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LETTER TO THE EDITOR

Long-Distance Trafficking: Lost in Transit or Stopped at the Gate? OPEN

Using *Arabidopsis thaliana* micrografts, a recent Breakthrough Report by Paultre et al. (2016) showed scion-expressed, fluorescent protein-tagged signal peptides in the root unloading zone of wild-type rootstocks. This finding indicates that tagged proteins formed in the companion cells entered neighboring sieve elements through the pore-plasmodesma units (PPUs) and were swept along with the phloem stream across the graft union into the root. The coincidence of these mobile peptides with proteins found in phloem exudate proteomes led the authors to conclude that companion cells constitutively lose soluble and targeted proteins to the translocation stream. However, this conclusion may be premature, as it is possible that the pressure release introduced by cuts into the phloem could contaminate the phloem sap with companion cell-derived macromolecules, explaining their appearance in phloem proteomes. Indeed, we find that the stress evoked by wounding has effects on both sieve elements and companion cells, as indicated by ultrastructural comparison of exuding phloem with intact controls. In addition, further experiments are needed to exclude alternative possibilities that overexpression, saturation of intracellular binding sites, and/or lack of recognition at the PPUs of the tagged signal peptides can lead to contamination of the phloem stream and displacement of the signal peptides into the root.

MOBILITY OF MACROMOLECULES FORMED IN COMPANION CELLS

The extent to which entry into and exit from phloem sieve tubes is subject to control has been an unsolved question for decades. Sieve tube elements (SEs) and companion cells (CCs) are coupled by sieve pores that branch toward the CC into many plasmodesmata, called pore-plasmodesma units (van Bel, 1996). Such PPUs form the decisive interface between the stationary phase in CCs and the mobile phase of the "running river" in sieve tubes (Sjolund, 1997). It is a prerequisite for phloem loading and transport that PPUs in minor vein phloem are able to convey sugars and other small molecules freely (Schulz, 2015). However, active phloem loading at the same time depends on retention of energy-rich compounds formed in CCs (UDP-galactose and ATP, respectively; Beebe and Turgeon, 1992; DeWitt and Sussman, 1995). Phytohormones, nucleotides, and molecules of similar molecular mass and hydrodynamic radius are detected in phloem exudate, but it is not clear to what extent their entry and exit is regulated.

In long-distance signaling, an exciting question is whether cytosolic proteins or proteins targeted to the nucleus or other organelles can freely pass PPUs. This question was experimentally addressed in the recent work of Paultre et al. (2016). The authors used Arabidopsis micrografts to test whether transit peptides tagged with GFP variants are phloem mobile. Grafting is the gold standard for testing phloem mobility, since it can discriminate the presence of a given macromolecule in phloem cells from its long-distance transport. In this way, the structural P-proteins PP1 and PP2 previously were shown to be phloem-mobile in cucurbits (Golecki et al., 1998, 1999). Grafting also provided the first bona fide evidence that selected RNAs and RNA binding proteins can move in the phloem (Ruiz-Medrano et al., 1999; Xoconostle-Cazares et al., 1999). Paultre et al. (2016) expressed tagged peptides in the scion under strong promoters and checked for their appearance in the nontransgenic rootstock. Surprisingly, the majority of the peptides not only crossed the graft interface and were unloaded from the phloem, but also adequately targeted the reporter to plastids, nuclei, and peroxisomes of the apical root stele. The largest construct unloaded was the cytosolic actin binding domain2 of fimbrin (67 kD). By contrast, fluorescent proteins that were targeted to the secretory pathway did not cross the graft interface (Paultre et al., 2016). The authors concluded that organelle-targeted and cytosolic proteins were constitutively lost to the translocation stream, making it a challenge to discriminate systemic phloem signals against background.

The article raises the question of whether any given cytosolic macromolecule in the CCs can be "lost in transit" without specificity. Alternatively, PPUs might have a mechanism that selectively retains some macromolecules and permits others to escape. While the article indeed shows that the chosen reporter constructs escaped into the phloem stream, it only touches upon the question of a selective retention mechanism. This question deserves more research, the background of which is reviewed below.

NATIVE PROTEINS ARE RETAINED IN, RATHER THAN RELEASED FROM, COMPANION CELLS

A CC-formed protein is predisposed to escape and enter long-distance trafficking when (1) it is strongly expressed on free ribosomes in the cytosol, (2) its binding sites at intracellular targets in CCs are saturated, and (3) it is not recognized by PPUs and is smaller than their size exclusion limit. Strikingly, the phloem-mobile signal peptides reported by Paultre et al. (2016) meet each of these conditions.

(1) The tagged transit peptides were strongly expressed in the transgenic shoot under the 35S promoter or the CC-specific *SUC2* promoter (Paultre et al., 2016). None of the tested transit peptides was expressed under its native promoter.

(2) The chosen peptides primarily guide the fluorescent proteins to their target organelles (Paultre et al., 2016) and bind to the respective import receptors. In case of chloroplast proteins associated with photosynthesis (FNR, plastocyanin, and RBCS1a), this would be the TOC159 receptor, which guides the preprotein to the translocon TOC75 (Bauer et al., 2000; Bölter and Soll, 2016). Competition between the overexpressed

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transit peptides and the endogenous, highly abundant photosynthesis-related preproteins (Jarvis and López-Juez 2013) could lead to a shortage of binding sites. When overexpressed, H2B-GFP and the actin binding site of fimbrin are also likely to saturate the NLS receptors and G-actin, respectively. Interestingly, the chloroplast-targeted RecA homolog1 peptide derived from a DNA repair protein was not detected at the root tip (Paultre et al., 2016). Like other housekeeping plastid preproteins, it would bind to the TOC132 receptor (Broad et al., 2016) rather than to TOC159. It can be speculated that TOC132 is less occupied by endogenous preproteins and thus able to bind the tagged RecA signal peptide and retain it in CCs.

(3) If PPUs have a retention mechanism for macromolecules, it does not appear to recognize free fluorescent proteins or those with an N-terminal transit peptide: Up to 67-kD large fluorescent protein constructs, expressed under the *SUC2* promoter in CCs, were unloaded from the root protophloem (Stadler et al., 2005) as were the fluorescent protein-tagged transit peptides (Paultre et al., 2016). By contrast, the transcripts of these peptides were not detectable by RT-PCR, indicating that they were retained in the CCs.

Thus, the results of Paultre et al. (2016) might be explained by an imbalance between the expression level and availability of intracellular binding sites and/or by a lack of PPU retention signals that could distinguish native CC proteins from the analyzed constructs.

PPUs ARE ABLE TO CONTROL THE EXIT OF ENDOGENOUS MACROMOLECULES

CCs cannot afford to lose cytosolic proteins to the phloem stream constitutively. Rather, a specific gating mechanism opens the PPU for passage of protein monomers that are needed for turnover in SEs, such as PP1 and PP2 (88 and 24 kD, respectively; Leineweber et al., 2000). Transcripts of these proteins localize to CCs, and any loss of the monomers to the assimilate stream is compensated for by transcription and translation in CCs (Bostwick et al., 1992; Dannenhoffer et al., 1997). RT-PCR confirmed that it is the protein, and not the transcript, that is mobile across a graft boundary (Golecki et al., 1999). These and later findings, such as movement of the flowering locus T (FT) protein, but not its transcript (Corbesier et al., 2007), can be explained only by a gating mechanism in the PPUs that retains certain macromolecules (like the *PP1* and *PP2* transcripts) but allows passage of others (like PP1 and PP2 protein monomers, FT, CmPP16 protein, and *CmPP16* or *CmNACP* transcripts; Ruiz-Medrano et al., 1999; Xoconostle-Cazares et al., 1999).

A filter mechanism at PPU sites might be questioned, considering the surprisingly large number of mobile RNAs that were recently identified in grafts between Arabidopsis accessions (Thieme et al., 2015). However, the exit of mobile RNAs from CCs into the phloem stream is directly or indirectly gated by tRNA-like structures (Zhang et al., 2016). In case of the FT protein, its exit from CCs depends on presence of the endoplasmic reticulum- and plasmodesma-localized FTIP1 protein (Liu et al., 2012). The given examples of native, phloem-mobile macromolecules indicate presence of a gating mechanism in PPUs that controls the passage of macromolecules from CCs into SEs, and possibly also vice versa.

PHLOEM PROTEOME DATA CONTAIN BOTH SIEVE ELEMENT AND COMPANION CELL PROTEINS

Paultre et al. (2016) analyzed proteome data to reject the argument that the tagged peptides access the phloem and reach the root tip just because of a strong overexpression in scion CCs. These data originated from laser microdissection and exudation (Deeken et al., 2008; Batailler et al., 2012). As in other proteome studies, exudate was collected after cutting into the phloem and facilitating exudation with EDTA (Giavalisco et al., 2006; Lin et al., 2009). The analysis showed a preference for small proteins (below 70 kD; Paultre et al., 2016). The authors correlated small size and high gene expression with the probability of a protein to escape CCs and suggested that mobile proteins represent the routine transfer of small proteins from CC to SE.

The relevance of the data is debatable since the microdissection did not discriminate between SEs, CCs, and phloem parenchyma cells (Deeken et al., 2008), and exudate is easily contaminated by CC contents. Even if the first exudate droplets—and thus possible contributions from other cells at the cut surface—were removed with filter paper (Lin et al., 2009), CC contents might indirectly contaminate the exudate. It can be proposed that the turgor loss, propagating upstream in a sieve tube, nearly instantaneously leads to pressure release in the intimately coupled CCs and subsequent loss of CC contents into the lumen of the exuding sieve tube.

All articles on phloem proteomics discuss the possibility that proteins found in the phloem sap originate in CCs, in particular, proteins related to energy conversion, protein biosynthesis, and nuclear import (Giavalisco et al., 2006; Lin et al., 2009; Batailler et al., 2012). These proteins would be expected to be highly abundant in CCs, but not present in SEs, since the latter are devoid of nuclei, protein biosynthesis machinery, Golgi apparatus, chloroplasts, and vacuoles (Froelich et al., 2011; Jensen et al., 2016). However, based on their comparative proteome analysis, Lin et al. (2009) hypothesize that some proteins might be synthesized in the SE which even might contain Golgi, endosomes, and small vacuoles. This hypothesis is supported neither by electron micrographs fixed under optimal conditions (Froelich et al., 2011) nor by the fact that exudate suppresses ribosomal activity (Zhang et al., 2009). This makes the preparation-induced loss of CC contents the most probable source of the above mentioned proteins in exudate proteomes.

PRESSURE RELEASE BY EXUDATION CUTS AFFECTS SIEVE ELEMENTS AND COMPANION CELLS

For an impression of what sudden pressure release does to conducting SEs and their CCs, we studied the ultrastructure of exuding castor bean (*Ricinus communis*) seedlings. Castor bean exudes spontaneously when the phloem is cut. In connection with a previous study (Orlich et al., 1998), we fixed well-exuding seedlings and controls. Like any other invasive preparation method, fixation can lead to the loss of phloem turgor and thus artifacts at the incision site. Therefore, our fixation method for vascular tissues routinely includes discarding the ends of longitudinal sections before embedding, thereby minimizing artifactual changes in the phloem distant to the incision sites. Figure 1A shows a control with the typical phloem ultrastructure: The SE is electron-translucent and its organelles are found at the cell periphery rather than covering the sieve plates. This SE has five CCs (CC

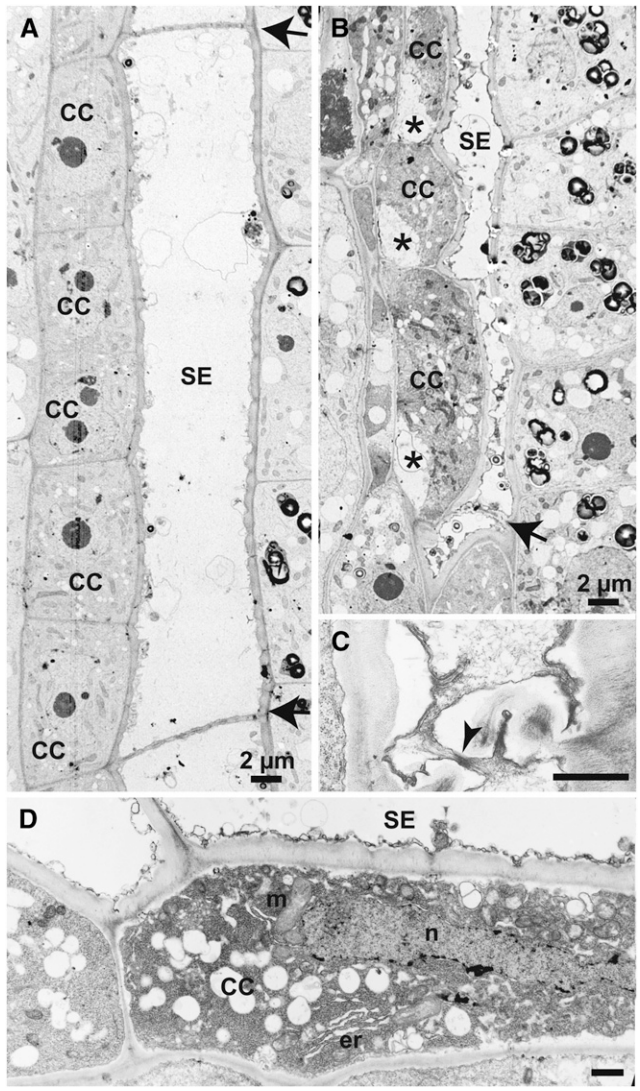

Figure 1. The Ultrastructure of Intact and Exuding Hypocotyl Phloem of Castor Bean.

(A) Control. SE with peripheral organelles, sieve plates (arrows), and a CC strand with five CCs, the cytoplasm of which is slightly denser than that of neighboring cells and contains spherical nuclei with prominent nucleoli.

(B) to **(D)** Exuding phloem.

(B) SE 1.15 mm from the exudation cut with distal sieve plate (arrow) and three CCs bulging out toward the SE and showing retraction of the plasma membrane from the cell wall (asterisk).

(C) Proximal sieve plate of the SE in **(B)** with moderate callose (white cell wall areas) and open pore channels, loosely filled with P-protein filaments (arrowhead). Bar = 1 μm.

(D) CC 0.25 mm from the exudation cut, with dense cytoplasm, vesiculating endoplasmic reticulum (er), mitochondria (m) that are less dense than the cytoplasm, and an elongated and invaginated nucleus (n). Bar = 1 μm.

All experiments were performed with 5-d-old castor bean seedlings. The cotyledons, but not the rest of the seedling, were incubated in buffered sucrose for 4 h. This preincubation provides a steady state in internal sucrose concentration important for phloem loading and export from the cotyledons. Controls: 10-mm longitudinal hand sections of hypocotyls were taken at the hook and fixed with paraform and glutaraldehyde according to Orlich et al. (1998). To exclude preparation artifacts, the apical and basal 2.5-mm ends were discarded before embedding. Exuding seedlings: The hypocotyls were cut in the hook and exudate collected with a microcapillary every 5 min. Well-exuding hypocotyls were fixed like the controls, but without discarding the basal end at the exudation cut.

strand; Esau, 1969) with spherical nuclei, prominent nucleoli, and slightly denser cytoplasm than the neighboring parenchyma cells. Figure 1B depicts a sieve element 1.15 mm from the exudation cut that appears squeezed out, with the liberated space taken by three CCs. Each of these CCs is plasmolysed; the plasma membrane distal to the SE-CC interface is retracted from the cell wall (Figure 1B, asterisk) and the dense cytoplasm is displaced toward the SE. Both sieve plates of this SE are moderately covered with callose without constricting the pore channels, which are loosely filled with P-protein filaments (Figure 1C, arrowhead). Figure 1D depicts a CC 0.25 mm from the exudation cut. Changes in the ultrastructure include the shape of the nucleus from spherical to elongated with invaginations, reduced density of mitochondria, increased density of the cytoplasm, and a vesiculation of the endoplasmic reticulum, all of which are indicative of cellular stress (Figure 1D). These micrographs are representative of samples from four different, well-exuding castor bean seedlings. Generally, sieve pores of exuding sieve tubes showed only moderate callose deposition and/or plugging with P-protein filaments, compatible with the view that they were not totally blocked, thus allowing prolonged exudation of phloem contents.

Collectively, the ultrastructural changes of the phloem show that the exudation cut leads to an immediate pressure release in each severed sieve tube that is conveyed via the PPUs to the CC protoplasts that plasmolyze and lose contents, including macromolecules. This implies that exudate proteomes are excellent sources for the contents of the phloem, but do not necessarily allow discriminating the mobile phloem sap fraction from the immobile CC fraction.

CONCLUSION

P-proteins and FT are translated in CCs, conveyed by PPUs into SEs, and swept along with the phloem stream. Some, but not all, transcripts formed in CCs are specifically targeted to the sieve tube lumen for long-distance trafficking. By contrast, the origin of the vast amount of proteins documented in the phloem exudate cannot unambiguously be assigned to either SE or CC. The exudation cut leads to a pressure release and compromises not only SEs, but

also CCs at a considerable distance to the cut as shown in electron micrographs of exuding phloem (Figures 1B to 1D). Any retention mechanism at PPUs, working in the intact phloem, seems to be overruled by the sudden turgor loss.

To test the specificity and efficiency of this retention mechanism, the graft-transmitted proteome should be analyzed in heterografts sensu strictu (grafts between plants of different genotypes), as done for the phloem transcriptome (Thieme et al., 2015). This requires the native promoter and the entire preprotein, since it might be recognized by PPUs in its folded state only. Proteomic (or immune) detection depends on sufficient specificity of the amino acid sequence to identify the graft partner. Fluorescent protein tags are less suitable for these analyses as they may increase the protein size above the size exclusion limit of PPUs, which lies between 56 and 67 kD (Itaya et al., 2002; Stadler et al., 2005). With this approach, proteins that traffic in the phloem can be unambiguously identified and the question of whether proteins can freely pass PPUs can be answered more definitively.

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AUTHOR CONTRIBUTIONS

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