Differentiation of conductive cells: a matter of life and death

Jung-ok Heo[1,2], Bernhard Blob[1], Yka Helariutta[1,2]

[1] Sainsbury Laboratory, Cambridge University, Bateman Street, Cambridge CB2 1LR, UK

[2] Institute of Biotechnology, University of Helsinki, 00014 Helsinki, Finland

Corresponding author: Yka Helariutta (yrjo.helariutta@slcu.cam.ac.uk)

Co-first authors: Jung-ok Heo (jungok.heo@slcu.cam.ac.uk) and Bernhard Blob (bernhard.blob@slcu.cam.ac.uk)

Abstract

Two major conducting tissues in plants, phloem and xylem, are composed of highly specialized cell types adapted to long distance transport. Sieve elements (SEs) in the phloem display a thick cell wall, callose-rich sieve plates and low cytoplasmic density. SE differentiation is driven by selective autolysis combined with enucleation, after which the plasma membrane and some organelles are retained. By contrast, differentiation of xylem tracheary elements (TEs) involves complete clearance of the cellular components by programmed cell death followed by autolysis of the protoplast; this is accompanied by extensive deposition of lignin and cellulose in the cell wall. Emerging molecular data on TE and SE differentiation indicate a central role for NAC and MYB type transcription factors in both processes.

Introduction

Plants have evolved highly specialized vascular tissues which enable the translocation of various molecules that are vital for their growth and survival. Phloem, one of principal components of the vascular system, transports sucrose from photosynthetic organs to actively developing ones, such as flowers, fruits, buds, and roots. Another major conduit is the xylem, a path through which water absorbed by the root is transported to the above-ground tissues. Recent studies have shed light on the role of phloem and xylem as conduits mediating long-distance communication. Various plant growth substances, such as phytohormones, small non-coding RNAs, proteins, and peptides were shown to be transported through these conduits [1-5]. Both conduits undergo specialized differentiation by which internal flow resistance is minimized and the cell walls become bolstered, enabling efficient molecular trafficking through the conduits. During the differentiation of phloem sieve elements (SEs), some organelles are selectively degraded and others are retained in a modified form. This differentiation process allows phloem to transport molecules through their symplastic space. By contrast, xylem tracheary elements (TEs) undergo complete clearance of their cellular components during differentiation, leaving only an apoplastic transport route available. In addition, the thick and lignified cell walls of xylem vessels and fibres provide mechanical support for the plant.

Recent experiments have lent support to pressure flow theory, a long-standing hypothesis which explains the transport mechanism in the phloem [6]. According to the theory, bulk flow in SEs is driven by a difference in osmotic pressure, which is high in source tissues, where sucrose is loaded, and low in sink tissues, where sucrose is unloaded. Cohesion-tension theory offers an explanation for the upward translocation of water through the xylem, proposing that the evaporation of water at the leaf surface due to transpiration creates a tension (negative hydrostatic pressure) which drives the ascent of sap. A stream of water is maintained in xylem TEs by the cohesive and adhesive properties of water [7]. In the following sections, we will describe the ontogeny of phloem and xylem tissues and discuss the molecular mechanisms regulating the differentiation of each conduit.

Specification and maintenance of the vascular tissue pattern

In *Arabidopsis*, vascular cell fate is determined very early in embryogenesis, at the globular stage. Subsequent periclinal divisions give rise to xylem, phloem, and procambium precursors in a spatially organized manner. The spatial coordination by oriented cell divisions is attributed to the interplay between two phytohormones, cytokinins and auxin [8], which are also crucial to maintain a stable vascular pattern in the post-embryonic root by establishing two distinct hormone signalling domains [9-11]. The diverse patterns of vascular tissue organization in different species have been reviewed in [12,13].

Differentiation of phloem and xylem – cytological changes

In contrast to xylem TEs, in which programmed cell death (PCD) followed by autolysis results in the total loss of protoplasmic contents, sieve tube elements undergo selective autolysis, retaining a few organelles and the plasma membrane (Figure 1). Before the onset of the differentiation, sieve element precursor cells do not show any obvious cytological differences compared with neighbouring cells. As the sieve tubes begin differentiating, the stroma of their plastids become electron-transparent, making the plastids discernible [14] (Figure 1a). Sieve element plastids are divided into two types: S-type plastids, which contain starch inclusions, and P-type plastids, which contain proteinaceous inclusion bodies and are typical of monocots [15]. Another feature noticeable early in SE differentiation is the presence of phloem protein (P-protein) bodies, agglomerates of protein tubules or filaments. In mature SEs, P-proteins are found in various forms, such as tubules, filaments, and amorphous or crystalline structures [16]. In most angiosperms, P-protein bodies disperse into filaments or a bundle of filaments and eventually align with the plasma membrane at the cell's side walls or on the sieve plates [17] (Figure 1a).

SE differentiation culminates in nuclear degradation. In protophloem SEs in the *Arabidopsis* primary root, enucleation is marked by the deformation of the nucleus. Prior to degradation, the nuclear envelope becomes disorganized and the nucleus shrinks. During enucleation, the nuclear contents diffuse into the cytoplasm, where they are degraded, leaving behind a remnant of the nuclear envelope (Figure 1a). The entire process takes 10 minutes [18]. When the nucleus changes its shape, the nucleolus becomes fragmented. The mitochondria also gradually change shape during nuclear degradation [18], and the ER undergoes structural changes by making stacks, losing ribosomes and migrating toward the wall [14] (Figure 1a). The appearance of perinuclear complexes composed of vacuoles and many other organelles was shown to coincide with nuclear disintegration (Figure 1a). No signs of a large central vacuole were observed, but small lytic vacuoles were shown to persist before and after enucleation, suggesting that the mechanism is slightly different from PCD [18]. In mature SEs, modified organelles such as the plastids and mitochondria are eventually aligned with the parietal walls of the cell (Figure 1a).

In angiosperms, SEs are functionally associated with specialized parenchyma cells named companion cells (CCs). Unlike SEs, which lose many of their organelles, CCs are characterized by their increased cellular density. A large nucleus and an increased number of ribosomes, mitochondria, ER, and plastids are observed in CCs [19] (Figure 1a). It has long been proposed that CCs support SEs to keep them viable. However, details about how they are functionally related remain far from being understood.

A detailed picture of the cytological changes during TE differentiation have been pieced together by studying an *in vitro* cell culture system of *Zinnia elegans*. The start of TE differentiation is marked by a swelling of the vacuole, which leads to altered tonoplast permeability and eventually vacuole collapse. The release of hydrolytic enzymes into the cytoplasm increases its acidity, activating cytoplasmic enzymes, which results in dilation of the ER and Golgi and the complete degeneration of other organelles [20,21] (Figure 1b). Cytoplasmic streaming is no longer active after the vacuole collapses. It has been proposed that the degradation of nucleus in TE differentiation is related to a post-mortem autolysis event rather than being a controlled degradation [22] (Figure 1b).

Differentiation of phloem and xylem – cell wall elaboration

The initiation of differentiation in both SEs and TEs is delineated by the thickening of their walls. Although there is considerable variation in the walls' structure and composition, the walls of mature SEs are thought to consist of primary cell wall components such as cellulose and pectin. However, some species were shown to have lignified thick cell walls in SEs [14]. The ends of SE cells undergo significant modification in the formation of sieve plates, when the plasmodesmata (PD) at the junctions of SEs become enlarged and perforated. These perforated pores begin to be visible once the nucleus has degraded (Figure 1a). Callose (beta-1,3-glucan), a constituent of cell walls, is deposited on sieve plates as well as at PD on the lateral cell wall [23].

Lateral sieve areas in mature sieve tube elements provide a symplastic path to CCs through branched PD. These modified PD, also known as phloem pore units (PPU), link multiple pores from the side of companion cell to a single channel from the sieve tube element (Figure 1a). PPUs allow short-range cell-to-cell communication. In fact, a wide range of molecules, such as small RNAs and even exogenous protein such as GFP have been shown to be transported from CCs to SEs through PPUs. The size exclusion limit (SEL) of these modified PD is slightly larger than other PD, allowing proteins as large as 67 kDa to move through them [4,24]. Some RNA molecules have been shown to need RNA-binding proteins for symplastic transport, suggesting that molecular trafficking between SEs and CCs is selective [4].

Unlike phloem SEs, which exhibit thick walls consisting mainly of primary cell wall material, the diverse xylem elements are distinguished by unique patterns of secondary cell wall (SCW) deposition [25]. In addition, while tracheids, an evolutionarily ancient form of TEs, are connected only by pit-pairs on their common walls, vessel elements are connected by perforation plates at their junctions [26]. The secondary walls of vessels and fibres are composed of cellulose, hemicelluloses and phenolic lignin polymers deposited in special patterns which can be used to clearly distinguish the vessel types [27,28]. SCW patterning depends on the orientation of cellulose microfibrils, which is directed by the arrangement of microtubules [29,30] (Figure 1b). Following cellular autolysis, the primary cell walls are partially hydrolysed, especially at positions free of secondary cell wall [22]. Details about secondary cell wall formation and fibre differentiation are well described in [22,31].

Molecular regulation of phloem and xylem differentiation

Despite general similarities in SE and TE differentiation, such as cell wall thickening, autolysis and the loss of the nucleus, most of regulatory components vary.

The MYB transcription factor (TF) *ALTERED PHLOEM DEVELOPMENT (APL)* is a key regulator and promoter of SE differentiation [32]. The loss-of-function mutant has cells with mixed SE and TE identity in the position of SEs and is seedling lethal [32,33]. Two members of the plant-specific NAC TF family, *NAC45* and *NAC86*, are downstream targets of *APL* and are expressed in differentiating SEs and in the phloem pole pericycle cells. They redundantly regulate the expression of *NAC-DEPENDENT EXONUCLEASE 1*, *2* and *4 (NENs)* and control the translocation of NEN2 from the cytosol to the nucleus. The NAC-NEN pathway was shown to regulate nuclear degradation during SE differentiation (Figure 2a). Similar to the *apl* mutant, a double knockout mutant, *nac45-2 nac86*, exhibited seedling lethality and retained nuclei in SEs, while mitochondrial shape changes and the formation of sieve plates occur normally [18]. Proper transformation of PD into SPPs connecting the SEs is affected by the level of choline, which depends on the expression of *CHOLINE TRANSPORTER LIKE 1* (*CHER1)* [34]. However, the detailed cellular processes influenced by CHER1 and the choline level have yet to be revealed. In addition, callose deposition by CALLOSE SYNTHASE 7 (CALS7) is required for correct formation of SPPs [35].

The recent development of a culture system with inducible ectopic phloem differentiation has facilitated the identification of new regulatory elements. In this system, *NAC020* was identified as an early, SE-specific NAC TF that potentially acts as a negative regulator of *APL* [36] (Figure 2a). Furthermore, several genes have recently been found to regulate early SE development, including *BREVIX RADIX (BRX), OCTOPUS (OPS), CLAVATA3/ENDOSPERM SURROUNDING REGION 45 (CLE45)* and its receptor *BARELY ANY MERISTEM3 (BAM3)*. So far, the evidence implicates *BRX* as an upstream regulator of *CLE45/BAM3* signalling, while *MEMBRANE-ASSOCIATED KINASE REGULATOR 5* was recently shown to be a downstream amplifier of the *CLE45* signal [37]. In the *Arabidopsis* root, BRX and OPS are plasma-membrane-associated proteins which are polarly localized rootward and shootward, respectively, but their regulatory interaction is not yet fully understood [38,39]. BRX is thought to translate auxin signalling in a contextual manner, while OPS is a positive regulator of SE differentiation and might activate *BRI1-EMS-SUPPRESSOR 1 (BES1)* by sequestering its repressor BRINSENSITIVE 2 [38,40,41]. The small mobile peptide CLE45 suppresses SE differentiation in SE precursor cells [42] (Figure 2a). Loss-of-function or over-expression of these genes results in differentiated SE strands containing undifferentiated cells which interrupt the continuity of the cell file [39-43]. In addition, *COTELYDON VASCULAR PATTERN2 (CVP2)* acts genetically upstream of OPS by controlling the level of phosphatidylinositol-4,5-biphosphate (PIP_2), although the exact mechanism of this control is not yet understood (Figure 2a). A double mutant of *CVP2* and its homolog *CVP2-like1 (CVL1)* has discontinuous SE cell files, similar to the *ops* and *brx* mutants [44].

The role of auxin response in TE fate determination and the initiation of differentiation, and the tight control both spatially and temporally are well described [45-50]. CLE41 and CLE44, small peptides which contain the TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR sequence in their C-terminus, were shown to suppress TE differentiation in *Arabidopsis*, similar to the effect of CLE45 on SE differentiation [51]. Interestingly, this signalling involves the suppression of *BES1*, which is required for the differentiation not only of SEs but also of TEs from procambial and cambial cells [52]. TE differentiation is controlled by two other members of the NAC TF family,

VASCULAR-RELATED NAC-DOMAIN6/7 (VND6/7), which were both shown to be sufficient to induce differentiation of TEs in other cell types. *VND6/7* control the expression of genes responsible for secondary cell wall thickening and PCD, resulting in temporal co-occurrence of these two events [53-55]. The MYB TFs *MYB46* and *MYB83* act downstream of *VND6/7* to regulate secondary cell wall synthesis [56,57]. Another NAC TF, VND-INTERACTING2 interacts with VND7 and represses TE differentiation [58]. Furthermore, the level of the polyamine thermospermine, which is controlled by the essential biosynthetic enzyme *ACAULIS5 (ACL5)*, regulates the patterning of SCW and delays cell death [59]. Thermospermine and *ACL5* are thought to act through *SUPRESSOR OF ACAULIS51 (SAC51)*, which is also a direct target of *VND7* [60] (Figure 2b). Xylogen, a secreted proteoglycan first isolated from *Zinnia* cultures and active in both monocots and dicots, is another important signal in TE differentiation. It is polarly localized towards the shoot apex on the plasma membrane, and only the homolog ARABIDOPSIS THALIANA XYLOGEN PROTEIN 2 (XYP2) but not XYP1 is specifically expressed in vascular tissues. The double loss-of-function mutant of *xyp1/2* showed discontinuous TE formation, indicating that communication between TE is important for the correct formation of TE cell files [61,62].

XYLEM CYSTEINE PROTEASE1 (XCP1) and XCP2 contribute to micro-autolysis within the tonoplast, as well as the mega-autolysis after the tonoplast ruptures [63]. The S1-type nuclease ZINNIA ENDONUCLEASE 1 (ZEN1) was shown to be necessary for post-mortem DNA degradation during TE differentiation in *Z. elegans* cell cultures [64]. In *Arabidopsis*, BIFUNCTIONAL NUCLEASE1 (BFN1), which shows a high sequence similarity to ZEN1, is expressed in differentiating xylem and exhibits DNase activity, suggesting that it may have a similar function [65,66]. In *Arabidopsis*, *METACASPASE9 (MC9)* is required for the clearance of cellular contents after cell death in xylem vessel elements, which is likely mediated by papain-like cysteine proteases [67,68] (Figure 2b).

Conclusion

Improvements in imaging and tissue culture techniques have led to an improved understanding of both SE and TE differentiation. For example, serial block-face scanning electron microscopy has recently been used to study the morphological changes during SE differentiation in greater detail [18,34]. Many similarities have between the two process have been revealed. NAC TFs serve as key regulators of both SE and TE differentiation, with the couplets *NAC45/86* and *VND6/7* respectively controlling the two processes [18,56,58]. Both differentiation programs appear to require active *BES1* signalling and are inhibited by CLE peptides [40,42,51,52]. In addition, polarly-localized signalling components on the plasma membrane, including BRX, OPS and xylogen, are necessary to ensure the correct pace and continuity of differentiation in both types of conduit [39,41,62]. Despite these similarities, the differentiation processes are executed by different functional components in these conductive cells. Furthermore, the regulatory networks known to be involved in SE differentiation are relatively incomplete, whereas those controlling TE differentiation are better understood. This difference may be due to the early discovery of the key regulators *VND6/7*, which allowed experimental initiation of TE differentiation from other cell types, greatly advancing research in this field [56]. Similar regulators of SE differentiation, *NAC45/86*, have only been recently discovered, and their capacity to induce cytosol degradation is not yet fully understood [18]. Nonetheless, ectopic overexpression of *NAC45/86* in an inducible manner could offer improved avenues for the study of

the cellular changes during SE differentiation, particularly with regard to the temporal order of events. Further advances may also become possible by exploiting the ability of the VISUAL tissue culture system to induce ectopic SE-like differentiation [36]. However, a full understanding of SE differentiation and function can only be achieved by incorporating the insights gleaned from these approaches into *in planta* experiments.

Figure captions

Figure 1.

Overview of the vascular tissue differentiation. (a) Phloem sieve elements undergo a cellular rearrangement in which organelles, such as plastids, mitochondria, endoplasmic reticulum are modified and reoriented toward the parietal walls of the cell. The nucleus changes its shape and eventually becomes degraded. P-proteins are formed in early stage and retained after nuclear degradation. In mature SEs, P-protein filaments are found at the side walls of the cell. At maturity, SE forms sieve plates - highly perforated pores between SEs. SEs are associated with companion cells (CCs) through branched plasmodesmata, also known as phloem pore units. CCs are characterized by their dense cytoplasm as they contain increased number of organelles, such as plastids and mitochondria. (b) Xylem tracheary elements undergo programmed cell death. After vacuole ruptures, organelles including the nucleus are degraded. The deposition of secondary cell walls occurs before and after the vacuole rupture. Lignins are deposited in a specific pattern at lateral walls of TEs. The end walls of a vessel element become modified to form perforation plates. However, the timing of when it is formed is unclear.

Figure 2.

Regulatory networks in SE and TE differentiation. (a) OPS regulates SE differentiation by suppressing activity of BIN2, a putative suppressor of *BES1*. BRX restricts the expression of BAM3, the receptor of CLE45 signalling which inhibits SE differentiation. The interplay of *BRX* and *OPS* is not fully understood yet. In addition, the PIP_2 level regulated by CVP2 has a major impact on SE differentiation. The MYB type TF APL regulates the expression of *NAC45/86*. NENs as downstream targets of the APL-NACs pathway play a key role in SE enucleation. *APL* expression is downregulated by *NAC20*. (b) Thermospermine levels controlled by ACL5 regulate the expression of the TE differentiation suppressor *SAC51*. At the same time, the key regulators of TE differentiation, VND6/7, also directly control expression of *SAC51*. VND6/7 control the expression of *MYB46/83*, key regulators of secondary cell wall thickening, and MC9 that is required for autolytic degradation of cellular contents. The expression of XCP1/2 proteases are also controlled by VND6/7 and participate in both vacuolar rupture and subsequent autolysis. VNI2 interacts with VND7, thus repressing its function. ZEN1/BFN1 are thought to facilitate the breakdown of the nucleus. Xylogen is a positive signal for TE differentiation, while CLE41/44 signaling has an inhibitory effect. Uncertain interactions are represented with dotted lines.

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