THE GREAT ESCAPE: Phloem Transport and Unloading of Macromolecules

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Abstract The phloem of higher plants translocates a diverse range of macromolecules including proteins, RNAs, and pathogens. This review considers the origin and destination of such macromolecules. A survey of the literature reveals that the majority of phloem-mobile macromolecules are synthesized within companion cells and enter the sieve elements through the branched plasmodesmata that connect these cells. Examples of systemic macromolecules that originate outside the companion cell are rare and are restricted to viral and subviral pathogens and putative RNA gene-silencing signals, all of which involve a relay system in which the macromolecule is amplified in each successive cell along the pathway to companion cells. Evidence is presented that xenobiotic macromolecules may enter the sieve element by a default pathway as they do not possess the necessary signals for retention in the sieve element–companion cell complex. Several sink tissues possess plasmodesmata with a high-molecular-size exclusion limit, potentially allowing the nonspecific escape of a wide range of small (<50-kDa) macromolecules from the phloem. Larger macromolecules and systemic mRNAs appear to require facilitated transport through sink plasmodesmata. The fate of phloem-mobile macromolecules is considered in relation to current models of long-distance signaling in plants.
INTRODUCTION

The phloem of higher plants forms an extensive conduit for the long-distance transport of a diverse range of compounds. The elongated conducting elements of the phloem, the sieve elements (SEs), are joined by perforated end walls known as sieve plates to form sieve tubes, a functional continuum of cells that is closely connected with adjoining, nucleate companion cells (CCs) and a range of associated parenchyma elements (2, 3, 58, 60, 80, 82). During differentiation, SEs lose many of their organelles and at maturity, function as enucleate pipes through which solutes are transported from source (net carbon exporting) to sink (net carbon importing) regions of the plant. During maturation of SEs, partial autolysis of the protoplast results in a reduced component of organelles comprising the plasma membrane (PM), smooth endoplasmic reticulum (ER), mitochondria, and starch-storing plastids (2, 3, 58, 60, 80, 82). This thin layer of organelles, appressed against the SE wall, is referred to as the parietal layer and appears to provide the only means of membrane continuity between the SE and CC (63, 82). Because of the intimate structural and functional connections between SE and CC, the SE-CC complex is frequently viewed as a single functional entity within the phloem (63, 82, 92–94). Although considerable research has been devoted to unraveling the mechanism(s) of long-distance solute movement through the phloem, the pressure-flow hypothesis of Münch (55) is now commonly accepted (63, 82, 92, 93). According to this model, solute movement through the sieve tubes occurs by mass flow and is driven by a pressure gradient between source and sink regions of the plant (55). In the 1980s, attention turned to the mechanisms by which solutes, particularly sucrose, were loaded and unloaded from the phloem (for review, see 80). This sharp focus on sucrose movement to a large extent detracted from the fact that the phloem carries a wide range of macromolecules, in addition to solutes, throughout the plant. These include proteins, systemic wound signals, and pathogens (6, 10, 14, 27, 50, 58, 75, 80, 86, 103). Studies of phloem exudate, collected from aphid stylets, highlighted the enormous diversity of materials moving in the translocation stream (23, 25, 76, 78, 103), but unfortunately offered little insight as to their role.
Recent research has depicted the phloem as a dynamic transport system capable of carrying several macromolecular signals, including RNA information molecules, from source to sink (14, 27, 41, 53, 70, 75, 78, 79, 87). For example, during systemic posttranscriptional gene silencing, small RNA signal molecules are believed to be transported from their sites of generation in source tissues to induce gene silencing in distant sink tissues (31, 41, 64, 95, 96). Research on phloem exudates has also demonstrated the presence of numerous proteins (25, 28, 29, 32, 34, 76, 79, 103) and endogenous mRNAs (70, 78, 101) in sieve tube exudate. Together with a large body of evidence that viral genomes, either as nucleoprotein complexes or intact virions, may be translocated in the phloem (4, 10, 27, 50, 58, 65, 69, 72, 75, 77, 81, 91), the picture that emerges is one in which the transport phloem functions as an information superhighway (41, 70), translocating a wide range of macromolecular traffic throughout the plant. What is the role of the many diverse macromolecules in the phloem and what is their final destination? The following discussion focuses attention on how macromolecules enter and exit the translocation stream and examines their potential fate within sink tissues. In particular, we examine critically the evidence for macromolecular signaling through the phloem and address the problems inherent in unloading macromolecules from the terminal phloem of sink organs. Space constraints restrict us only to case histories in which specific studies have begun to shed light on the origin and destination of phloem-mobile macromolecules.

ENTERING THE SE-CC COMPLEX

Before macromolecules gain access to the plant’s long-distance transport pathway, they must enter the SE-CC complexes in source tissues. In exporting leaves this is assumed to occur in the phloem of minor veins, where the bulk of solute loading is thought to occur (46, 69, 80, 89, 92) (Figure 1a). However, larger vein classes could also potentially be entry points for systemic macromolecules, and there is no a priori case as to why macromolecules should enter the phloem at the same sites as solutes (58). Minor veins of dicotyledons usually have a characteristic architecture, comprising two or three mature sieve elements, associated companion cells, and phloem parenchyma elements (4, 19, 26, 46, 58, 69, 75, 76, 92) (Figure 1a). Prior to the sink-source transition these minor vein complexes are immature (69, 89) and symplastically coupled to the mesophyll. Some macromolecules may enter the SE-CC at this early stage of differentiation and subsequently be translocated out of the leaf when the SE-CC matures. Early invasion of the SE-CC complex can occur with systemic viruses, which may enter the immature phloem and replicate prior to maturation of the SE (58, 69) (Figure 1b). At this early stage of SE-CC differentiation, macromolecules could enter SEs either from the CC or directly from phloem parenchyma elements, as at this stage all cell types are symplastically coupled to the mesophyll (58, 69, 92).
SYMPLASTIC VERSUS APOPLASTIC LOADING

The mechanism of phloem loading appears to differ among plant species (26, 58, 80, 92), suggesting that the pathways of macromolecular trafficking into the SE might also vary. To date, two main pathways of phloem loading have been identified, apoplastic and symplastic (80, 92). In putative apoplastic loading species, sucrose is loaded into the SE-CC actively by transmembrane carriers (80, 92), and the numbers of plasmodesmata around the SE-CC complex become progressively reduced during development (92). However, a few plasmodesmata remain at this interface (58, 69, 92), and these become potential routes through which systemic macromolecules may enter the SE-CC complex. During maturation of the SE-CC complex, the plasmodesmata that connect the SE and CC become branched on the CC-side of the wall and open up into the sieve element via a single pore (2–4, 45, 58, 80, 82, 92–94) (Figure 1c). Callose is associated with the entrances of these plasmodesmata (Figure 2b, see color plate), and they have been termed pore-plasmodesma units (PPUs) to reflect their unique architecture (94). In putative symplastic loading species, solutes enter the SE via specialized CCs known as an intermediary cells (ICs; 80, 89, 90, 92). In symplastically loading Cucurbitaceae, branched plasmodesmata increase in numbers between the ICs and bundle sheath cells prior to the sink-source transition (97). However, the PPUs that form between SE and CC are similar in architecture regardless of whether the mode of loading is apoplastic or symplastic (94). Intuitively, one might expect that apoplastic loaders would be less susceptible to invasion by macromolecular pathogens such as viruses. However, both apoplastic and symplastic loaders can be infected by a plethora of host-specific viruses (10, 19, 58, 75), suggesting that the absolute number of plasmodesmata around the SE-CC complex is not the sole determinant of virus invasion. Macromolecules that enter the translocation stream may originate within or outside the SE-CC complex (Figure 3, see color plate). In the former,
Figure 2 (a) Targeting of the MP of CMV (expressed as a MP-GFP fusion) to minor vein sieve elements. Note the punctate fluorescence arising from plasmodesmata. (From Reference 4, courtesy of *The Plant Cell*). (b) Sieve element stained with aniline blue to reveal callose associated with the sieve plates (darts) and pore-plasmodesma units (arrows). (c) Targeting of pore-plasmodesma units by a CMV MP-GFP fusion. The left panel shows the plasmodesmal entry sites of the MP-GFP, imaged under a confocal microscope. The right panel, taken at higher gain, reveals a labeled reticular structure within the SE parietal layer (arrows) (From Reference 4, courtesy of *The Plant Cell*). (d) The crease region of a developing wheat caryopsis showing the cell-cell transport of fluorescein dextran through chalazal tissues. Red autofluorescence is generated from chlorophyll-containing pericarp tissues. (From Reference 99, courtesy of *The American Society of Plant Physiologists*.)
Figure 3 Schematic representation of macromolecular trafficking between source and sink regions of the plant. In source tissues: 1. Most mesophyll-synthesized proteins (purple squares) remain in this cellular domain and do not traffic through the plasmodesmata that connect the mesophyll with the SE-CC complexes. 2. CC-synthesized proteins, such as P-proteins (blue circles), may traffic into the SE. Some of these remain anchored to the parietal layer although a proportion may enter the translocation stream. 3. Some CC-specific mRNAs (yellow strands) may also enter the SE and be translocated to sink tissues. 4. Some CC-specific proteins may be synthesized from amino acids that enter the CC by carrier-mediated transport from the mesophyll. Xenobiotic proteins, such as GFP (green circles), synthesized within the CC may also enter the translocation stream. 5. Systemic RNA signals (blue strands) that originate outside the SE-CC complex are amplified as they move between cells. (Legend continues on the next page.)
they are synthesized specifically within companion cells (or ICs) and enter the SE via the PPUs that connect the two cell types, whereas in the latter they are synthesized in cells outside the SE-CC complex (e.g. in mesophyll cells) and enter the SE-CC complex by the plasmodesmata that connect the SE-CC complexes with the mesophyll. For a given systemic macromolecule, it is important to establish which of the above routes is taken. As discussed below, the site of origin of macromolecules will to a large extent determine their ability to enter the translocation stream.

SYNTHESIS OF MACROMOLECULES WITHIN THE CC

Amino acids may enter the CC symplastically; alternatively, they may be transported into the CC by transmembrane carriers (Figure 3). In the case of proteins, synthesized on the ER of the CC, at least three fates are possible: (a) The protein may be retained within the CC cytoplasm or targeted to specific CC organelles, (b) it may be targeted to the SE parietal layer, or (c) it may be exported in the translocation stream (Figure 3). Over the past decade, a substantial body of evidence has accumulated to suggest that macromolecules synthesized within CCs may be transported into enucleate SEs. The ability of a given protein to enter the SE from the CC depends on a number of features unique to the PPUs. One of these is the unusually high size exclusion limit (SEL) of these plasmodesmata, which have the ability to traffic macromolecules that are normally too large to pass between source-leaf mesophyll cells (25, 32, 43, 58). The SEL of PPUs has been probed in a number of ways. First, microinjected fluorescent dextrans of at least 10 kDa have been shown to move between SE and CC (43). Second, labeling of wheat leaves with radioactive methionine established that wheat phloem sap, collected as exudate from aphid stylets, contained more than 200 proteins within the size range 10–40 kDa, as well as a number of larger proteins (60–79 kDa; 25). A similar range of proteins has been detected in sieve-tube exudate of rice (34) and castor bean (77). An important feature noted in the wheat study was a constant exchange of proteins between SEs and CCs along the translocation pathway (43). A third demonstration of macromolecular exchange between CC and SE was provided recently by Imlau et al (32). These authors used the CC-specific promoter of the Arabidopsis sucrose transporter, SUC2, to drive the production of the jellyfish green fluorescent protein (GFP) within source CCs. The 27-kDa GFP moved into the SE and was translocated to sink regions of the plant (Figure 4c, see color plate). In the case of xenobiotic macromolecules such as microinjected dextrans and GFP, one could argue that such macromolecules are not normal constituents of the CC and may not reflect the normal trafficking behavior of proteins between SE and CC. However, recent evidence suggests that several SE-specific proteins are also trafficked from the CC to the SE. The following section catalogues some examples of this phenomenon.
Figure 4 Phloem unloading of systemic macromolecules is restricted to major veins. (a) Control leaf of tobacco showing three distinct vein classes. (b) Systemic silencing of the nitrite reductase gene is first seen as chlorosis around the class III veinal network. (Figure courtesy of Hervé Vaucheret.). (c) Phloem unloading of GFP from class III veins in a tobacco leaf. The GFP was expressed in source leaves under the CC-specific promoter SUC2, and was subsequently translocated to, and unloaded within, the sink leaf (See Reference 62). (d) Phloem unloading of a tobacco etch virus (TEV) construct expressing GFP. Virus exit (seen as green fluorescence) is first seen from the class III veinal network. The xylem of this leaf was allowed to transpire Texas red in order to reveal the minor vein networks. Note that virus unloading does not occur from minor veins. (Figure courtesy of Sophie Haupt.)
SUCROSE CARRIER PROTEINS

Given the role of the SE-CC complex in apoplastic phloem loading, it is perhaps not surprising to find that proteins responsible for carrier-mediated sucrose transport are trafficked between CC and SE. If such proteins are destined for the SE parietal layer, targeting signals are envisaged that direct the protein to the correct location within the SE (63). Although the sucrose transport protein, SUC2, of *Arabidopsis* is localized exclusively in CCs (32, 84), the SUT1 protein of tobacco is synthesized in CCs and subsequently moves into SEs (46, 45). It is unclear whether both the SUT1 protein and its mRNA move into SEs. In situ hybridization showed that SUT1 mRNA was localized to both SEs and CCs and was associated strongly with the orifices of PPU, leading to the suggestion that the protein may be translated in the SEs (45). However, this conflicts with the dogma that enucleate SEs contain only parietal sheets of smooth ER (2, 3). Polysomes have been detected in enucleate protophloem SEs of rice (59), and polysomes have also been detected on the outermost (lumen-facing) cisternae of the stacked ER in the parietal layer in recently matured SEs (2, 3). However, polysomes are not thought to persist in mature, translocating SEs (2, 3, 25, 80). The correct insertion of SUT1 to the SE plasma membrane clearly requires a targeting mechanism that prevents loss of this protein to the translocation stream. This could occur by transport along the plasma membrane or ER, both of which are continuous through the PPU (53, 63, 80, 82). Whatever the means by which SUT1 reaches SEs, its function in sucrose retrieval requires that the protein is retained at the SE plasma membrane and that it is not lost to the translocation stream. Oparka & Turgeon (63) have suggested that some proteins destined for the SE parietal layer may be transported beneath the blanket of smooth ER that lines the SE plasma membrane. This ER is separated from the PM by an electron-dense material, as are the stacked sheets of ER cisternae (2, 3, 63, 82). Conceivably, these canals may provide a transport pathway for proteins along the parietal layer of the SE.

P-PROTEINS

The SEs of most plant species contain phloem-specific proteins (P-proteins) that occur in a variety of forms including filaments, tubules, and crystalline aggregates (2, 3, 15, 44, 74, 80). The biochemistry of P-proteins is best characterized in the Cucurbitaceae, where phloem exudate is easily collected from several species. *Cucurbita maxima* exudate contains two prominent polypeptides: phloem protein 1 (PP1; 96 kDa), which forms the P-protein filaments seen in electron micrographs (2, 3, 15, 74, 82); and PP2 (a 48-kDa dimer), which functions as a lectin (74, 83). PP1 and PP2 transcription and translation occur in CCs (5, 11, 28, 29) (Figure 3), and therefore the subunits of these proteins are probably transported through the PPU prior to final protein assembly in the SE parietal layer. It has been well established that one of the roles of P-proteins is to seal the sieve-plate pores upon
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wounding (2, 3, 15, 44, 74, 80). Traditionally, it has been thought that in intact, translocating SEs, P-proteins are anchored to the parietal layer (2, 3, 44, 74), with the phloem lectin protein perhaps attaching the P-protein filaments to the SE reticulum or plasma membrane (83). However, recent evidence suggests that both PP1 and PP2 may be translocated across both interspecific and intergeneric grafts of the Cucurbitaceae (28, 29, 70, 87). Aside from their function in sealing damaged SEs, P-proteins might also function as part of a long-distance signaling system (87), although such a role has yet to be confirmed. Immunolocalization studies of PP1 and PP2 revealed the exclusive localization of these proteins in the SE-CC complexes and showed that the proteins, but not their respective transcripts, were detectable in the scions of interspecific grafts (28, 29). The presence of PP1, but not PP2, in scion CCs demonstrated that the SEL of the PPUs was sufficiently large to permit the passage of PP2 dimers (or monomers) between SE and CC while limiting the larger 96-kDa filament protein to the SE (29) (Figure 3). A significant finding was that neither PP1 nor PP2 was detected outside SE-CC complexes by either light or electron microscopy. Thus, while some macromolecules, such as viruses, can exit the phloem (see below), P-proteins do not exit the SE-CC complex in sink tissues.

Curiously, both PP1 and PP2 increase the SEL of mesophyll cells in C. maxima cotyledons. FITC-labeled PP2 moved extensively while unlabeled PP2, co-injected with dextrans, facilitated the cell-cell movement of 20-kDa but not 40-kDa dextrans (1). Other phloem exudate proteins, such as RPP-13-1, a thioredoxin h protein, also modify the SEL of mesophyll plasmodesmata (33). Given that PP1 and PP2 are synthesized exclusively in CCs and do not leave the SE-CC complex in sink tissues, the biological significance of these observations is obscure. One possibility is that these proteins possess motifs that normally modify only PPUs and that their behavior in mesophyll cells mimics this role (53). It appears, however, that P-proteins are incapable of modifying the plasmodesmata between the SE-CC complex and surrounding bundle sheath cells to mediate their escape to the mesophyll.

CmPP16

Recently, Lucas and co-workers (102) isolated a 16-kDa phloem protein from C. maxima (CmPP16) that appears to share several features with viral movement proteins. CmPP16 was isolated using antibodies to the MP of red clover necrotic mosaic virus (RCNMV) and has been shown to bind to and mediate the cell-cell trafficking of nonsequence-specific RNA between mesophyll cells (102). Like other phloem proteins, CmPP16 has been immunolocalized to the periphery of sieve elements (102). Significantly, high-resolution in situ PCR detected CmPP16 mRNA in CCs and also in mature SEs in petioles and stems, indicating that the mRNA as well as the protein may be translocated in the phloem (Figure 3). Movement of CmPP16 protein and its mRNA across interspecific grafts confirmed this mobility (102). Although injection experiments demonstrate that CmPP16 can
modify mesophyll plasmodesmata, the extent to which CmPP16 is found naturally in the mesophyll has not been determined, nor has its ability to traffic mRNA molecules into and out of the phloem. Thus, the suggestion that CmPP16 is a plant paralog of a viral MP (102) requires unequivocal demonstration.

SYNTHESIS OF MACROMOLECULES OUTSIDE THE CC

Reports of proteins synthesized outside the SE-CC complex that are capable of subsequent entry into the translocation stream are scant in comparison with those that document synthesis within the CC. To date, no direct evidence has been produced to suggest that endogenous plant proteins traffic from mesophyll into the SE-CC complex. If macromolecules are synthesized in tissues outside the phloem, the problem faced is one of entering the SE-CC complex to gain phloem mobility. Such putative trafficking molecules require not only the capacity to move from cell-to-cell through mesophyll tissues but also the ability to breach the plasmodesmata around the SE-CC complex (63).

VIRUSES

Plant viruses have been studied extensively with respect to their ability to move from cell to cell and to enter the phloem. Most viruses encode movement proteins (MPs) that facilitate cell-cell movement of the viral genome, either as virions or some form of nucleoprotein complex, through plasmodesmata (10, 17, 27, 48, 58, 75). A number of viral MPs bind nucleic acid (98), target plasmodesmata (4, 35, 61, 71, 76, 88) (Figure 2c), and gate the plasmodesmal pore to a higher than normal exclusion limit (35, 61, 76, 101). Despite extensive evidence for modification of mesophyll plasmodesmata by viral MPs, only recently has it been shown that a viral MP can traffic directly into SEs. Blackman et al (4) made a fusion of the MP of cucumber mosaic virus (CMV) to GFP and expressed this fusion protein from a potato virus X vector. The MP-GFP fusion entered minor vein SEs (Figure 2a) and became associated with a reticular structure in the SE parietal layer (Figure 2c, d). Bright punctate accumulations of MP-GFP were probably associated with the entrances of PPUs (Figure 2c, d). Immunogold cytochemistry of MP distribution during a wild-type CMV infection confirmed that the MP entered the SE (4). Much of the MP detected within the SE remained associated with the parietal layer, close to the point of MP entry (Figure 1c). Given that viral MPs may enter SEs, some of the MP could be translocated to distant sites in the plant and exert an influence on plasmodesmata in these regions. Given the small size (>50 kDa) of many viral MPs, and the known ability of these proteins to traffic between cells, large quantities of virus movement protein may be exported from infected CCs in source tissues and exit into the postphloem pathway of sink tissues. Although undemonstrated, such an efflux of MPs could prime sink tissue for the subsequent export and transport of virus.
An extensive study of virus accumulation in minor veins suggested that the route taken to the SE invariably involves phloem parenchyma elements (19), as these cells often directly abut minor-vein SEs (Figure 1a). In all cases in which CCs became infected, the phloem parenchyma elements were also infected (19). Furthermore, in apoplastic loading species in which the CCs are specialized transfer cells (92), the symplastically isolated CCs did not become infected, suggesting that these cells are circumvented in establishing phloem-mediated infection (19). Thus, not all viruses may enter SEs across PPUs, and the plasmodesmata that link phloem parenchyma directly to SEs may provide a potential Achilles’ heel for SE invasion.

For many viruses, encapsidated virions appear to represent the functional long-distance movement complex (4, 27, 57, 58, 81, 91) (Figure 1c). However, the preceding cell-to-cell movement steps through mesophyll cells may occur as a ribonucleoprotein complex (4, 18, 27, 58, 71) or as an intact virion (58, 76), depending on the virus. In CMV, virions have not been detected in mesophyll plasmodesmata nor in the PPUs that connect the SE and CC (4). For CMV, it appears that final viral assembly prior to long-distance transport occurs in the SE parietal layer, subsequent to cell-to-cell transport of complexes of viral nucleic acid, MP, and coat protein through the PPUs (4) (Figure 1c). In contrast, other spherical viruses have been detected within PPUs in an encapsidated form (56), suggesting considerable plasticity of the PPUs in accommodating viral trafficking. The exact site of encapsidation may differ among different viral groups. For example, tobacco mosaic virus (TMV), type member of the tobamoviruses, can move cell to cell without its CP. However, CP is an absolute requirement for long-distance movement of TMV, suggesting that phloem transport of TMV involves virions (reviewed in 10, 27, 58). A related tobamovirus, cucumber green mottle mosaic virus (CGMMV), has been detected as intact particles in *Cucurbita* sieve tube exudate (81). In this study, no evidence of free viral RNA or other CGMMV-related structures was found in exudate, suggesting that movement through the phloem occurs exclusively as virus particles. Some infectious viral agents do not encode a CP and yet move long distances through the phloem. In the case of viroids, small pathogenic RNAs with no protein-coding capacity, both cell-to-cell (18) and long-distance movement (65) occur, indicating that any protein(s) involved in viroid movement must be encoded by the host. The umbravirus, groundnut rosette virus (GRV), also moves via the phloem without encoding a CP (71, 72). The ORF3 protein of this virus encodes a protein that is essential for long-distance movement (72), whereas a second protein (ORF4), which shares sequence homology with several known viral MPs (71), is essential for cell-cell movement. In GRV, the respective short- and long-distance movement components appear to be under the control of separate gene products. Significantly, the ORF3 protein can replace the CP of TMV for long-distance movement, providing evidence that the ORF3 product represents a class of trans-acting long-distance movement factors that can facilitate trafficking of an unrelated viral RNA (72). It will be interesting to determine if this viral protein can traffic endogenous plant RNAs over long distances.
TRANSCRIPTION FACTORS

Although the subject of this review concerns the systemic transport of macromolecules, it is worth considering the available evidence for intercellular transport of endogenous plant proteins, peptides, and nucleic acids in order to discriminate between macromolecules capable only of intercellular movement and those capable of combined intercellular and systemic movement.

The first class of plant proteins to be ascribed a cell-to-cell transport function were transcription factors involved in plant meristem identity. Experiments using periclinal chimeras between wild-type and mutant plants demonstrated that several transcription factors functioned in a non-cell-autonomous fashion (9). Subsequently, elegant studies, based on in situ hybridization of mRNA and proteins, suggested that this nonautonomous behavior was due to an ability of these transcription factors to traffic from cell to cell (37, 38, 67). Notably, the intercellular transport of transcription factors was directional, restricted to meristems, and occurred only between one or two cell layers (36, 67). Surprisingly, however, a recombinant form of the maize transcription factor KNOTTED1 (KN1; 25 kDa), purified from *Escherichia coli*, moved between both maize and tobacco mesophyll cells following microinjection of fluorescently labeled protein (52). Moreover, microinjected KN1 increased the plasmodesmal SEL in mesophyll cells and also mediated the selective plasmodesmal trafficking of *kn1* sense RNA (52). Curiously, in microinjected mesophyll tissue, KN1 showed no affinity for nuclei, the predicted site of transcription factor localization (52). Furthermore, despite the ability of recombinant KN1 to traffic its own mRNA, previous in situ hybridization studies had indicated that only KN1 and not KN1 transcripts were present in the L1 layer of the maize epidermis. These discrepancies between the behavior of KN1 in mesophyll and meristematic tissue suggest that additional mechanisms exist to regulate the trafficking of KN1 and other transcription factors when expressed in their native context. A curious paradox emerges; endogenous proteins of nonphloem origin, such as KN1, which can modify mesophyll plasmodesmata, remain restricted to limited cellular domains, whereas some CC-synthesized proteins, such as PP2, which can also modify mesophyll plasmodesmata, do not exit the phloem.

SYSTEMIN

The ability of viruses to move from mesophyll to phloem tissue is undisputed. In contrast, the evidence that endogenous proteins, synthesized in the mesophyll, enter the phloem is less compelling. The 18-amino acid polypeptide, systemin, is a powerful inducer of defense-related genes and has been suggested to be the primary systemic signal of gene induction (6, 20, 73). Whole-leaf autoradiographs (57) showed that when $^{14}$C systemin was applied to fresh wound it was delivered to upper leaves within 2–4 h, showing a distribution pattern similar to $^{14}$C-labeled sucrose (57). Over short time periods, the labeled systemin was found in both
xylem and phloem of leaf veins, suggesting that systemin may move initially in the apoplast and from here be loaded into the phloem (57, 73). However, as pointed out by Bowles (6), there is no direct evidence that endogenous systemin is mobile in the wounded plant. The initial site of systemin synthesis in wounded tissues has not been clearly demonstrated. For example, it has yet to be established whether systemin moves from cell to cell through mesophyll plasmodesmata, and it is possible that phloem-mobile systemin is manufactured in CCs prior to movement into SEs for long-distance transport. Using expression of a reporter gene (GUS) driven by the prosystemin promoter, activity was found to be located only in the CCs and associated parenchyma (36). When up-regulated upon wounding, this high cell specificity was maintained. This evidence strongly suggests that other signals, originating outside the SE-CC complex, give rise to systemin synthesis specifically within CCs.

SYSTEMIC RNA SIGNALING

Recent studies have provided evidence that the systemic signal(s) involved in gene silencing can enter the translocation stream and be transported and unloaded in sink regions of the plant (Figure 4a, b). Using grafts, Vaucheret and coworkers demonstrated that signals specifying silencing of both nitrate reductase and nitrite reductase could be transmitted from silenced stocks to nonsilenced scions (64). In studies performed in the Baulcombe laboratory, a stably integrated gfp transgene was silenced by infiltration with A. tumefaciens carrying gfp in the T-DNA of a binary vector (95). At 18 days after infiltration of lower leaves, silencing (indicated by loss of GFP fluorescence) was observed in young developing leaves and shoot tips (95). When a single source leaf was similarly inoculated, silencing of the gfp transgene was observed on the stem one month after treatment and was restricted to shoots that emerged from the same side of the stem as the inoculated leaf (96). Although interpreted as evidence for phloem transport of the systemic signal (96), the time scale for systemic silencing to occur is particularly long when compared with the movement of photoassimilates (minutes to hours; 23, 44, 50, 54, 69) and systemic viruses (3–10 days; 10, 19, 50, 58, 69, 72). The fact that several viruses trigger a response in their hosts that leads to a recovery from virus infection, together with the observation that some viruses possess mechanisms to suppress gene silencing, have led to the suggestion that systemic gene silencing may have evolved as a host response to viral attack (8, 13, 42, 68). If so, then it might be expected that systemic gene silencing would occur at a rate greater than that observed for virus movement, a feature not observed. Clearly, further characterization of the long-distance signal is required before functional relationships between gene silencing and virus movement can be established.

Despite the long time scale for the establishment of systemic gene silencing, the progression of silencing in young sink leaves mirrors the pattern of phloem unloading of GFP and viruses (75, 96) (Figure 4b, c, d). Furthermore, the systemic signal moved through a three-way graft in which the middle section did not contain
a GFP transgene (96), strongly suggesting that transport of the silencing signal was truly phloem dependent. The case for phloem-transmission of gene silencing will be strengthened substantially if the systemic signal responsible for gene silencing can be isolated from phloem exudates.

A further unresolved question regarding gene silencing is whether the systemic signals are initiated in CCs or originate outside the phloem prior to entering the SE-CC complex. Although cell-to-cell propagation of gene silencing clearly occurs in sink tissues (95, 96), evidence for cell-to-cell transmission of silencing in source tissues is less clear. In studies where silencing is initiated by either stably integrated transgenes or agroinfiltration, the trigger responsible for gene silencing is likely to be present in both CCs and mesophyll cells (Figure 3). Evidence for intercellular transmission of gene silencing in source tissue is provided by agroinfiltration experiments in which the silencing of an integrated gfp transgene by infiltration with Agrobacterium carrying the gfp gene was seen to extend beyond the margin of the infiltrated tissue (95). In addition, these authors demonstrated systemic silencing of a gfp transgene following biolistic bombardment of gfp-carrying plasmids into single leaf cells. Cobombardment of seedlings with a 35S-GUS plasmid revealed, on average, less than eight randomly distributed individual cells that exhibited blue staining (96). Thus, very localized events can apparently initiate production and spread of the sequence-specific signal of gene silencing, and at least limited cell-to-cell movement of the silencing signal may occur in source tissue.

What is the nature of the transported signal? RNA molecules seem the most likely candidate for transmission of the cosuppressed state between cells (40) (Figure 3). Recent studies examining both transgene and viral-induced posttranscriptional gene silencing have identified small (25-nucleotide) RNA molecules whose accumulation required either transgene sense transcription or RNA virus replication (31). Note that endogenous and viral RNA movement both involve a relay system in which the signal is produced and amplified in each successive invaded cell (10, 68, 75, 95, 96). One problem faced by the plant in utilizing an RNA-based signaling system is the potential for RNA degradation as it moves between cells and within the phloem. It has been suggested that the systemic RNA signal may be protected in transit by a host protein that also facilitates its movement, similar to the long-distance movement of viral RNA (41, 53, 70).

**TRANSLOCATION OF mRNAs**

The presence of endogenous RNAs in the phloem was first reported in the literature in 1975 (103). Recent reports have noted the presence of thioredoxin h, oryzacystatin-I, and actin mRNAs in rice phloem sap collected by an insect laser method (78). Thioredoxin h mRNA has been immunolocalized to the CCs of the leaf sheath of rice (34); hence, the most likely origin of these mRNAs in phloem sap is the CC (Figure 3). An analysis of the phloem sap of *Cucurbita maxima* has disclosed several mRNA species, some with putative roles in meristem identity (70). Using RT-PCR, Ruiz-Medrano et al (70) showed that NACP, a member of the
NAC domain gene family involved in apical meristem development, was present in sieve elements and companion cells of stem and root phloem. Significantly, longitudinal sections of root and shoot apices showed transcript continuity between meristems and sieve elements of the protophloem, the presumed exit point of the mRNA, suggesting that NACP was transported over long distances and accumulated subsequently in vegetative and floral meristems. In grafting experiments in which *Cucurbita maxima* acted as the stock and *Cucumis sativus* as the scion, the *C. maxima* NACP mRNA moved through the phloem and accumulated in apical tissues of *C. sativus*. An additional significant observation was that only NACP mRNA, and not NACP protein, entered the translocation stream, consistent with the view that the transcript may have a role in long-distance signaling (41, 53). Ruiz-Medrano et al (70) have suggested that many phloem-specific transcripts or their proteins may play a general role in physiological events within developing leaves, as well as developmental events taking place in meristems.

Do all the mRNAs detected in the phloem have a signaling function? Conceivably, some translocated mRNAs are translated in sink tissues or act as signals to regulate the transcription of related genes (53). The signal that induces flowering has been well documented to be translocated to the vegetative apex via the phloem (12, 47) and, although not yet identified, speculation continues to grow that this signal may be RNA or an RNA-protein complex (70). At present, a direct functional link is lacking between the presence of mRNA species in the phloem and the regulation of specific cellular functions within meristems. In particular, if mRNAs are unloaded from the phloem, what factors mediate their selective transport and targeting to apical meristems?

**SUPERHIGHWAY OR SEWAGE SYSTEM?**

The phloem provides an ideal conduit for long-distance signals. However, very few of the 200 soluble proteins detected in sieve tube exudate have been identified, much less attributed a function, and of the RNA species identified in exudate, not all have an obvious function in sink tissues (70, 78). Some of the macromolecules present in the translocation stream may indeed have entered by default rather than design. The indiscriminate movement of large dextrans (43) and proteins such as GFP (32, 62) between SE and CC provide clear evidence that at least some macromolecules may enter the SE freely through the PPUs. This raises the possibility that unless a protein has a retention signal for the CC, or a targeting signal that directs it the SE parietal layer (see above), it will be exported in the translocation stream (63). Fisher et al (25) provided compelling evidence for protein turnover by CCs along the transport pathway following the radiolabeling of wheat leaves with amino acids. These authors proposed highly selective regulation of protein removal from SE in the transport phloem and nonselective protein removal from the SEs in sink tissues (25). As the SEs alone do not possess the machinery to degrade proteins in the translocation stream (see 87), many of the proteins detected in sieve tube exudate may reflect the flotsam produced by CCs along the phloem.
transport pathway. In the above model, loss of small proteins (and possibly nucleic acids) to the SE would be an inevitable consequence of the intimate symplastic continuity between SE and CC (43, 94). Distinguishing between macromolecules that enter SEs for a signaling purpose and those that enter by a default pathway may prove to be a difficult task for the future.

An additional problem in sampling the phloem is whether sieve-tube exudate represents an accurate reflection of the moving translocation stream. When the phloem is severed, the sudden loss of turgor pressure from the sieve tubes can lead to the indiscriminate movement of macromolecules between CCs and SEs (63). This seems less likely to be a problem during aphid feeding (23) but may give rise to potential artefacts when collecting exudate from direct incisions made into the phloem (63). In the case of CC-specific enzymes such as dehydrogenase (49), this can result in rapid displacement of the enzyme into the SE. Given the high natural SEL between SE and CC, one is left wondering if a little bit of everything enters SEs during the collection of phloem exudate from cut tissues. Clearly, stringent controls are required to ensure that the macromolecules present in sieve-tube exudate are normal constituents of the translocation stream.

SE UNLOADING

A growing body of evidence suggests that in rapidly growing sink tissues the pathway of unloading from SE-CC complexes is symplastic (22, 24, 66, 80). Apoplastic unloading, involving the loss of solutes across the SE-CC membranes (24, 66, 80), appears to be restricted to pathway phloem, where routine solute retrieval occurs into the SE-CC complexes by carrier-mediated transport (54, 63, 66, 80, 93). In terminal sinks, such as root tips, fruits, and seeds, symplastic unloading of the SE-CC complexes appears to be almost universal (63, 66, 80). Teleologically, to achieve efficient and rapid unloading, the simplest solution is that the postphloem symplast does not place major constraints on the exit of solutes from the SE-CC complex (63). This could be achieved by increasing the number and permeability of the plasmodesmata in the postphloem pathway (22, 24, 66). For symplastic phloem unloading, it is commonly assumed that the limiting path cross-sectional area is set by the contiguous walls containing the least number of plasmodesmatal connections (66). Such studies have assumed an almost universal plasmodesmal SEL of < 1 kDa for sink tissues (66). For example, it has been calculated that plasmodesmata in unloading root tips of corn may not accommodate the observed fluxes of sucrose, necessitating apoplastic postphloem transfer (7). However, recent evidence from a range of species suggests that plasmodesmata in the postphloem pathways of sink tissues may have a higher than normal SEL. For example, Fisher & Wu (25) applied fluorescent dextrins to the postphloem pathway in wheat grains and found the SEL of plasmodesmata to be in excess of 10 kDa (25) (Figure 2d). As discussed below, such a large SEL of plasmodesmata in sink tissues forces a re-evaluation of the pathways and mechanisms of postphloem transport.
MOLECULAR MASS VERSUS STOKES RADIUS

Most studies of plasmodesmal function cite the SEL as a function of molecular mass ($M_r$; see 16, 30, 43, 51, 53, 61, 101). Early studies, utilizing microinjected fluorescent probes, estimated the SEL of plasmodesmata to be about 0.8–1 kDa (30, 86). Terry & Robards (86) emphasized the role of the Stokes radius ($R_s$) determining molecular mobility through plasmodesmata. The $R_s$ is the molecular dimension of an equivalent sphere with the same hydrodynamic drag as the molecule in question (22, 40). The larger molecules (~1 kDa) that pass through plasmodesmata had an $R_s$ of 0.9 nm, suggesting functional channel diameters in the plasmodesmal pore of about 3 nm (86). This value has now been shown to be closer to 4 nm (21). Unfortunately, as pointed out by Fisher & Cash-Clark (22), there is no unique relationship between $M_r$ and molecular dimensions. The $R_s$ of transported molecules assumes greater importance with the demonstration that some macromolecules may be transported through sink plasmodesmata (22, 24, 32, 62, 99). It should be stressed that fluorescent dextrans, which have been used extensively in microinjection experiments, do not have the same $R_s$ of proteins of identical $M_r$ (22, 40). In considering the estimated functional SEL of a plasmodesmal pore, it should be noted that a 20-kDa dextran has the same $R_s$ as a 51-kDa globular protein (22, 40). By infiltrating fluorescent dextrans into the postphloem pathway of wheat grains, Wang & Fisher (99) demonstrated the passage of 10-kDa dextran through plasmodesmata, equating to a functional channel diameter of 7 nm (Figure 2d). The effect of channel dimensions on the conductance for diffusive transport of low-molecular-weight solutes is very high (21, 22, 24, 66). For example, in the case of sucrose, the per channel conductance of a 7-nm channel would be 12 times that of a standard 3-nm channel (24). Although evidence favors diffusive transport in the postphloem pathway of wheat (22, 99), the high conductance of sink plasmodesmata could also facilitate bulk (convective) flow, rather than diffusion, of solutes from the phloem in cases where import rates are exceptionally high (66).

UNLOADING OF MACROMOLECULES

The diversity of macromolecules moving in the phloem poses a potential problem for the phloem located at the terminus of the translocation stream: How do the SE-CC complexes located in sink tissues discriminate between the different solutes and macromolecules arriving in the phloem? The simplest answer is that they do not. As pointed out by Oparka & Turgeon (63), apoplastic SE unloading would necessitate the presence of carriers for an enormous range of low-molecular-weight solutes. Furthermore, proteins moving in the translocation stream would have to be rapidly degraded into smaller moieties in sink CCs so that these could be unloaded by membrane carriers. To achieve efficient and rapid unloading, the simplest scenario is that the postphloem symplast does not place major constraints on the exit of solutes or small proteins from the SE-CC complex. An analysis of unloading patterns in sink leaves shows a remarkably similar pattern for a diverse range of
phloem-transported compounds. For example, radioactive solutes (50, 89), fluorescent solutes (69, 75), GFP (32, 62), and systemic RNA signals (62, 75, 96) all exit the phloem from major veins in similar patterns (Figure 4b, c, d), suggesting that the same vein classes utilized in solute unloading are also involved in the unloading of some phloem-mobile macromolecules (75). Given the relatively small size of RNA molecules involved in systemic posttranscriptional gene silencing (31), it would appear that the postphloem pathway could accommodate the diffusional transport of these signals through the symplast.

Recently, Fisher & Cash-Clark (22) injected a range of fluorescent proteins and dextrans into wheat grains through severed aphid stylets and observed the SE unloading and postphloem transport of dextrans up to M_r 16 kDa (R_s 2.6 nm), predicting aqueous channel diameters in the plasmodesmata of 8–9 nm. To probe the SEL of sink-leaf tissues of tobacco, Oparka et al (62) biolistically bombarded sink and source leaves with plasmids encoding GFP-fusion proteins. Free GFP and a GFP-sporamin fusion (47 kDa; R_s 2.21) moved freely through sink (but not source) leaf plasmodesmata whereas larger fusion proteins (61 kDa; R_s 2.6 and 67 kDa; R_s 2.8) failed to move in both sink and source leaves. The decrease in plasmodesmal permeability during the sink-source transition was correlated with a change in architecture from simple to branched plasmodesmata (62). When considered in terms of R_s, the estimates of aqueous channel diameters in postphloem tissues of wheat grains and in sink tobacco leaves give similar values of about 8–9 nm, considerably larger than previously published values for source tissues (30, 51, 61, 86, 101). Since many of the proteins detected in sieve tube exudate fall in the range 10–40 kDa, the postphloem pathway in sink leaves could potentially accommodate the symplastic transport of many of the small proteins present in the translocation stream.

As pointed out, some of the large macromolecules present within the SE-CC complexes, such as the P-proteins PP1 and PP2, do not exit the SE-CC complex in sink tissues (29) (Figure 3). It remains to be demonstrated whether such molecules are retained within the SE-CC complex by specific targeting mechanisms, or whether they are simply too large to pass through the plasmodesmata that surround the SE-CC complex. Fisher & Cash-Clark (22) have suggested that the larger proteins present in sieve tube exudate (up to 70 kDa in wheat; 25) may require recognition factors that facilitate a specific interaction with plasmodesmata if they are to move through the postphloem pathway. Similarly, if sink plasmodesmata impose an SEL of <50 kDa (62), the mRNA molecules transported from the phloem to meristematic tissues (70) would also require a facilitated transport mechanism.

UNLOADING OF VIRUSES

Some systemic viruses facilitate their escape in sink tissues, although the mechanism of exit from the SE-CC complex remains unknown. It is perhaps significant that very few viruses fail to be unloaded from the SE-CC complex. In
phloem-limited viruses, restriction of virus movement usually occurs at some point outside the SE-CC complex (10, 58, 85), suggesting that the limiting steps in tissue invasion lie in cell-to-cell postphloem movement rather than in the ability to escape the SE-CC complex. Since many viruses appear to move in an encapsidated form, one possibility is that viral disassembly occurs within the SE prior to movement of the viral RNA into the CC. How this might occur in the translocation stream in the absence of viral MPs is unclear, unless MP is also translocated in some form to permit RNA trafficking into the sink CC. A second possibility is that the SEL of PPsUs, in common with the majority of sink plasmodesmata (22, 62, 99), is considerably larger than in source tissues, allowing intact virions to pass freely between SE and CC. In the CC, disassembly could occur followed by reinitiation of the viral replication and movement cycles (Figure 3). Oparka et al (62) examined the possibility that, due to the high SEL of sink-leaf tissues, viral MPs may not be required for postphloem movement of the viral genome through plasmodesmata. A GFP-expressing PVX mutant was constructed that lacked the 25-kDa protein of the triple gene block, a gene product shown to be essential for cell-cell movement of PVX (76). This mutant virus was restricted to single cells on sink as well as source tissues, indicating that an interaction is still required between the viral MP and sink plasmodesmata before the viral genome can pass between cells.

POSTPHLOEM SORTING OF MACROMOLECULES

The symplastic unloading of solutes from the phloem in sink tissues may be a mechanism that ensures the efficient delivery of carbon to rapidly importing sinks (24, 66). Implicit in this unloading mechanism is the accompanying exit of a range of low-molecular-weight macromolecules into the postphloem pathway. By allowing both solutes and macromolecules to exit the phloem freely the plant overcomes the immediate problem of fouling the terminal transport pathway, but shifts the sorting problem to the cells of the postphloem pathway. In several types of reproductive sinks, specialized parenchyma elements surround the terminal phloem elements (24, 60, 66, 99, 100). In cereal caryopses, the terminal sieve elements are separated from the endosperm by specialized chalazal tissues. These tissues have very high frequencies of simple plasmodesmata (60, 100) and accumulate a wide range of compounds during grain development (60, 100). Such postphloem tissues are most likely sites of degradation and recycling of macromolecules within the postphloem pathway, breaking down and/or storing those materials that are unable to be used by the seed and passing on only those that can be utilized (63) (Figure 3). The postphloem pathway in several sinks may thus have a dual function; facilitation of symplastic transport and retrieval/degradation of unloaded macromolecules.

A common feature of symplastic phloem unloading in reproductive structures is a complete symplastic barrier between maternal and filial generations of the caryopsis, necessitating the exclusive apoplastic transfer of solutes by carrier-mediated transport at this interface (24, 32, 60, 66, 99, 100) (Figure 3). The judicious
placement of this symplastic barrier at a location distant to the SE-CC complexes may be a mechanism that allows the postphloem sink tissues both time and space in which to process the cargo being unloaded from the translocation stream, as well as a mechanism for preventing the movement of phloem-unloaded pathogens into the seed (Figure 3). In the case of GFP unloaded into the seed coat of *Arabidopsis*, the protein is translocated extensively around the seed coat without entry into the endosperm (32). Presumably, with time GFP is degraded by these tissues and recycled, as without continued turnover the protein appears to have a relatively short biological half-life (32, 62). Several small solutes, particularly amino acids, produced as degradation products from unloaded macromolecules, could be recycled to the shoot (39), along with phloem-unloaded water, via the xylem (Figure 3).

**CONCLUSION**

Of the several macromolecules detected in the phloem exudates of plants, it appears that most originate in the CC, with subsequent movement into the SE occurring by the PPUs that connect these cell types. Unusually, several phloem-mobile proteins possess the capacity to traffic through mesophyll cells, although such proteins are not normally present in this cellular domain. Conversely, endogenous mobile proteins such as the transcription factor KN1 appear to be able to modify nonphloem plasmodesmata but have not been detected in the translocation stream. These observations may point to the selective operation of endogenous plant mobile proteins within specific tissue domains. Identifying those phloem-mobile macromolecules that act as long-distance signaling agents is likely to prove a difficult task for the future. The ability of xenobiotic macromolecules to enter the SE and be subsequently unloaded in sink tissues suggests that some macromolecules may be present in the translocation stream by default rather than design. To assess the role of macromolecules in long-distance signaling will thus require more than their mere presence in sieve-tube exudate. For a given phloem-mobile macromolecule, number of criteria must be met before a signaling role can be attributed.

1. The macromolecule must be demonstrated to move from source to sink in the translocation stream. In this respect, grafting experiments are likely to play a major role in determining uniqueness of the macromolecule in sink tissues.

2. It must be demonstrated that the macromolecule leaves the SE-CC complex in sink tissues. As detailed above, several sink tissues possess plasmodesmata with a SEL sufficiently large to accommodate the bulk of proteins that have been detected in sieve-tube exudate. On the other hand, several large proteins, including P-proteins, appear to be phloem-mobile across graft unions but are not unloaded from the SE-CC complex. Some of these macromolecules may initiate a signal cascade from within the sink CC, involving a second signal that transmits information through the
postphloem pathway (53), but such a mechanism remains hypothetical. It seems equally possible that several of the macromolecules unloaded from the phloem are targeted for degradation in postphloem tissues.

3. The macromolecule must be shown unequivocally to target and modify specific cell(s) following unloading from the phloem. To assess this capacity, it will be essential to follow the putative phloem-unloaded signal to its destination and to demonstrate an unequivocal and unique effect on cellular function.

Of the above criteria, the last is clearly the most important (and probably the most difficult) to demonstrate. The observation that several macromolecules are present in phloem exudate is an intriguing discovery that warrants further detailed study. However, it may be premature to attribute a signaling function to each new species of macromolecule identified in the translocation stream. In this respect, it is essential to move from a situation of guilt by association to one in which long-distance macromolecular signals can be identified and characterized in detail. Such experiments may prove conceptually challenging but should provide important insights into long-distance communication in plants.

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PHLOEM TRANSPORT


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