance of confocal microscopy instruments, staining protocols that permit routine identification of protophloem sieve elements in whole-mount imaging, and fixation procedures that not only preserve cellular structure in meristematic as well as mature tissues but also reporter protein fluorescence have overcome the major technical hurdles (Truernit *et al.*, 2008; Kurihara *et al.*, 2015; Graeff & Hardtke, 2021). Combined with genetic analyses, these techniques allow the routine observation of protophloem development and thereby aided in the isolation and characterization of numerous protophloem mutants and molecular markers.

1. The trajectory of phloem pole development

In the Arabidopsis root meristem, apical stem cells that reside adjacent to the quiescent center produce two separate protophloem poles (Fig. 3a). Each of them consists of a protophloem sieve element cell file that is flanked by two incipient companion cell files, an incipient metaphloem sieve element cell file to the inside, and two protophloem pole pericycle cell files to the outside (Fig. 3b). The protophloem and metaphloem sieve element cell files derive from the same precursor, which itself derives from a stem cell daughter that also gives rise to the procambial file (Mahonen *et al.*, 2000; Furuta *et al.*, 2014a; Rodriguez-Villalon *et al.*, 2014; Fig. 4a). Thus, close to the stem cell niche, the phloem sieve element lineage undergoes two subsequent periclinal formative divisions. Thereafter, the development of the protophloem sieve elements can be followed along a spatio-temporal gradient of *c*. 20–30 cells (depending on root meristem age/size) from stem cell daughter to mature sieve element (Furuta *et al.*, 2014b; Rodriguez-Villalon *et al.*, 2014; Fig. 4b). Compared with other proximal tissues, protophloem sieve elements thus differentiate early, while neighboring cells still divide or undergo expansion growth (Esau, 1977; Lopez-Salmeron *et al.*, 2019; Graeff & Hardtke, 2021). Molecular markers indicate that the companion cells only differentiate once the protophloem sieve elements are fully elongated and functional (Graeff & Hardtke, 2021). Moreover, they also serve the metaphloem sieve elements, which replace the protophloem sieve elements as they eventually become

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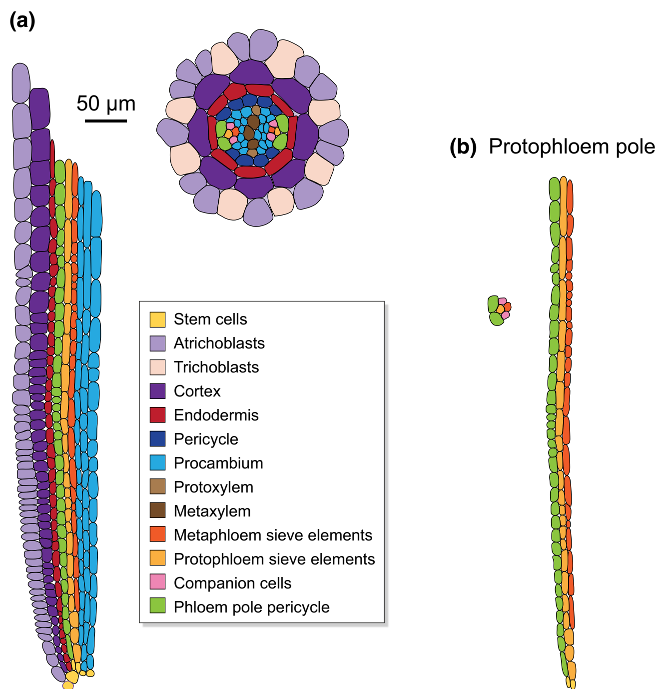

Fig. 3 Protophloem formation in the Arabidopsis root tip. (a) Schematic presentation of cell outlines and tissue types in a (semi-)longitudinal and a horizontal cross-section, drawn from an actual Arabidopsis primary root (without columella-root cap cells). The radial pattern in the stele is determined by mutual interaction between auxin and cytokinin signaling, which control the expression of distinct vascular cell type regulators. See Fukuda & Ohashi-Ito (2019) for a review of auxin–cytokinin interaction in vascular patterning. (b) Highlight of an isolated protophloem pole.

nonfunctional and are obliterated (Esau, 1977; Graeff & Hardtke, 2021). Metaphloem is retained as the main conducting phloem in mature parts of the root but can be replaced by secondary phloem once secondary growth sets in. Finally, the phloem-associated pericycle cells are the main conduit for phloem sap unloading and therefore sometimes considered part of the phloem pole (Ross-Elliott *et al.*, 2017; Otero *et al.*, 2022).

Defects in protophloem development typically have detrimental systemic consequences on root meristem growth and maintenance, and most mutants with such defects were therefore initially isolated because of their short root phenotype (Bonke *et al.*, 2003; Mouchel *et al.*, 2004; Ingram *et al.*, 2011; Anne & Hardtke, 2017; Wallner *et al.*, 2017). In general, they fall into one of three principal categories: mutants that fail to initiate the phloem (sieve element) lineage altogether; mutants in which a fraction of the sieve element precursors does not enter the differentiation program, resulting in not fully penetrant, seemingly stochastic differentiation failures; and mutants in which one or several fundamental aspects of sieve element morphogenesis are absent. In all three cases, the formation of protophloem sieve tubes or their continuity or functioning are disrupted and phloem sap delivery to the meristem is thus suboptimal. Nevertheless, with few exceptions (Ingram *et al.*, 2011), it is generally not possible to mend the impaired root growth of such mutants by providing nutrients in the culture media. This indicates that beyond its nourishing function, the protophloem also delivers or produces systemic signaling molecules and acts as an organizer of root meristem morphogenesis.

2. A transcription factor cascade connects sieve element specification with differentiation

Apart from mutants that generically affect meristem patterning or formative divisions close to the stem cell niche, and which are therefore not specifically required for protophloem formation (Bishopp *et al.*, 2011; De Rybel *et al.*, 2013; Crook *et al.*, 2020), the first category of mutants comprises relatively few players. Among them, the putative transcriptional regulators SUPPRESSOR OF MAX 2-1-LIKE 3 (SMXL3), SMXL4, and SMXL5 (SMXLs hereafter) act redundantly and in a dosage-dependent manner as cell-autonomous key promoters of phloem formation (Wallner *et al.*, 2017). Their central role in phloem initiation was not only demonstrated by the eventual failure of increasingly higher order *smxl* mutants to make root protophloem, but also supported by the observations that viable *smxl* mutant combinations with suboptimal SMXL dosage fail to produce transdifferentiated phloem in the VISUAL assay or secondary phloem in stems (Wallner *et al.*, 2017, 2020).

A combination of live imaging, stage-specific cell sorting, and single-cell mRNA sequencing has recently characterized protophloem sieve element maturation at extraordinary spatial and temporal resolution (Roszak *et al.*, 2021). This study found that a new sieve element appears around every 2 h, whereas the entire trajectory from stem cell daughter to mature sieve element takes *c.* 3–4 d (Fig. 4c). After an initial stem cell division, which only happens every 5–6 d, this trajectory comprises a nearly 3-d-long phase of repeated cell division that also covers the two formative divisions. This is followed by a *c.* 12 h period during which the transition to differentiation and initial cell elongation take place, and finally a *c.* 8 h period of terminal differentiation that encompasses the remodeling of cellular contents including enucleation (Roszak *et al.*, 2021).

The regulatory cascade that drives this developmental trajectory could be deciphered through pseudo-time analysis of the spatially ordered single-cell mRNA sequencing data buckets and its integration with prior knowledge from mutant and gene analyses. This approach revealed that the PLETHORA (PLT) transcription factors, which are strongly expressed in the stem cell niche and required for its maintenance (Aida *et al.*, 2004), are also early key determinants of the protophloem lineage (Fig. 4c). Their activity intersects with the DNA-BINDING ONE ZINC FINGER (DOF) transcription factors PHLOEM EARLY DOF 1 (PEAR1) and PEAR2, which act redundantly with additional DOF transcription factors (collectively name PEAR proteins in the following) to control formative cell divisions in and around developing protophloem sieve elements (Miyashima *et al.*, 2019). At least in the case of PEAR1 and PEAR2, this involves noncell-autonomous action, because whereas the two genes are specifically expressed in developing sieve elements, the proteins are also detected in neighboring cell files and form a short-range gradient, presumably by trafficking via plasmodesmata. The spatial restriction of PEAR expression to the phloem pole is likely due to

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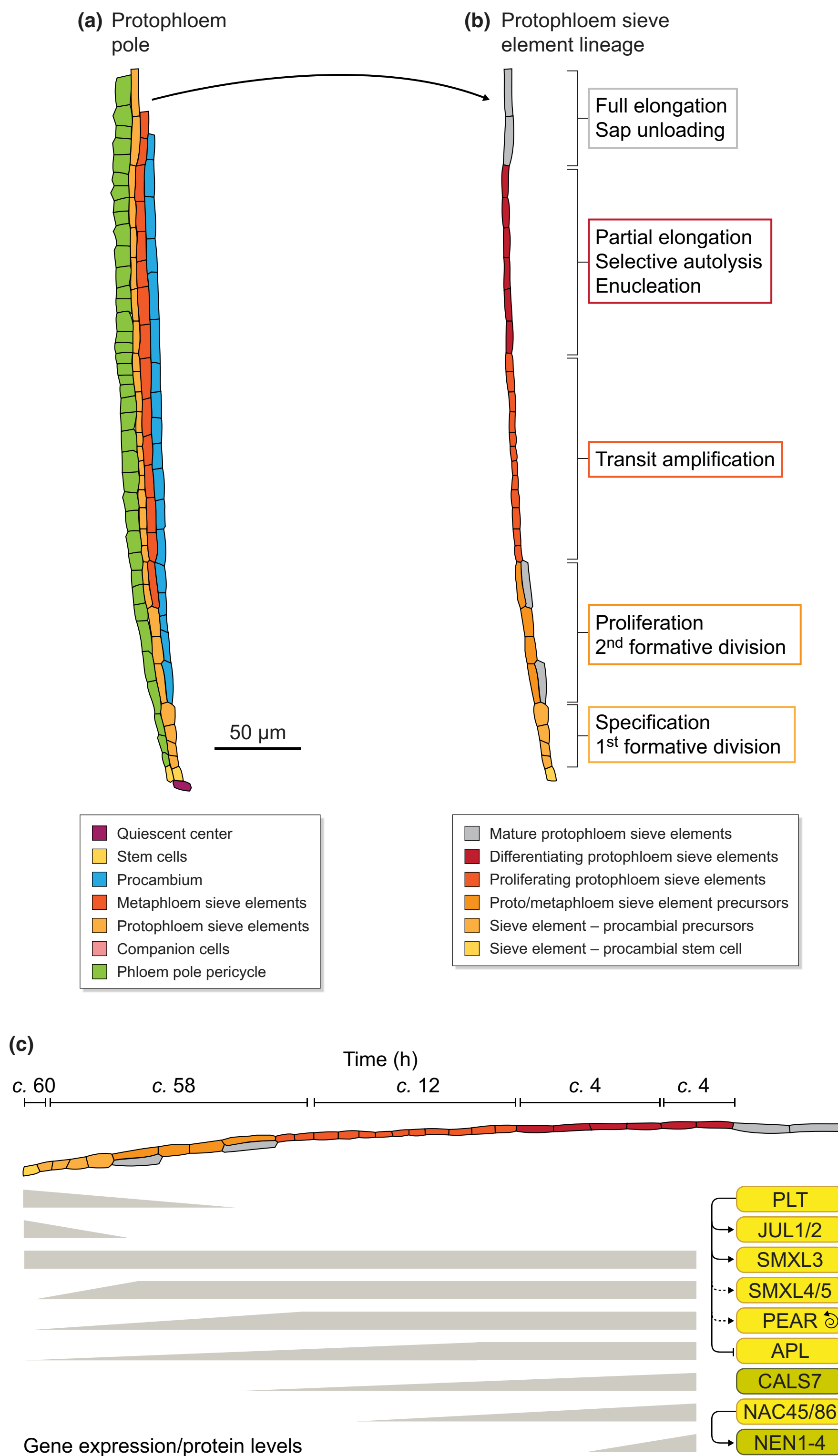

Fig. 4 Transcriptional control of the protophloem sieve element trajectory. (a, b) Schematic longitudinal presentation of an actual protophloem pole (a) and detailed developmental trajectory of the sieve element lineage (b). (c) Spatio-temporal model sketch of the transcription factor cascade that connects stem cell regulators (PLT) with effectors of terminal differentiation (enucleation; NEN1-4). High expression of PLT in the stem cell niche drives expression of the phloem lineage promoter SMXL3, and possibly also of its homologs SMXL4 and SMXL5, as well as of the post-transcriptional regulators JUL1 and JUL2. Parallel expression of PEARs further promotes formative divisions and phloem fate, whereas commitment to the sieve element differentiation program is put on hold by JUL1/2-mediated suppression of *SMXL4/5* translation and antagonistic competition between PLT and PEARs for control of *APL* expression. As inherited PLT protein is gradually diluted across the two formative divisions of the sieve element lineage precursors, PEARs eventually win and stimulate *APL* expression. This triggers the transition toward differentiation and the APL-mediated activation of structural effectors of sieve element formation via another layer of intermediate transcription factors such as NAC45 and NAC86. Expression levels and spatial profiles are indicated approximate and relative based on the literature (see Roszak *et al*., 2021 for details).

interaction with vascular patterning cues emanating from the xylem axis (Miyashima *et al*., 2019). The initial radial pattern in the stele (Fig. 3a) is determined by mutual interactions between auxin and cytokinin signaling (Bishopp *et al*., 2011; De Rybel *et al*., 2014) and possibly reinforced by mechanical constrains emerging from formative cell divisions (Fujiwara *et al*., 2023). Moreover, the pattern is likely stabilized by *PEAR* genes because they are cytokinin-inducible, since the auxin-cytokinin interactions set up high cytokinin signaling in the early protophloem lineage (Fukuda & Ohashi-Ito, 2019; Miyashima *et al*., 2019). Thus, longitudinal patterning by PLT proteins, which form a gradient due to their relatively high stability as they are progressively diluted over cell

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divisions (Mahonen *et al.*, 2014), may intersect with radial patterning to set up phloem pole position.

Whereas PEARs are apparently not directly controlled by PLTs, at least *SMXL3*, but probably also *SMXL4* and *SMXL5*, are likely targets of PEARs and might convey the latter's additional cell-autonomous role in sieve element specification (Miyashima *et al.*, 2019; Qian *et al.*, 2022). Moreover, unlike *PEAR* genes, *SMXL3* expression in the phloem also responds to PLT induction (Santuari *et al.*, 2016). This suggests that both PLTs and PEARs synergistically promote *SMXL3* expression and thereby reinforce specification of the phloem lineage. However, at the same time, PLTs and PEARs antagonize each other in the control of *ALTERED PHLOEM DEVELOPMENT* (*APL*), a key transcriptional regulator of protophloem sieve element differentiation later in the trajectory (Bonke *et al.*, 2003; Furuta *et al.*, 2014b). Close to the stem cell niche, PLTs dominate this competition and prevent *APL* induction. However, as PLT levels gradually fade out, PEARs can take over *APL* control and induce its expression, which is one of the prerequisites for the transition to differentiation (Roszak *et al.*, 2021). Thus, the high levels of PLTs around the stem cell niche may permit transient amplification of stem cell daughters before their actual commitment to the sieve element differentiation program by *APL* induction via a hand-off to PEARs.

Transit-amplifying cells are apparently nevertheless committed to phloem fate very early because of PLT-mediated *SMXL3* induction, as suggested by molecular markers (Rodriguez-Villalon *et al.*, 2014, 2015; Wallner *et al.*, 2017). This commitment may be earmarked and put on hold however by post-transcriptional control of overall SMXL protein activity. Similar to *SMXL3*, both *SMXL4* and *SMXL5* are expressed in the phloem pole from very early on (Wallner *et al.*, 2017). Unlike *SMXL3* however, *SMXL4* and *SMXL5* contain a critical G-quadruplex in the 5′-UTR of their mRNA, which can be bound by the RNA-binding proteins JULGI 1 (JUL1) and JUL2 to prevent their efficient translation (Cho *et al.*, 2018). Both *JUL1* and *JUL2* respond strongly to PLT induction (Santuari *et al.*, 2016) and consistently show high levels of expression close to the stem cell niche (Roszak *et al.*, 2021). Thus, by controlling *JUL* expression, the PLT protein gradient may indirectly promote a SMXL protein counter-gradient (Fig. 4c). Because JUL regulation of *SMXL* mRNAs inhibits phloem differentiation (Cho *et al.*, 2018), this early surge of *JUL* expression might serve as a second regulatory layer to prevent premature entry into the differentiation program.

During the early stages, PEARs also stimulate the expression of rate-limiting RHO OF PLANTS (ROP) GTPase signaling components, which promote the transient cell division plane reorientations in the two successive formative divisions (Roszak *et al.*, 2021). Thereafter, PEARs win the fight for control over *APL* expression, which in turn activates genes that are required for sieve element differentiation (Furuta *et al.*, 2014b; Miyashima *et al.*, 2019). Confirmed APL targets include genes that encode effectors involved in structural remodeling of the cell, such as CALLOSE SYNTHASE 7 (CALS7), which is required for the development of properly sized sieve plate pores (Barratt *et al.*, 2011; Xie *et al.*, 2011), or AUTOPHAGY 8A (ATG8A), which promotes cellular autolysis (Chen *et al.*, 2019). However, APL also promotes the expression of additional transcription factors, NAC45 and NAC86, which act redundantly to control yet another set of effectors that are necessary for sieve element autolysis and enucleation (Furuta *et al.*, 2014b; Fig. 4c). Most prominent among the latter are the NAC45/86-DEPENDENT EXONUCLEASE-DOMAIN PROTEINs 1 to 4 (NEN1 to NEN4), which are necessary for the completion of the enucleation process (Furuta *et al.*, 2014b). In summary, a set of studies over the last two decades identified a spatio-temporal cascade of transcription factors that connects essential regulators of stem cell maintenance to essential structural effectors of protophloem sieve element differentiation.

3. Auxin canalization coordinates the progression of sieve element differentiation

Despite the astounding progress, the regulatory network is still incomplete. For example, how APL suppresses xylem vessel-type cell wall thickening in favor of phloem sieve element-specific cell wall thickening remains unclear (Truernit *et al.*, 2008). Moreover, not all aspects of sieve element differentiation are controlled by APL, for instance *apl* mutants as well as *nac45/86* double mutants still form sieve plates (Truernit *et al.*, 2008; Furuta *et al.*, 2014b; Kalmbach & Helariutta, 2019). Obvious candidates for the control of other aspects are the SMXLs. Although their direct target genes remain to be determined, transcriptome profiles and reporter analyses of *smxl* mutant backgrounds show that key regulators of phloem differentiation such as *APL*, *CALS7*, *NAC86*, or *NEN4* depend on them at least indirectly, consistent with the essential role of SMXLs in protophloem formation (Wallner *et al.*, 2017). Moreover, SMXLs are required for the expression of genes that constitute another regulatory layer, in the timing of sieve element differentiation (Wallner *et al.*, 2017, 2020). Corresponding loss-of-function mutants are characterized by a short root phenotype that can be traced back to not fully penetrant protophloem differentiation defects (Anne & Hardtke, 2017). For instance, in mutants of the *BREVIS RADIX* (*BRX*) and *OCTOPUS* (*OPS*) genes, which both encode distinct plant-specific plasma-membrane-associated proteins, individual protophloem sieve element precursors frequently display differentiation failures (Truernit *et al.*, 2012; Rodriguez-Villalon *et al.*, 2014). Such cells stand out as morphological 'gaps' among flanking, normally differentiating cells, because they do not build up the sieve element-specific cell wall and because their nucleus persists. These seemingly stochastic defects are sufficient to disrupt protophloem sieve tube function, because they prevent efficient phloem sap delivery to the root meristem (Cattaneo & Hardtke, 2017; Cattaneo *et al.*, 2019). The loss of differentiation markers, such as *APL* expression, confirms that the 'gap cells' do not enter the differentiation program (Depuydt *et al.*, 2013; Rodriguez-Villalon *et al.*, 2014). However, they still express other sieve element-specific markers, such as *COTYLEDON VASCULAR PATTERN 2* (*CVP2*), which indicates that 'gap cells' were initially correctly specified (Rodriguez-Villalon *et al.*, 2015; Marhava *et al.*, 2018; Moret *et al.*, 2020).

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BRX is primarily polar localized, at the rootward end of developing protophloem sieve elements, where it interacts with other plasma-membrane-associated proteins to dynamically regulate cellular auxin efflux (Marhava *et al.*, 2018, 2020; Fig. 5a). Together with the AGC-type kinase PROTEIN KINASE ASSOCIATED WITH BRX (PAX), it forms a regulatory module whose localization is reinforced by interaction with phosphatidylinositol-4-phosphate 5-kinases (PIP5Ks) and

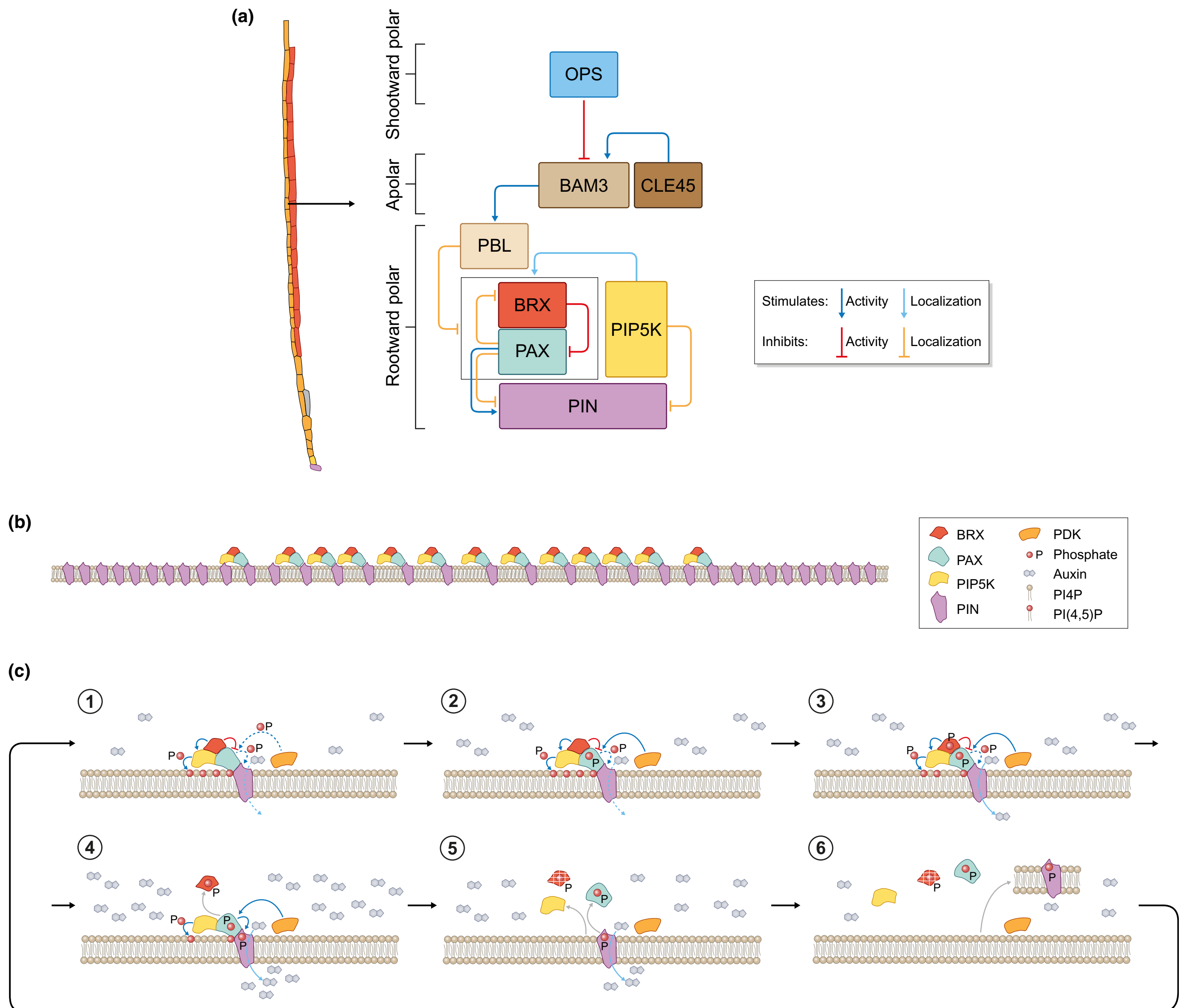

Fig. 5 Regulation of polar auxin transport in developing protophloem sieve elements. (a) Schematic presentation of the key regulatory players that guide sieve element development in the Arabidopsis root tip. Polar auxin efflux by rootward-localized PINs is subject to protophloem sieve element-specific regulation by a plasma-membrane-associated molecular rheostat (BRX & PAX). Its localization is reinforced by interaction with PIP5K, which thereby antagonizes dissociation of the rheostat by BAM3-mediated CLE45 signaling via PBLs. CLE45 sensing is also dampened by the OPS protein. (b) Subcellular localization of the PIP5K-rheostat module and PINs at the rootward plasma membrane of developing protophloem sieve elements. The combined activity of BRX-PAX-PIP5K in the center of the membrane results in depletion of PIN, which is therefore more abundant in the periphery. (c) Model of the dynamics of polar auxin efflux regulation in developing protophloem sieve elements. At low intracellular auxin levels, PAX, BRX, and PIP5K interact at the plasma membrane (1). Local production of PI (4,5)P (a promoter of PAX plasma membrane association) by PIP5K activity reinforces the localization of all three proteins. Because BRX inhibits PAX activity, auxin efflux is low. As intracellular auxin builds up, PAX activity is stimulated via PDK-mediated phosphorylation (2). PAX can now phosphorylate BRX (3), leading to its dissociation from the plasma membrane and allowing activation of PINs via PAX-mediated phosphorylation (4). The stimulation of auxin efflux is eventually reset as BRX is degraded, PIP5K dissociates from the plasma membrane (since its polar localization largely depends on BRX) and PAX association with the plasma membrane consequently decreases as well (5). Moreover, PIN activation by PAX eventually also entails its clathrin-mediated endocytosis (6) and auxin efflux ceases. Eventually, the system returns to its initial state (1) because of auxin-induced BRX and PIP5K transcription in the meantime. These steps play out in a dynamic steady equilibrium that coordinates auxin levels between adjacent cells, thereby prevents fate bistability, and canalizes auxin in the developing sieve element cell file (see Aliaga Fandino & Hardtke, 2022 for a detailed review of auxin canalization in the protophloem).

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controls the local abundance and activity of plasma-membrane-integral PIN-FORMED (PIN) auxin efflux carriers (Fig. 5b). In this regulatory module, PAX is thought to activate PIN-mediated auxin efflux by phosphorylation of target sites in the PIN hydrophilic loop, like other clade VIII AGC protein kinases (Zourelidou *et al.*, 2014; Fig. 5c). Moreover, PAX can phosphorylate its inhibitor BRX, which leads to BRX dissociation from the plasma membrane (Koh *et al.*, 2021; Wang *et al.*, 2023). However, PAX is a relatively poor PIN activator unless it is itself activated by 3-PHOSPHOINOSITIDE-DEPENDENT PROTEIN KINASE 1 (PDK1)- or PDK2-mediated phosphorylation (Zegzouti *et al.*, 2006; Gray *et al.*, 2013; Marhava *et al.*, 2018; Xiao & Offringa, 2020). Because PAX activity is induced by cellular auxin accumulation (Marhava *et al.*, 2018), the system works as a molecular rheostat: High auxin levels activate PAX, likely via PDKs, leading to BRX plasma-membrane-dissociation, PIN activation, and increased auxin efflux. Because auxin levels consequently drop, because BRX is required for efficient PIP5K polarity, and because PIP5K catalyzes the phosphoinositide species that promotes PAX polarity, the system eventually returns to its initial state (Barbosa *et al.*, 2016; Marhava *et al.*, 2018, 2020; Wang *et al.*, 2023; Fig. 5c). Moreover, this cycle is reinforced by PAX-triggered PIN internalization, likely through clathrin-mediated endocytosis (Marhava *et al.*, 2020; Wang *et al.*, 2023). Along a file of interconnected developing sieve elements, these local interactions are thought to play out in a dynamic steady equilibrium that coordinates auxin levels between adjacent cells through controlled polar auxin transport (Moret *et al.*, 2020; Aliaga Fandino & Hardtke, 2022). In addition, this machinery drains auxin from neighboring cell files because of auxin-responsive feed-forward expression of the apolar auxin influx facilitator AUX1 (El-Showk *et al.*, 2015; Moret *et al.*, 2020). Collectively, this multilayered system orchestrates local auxin accumulation in developing sieve elements, which instructs properly coordinated differentiation timing (Marhava *et al.*, 2018; Moret *et al.*, 2020; Aliaga Fandino & Hardtke, 2022). Loss of this coordination, for example in *brx* or *pax* mutants, leads to heightened auxin competition between adjacent sieve elements and triggers fate bistability, which results in the 'gap' differentiation failures that occur, indeed, in a nonrandom pattern (Moret *et al.*, 2020).

The importance of polar auxin transport regulation for sieve element development was recently directly demonstrated by inducible protophloem-specific PIN depolarization, which interfered with sieve element differentiation and root growth (Wang *et al.*, 2023). The notion that elevated auxin levels, albeit not the high levels required for stem cell niche formation or maintenance (Sabatini *et al.*, 1999), are associated with protophloem formation is further supported by observation of auxin sensors, which indicate that auxin levels are higher in developing protophloem sieve elements than in neighboring cell files (Santuari *et al.*, 2011; Moret *et al.*, 2020). Moreover, higher auxin levels in VISUAL favor trans-differentiation toward sieve elements over companion cells (Kondo *et al.*, 2016; Tamaki *et al.*, 2020), and intriguingly, primary root protophloem defects in mutants of the *LATERAL ROOT DEVELOPMENT 3* (*LRD3*; a.k.a. *DA1-RELATED PROTEIN 2* (Peng *et al.*, 2013)) gene can be rescued by adding auxin to the culture medium (Ingram *et al.*, 2011). Thus, protophloem sieve element differentiation may represent a variation of the classic concept of auxin canalization, which posits that auxin accumulation in narrow 'canals' promotes vascular tissue formation (Berleth *et al.*, 2000; Ravichandran *et al.*, 2020; Kneuper *et al.*, 2021; Aliaga Fandino & Hardtke, 2022; Hajny *et al.*, 2022).

4. CLE peptide sensing pathways inhibit sieve element formation

Loss-of-function mutants in *OPS* appear very similar to *brx* mutants (Truernit *et al.*, 2012). However, because the phenotype of *brx ops* double mutants is additive and because dominant *ops* alleles can (partially) rescue the *brx* phenotype, the two genes are thought to act independently (Rodriguez-Villalon *et al.*, 2014; Breda *et al.*, 2017). Indeed, although OPS is also primarily plasma-membrane-associated and polar localized, it is mostly found at the shootward end of cells, opposite BRX (Truernit *et al.*, 2012; Rodriguez-Villalon *et al.*, 2014; Fig. 5a). Yet, OPS polarity possibly only has minor functional significance (Breda *et al.*, 2017). A most fascinating aspect of OPS is its highly dosage-sensitive action. For example, *OPS* copy number increase can suppress the *brx* phenotype, and phosphomimic gain-of-function variants that render OPS protein hyperactive can even complement the severe *brx ops* double mutant phenotype (Breda *et al.*, 2017, 2019). Moreover, ectopic OPS overexpression severely impairs overall plant development, possibly by generically promoting premature cellular differentiation (Anne *et al.*, 2015; Breda *et al.*, 2019).

The exact molecular mode of action of OPS remains largely elusive (Breda *et al.*, 2017). However, various independent evidences suggest that OPS interferes with receptor kinase signaling pathways. For example, OPS may promote brassinosteroid signaling by inhibiting BIN2 activity (Anne *et al.*, 2015), although whether this reflects its genuine function in protophloem development remains unclear (Kang *et al.*, 2017). Interestingly however, brassinosteroid receptors are redundantly required for proper sieve element differentiation (Kang *et al.*, 2017), despite their possible sub-functionalization in other contexts (Fabregas *et al.*, 2018). Moreover, OPS gain-of-function inhibits sensing of the secreted CLE45 peptide ligand by its cognate receptor, the LRR-RK BARELY ANY MERISTEM 3 (BAM3), likely through direct interference with BAM3 signaling component interactions (Breda *et al.*, 2019; Fig. 5a). BRI1 hyperactivity in the protophloem also confers CLE45 resistance (Graeff *et al.*, 2020), and both *brx* and *ops* loss-of-function can be rescued by second-site *bam3* loss-of-function (Depuydt *et al.*, 2013; Rodriguez-Villalon *et al.*, 2015). Together with the finding that CLE45-BAM3 signaling is hyperactive in *brx* and *ops* mutants (Depuydt *et al.*, 2013; Breda *et al.*, 2019), these data suggest that CLE45 signaling must be dampened in developing sieve elements to permit their differentiation. Consistently, transgenic CLE45 dosage increase and external CLE45 application suppress protophloem formation (Rodriguez-Villalon *et al.*, 2014; Czyzewicz *et al.*, 2015).

Besides CLE45, other so-called 'root-active' CLE peptides also inhibit root growth when applied externally at nanomolar concentrations because they suppress protophloem sieve element

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specification (Ito *et al.*, 2006; Kinoshita *et al.*, 2007; Rodriguez-Villalon *et al.*, 2014; Hazak *et al.*, 2017). In the case of stimulus-induced CLE peptides, this might trigger an adaptive shutdown of an unproductive sink meristem (Fukuda & Hardtke, 2020). It remained puzzling however why a few of the most efficient root-active CLE peptides, CLE25, CLE26, and CLE45, are expressed in developing sieve elements then (Rodriguez-Villalon *et al.*, 2014; Anne *et al.*, 2018; Qian *et al.*, 2018; Ren *et al.*, 2019). Moreover, because both *CLE45* and *BAM3* and other rate-limiting components required for CLE(45) peptide sensing are expressed throughout the developmental trajectory (Depuydt *et al.*, 2013; Rodriguez-Villalon *et al.*, 2014, 2015; Kang & Hardtke, 2016; Hazak *et al.*, 2017; Anne *et al.*, 2018; Blumke *et al.*, 2021; Roszak *et al.*, 2021; DeFalco *et al.*, 2022; Hu *et al.*, 2022; Wang *et al.*, 2022), how sieve elements escape the proposed autocrine inhibition of their differentiation remained unclear.

A recent study found that CLE45 perception depends on apoplastic pH (Diaz-Ardila *et al.*, 2023). That is, roots do not sense externally applied CLE45 if they are grown on neutral or alkaline pH. Moreover, unlike neighboring cell files, developing sieve elements no longer respond to CLE45 once they transitioned toward differentiation (Rodriguez-Villalon *et al.*, 2014; Diaz-Ardila *et al.*, 2023). These findings match an observed pH gradient along the apoplast of sieve element cell files, which culminates in a marked alkalinization upon transition to differentiation (Diaz-Ardila *et al.*, 2023). Although a mechanistic explanation for this phenomenon is still missing, the data thus suggest that sieve element precursors self-organize their transition to differentiation by apoplastic alkalinization, which allows them to evade the CLE45 signal (Diaz-Ardila *et al.*, 2023; Fig. 6a). Interestingly, it has been reported that threshold cellular auxin levels at least transiently trigger apoplastic alkalinization (Du *et al.*, 2020; Li *et al.*, 2021). Thus, auxin canalization in developing sieve elements may be required for eventual desensitization against the autocrine CLE45 signal. A recent study, which proposes that CLE45-BAM3 signaling directly interferes with auxin efflux regulation by the

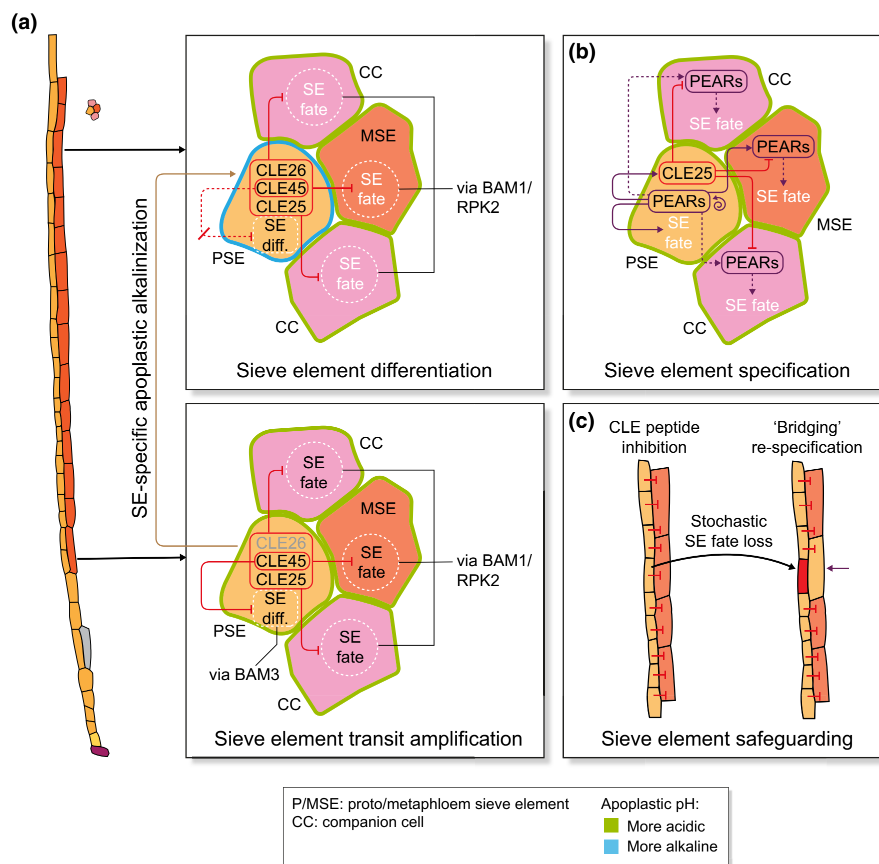

Fig. 6 Phloem patterning through PEARs and CLE peptides. (a) Schematic model of the autocrine and paracrine activities of CLE peptides expressed in and secreted by developing protophloem sieve elements. In the early, proliferation phase (bottom), CLE45 acts autocrine through BAM3 to delay sieve element (SE) differentiation, and paracrine together with CLE25 and CLE26 via BAM1 and RPK2 to suppress ectopic sieve element fate in the neighboring incipient metaphloem and companion cell files. Gradual alkalinization of their apoplast allows sieve elements to eventually evade the autocrine CLE45 signal and transition toward differentiation (top). (b) PEARs are redundantly required cell-autonomously for sieve element specification, but also act noncell-autonomously by trafficking to the neighboring cell files and inducing early formative divisions required for the formation of surrounding cell files. Positive autoregulatory feedback of PEARs on their own expression is dampened by paracrine action of (PEAR-stimulated) CLE25 via its high-affinity receptor BAM1, thereby contributing to the inhibition of sieve element fate in the neighboring cell files. (c) Quantitative easing of paracrine CLE peptide signaling upon stochastic sieve element specification failure relieves fate inhibition in the neighboring metaphloem/companion cells and permits safeguarding of sieve tube continuity, through bridging of the defect in the genuine sieve element cell file.

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BRX-PAX rheostat via the redundant downstream RLCK-VII/PBS1-LIKE (PBL) cytoplasmic kinases PBL34, PBL35, and PBL36 (Fig. 5a; DeFalco *et al.*, 2022; Wang *et al.*, 2023), provides a starting point to explore this notion mechanistically. This is because interference of CLE45-BAM3-PBL signaling with rheostat assembly is antagonized by PIP5K recruitment, and because auxin stimulates PIP5K activity (Tejos *et al.*, 2014).

V. Sieve elements control spatial protophloem pole patterning

CLE peptide signaling pathways fulfill various roles in the root meristem, for instance they contribute to stem cell maintenance and promote formative divisions (Berckmans *et al.*, 2020; Crook *et al.*, 2020). Whether CLE25/26/45 sensing is somehow required for protophloem development, for instance to prevent premature transition of sieve element precursors to differentiation, remains unclear because of redundancies between CLE peptides as well as compensatory cross-regulation between CLE sensing pathways (Nimchuk, 2017; Crook *et al.*, 2020; Kang *et al.*, 2022; Qian *et al.*, 2022; Wang *et al.*, 2023). Moreover, because the BAM3-related BAM1 and BAM2 receptor kinases are redundantly required for CLE-mediated formative divisions in the stem cell niche (Crook *et al.*, 2020) and can bind CLE25, CLE26, and CLE45 (Qian *et al.*, 2022), it cannot be excluded that CLE(45) peptide signaling initiates the sieve element lineage by timing early formative divisions (Ren *et al.*, 2019). However, recent studies also suggest another, paracrine and dosage-dependent role of CLE peptides in phloem pole patterning (Gujas *et al.*, 2020; Qian *et al.*, 2022).

1. CLE peptide signals pattern the phloem pole

Unlike CLE45, CLE25 and CLE26 are perceived independent of both pH and BAM3 (Diaz-Ardila *et al.*, 2023). However, CLE25 and CLE26 play an important paracrine role in phloem pole patterning that is partially redundant with CLE45, as revealed by the phenotype of *cle25 cle26 cle45* triple mutants. Such mutants form ectopic sieve element-companion cell complexes in procambial cell file positions neighboring the genuine protophloem (Qian *et al.*, 2022). Because no extra cell files were observed, this suggests that CLE25/26/45 action prevents ectopic phloem specification (Fig. 6b) and also reiterates the notion that companion cell specification is controlled by sieve elements. Intriguingly, CLE peptide action intersects with the activity of PEARs, which promote *CLE25* and *CLE26*, and less so *CLE45* expression (Qian *et al.*, 2022). Moreover, PEARs are post-transcriptionally down-regulated by CLE25 signaling (Qian *et al.*, 2022). Because PEARs are redundantly required for sieve element formation (Miyashima *et al.*, 2019), this raised the question how CLE signaling can prevent PEAR activity in procambial cell files but not interfere with it in the genuine developing sieve elements? One possible answer is differential expression of BAM1, which is expressed throughout stele tissues but only weakly in developing sieve elements (Crook *et al.*, 2020; Roszak *et al.*, 2021) and binds CLE25 with considerably higher affinity than CLE26 or CLE45 (Qian *et al.*, 2022; Fig. 6b). This could introduce a spatial gradient of CLE signaling effects, which may run counter to the gradient of PEAR activity and is amplified or sharpened by positive cross-regulation between PEARs (Miyashima *et al.*, 2019; Qian *et al.*, 2022). Such a CLE signaling gradient may also be amplified by differential expression of downstream effectors. For example, the CLE45 signaling component MEMBRANE-ASSOCIATED KINASE REGULATOR 5 (MAKR5) is expressed at higher levels in developing companion and metaphloem cells than in the sieve elements (Kang & Hardtke, 2016; Otero *et al.*, 2022; Diaz-Ardila *et al.*, 2023). In summary, PEARs promote their own expression as well as the expression of CLE25, which feeds back negatively on PEAR expression. Combined with quantitatively differential CLE25 perception in developing sieve elements vs adjacent cell files, this reinforces the positioning and specification of a single sieve element file, possibly also aided by PEAR-induced *OPS* expression (Qian *et al.*, 2022; Fig. 6b).

2. Quantitative easing of CLE peptide signaling safeguards the phloem lineage

Besides this patterning aspect, paracrine CLE peptide signaling also performs a safeguarding function for the phloem lineage. Yet another alternative CLE peptide receptor, RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2; a.k.a. TOADSTOOL 2; Mizuno *et al.*, 2007; Nodine *et al.*, 2007), is expressed in the sieve element-surrounding incipient companion cell and metaphloem sieve element cell files (Gujas *et al.*, 2020; Fig. 6a). There it senses CLE45 (and likely also CLE25 and CLE26) to repress ectopic sieve element fate (Gujas *et al.*, 2020). *CLE45* expression is specifically observed in normally developing sieve elements but lost in 'gap' cells that do not enter the differentiation program (Marhava *et al.*, 2018; Gujas *et al.*, 2020; Moret *et al.*, 2020). Thus, lateral inhibition of sieve element fate in the neighboring cells is relieved if a genuine sieve element precursor fails to commit to differentiation by quantitative easing of the local CLE signal (Fig. 6c). Indeed, such re-specification of incipient companion or metaphloem cells could be induced by laser ablation of individual neighboring sieve element precursors (Gujas *et al.*, 2020). The switch to the sieve element fate in the neighboring cell then permits bridging of the local differentiation failure, thereby safeguarding continuity of sieve tubes (Gujas *et al.*, 2020). Such bridging is occasionally observed in wild-type roots, corroborating the physiological relevance of this safeguard mechanism. The likely additive effects of CLE25/26/45 perception by BAM1, BAM3, and RPK2 in this spatial context remain to be precisely defined, but the existing data highlight once more the importance of finely tuned differential quantitative gene activity in protophloem development.

In summary, CLE peptide signaling in the protophloem apparently fulfills several, both autocrine and paracrine roles. It contributes to correct lineage and spatial patterning of the phloem by preventing ectopic sieve element fate acquisition, but also maintains plasticity of the incipient companion and metaphloem cell files to permit repair of rare stochastic sieve element differentiation failures. Once the sieve elements mature and enucleate, CLE peptide expression ceases and releases the

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neighboring cells to progress in their proper differentiation program, which may feedback to reinforce proper phloem pole patterning (Kim *et al.*, 2020).

VI. Beyond sieve elements

Compared with our knowledge on protophloem sieve element development, comparatively little is known about the molecular determinants of companion cell or metaphloem sieve element formation. Yet, despite apparent differences in the mode of development (e.g. unlike protophloem sieve elements, metaphloem sieve elements only start to differentiate once they have fully elongated (Graeff & Hardtke, 2021)), substantial commonalities can be expected. Phloem-specific single-cell mRNA sequencing will likely drive advances in this area (Kim *et al.*, 2021; Roszak *et al.*, 2021; Otero *et al.*, 2022) and already indicates resemblance of transcriptomic profiles between the different phloem pole cell files (Otero *et al.*, 2022). Furthermore, one could expect a significant level of genetic redundancy or tissue-specific subfunctionalization of gene family members. For instance, it was found that *OPS* is also required for metaphloem sieve element differentiation, where it acts fully redundant with another *OPS* family gene (Ruiz Sola *et al.*, 2017; Graeff & Hardtke, 2021). Also, mutations in *VASCULATURE COMPLEXITY AND CONNECTIVITY* (*VCC*), which encodes an OPS interactor of unclear molecular function, enhance the vascular defects in *ops* cotyledons by impinging on PIN localization dynamics (Roschzttardtz *et al.*, 2014; Yanagisawa *et al.*, 2021). A potential role of *VCC* in the root may be masked by extensive redundancy with its homologs (Roschzttardtz *et al.*, 2014; Wilson-Sanchez *et al.*, 2018). Thus, root phloem mutants may instruct shoot phloem development and vice versa.

Single-cell mRNA sequencing could pinpoint redundancies and aid in the identification of novel players. For example, it already enabled the discovery of another set of DOF transcription factors, encoded by the redundant *PINEAPPLE* (*PAPL*) genes (Otero *et al.*, 2022). *PAPLs* mark the incipient companion and metaphloem cell files but are absent from the early phloem pole. Their expression depends on PEARs, but unlike PEAR gain-of-function, PAPL gain-of-function does not induce formative divisions. Moreover, higher order *papl* mutants do not display obvious defects in phloem development. Nevertheless, they display a short root phenotype that can however be rescued by sucrose addition to the medium (Otero *et al.*, 2022), similar to *lrd3* mutants (Ingram *et al.*, 2011). Thus, *PAPLs* and *LRD3* present physiologically relevant targets of phloem functioning.

In summary, the molecular-genetic and cell-biological analysis of phloem development has made impressive progress over the last decade. Collectively, this research field succeeded in establishing a network of transcriptional and cell biological regulators that connects the cell-autonomous and spatial aspects of root protophloem specification with spatio-temporally downstream structural and physiological effectors of sieve element differentiation and phloem function. These major anchor points should not only allow us to fill in the knowledge gaps between them but will likely also instruct molecular-level investigation of phloem formation in other organs.

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Competing interests

None declared.

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