Phloem Loading: How Leaves Gain Their Independence

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The transition from sink to source status is one of the key events in leaf development. When a leaf is about half grown, it stops importing phloem-mobile nutrients from the rest of the plant and begins to export its own products of photosynthesis. This shift in transport direction, which is largely irreversible, involves major changes in the way metabolites are transported to and from the mesophyll through plasmodesmata and via transporters. The import of nutrients ceases when plasmodesmata in large veins are lost or narrowed, preventing phloem unloading. Export begins when the minor veins mature and begin to load sugars and other compounds into the phloem. The unidirectional nature of loading is a consequence of sucrose transporter orientation in the plasma membrane of phloem cells, or of the trapping of raffinose-family sugars in those species that load through plasmodesmata.

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When adolescent people, or leaves, make the transition to independence and adulthood, they lose the security of a steady food supply and start fending for themselves. Leaves begin their development as entirely heterotrophic organs at the shoot apex, relying on the rest of the plant for carbohydrate and other nutrients that they unload from the long-distance transport tissue, the phloem. But as the leaves expand, their photosynthetic capacity develops. By the time a leaf of a dicotyledonous plant is approximately half grown, it has reached positive carbon balance and is, in this sense at least, independent. Thereafter, it acts as a nutrient source, more than repaying its debt over the rest of its lifetime by exporting photoassimilate (the products of photosynthesis) to sink tissues, including roots, fruits, and newly emerging leaves.

Although a young human who has left home might ask for help from parents at some point, development imposes stricter constraints on a leaf. A large body of evidence indicates that mature leaves that are no longer productive, as a result of shading, age, or nitrogen deficiency, do not become a burden on the rest of the plant; rather, they retranslocate mobile nutrients and senesce (Hikosaka 2005).

How is such independence enforced? An important constraint on phloem transport to and from leaves is that it is essentially unidirectional. Once the source phase has begun, there is no going back. This is like locking the door behind a young adult who has just left home. If mature leaves are darkened for long enough, a small amount of photoassimilate from other leaves will enter the veins, but little, if any, will unload and reach the mesophyll (Turgeon 1989).

The irrevocability of this dissociation is evident in experiments in which the shoot of a nonphotosynthetic (albino) tobacco plant is grafted to a wild-type stock (Turgeon 1984). Once the graft takes, emerging albino leaves thrive on nutrients they import from the stock plant. However, when these albino leaves reach the stage of development at which green leaves stop importing carbohydrate, they do, too. Unfortunately for albino leaves, they cannot make their own food, and they starve. Clearly, transport is under developmental control and is not simply a mass-action response to the relative availability of sugars at one end of the network or the other. A valve of some sort prevents backflow. To understand how unidirectionality of phloem transport is imposed on leaves at the physiological level, we must consider the mechanisms of intercellular transport, and the way these mechanisms are employed in different vein orders to determine transport direction and mediate phloem loading and unloading.

From one cell to another

Two routes are available for the passage of metabolites between plant cells: across the cell wall space (apoplastic transport) and through plasmodesmata (symplastic transport). Apoplastic transport requires passage of the transported molecular species across two plasma membranes. Little is known about the efflux step into the apoplast, but considerable progress has been made in studying transporters that mediate uptake from the apoplast.

Sucrose transporters fall into three subfamilies, on the basis of phylogenetic analysis: *SUT1, SUT2,* and *SUT4* (Kühn

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2003). Seven functional genes code for active sucrose transporters in *Arabidopsis* (Sauer et al. 2004). Within subfamilies, the expression patterns of the genes differ, as do the kinetic properties, substrate specificities, and amounts of protein produced. Most sucrose transporter genes are expressed in the phloem, but some of the proteins are found in sink tissues such as anthers, pollen, and root tip.

Sugar alcohol transporters have also been discovered (Noiraud et al. 2001, Ramsperger-Gleixner et al. 2004, Watari et al. 2004). Three sugar alcohols are transported in large quantities in phloem sap, although only in specific plant families (Zimmermann and Ziegler 1975). These are mannitol in the Apiaceae and Oleaceae, glucitol (sorbitol) in the Plantaginaceae and Rosaceae, and galactitol (dulcitol) in the Celastraceae. Two sorbitol transporters have been localized to companion cells of minor veins in the leaves of common plantain (Ramsperger-Gleixner et al. 2004).

Symplastic transport is mediated by plasmodesmata (figure 1). These narrow, cylindrical structures penetrate the common walls between plant cells, and are lined by continuous plasma membrane (Cilia and Jackson 2004, Schulz 2005). A fine tube, the desmotubule, runs through the center of each plasmodesma and is continuous with the endoplasmic reticulum of the two cells. In favorable sections, an apparently proteinaceous rod can be seen in the middle of the desmotubule, and "spokes" of protein cross the space, known as the cytoplasmic annulus, between the desmotubule and the plasma membrane. Most investigators believe that the cytoplasmic annulus is the space that is open to the flux of solute molecules. There are no membranes across the cytoplasmic annulus. Therefore, active transport through this space, in the usual sense of requiring membrane-based transporters, is not possible. Instead, metabolite movement through the cytoplasmic annulus is passive.

Plasmodesmata constitute the path of least resistance for intercellular transport of ions and small organic molecules, such as sucrose, at most cellular interfaces. Their functional size is usually measured by monitoring the movement of fluorescent dyes of various dimensions that have been injected into cells. This function is best expressed as the Stokes radius of the penetrating molecule, but is more often defined by its mass. The mass exclusion limit (MEL) for solute in many plasmodesmata is in the range of 1 kilodalton (kDa), which corresponds to a pore diameter of approximately 4 nanometers (nm) (Fisher 1999). In figure 1, the diameter of the open spaces in the cytoplasmic annulus is approximately 2.5 nm (Ding et al. 1992). The discrepancy between the calculated and measured pore sizes in electron micrographs may be due to the limitations of microscopy when viewing such complex structures. It could also be due to an oversimplification of pore structure in mathematical treatments. For example, to calculate pore diameter from the MEL, the cytoplasmic annulus is treated as a ring of ideal, cylindrical tubes, approximately nine in number. However, this is not the physical reality. The structure appears to be more like a sieve, providing a tortuous pathway for solute travel around and between protein particles.

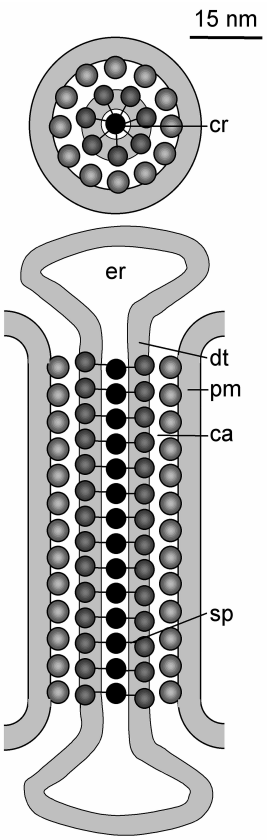

Figure 1. A model of plasmodesmatal structure shown in transverse (top) and longitudinal (bottom) sections. A plasmodesma is lined by continuous plasma membrane (pm) between adjacent cells. A central rod (cr), composed of globular proteins, runs through the center of the desmotubule (dt), formed from compressed endoplasmic reticulum (er). Proteins are embedded in the plasma membrane and desmotubule. Proteinacous spokes (sp) extend between the central rod and desmotubule proteins. Solutes and larger molecules are thought to pass between cells through the cytoplasmic annulus (ca), the continuous space between the plasma membrane and desmotubule. In some plasmodesmata, especially in older tissue, the cytoplasmic annulus widens considerably to form a large central cavity (not shown), and within the central cavity spokes are visible between the plasma membrane and desmotubule proteins. In many microscope images, the plasmodesmata are visibly narrowed at each end, in the neck regions (not shown). Redrawn from Ding and colleagues (1992).

Although a MEL of 1 kDa would seem to limit the passage of all but small molecules, green fluorescent protein (GFP, with a mass of 27 kDa) that has been introduced to the phloem unloads in sink leaves and passes from cell to cell through the mesophyll (Imlau et al. 1999). More surprisingly, certain endogenous macromolecules, including transcription factors and

RNA, can move between cells through plasmodesmata (Cilia and Jackson 2004). This discovery, which is creating a sea change in researchers' understanding of the principles of plant development, followed from the study of virus movement. Viruses apparently hijack the mechanisms used to carry endogenous macromolecular signals from one cell to another.

How do such large molecules pass through plasmodesmata? The measured MEL of plasmodesmata at some interfaces is considerably larger than the standard 1 kDa (Fisher and Oparka 1996). One such interface is the common wall between sieve elements and companion cells. Another explanation is that some endogenous macromolecules, and viral genomes, are targeted to plasmodesmata and are drawn through them by an active mechanism separate from the passive movement of metabolites (Itaya et al. 2002).

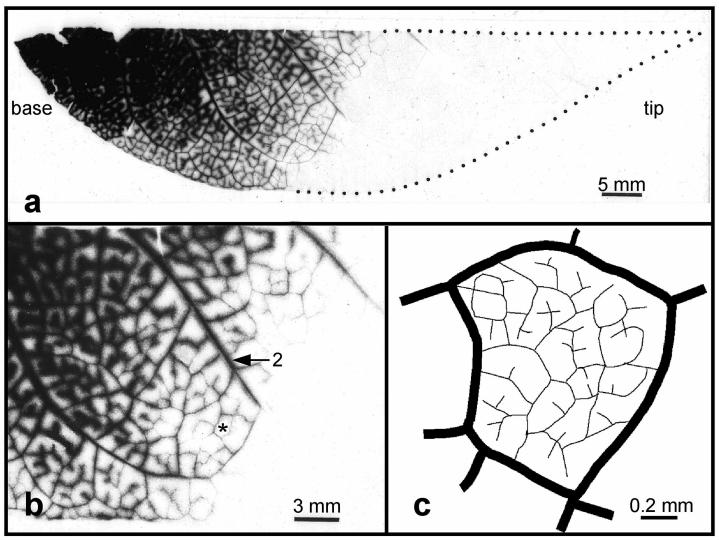

Figure 2. Autoradiographs of tobacco sink leaves that have imported ^{14}C-labeled photoassimilate from mature leaves. (a) One lateral half of a leaf, showing the basipetal pattern of the sink–source transition. There is a clear demarcation between the importing base and the nonimporting tip of the lamina, and this demarcation moves basipetally as the leaf matures. Import and unloading are limited to the largest vein orders. The first-order vein (midrib) is not included. (b) A higher-magnification image from another autoradiograph, showing the vein orders more clearly. The arrow indicates a second-order vein and the asterisk an island delimited by third-order veins similar to the one drawn in the next panel. (c) A single island delimited by third-order (thick) veins. This is the same island drawn in figure 3b. Minor veins within it (thin lines) are immature at this stage of development and do not import nutrients. Autoradiographs in panels (a) and (b) are from Turgeon (1987), reprinted with permission from Springer Science and Business Media.

Closing the door in sink leaves

To determine how the door is closed on the import of photoassimilate during the sink–source transition in developing leaves, we need to know which pathway these compounds take as they arrive in the leaf and unload into the mesophyll. Early studies indicated that the unloading pathway is through plasmodesmata in sink leaves (Schmalstig and Geiger 1985, Turgeon 1987), and the GFP experiments described above substantiate this conclusion. Clearly, the plasmodesmata provide an open avenue into the mesophyll. This suggests that termination of import during the sink–source transition in leaves involves the developmentally regulated closure or loss of these pores, or both.

However, we must be careful to ask which veins are involved, since there is no reason to assume a priori that photoassimilates unload from all veins. The veins in question can be visualized by following the movement of radiolabeled phloem-mobile sugar. In these experiments, mature leaves are provided with $^{14}CO_2$, and the radiolabeled molecules entering the sink leaf are identified by autoradiography (Turgeon 1987). Phloem-mobile fluorescent dyes can be used for the same purpose (Roberts et al. 1997). These experiments indicate that only large (major) veins in tobacco leaves are used for the distribution and unloading of imported photoassimilate (figures 2, 3). The major veins block off the lamina into discrete islands, inside which an extensive set of minor veins can be seen when the tissue is cleared (figures 2c, 3b). The major veins are generally referred to as first-order (midrib), second-order, and third-order; however, a close examination of figure 2b reveals that some are fourth- or even fifth-order. This illustrates the limitations of describing the size and function of veins on the basis of how they branch. Consider, for example, that the fine terminations of second-order veins near the leaf margins are smaller than the third-order veins near the midrib. Although branching order serves as a convenient reference point, in the context of nutrient transport it is more important to focus on function. In this respect, there is a clear distinction between the major veins that import nutrients, no matter what their branching order, and the minor veins that are used later as the primary collecting veins for export. Minor veins are still immature during the import phase (Turgeon and Webb 1976). Another important consideration is that veins of the same size or the same branching order may not have the same functions in all plants. In *Arabidopsis thaliana,* for example, there are fewer vein orders than in most other species (Haritatos et al. 2000a).

Does symplastic continuity decline in the major veins at the sink–source transition, as predicted by the arguments above? Yes. In tobacco, plasmodesmata at the critical interface between companion cells and phloem parenchyma cells are much less frequent after the sink–source transition than they are when the tissue is still in the sink phase (Ding et al. 1988). This loss is accompanied by an overall decline in plasmodesmatal frequencies in the mesophyll and epidermal cells and, at the same time, by the appearance of branched plasmodesmata (Roberts et al. 2001). The MEL of original, unbranched plasmodesmata is much higher than that of branched plasmodesmata (Oparka et al. 1999). This means that symplastic

continuity is reduced during the sink–source transition, not only because many plasmodesmata are eliminated but also because the channels are narrowed.

It is important to note that termination of import does not occur all at once throughout the leaf blade (Turgeon 1989). Although initial leaf development in dicotyledonous species occurs acropetally (from leaf base to tip), this is followed by a basipetal (tip-to-base) wave of leaf maturation—including loss of import capacity, development of minor venation, and changes in growth rate and photosynthetic capacity—that establishes source status. As a result, the sink–source transition occurs basipetally as leaves mature (figure 2a). There is a brief period of bidirectional transport in dicotyledonous leaves; the relatively immature base continues to import nutrients for a period after the tip has begun to export. Note, however, that transport is unidirectional at the local level in the lamina.

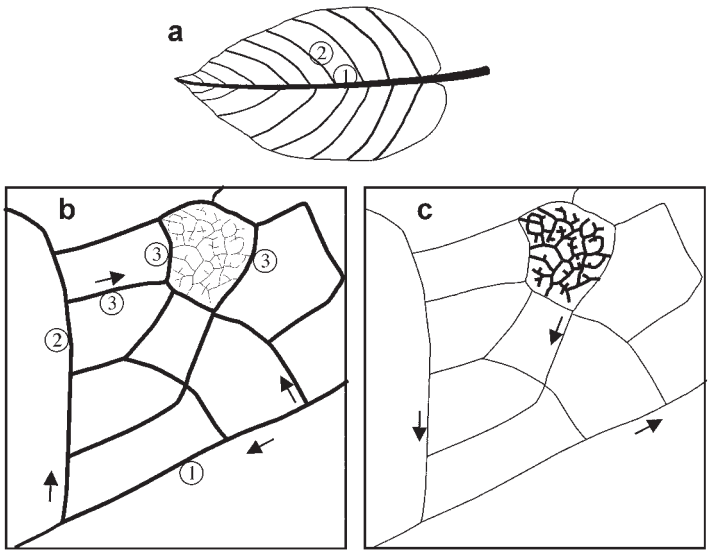

Figure 3. (a) Division of labor in the veins of a tobacco leaf. (b) When the leaf is immature and still in the sink phase, photoassimilate is imported from mature leaves and distributed (arrows) throughout the lamina via the major veins (numbered; the midrib is the first-order vein). The imported photoassimilate unloads from these same vein orders (thicker lines) into the mesophyll. Minor veins are visible within areoles formed by third-order veins. The minor veins do not function in import and unloading because the phloem is immature. (c) In a source leaf, import has ceased and export has begun. Photoassimilate loads into the minor veins (thicker lines), while the larger veins serve only in long-distance transport (arrows); they can no longer unload. Although (b) is drawn to scale from an autoradiograph, (c) is not to scale, or in correct proportions, since the lamina grows considerably as the leaf matures.

Opening the door in mature leaves: The role of minor veins

Once the door has been locked behind the young adult, another door must be opened to allow communication with the outside world. Although that communication is essential, it could provide an opportunity for a freeloader to become a burden on the household again. In the case of a green leaf, an avenue must be established for photoassimilate export without providing an unloading route through the same cells in prolonged darkness. Where is the valve, and how does it work?

The valve must be located wherever photoassimilate enters the phloem from the mesophyll. It is possible that some photoassimilate enters in the major veins, but minor vein phloem has more intimate contact with the mesophyll, and the minor veins are far more extensive. In sugar beet leaves, they measure 70 centimeters (cm) per cm^2 of leaf lamina (Geiger and Cataldo 1969). No mesophyll cell is more than six or seven cells away from a minor vein.

Up to this point, we have used the term "minor vein" loosely. It is worth asking more specifically how this term is defined. Are the minor veins a distinct class of vascular bundle, or just a subjective construct, the arbitrarily defined terminals of a continuous transport network?

In anatomical texts, minor veins are often described as being embedded in the mesophyll, while major veins are surrounded by a layer of parenchyma that sometimes forms a visible ridge (rib) on the abaxial side of the lamina. Although this definition is convenient, it is arbitrary, and it ignores the fact that vein ribs are more prominent in some species than in others. Furthermore, it begs the question of function.

One may also ask if vein classes have distinct molecular genetic regulatory systems. Indeed, this is the case. For example, galactinol synthase gene expression is specifically localized to the minor vein companion cells of mature leaves. Galactinol synthase catalyzes the first committed step in the raffinose and stachyose synthetic pathway. These sugars are synthesized in specialized companion cells of certain plants, such as melon, as part of the loading mechanism. The galactinol synthase promoter from melon drives reporter gene function in transgenic tobacco only in the small veins within the islands formed by major veins (Haritatos et al. 2000b). Thus, the function of the galactinol synthase gene is loading related, and the promoter responds to a regulatory system that defines minor veins on a physiological and genetic basis.

The sucrose transporter SUT1, from potato, is also localized preferentially, though not exclusively, in the minor veins. The lack of complete specificity is not surprising given that transporters also serve the general function of retrieving sucrose that has leaked into the apoplast. Indeed, SUT1 is also found in sink tissues (Truernit 2001).

It is interesting that the galactinol synthase promoter is active in minor veins of tobacco even though tobacco does not synthesize raffinose and stachyose in these cells. The melon galactinol synthase promoter must be responding to an evolutionarily conserved regulatory system that controls other minor vein functions. This conclusion is supported by experiments in which the activity of the *SUC2* promoter is visualized by GFP expression (Wright et al. 2003). When the leaf tissue of these plants is kept shaded during the sink–source

transition, GFP synthesis is activated in major, but not minor, veins, even though the minor veins mature structurally. Evidently, the signals that activate the *SUC2* promoter differ in the different vein classes.

Is phloem loading necessary?

The minor vein valve (or valves) that keeps transport unidirectional can be understood only by carefully detailing how photoassimilate enters the phloem. The route that sucrose takes from one mesophyll cell to another on its way to the veins is not well understood, but is thought by most investigators to be largely, or entirely, symplastic (Stitt 1996, Lalonde et al. 2003, Schulz 2005, Turgeon and Ayre 2005, van Bel and Hafke 2005). Given a symplastic pathway, is it possible that sucrose simply continues along the same route and diffuses into the minor vein phloem, with no increase in concentration?

This is an unorthodox proposition, because it would mean that there is no true phloem-loading step. However, such a step may not be needed. Ernst Münch (1930) did not postulate phloem loading in his classic, and widely accepted, model of long-distance transport. Münch reasoned that, as a result of photosynthesis, the carbohydrate concentration in mature leaves is higher than it is in sink tissues, such as immature leaves, fruits, and roots. In his scheme, the mesophyll cell (actually the chloroplast) is the source, and not the minor vein phloem. He did not envision any additional pressure in the leaf phloem above that generated by the accumulation of photosynthate, itself an active process (figure 4a).

On an even more fundamental level, the common assumption that mass transport of solution through the phloem is driven by a pressure differential between source and sink is misleading. The phloem acts as a rigid-walled manifold that accumulates and loses solute in response to source and sink activities. The pressure differential is a manifestation of resistance to flow, not a cause; in an ideal system without resistance, flow would occur without a pressure differential.

It may also be misleading to think of the pressure differential as controlling flow. It is probable that control is exercised instead by the local solute exchange activities of sources and sinks (Thompson and Holbrook 2003a). This makes sense from an engineering perspective and helps us understand the benefits a plant enjoys by maintaining high phloem pressure. Operating the phloem "manifold" at high pressure permits fine regulation of unloading throughout the plant, regardless of position.

For a familiar illustration of this principle, consider water distribution in a house. If the pressure in the pipes is high, the faucets in various locations behave similarly, even those on different floors. This is because the difference in pressure between the pipes and the atmosphere is greater than the relatively minor differences in pressure within the pipes themselves, and is therefore approximately the same in all locations. In the same way, high pressure in the phloem facilitates controlled unloading of solutes into sink cells all over the plant. Furthermore, there are reasons to believe that phloem transport is more easily regulated if the pressure drop along the pathway is small, since a large pressure differential may slow the rate of communication of local disturbances in pressure and concentration between source and sink (Thompson and Holbrook 2003b).

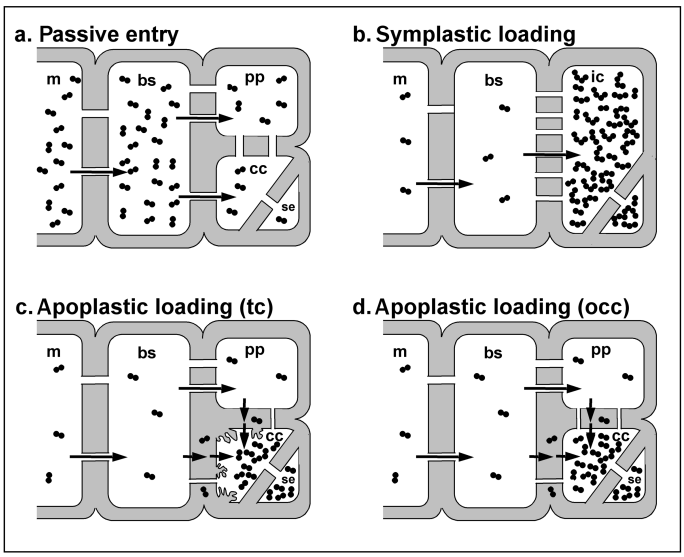

Figure 4. Schematic diagrams of photoassimilate entry into the minor vein phloem of leaves. (a) Passive entry. Sucrose (double circles) in mesophyll cells (m) passes through plasmodesmata to the bundle sheath (bs) and phloem parenchyma (pp) cells and by the same routes into the companion cells (cc) and sieve elements (se). Sucrose concentrations in all cell types is approximately the same. Continued delivery to the phloem depends on removal of sucrose by export. Sucrose concentrations in nonphloem cells may be higher than in other species. This is the process envisioned by Münch (1930), exemplified by willow. (b) Symplastic loading by the polymer trap. Sucrose enters intermediary cells (ic) (a specialized form of companion cells) passively from the bundle sheath through the numerous plasmodesmata that connect these two cell types. Inside the intermediary cell, raffinose (triple circles) and stachyose (quadruple circles) are synthesized from sucrose, keeping the concentration low. Raffinose and stachyose do not diffuse back to the bundle sheath because they are apparently larger than the mass exclusion limit of the branched plasmodesmata. These sugars enter the sieve elements through the large plasmodesmata that typically join these two cell types. (c and d) Apoplastic phloem loading. Sucrose enters the cell wall space and is pumped, against a concentration gradient, into the companion cells, or in some cases the sieve elements (tandem arrows). Plasmodesmata are always present between the companion cells and both the phloem parenchyma and the bundle sheath, in numbers that range from sparse to abundant, but they are apparently too narrow to accommodate flux of sucrose. (c) Some species have transfer cells (tc), that is, companion cells with wall ingrowths that increase uptake capacity from the apoplast. (d) Other plants have ordinary companion cells (occ), with smooth walls.

In keeping with Münch's description, the entry of photoassimilate into the phloem of willow leaves apparently follows a downhill gradient in sucrose concentration (Turgeon and Medville 1998). Thus, there is no loading, in the sense that this term implies a thermodynamically active step. The mesophyll and minor vein phloem plasmolyze at approximately the same concentration of osmoticum, and the turgor in both is greater than it is in the phloem of the stem, which is lower than in other species that have been analyzed. Since plasmodesmata are common between minor vein companion cells and surrounding cells in willow (Gamalei 1989), it is reasonable to assume that photoassimilate migrates passively through these plasmodesmata and into the sieve element/companion cell (SE/CC) complexes of the veins. It is not yet clear how widespread the passive access of photoassimilate to the phloem will prove to be in angiosperms and other plants.

How might a valve work to prevent import of photoassimilate in a mature leaf if the process is passive? There is no direct information available, but one possibility is that the plasmodesmata joining minor vein companion cells to adjacent phloem parenchyma and bundle sheath cells are pressure regulated. Pressure regulation of plasmodesmatal transport has been documented in trichomes (Oparka and Prior 1992). According to this scenario, if the leaf is darkened or for some other reason cannot maintain positive carbon balance, and the sugar concentration of the leaf drops, the plasmodesmata will sense the pressure difference between the phloem (higher) and adjacent cells (lower) and close.

Phloem loading of sucrose via the apoplast

In the 1940s, workers began to realize that, at least in the plants they were studying, the solute content of the phloem was too high to be accounted for by passive accumulation (see Grusak et al. 1996 for a review of the older literature). In one elegant and critically important experiment, Brunhild Roeckl (1949) demonstrated that phloem exudate from *Robinia pseudoacacia* will plasmolyze mesophyll cells in leaves of the same plant. By this simple test, Roeckl added significantly to the accumulating evidence that the concentration of metabolites is higher in the phloem than in the mesophyll cells, where photosynthesis occurs. A thermodynamically active pump must be involved. In the 1950s, the term "phloem loading" was coined to describe this phenomenon.

Apoplastic loading (figure 4c, 4d) was first proposed in the 1970s (Geiger et al. 1973). Physiologists focused on this hypothesis because an extracellular step, with retrieval into the SE/CC complex via transporters situated on the plasma membrane, was the simplest way to envision active accumulation of solute.

One way to test this model is to determine whether sucrose is present in the apoplast (see Grusak et al. 1996 for a review). Although this has consistently been shown to be the case, the proof is not secure, because the sampling methods extract sucrose from the entire extracellular space of the lamina, whereas the apoplastic step in phloem loading may be limited to the interface between the SE/CC complex and surrounding cells. Most of the sucrose detected in the apoplast may not be on its way to the phloem.

A more compelling experimental strategy has been to localize and study the function of transporters. However, the presence of transport proteins in the minor vein phloem does not provide unequivocal proof of apoplastic loading either. It has long been recognized that various compounds leak from plant cells and are actively retrieved from the apoplast (Ayre et al. 2003, van Bel and Hafke 2005). Therefore, transporters act as bilge pumps and are widespread in different tissues. Apoplastic loading may have evolved by the up-regulation of a general retrieval mechanism in the phloem. Sucrose transporters are found throughout the phloem (Lalonde et al. 2003), where continual retrieval of leaked sugar is needed to maintain effective transport rates.

To prove that transporters are used for loading, it is necessary to experimentally inhibit, or genetically down-regulate, them and observe the effects on loading and long-distance transport. Inhibition of loading was first accomplished by exogenous application of the sulfhydryl-modifying compound *p*-chloromercuribenzenesulfonic acid (PCMBS). PCMBS effectively inhibits sucrose transporters. Unfortunately, mercury inhibits most (Tyerman et al. 1999), although not all (Daniels et al. 1994), aquaporins. This complicates interpretation of the experiments, since water must accompany sucrose into the phloem. Also, this approach does not work with sugar alcohol transporters, since, with the exception of PmPLT1 (Ramsperger-Gleixner et al. 2004), they are not sensitive to PCMBS.

Several successful attempts have been made to genetically down-regulate transporters. Antisense inhibition (Riesmeier et al. 1994) and insertional mutagenesis (Gottwald et al. 2000) of *SUT1* lead to severe symptoms of chlorosis due to inhibition of export and the resulting accumulation of large amounts of carbohydrate in the mesophyll cells.

Another way to inhibit apoplastic loading is to target yeast invertase to the apoplast (Von Schaewen et al. 1990). Indeed, these were the first transgenic experiments that tested the apoplastic model of phloem loading. Since invertase hydrolyzes sucrose to glucose and fructose, neither of which is recognized by sucrose transporters, loading should be, and is, severely curtailed in these plants. Transgenic plants exhibit the usual symptoms associated with export inhibition: leaf curling, diminished photosynthesis, chlorosis, slow growth, and accumulation of large amounts of soluble carbohydrate and starch.

Examination of figure 4c and 4d makes it clear why export is unidirectional in species that load via the apoplast. If a leaf is darkened for a prolonged period, even small amounts of stored photoassimilate in the mesophyll can still be pumped into the SE/CC complex to maintain the source–sink pressure gradient. With further light deprivation, the blade as a whole might become a sink, but an ineffective one, since the entering photoassimilate will be confined to the veins. If sucrose leaks out of the phloem across the plasma membranes of sieve elements or companion cells, it will be retrieved immediately

by transporters and returned to the phloem. Leaf tissue that has been maintained in the dark for long periods is still capable of sucrose uptake from the apoplast into the phloem (Wright et al. 2003).

An assumption in this analysis is that leakage cannot occur through the plasmodesmata that link the minor vein phloem to surrounding cells and the mesophyll. Leakage from sieve elements is not an issue, since they have only rare symplastic contact with any cell except their associated companion cell. One might also expect that plasmodesmata are uncommon between companion cells and phloem parenchyma cells or bundle sheath cells; otherwise, sucrose would leak back toward the mesophyll while loading is going on, setting up a futile transport cycle. However, contrary to these expectations, there is a range of plasmodesmata frequencies at these locations (Gamalei 1989). Originally, it was thought that the spectrum from abundant (type 1) to sparse (type 2) companion cell plasmodesmata reflected a spectrum of loading mechanisms, from symplastic to apoplastic. One observation consistent with this interpretation is that species with very few such plasmodesmata often appear specialized for apoplastic loading. The specialization referred to here is the presence of elaborate wall thickenings that increase the surface area of the plasma membrane and the uptake capacity from the apoplast (Amiard et al. 2004). These specialized companion cells are called *transfer cells* (figure 4c). On the other hand, many species of apoplastic loaders have no such wall elaborations. Their companion cells are often referred to as "ordinary" (figure 4d). Why some apoplastic loaders have transfer cells (e.g., *Pisum sativum*), while others have ordinary minor vein companion cells (e.g., *Nicotiana tabacum* and *A. thaliana*), is unclear. Confusing the issue even more is the fact that several type 1 species load apoplastically (Goggin et al. 2001, Turgeon and Medville 2004).

Why does sucrose not leak out of the phloem in these apoplastic loaders, especially the ones that have abundant plasmodesmata in the companion cells? It seems probable that the plasmodesmata are especially narrow, if not sealed. This is the case in phloem along the long-distance transport pathway, where microinjection studies with fluorescent dyes indicate that the SE/CC complexes are symplastically isolated (van Bel and Kempers 1991). Several attempts have been made to measure the MEL of plasmodesmata between the minor vein companion cells and either the adjacent phloem parenchyma or the bundle sheath cells. However, these efforts have been thwarted by the secluded location of the veins, which makes microinjections taxing, and by the difficulty of distinguishing minor vein companion cells from phloem parenchyma cells in fresh tissue (see discussion in Beebe and Russin 1999). In *Moricandia,* plasmodesmata at this interface appear to be actually plugged with amorphous material (Beebe and Evert 1992), but in other cases there is no visible alteration in plasmodesmatal structure. Perhaps the plasmodesmata close when they sense a pressure difference between the phloem and surrounding cells, as described above in the section on passive photoassimilate accumulation.

Finessing the second law: Symplastic phloem loading

Yet another explanation for abundant plasmodesmata in the phloem of minor veins is that the plasmodesmata provide an avenue for phloem loading, leading to the accumulation of photoassimilate in the SE/CC complex against a concentration gradient (figure 4b). Early suggestions to this effect met with skepticism, for the obvious reason that energetic loading of molecules through open pores appears to violate the second law of thermodynamics. However, a solution to this problem was found when it became apparent that the plants with the most numerous plasmodesmata in the minor vein phloem have a specialized form of minor vein companion cell, called the intermediary cell.

Intermediary cells have dense cytoplasm, small vacuoles, and proplastids rather than chloroplasts. The plasmodesmata that link them to the bundle sheath are not only plentiful but highly branched, more so on the intermediary cell side. In members of the Lamiales, the plasmodesmatal branches on the intermediary cell side are narrow (Fisher 1986, Turgeon et al. 1993). Plasmodesmatal branching occurs just before the sink–source transition, strengthening the argument that the plasmodesmata are involved in phloem loading (Volk et al. 1996). Intermediary cells have been found in many families of angiosperms (Acanthaceae, Bignoniaceae, Buddlejaceae, Celastraceae, Cucurbitaceae, Hydrangiaceae, Juglandaceae, Lamiaceae, Oleaceae, Scrophulariaceae, and Verbenaceae), and this list will undoubtedly grow.

Although an extensive electron microscopy analysis has not been undertaken, it appears that the trait is widespread, and perhaps even uniform, in the families in which it is found. Intermediary cells are not present in all of the Scrophulariaceae (figworts), but this is a polyphyletic family. When the figworts are analyzed by more recent phylogenetic criteria (Olmstead et al. 2001), the presence of intermediary cells is found to be uniform within monophyletic groups. Symplastic loading has evolved independently at least four times (Turgeon et al. 2001).

The other distinguishing characteristic of species with intermediary cells is that they translocate high concentrations of raffinose-family oligosaccharides (RFOs; raffinose and stachyose). Some plants that load apoplastically also translocate RFOs, especially raffinose (Zimmermann and Ziegler 1975), but only in small, and in some cases trace, amounts. For example, *A. thaliana* transports sucrose predominately, with a much smaller amount of raffinose (Haritatos et al. 2000a). In contrast, plants with intermediary cells transport most of their sugar as RFOs, with stachyose dominating. Indeed, all species known to have intermediary cells use RFOs as their primary transport sugars, and all species known to use RFOs as their primary transport sugars have intermediary cells. Clearly, this is not a coincidence; it is a mechanistic association.

The clearest experimental evidence that RFO species load through the symplast is that loading is not inhibited by PCMBS. Several complementary techniques have been used

to verify these results, including macroautoradiography, exudation into EDTA (ethylenediaminetetraacetic acid) solutions, and plasmolysis (Turgeon and Medville 2004). Of these, plasmolysis has the advantage that it directly visualizes loading. Plasmolysis is the retraction of the plasma membrane and cytoplasm away from the wall that occurs when plant cells are placed in a hypertonic solution (figure 5). It is the most direct way of providing evidence for the presence or absence of a solute concentration gradient between specific cell types (Geiger et al. 1973).

In experiments with *Coleus blumei* (*Solenostemon scutellarioides*), an RFO-transporting species, plants were placed in the dark until the contents of the phloem were depleted, and then fragments of leaf tissue were exposed to light. As measured by plasmolysis, the solute content of the phloem rose dramatically, even when the tissue had been pretreated with PCMBS (Turgeon and Gowan 1990).

The strict association between intermediary cells and the translocation of RFOs led to the development of a thermodynamically feasible model of symplastic loading, termed the "polymer trap" (Turgeon 1991). According to this model, sucrose diffuses into intermediary cells *down* its concentration gradient and is converted to the larger oligosaccharides, raffinose and stachyose. Synthesis of these sugars reduces the concentration of sucrose to permit continued diffusion. A fundamental assumption of the model is that the branched plasmodesmata are functionally narrower than those at many other interfaces, with a MEL that allows sucrose to enter the cell but does not permit diffusion of the larger sugars, raffinose and stachyose, in the opposite direction. Raffinose and stachyose are tri- and tetrasaccharides, respectively. Small amounts of the pentasaccharide verbascose are also transported in some plants. The concentration of raffinose and stachyose in the phloem is approximately 400 to 600 millimolar as measured by microdissection of minor veins (Haritatos et al. 1996) and analysis of aphid stylet exudate from the stem (Knop et al. 2001). The total solute concentration in the intermediary cells is far above that in the adjacent bundle sheath cell, as visualized by plasmolysis (figure 5).

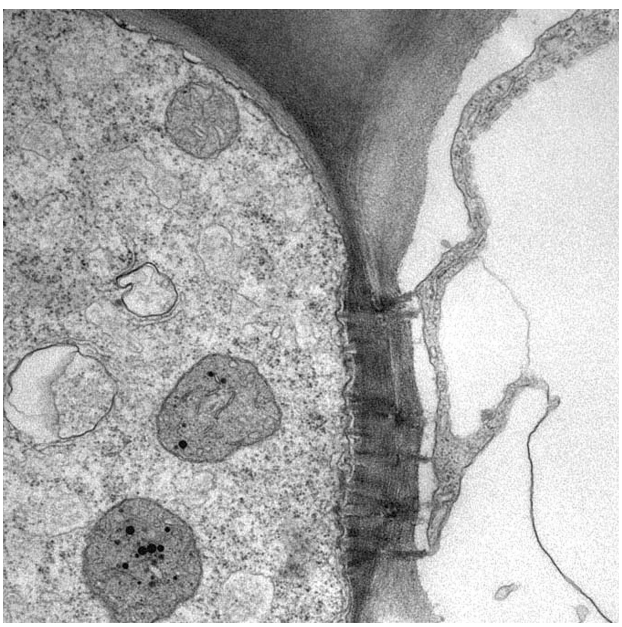

Figure 5. Electron micrograph of an intermediary cell (left) and a bundle sheath cell (right) in* Euonymus fortunei *leaf tissue.* E. fortunei *translocates raffinose-family oligosaccharides (Turgeon et al. 2001). Sorbitol was added to the fixative to a total osmolality of 1200 milliosmols per kilogram. The bundle sheath cell is plasmolyzed, but the intermediary cell is not. Note the connections of bundle sheath cytoplasm to the numerous branched plasmodesmata that join the two cell types. Micrograph by Véronique Amiard and Kristine E. Mueh (Department of Ecology and Evolutionary Biology, University of Colorado, Boulder).

As predicted by the model, the RFO pathway in the leaves of cucurbits is localized in intermediary cells. In some species, such as *Ajuga,* RFOs are also produced in the mesophyll, but the two pools are separate; export occurs from the intermediary cell pool (Sprenger and Keller 2000).

All plants with intermediary cells have standard ("ordinary") companion cells in the minor vein phloem as well. This has led to the suggestion of mixed loading: concurrent introduction of sucrose and RFOs to the phloem stream by apoplastic and symplastic pathways, respectively (van Bel and Gamalei 1991). If this occurred, one would predict that treating leaves with PCMBS would alter the ratio of sugars transported, decreasing the proportion of sucrose. Experiments to test this hypothesis have met with varied results (Flora and Madore 1996, Knop et al. 2004) and have been confounded by systemic problems in determining the exact composition of the phloem sap. Workers have relied heavily on a simple technique in which leaves are excised and phloem exudate from the cut ends of the petioles is collected in a vial containing an EDTA solution (King and Zeevaart 1974). EDTA presumably prevents callose from forming at the sieve plates. While this technique can sometimes provide a reasonable picture of transported solute, this is not always true. For example, in *Lolium perenne,* phloem exudate obtained by the EDTA method contains monosaccharides and loliose, although these sugars are not detected in aphid stylet exudate (Amiard et al. 2004). It is possible that under some circumstances EDTA extracts nonmobile compounds from cells surrounding the SE/CC complex. Mixed loading is an intriguing possibility, and it opens the possibility that different loading strategies could be used by the same plant under various conditions, but it has not yet been rigorously proved.

Now, how is unidirectional transport enforced in the darkened leaves of plants with intermediary cells? It is unlikely that the plasmodesmata of intermediary cells are pressure regulated, because, if they were, they would always be closed and loading would not occur. Instead, according to the polymer trap model, they are constitutively too narrow to allow the RFO sugars in the phloem to escape into the mesophyll (figure 4b). Therefore, leakage through the symplast should be minimal. As in apoplastic loaders, some sugar will cross the

plasma membrane into the apoplast, but it will be retrieved by transporters that appear to be ubiquitous in the phloem, and are present in species that load symplastically (Knop et al. 2001). Note also that RFOs do not leak out of cells to the same extent as sucrose (Ayre et al. 2003).

Where to go from here

The hypotheses put forward in this article are, of course, hypotheses only and require testing. Some aspects of this subject are better supported by data than others. The apoplastic model of phloem loading seems secure in its broad outlines, although researchers are only beginning to understand how this mechanism is regulated. Although available data support the polymer trap model, they are limited and much more needs to be done. In particular, the MELs of plasmodesmata in the minor vein phloem are not known for any species. Pressure regulation of plasmodesmata in minor vein phloem could be important in terms of how they operate, but that has not been experimentally verified. I have made only passing reference here to sugar alcohol loading and transport, which is an area of study in its infancy. It is far from clear how widespread passive entry of photoassimilate into the phloem will prove to be. Last but not least, the ecological advantages conferred by different loading mechanisms are still obscure, and the phylogenetic distribution of the loading types is only partially understood. Although comparative studies indicate that different plants use different loading strategies, only a few model species are used in most transport experiments. Even the large data sets of Gamalei (1989) and Zimmermann and Ziegler (1975) include only a limited number of plant families. Sadly, information on phloem loading in nonangiosperms is almost entirely lacking.

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