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Review

Sieve elements and their cell neighbours in the Arabidopsis root – Roles and relationships

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1. Introduction

The phloem is the part of the plant vascular tissue that transfers sugars from sites of photosynthesis *i.e.* "source tissues", especially leaves, to sites of carbohydrate consumption *i.e.* "sink tissues", including roots, flowers, and fruits. In addition, the phloem is also responsible for the long-distance transport of several other compounds, such as amino acids and signalling molecules. Thus, phloem transport is crucial for fuelling the growth of sink tissues as well as for the systemic adaptation and coordination of the plant response to external and internal stimuli.

The phloem can be divided approximately into three functional parts; (i) where solutes get loaded into the phloem from source tissues, (ii) a transport section, and (iii) where solutes get unloaded from the phloem into sink tissues. In this review, we primarily focus on the phloem in Arabidopsis roots that serve as a typical sink tissue dependent on carbohydrate import. We describe the role and identity of the different root phloem cell types, and summarize our current understanding of how these different cell types interact, resulting in the establishment of a functional phloem tissue.

2. Phloem sieve tubes

The long-distance transport of solutes occurs in the phloem sieve tubes. These are made up of individual sieve elements, which are elongated cells joined together by their perforated end walls to establish a symplasmic continuum. To enable efficient solute transport, sieve elements are highly specialized cells. In the fully differentiated state, they are enucleate and are also devoid of a vacuole and several other organelles. However, they still possess, for example, mitochondria, plastids and a smooth endoplasmic reticulum (Behnke and Sjolund, 1990). They are thus functional cells but require support of neighbouring cells for long-term survival (see below).

3. Sieve elements in roots

In roots, new cells are produced from cell divisions in the root apical meristem located at the root tip. These cells get added to the existing root cell files, which are arranged in a stereotypical radial pattern. Through the continuous activity of the root meristem, the newly produced cells eventually get displaced from the root tip, elongate and finally differentiate into the respective root cell types (Fig. 1) (Dolan et al., 1993; Williams, 1947).

As typical sink organs, roots depend on carbohydrate import for sustaining the activity of the meristem. This is certainly the reason why sieve elements differentiate closest to the root meristem, prior to all other root cell types (Williams, 1947). The early differentiating sieve element file is referred to as the protophloem sieve tube. Since the protophloem sieve elements are already differentiated in a region where the surrounding root cells still divide and elongate, they are subjected to extensive stretching and, therefore, they remain only functional for a short period of time (Esau, 1939). Located next to the protophloem file are the metaphloem sieve elements (Fig. 1a and b). These differentiate further away from the root tip, where surrounding cells have completed rapid elongation. Thus, they can stay functional for longer time periods (Esau, 1939).

Due to its transparency and small size, the *Arabidopsis thaliana* root is a useful organ for studying the differentiation process of sieve elements, which are located in the stele in the center of the root. The Arabidopsis root stele exhibits two phloem poles (Fig. 1a) (Dolan et al., 1993). In the primary state, each phloem pole consists of one protophloem and one metaphloem sieve element file. These originate from the same stem cell in the root apical meristem (Fig. 1b and c) (Mähönen et al., 2000). This stem cell first produces a daughter cell by anticlinal division, which subsequently divides periclinally to give rise to two cell files - an inner procambial cell file and an outer sieve tube cell file. The sieve tube cell file then divides to produce the proto- and metaphloem files (Fig. 1b) (Rodriguez-Villalon et al., 2014). In Arabidopsis, the protophloem sieve elements start differentiating approximately 120 μm above the root

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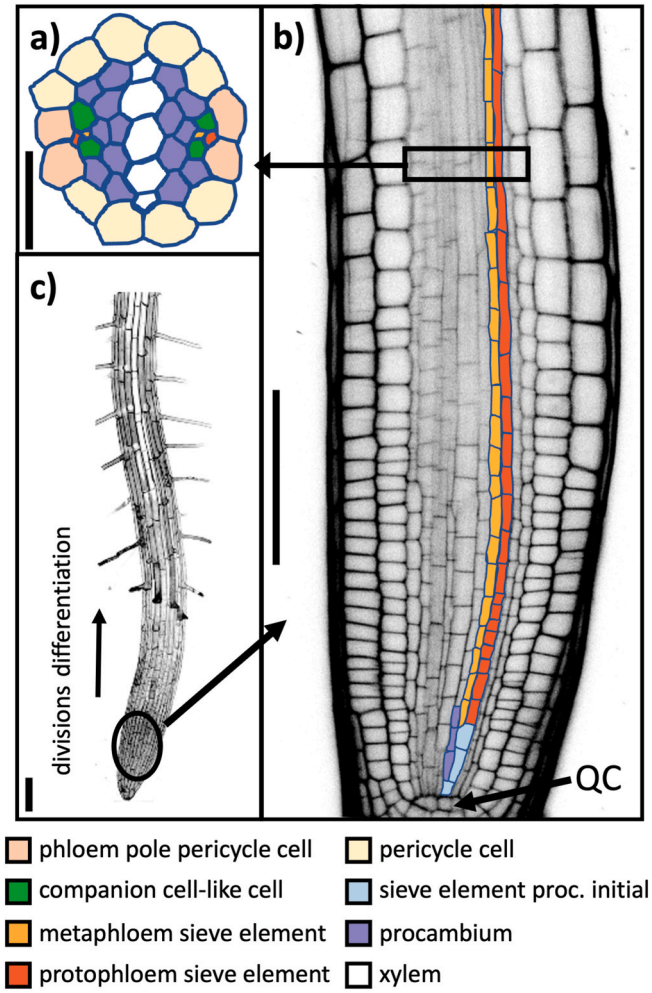

Fig. 1. Phloem in the Arabidopsis root with relevant cell types coloured in (see figure annotation for colour coding). a) Sketch of a cross section through the Arabidopsis stele at the position indicated with a box in b). b) Longitudinal optical section taken with a confocal laser-scanning microscope through an Arabidopsis root tip. The quiescent center (QC) is labelled. One of the phloem sieve tube files is visible in this section. c) Overview of an Arabidopsis root. The circle indicates the position of the root apical meristem. Scale bars: 20 μm in a), 100 μm in b) and c).

quiescent center, the metaphloem sieve elements at approximately 1400 μm (Fig. 1b) (Graeff and Hardtke, 2021).

4. Sieve element neighbours

In addition to the metaphloem sieve tube in the more central position, the Arabidopsis protophloem sieve elements have usually four more neighbouring cells: Two pericycle cells towards the outside of the root (called phloem pole pericycle cells), and two lateral cells (Fig. 1a). These lateral cells are adjacent to both, proto- and metaphloem sieve elements and are now more commonly referred to as companion cells (*e.g.* Otero and Helariutta, 2017 and references therein). However, these cells do not meet one important criterion for this denomination, which is that they are apparently not derived from the same initial as the adjoining sieve tube(s), but from a neighbouring initial (Mähönen et al., 2000). For this reason, in the more classical literature it is often stated that in some plant species root protophloem may lack companion cells (Esau, 1939; van Bel, 1996), and *sensu stricto* this is also true for Arabidopsis (Oparka and Turgeon, 1999). As such, it may be more appropriate to name these cells "companion cell-like" (CC-like) cells to distinguish them from the "true" companion cells in the shoot or during secondary growth in all organs. These true companion cells derive from a common precursor cell, the sieve element - companion cell mother cell. Keeping this in mind may be relevant for developmental studies. For instance, it was recently suggested that GLYCOGEN SYNTHASE KINASE 3 (GSK3) activity is an important determinant of the sieve element: companion cell ratio during secondary growth (Tamaki et al., 2020). Whether GSK3 also plays a role in the development of the protophloem CC-like cells remains to be investigated.

5. The roles of sieve element neighbours

From a physiological perspective, the CC-like cells in the Arabidopsis root tip are most likely very similar to true companion cells. The fact that many genes expressed in these cells are also expressed in true companion cells supports this claim (*e.g.* Graeff and Hardtke, 2021; Smetana et al., 2019; Stadler et al., 2004). Moreover, the plasmodesmata connecting the CC-like cells to the protophloem sieve elements have the typical structure also seen in the shoot phloem; they are branched on the companion cell side and lead into one single pore on the sieve element side (Ross-Elliott et al., 2017).

Maintaining the functionality of enucleate sieve elements is one of the important roles of companion cells (Esau, 1939). It is of course debatable if this function is necessary for the short-lived protophloem sieve elements. However, as mentioned earlier, in the primary growth state, the CC-like cells also appear to be the only cells with this identity next to the differentiated metaphloem sieve elements (Graeff and Hardtke, 2021). Thus, they may well have a sustaining function for these cells.

Loading of sugars and other macromolecules into the phloem is another important function of companion cells. In Arabidopsis, SUCROSE TRANSPORTER 2 (SUC2) is chiefly responsible for the transport of sucrose across the companion cell membrane into the sieve element - companion cell complex (Gottwald et al., 2000). SUC2 is, however, not only expressed in companion cells of source tissue, but also in those of the transport phloem and in the CC-like cells of the root, probably approximately from the point at which the metaphloem starts differentiating (Stadler et al., 2004; Truernit and Sauer, 1995). There is good evidence that in these tissues SUC2 plays a role in the retrieval of sugars leaking out of the phloem as they are transported to the sink tissues (Gould et al., 2012).

How sugars get unloaded from the phloem into sink tissues may depend on the plant species and also on the tissue type (Patrick, 1997). For Arabidopsis roots, it was nicely shown that phloem-mobile fluorescent probes move down through the metaphloem sieve tube until they reach the location where the metaphloem sieve elements are not yet fully differentiated. At this developmental stage, solutes move into the adjacent protophloem and then further down until they arrive in the first differentiating protophloem cell. Unloading of solutes then occurs into the phloem pole pericycle cells (Ross-Elliott et al., 2017; Truernit, 2017). The fact that the CC-like cells apparently do not play a role in unloading was unexpected, and a contribution of these cells to the unloading process of specific molecules, for example signalling macromolecules which should evade degradation, was discussed (Lee and Frank, 2018).

6. Phloem cell differentiation

In the last two decades, several molecular regulators of protophloem differentiation were identified in Arabidopsis (for reviews see *e.g.* Anne and Hardtke, 2017; Blob, 2018; Rodriguez-Villalon, 2016; Seo et al., 2020). Loss-of-function of many of these regulators results in mild phenotypes, which likely reflects the essential role of phloem tissue in that disruption to crucial regulators would result in plant death. One of the most documented loss-of-function phenotypes is the so-called "gap-cell" phenotype, where individual cells within a developing protophloem file fail to differentiate in a seemingly stochastic way (Fig. 2a).

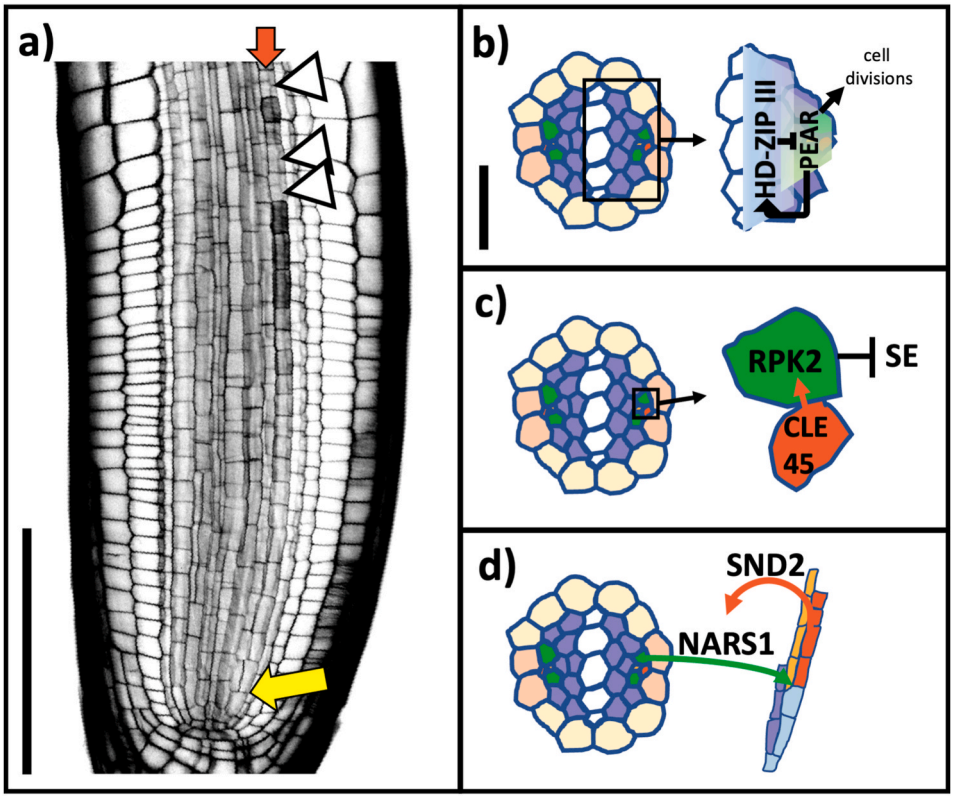

Fig. 2. Patterning the phloem pole a) Gap-cell phenotype. The protophloem file is indicated with an orange arrow. Due to cell wall thickening, differentiating protophloem cells appear darker. Gap-cells are indicated with white arrowheads. The first asymmetric cell division is indicated with a yellow arrow. The second division is missing. Shown is a confocal laser-scanning image of an *ops* root tip stained with propidium iodide. b) Regulation of cell division by HD-ZIP III and PEAR transcription factors. PEAR proteins moving away from the developing phloem promote cell division, while HD-ZIPIII proteins in the xylem and the procambium area prevent further PEAR movement. c) Lateral restriction of sieve element cell fate. CLE45 moving out of the developing protophloem sieve element inhibits sieve element specification in the neighbouring cell by binding to RPK2. d) Longitudinal regulation of cell divisions in the phloem file. NARS1 generated in companion cell-like cells of the differentiation zone promotes the second periclinal division in the phloem cell file. SND2 in the developing protophloem file promotes NARS1 expression. Scale bars: 100 μm in a), 20 μm in b).

The first mutants identified displaying this phenotype were *octopus* (*ops*), *brevis radix* (*brx*), and *cotyledon vascular pattern 2* (*cvp2*) *cvp2-like 1* (*cvl1*) (Depuydt et al., 2013; Rodriguez-Villalon et al., 2014, 2015; Truernit et al., 2012). These mutants all exhibit a short root phenotype, likely a consequence of the interruptions in the protophloem file, which were shown to impinge on solute transport towards the root tip.

In contrast to protophloem differentiation, our knowledge about metaphloem sieve tube differentiation is very poor. First, compared to the protophloem, it is even more difficult to obtain microscopic images of the metaphloem. However, with the development of novel techniques for plant tissue preparation for non-invasive microscopy there is hope this challenge could be overcome soon (Truernit, 2019). Indeed, a successful visualization of the early stages of metaphloem development in the Arabidopsis root using confocal laser-scanning microscopy was published recently (Graeff and Hardtke, 2021). Second, lethal mutants of metaphloem, as with the protophloem, hampers progress in research and our ability to investigate phloem development. Nevertheless, it was recently reported that *OPS* together with its homolog *OPS-LIKE 2* (*OPL2*) also have a role in metaphloem sieve tube differentiation (Ruiz-Sola et al., 2017). In the *ops opl2* mutant, gap-cells can be seen also in the metaphloem sieve tube, and root growth is severely impaired. To date, the molecular mechanism of the action of these proteins in the metaphloem remains unknown.

How the CC-like cells develop their identity remains equally unknown. The genes known to be expressed earliest in developing CC-like cells of the root are *ALTERED PHLOEM DEVELOPMENT* (*APL*) and *SISTER OF APL* (*SAPL*) (Bonke et al., 2003; Ross-Elliott et al., 2017). *APL*, however, is expressed throughout the developing phloem pole, and *SAPL* is weakly expressed also in the developing metaphloem file. Thus, these genes cannot be used easily as markers for the isolation of developing CC-like cells. For differentiated companion cells, however, several transcript data are available (Brady et al., 2007; Jean-Baptiste et al., 2019; Otero and Helariutta, 2017). These do, however, not distinguish between the different companion cell types.

7. Patterning the phloem pole

Several lines of evidence suggest that the developing protophloem sieve tubes are central to the patterning of the phloem pole. High cytokinin signalling in the developing phloem pole induces expression of a family of PHLOEM EARLY DOF (PEAR) transcription factors. This occurs already in the sieve element/procambium initial, but after the formative cell divisions described earlier, highest PEAR expression is found in the developing protophloem (Miyashima et al., 2019). The PEAR transcription factors move from the site of their expression into the surrounding cells and trigger cell divisions in these cells (Fig. 2b). Movement and expression of PEAR proteins is antagonized by the auxin and PEAR upregulated HD-ZIP III transcription factors PHABULOSA (PHB), CORONA (CNA), and REVOLUTA (REV). These HD-ZIP III proteins are present in the xylem axis and procambium of the stele, but they are cleared from the phloem pole by mobile micro-RNAs mir165 and mir166 moving in from the root cell layer surrounding the stele, the future endodermis. In this way, a robust phloem domain is established in the developing root (Miyashima et al., 2019).

Another line of evidence for the organizing capacity of the developing protophloem sieve tubes comes from a recent study by Gujas and colleagues (Gujas et al., 2020) (Fig. 2c). They describe a contingency mechanism that may allow for some flexibility for re-patterning a functional phloem pole in case the protophloem would fail to form properly. Based on marker gene expression, the authors demonstrated that cells located next to the still proliferating protophloem cell file (*i.e.* very close to the root tip) were able to adopt protophloem cell identity in case the protophloem cells did not develop properly. In addition, the authors suggested that the small peptide CLAVATA3/EMBRYO SURROUNDING REGION 45 (CLE45), which is expressed in the developing protophloem cell file, moves laterally into adjacent cells. Perception of CLE45 by RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2), which is expressed in the cells surrounding the already differentiating protophloem sieve elements (*i.e.* further away from the root tip), would then result in repression of sieve tube identity in these cells. Consequently, in

loss-of-function mutants of RPK2, "identity switches" between the developing protophloem file and the adjacent developing CC-like files increased, and mutants with gap-cell phenotypes could be rescued.

The above-mentioned mechanism may also be part of a process that establishes the identity of the sieve element neighbours once the protophloem is specified. Single cell transcriptomics of developing phloem pole cells identified protophloem sieve element enucleation as trigger for switching on similar gene regulatory networks in the surrounding cell types (Otero et al., 2021). Further, these studies found transcriptional similarities between CC-like cells and phloem pole pericycle cells, as well as between metaphloem cells and CC-like cells in the very early stages of their development. This again highlights the flexibility of cell fates in the young phloem pole.

Mutants with gap-cells in the protophloem cell files are interesting candidates for studying the influence of the developing protophloem sieve elements on neighbouring cells. Using marker genes and transcript analysis, gap-cells were found to display a hybrid identity between companion cells and sieve elements (Gujas et al., 2020). Although highly speculative, this finding may suggest that signals from differentiating protophloem sieve tube cells may turn adjacent cells into CC-like cells, even if they are located in the same cell file. This hypothesis is supported by the fact that mutants with protophloem gap-cells show discontinuous expression of the SUC2 companion cell marker (Rodriguez-Villalon et al., 2014; Truernit et al., 2012). However, it remains to be investigated whether the missing companion cell identity in these mutants correlates with the position of the gap-cells in the studied plants.

Metaphloem sieve tube differentiation, on the other hand, seems to follow its own robust pathway. Metaphloem sieve tubes appear to develop normally next to protophloem gap-cells (Rodriguez-Villalon et al., 2014; Truernit et al., 2012). Moreover, even when the second division in the sieve tube cell file, which leads to the formation of proto- and metaphloem sieve tubes (Fig. 1b), is missing (a phenotype frequently occurring in gap-cell mutants), cells with metaphloem identity can be found next to the protophloem sieve elements (Anne and Hardtke, 2017). Thus, it is very likely that the procambial cells in the metaphloem position adopt metaphloem cell identity in these cases (Rodriguez-Villalon et al., 2014). Overall, it seems to be clear that metaphloem sieve tube identity develops largely independently from protophloem sieve tube identity, but the molecular mechanisms involved remain to be elucidated.

The missing second asymmetric division in the sieve tube cell file of gap-cell mutants has been interpreted as a secondary effect resulting from the reduced meristematic activity caused by the phloem transport defects in these mutants (Anne and Hardtke, 2017). However, taking into account that the developing sieve tube acts as a local organizer of cell divisions in the root stele (Miyashima et al., 2019), the effect could also be more direct. Another layer of complexity to this was recently added by the study of Kim et al. (2020) (Fig. 2d). The authors found that the transcription factor NAC-REGULATED SEED MORPHOLOGY 1 (NARS1) positively regulates the second cell division in the sieve tube cell file. The NARS1 promoter was active in the CC-like cells in the root differentiation zone, but, unfortunately, it was not possible to detect a tagged NARS1 protein when expressed under its native promoter. However, a series of experiments suggested that NARS1 directly controlled the division of the sieve element precursors. This would require movement of NARS1 from the CC-like cells through the developing sieve tubes towards the root tip, a process which may well be impaired in gap-cell mutants. In addition, SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN 2 (SND2), expressed in the developing protophloem sieve tubes and upregulated by NARS1, positively regulated expression of NARS1, thus establishing a positive feedback loop along the longitudinal axis of the root.

8. Conclusion and outlook

Collectively, a picture emerges in which the functionally intimately related cells of the root primary phloem also tightly influence each other's development to ensure efficient delivery of solutes towards the root tip. Most of the molecular players in these pathways still await to be discovered. Investigating phloem development is laborious, since phloem cells are very narrow and long and the tissue is deeply embedded into plant organs. To date, our knowledge about companion cell or metaphloem differentiation is especially limited. However, some recent publications suggest that the first steps towards a better understanding of the development of these tissues have been made.

CRediT authorship contribution statement

Elisabeth Truernit: Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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