

Phloem

Phloem: Molecular Cell Biology, Systemic Communication, Biotic Interactions

Edited by

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Cover images: False-color confocal laser scanning images of phloem cells in intact plants.

Upper left: CMEDA/CMFDA-stained sieve element (reddish) and two companion cells, Upper right: At the right-hand side of the picture, two consecutive sieve elements (blue) each with a companion cell in a staggered position. The lower sieve element contains an arrowhead-shaped forisome (green colored) near the sieve plate (not visible). The other longitudinal cells are phloem parenchyma cells. Lower left: ER-Tracker Green-stained intact phloem tissue. At the left-hand side of the picture a sieve element (black with blue traces of ER near the sieve plate) and a companion cell (blue and green). At the right-hand side, the ends of two adjacent phloem parenchyma cells with a broad margin of cytoplasm (green and white). Lower right: Same picture as upper right with a different false-color setting.

Courtesy of Dr. Jens B. Hafke

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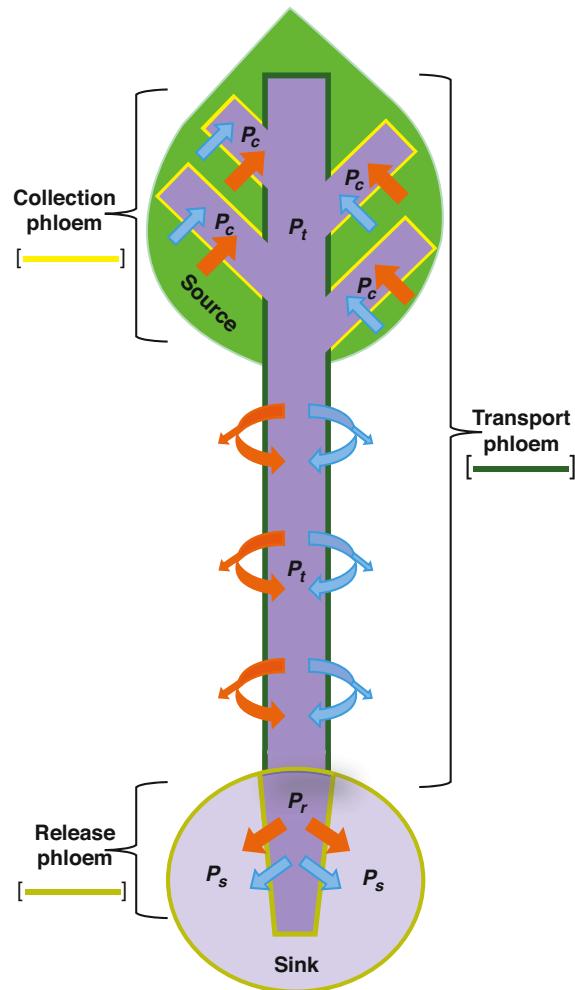


Plate 3.1 Münch pressure flow through three functional phloem zones: collection, transport and release phloem. Uptake and release of nutrients (brown arrows) into/from sieve elements are accompanied by concurrent fluxes of water (blue arrows). In collection phloem, major osmotic species are loaded into sieve tubes to high concentrations. As a result, their osmotic pressures (π_c) offset transpiration-induced tensions in the leaf apoplasm (P_o) to drive water uptake and hence generate hydrostatic pressure heads (P_c) in collection phloem (see Equation 3.3). Transport phloem retains a high hydrostatic pressure (P_t) by retrieval of leaked osmotica accompanied by water influx. At sinks, exit of phloem sap from sieve elements occurs by bulk flow through plasmodesmata and, as a consequence, hydrostatic pressure at the sink-end of the bulk-flow pathway (P_s) is located in the post sieve-element unloading pathway. Since sinks are hydraulically isolated from the remaining plant body, P_s is predominantly determined by $\pi_s - \pi_o$ with P_o exerting little influence (see Equation 3.3). The purple gradient represents the presumptive large hydrostatic pressure drop from release phloem (P_r) to sink cells (P_s). Resource loading/reloading and unloading set the magnitude of $P_c/P_t - P_s$ to drive volume flux ($\text{m}^3 \text{ m}^{-2} \text{ s}^{-1}$) through sieve tubes modulated by pathway geometry (largely dominated by plasmodesmal radii of postsieve element unloading pathways—see Equation 3.2). Phloem sap concentration (C) of a nutrient species is determined by loading/reloading and, together with volume flux, is a major regulator of bulk flow rates (R_f —see Equation 3.1).

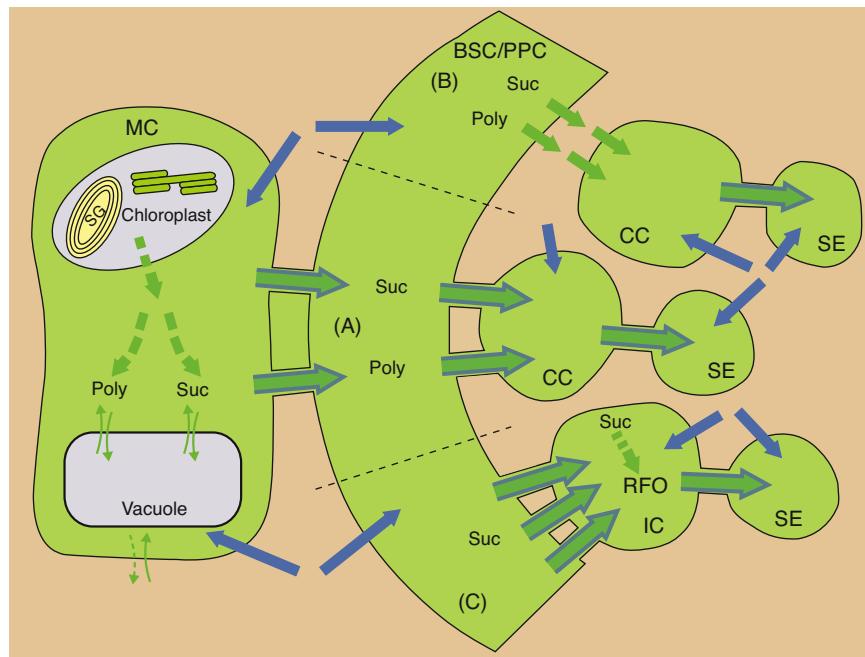


Plate 3.2 Phloem loading pathways for sugars (green arrows) and water (blue arrows) in source leaves. Cytoplasmic pool sizes of sucrose (Suc) or polyols (Poly) in mesophyll cells (MC) determine amounts available for loading. Metabolic transfers into these pools (green arrows with broken shafts) is primarily from current photosynthetically reduced carbon (C) crossing chloroplast envelopes with excess C stored as chloroplastic starch grains (SG) or as sugars sequestered in mesophyll cell vacuoles by transport across their tonoplast membranes (curved-green arrows). When C flows from current photosynthesis (e.g., during the night) do not meet demand for C, cytosolic sugar pools are buffered by remobilization of C from chloroplastic starch or sugars stored in vacuoles (green-curved arrows). Water flows (blue arrows) into MCs, down osmotically generated water potential gradients, create hydrostatic pressure gradients to drive bulk flow of cytosolic sugars (green arrows for sugar; water underlying blue arrow) through interconnecting plasmodesmata to reach bundle sheath/phloem parenchyma cells (BSC/PPC). During this passage, sugars passively leaked (curved green arrow with broken shaft) from the MC/BSC/PPC symplasmic domain are retrieved by sugar symporters (curved-green arrow). Sugar movement from BSC/PPC to sieve element–companion cell complexes (SE–CCs) fall into three categories that are species specific. (A) Continued passive movement through a symplasmic route by bulk flow down hydrostatic pressure gradients is prevalent in woody plants transporting sucrose or polyols. (B) Sucrose or polyols released to the phloem apoplasm from BSC/PPCs are retrieved by energy-coupled transporters (green arrows) to accumulate in SE–CCs to concentrations a magnitude higher than those in MC cytosolic pools. Osmotic water uptake generates hydrostatic pressure heads to drive bulk flow through sieve tubes to exit source leaves and flow onto sinks. (C) Sucrose symplasmically enters specialized CCs, intermediary cells (IC), where it is metabolized (green arrow with broken shaft) to raffinose family oligosaccharides (RFOs). RFO molecular dimensions exceed size exclusion limits of plasmodesmata interconnecting BSC/PPC with ICs effectively trapping RFOs in ICs. RFOs accumulate to high concentrations in IC/SEs to drive osmotic water uptake that create hydrostatic pressure heads to drive bulk through sieve tubes.

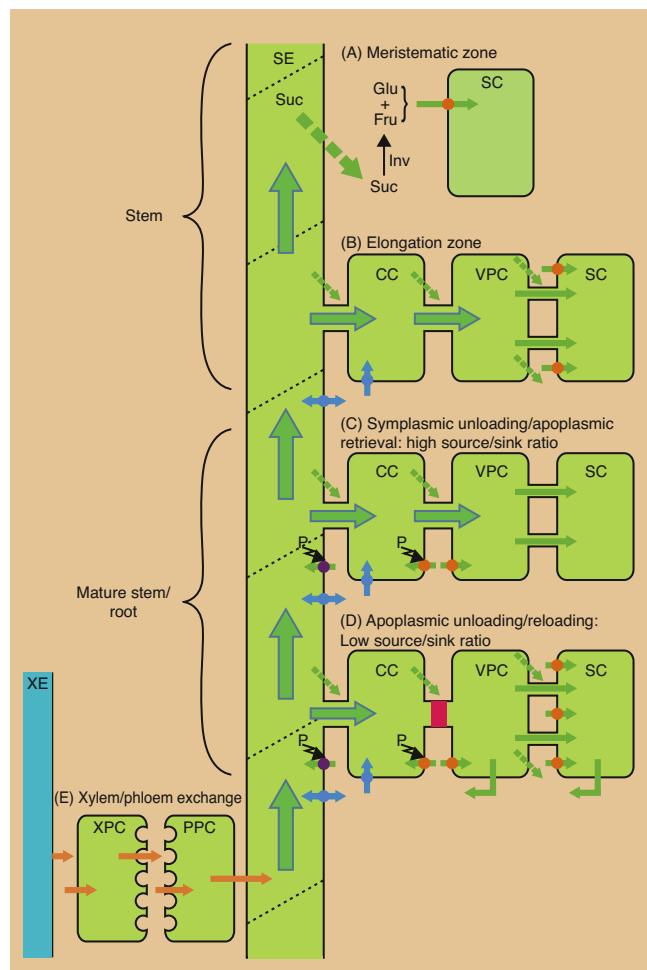
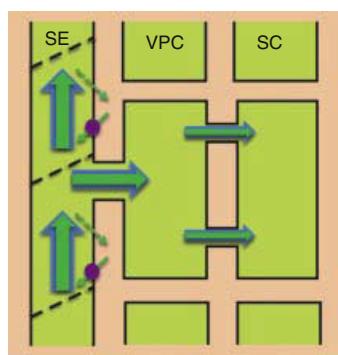
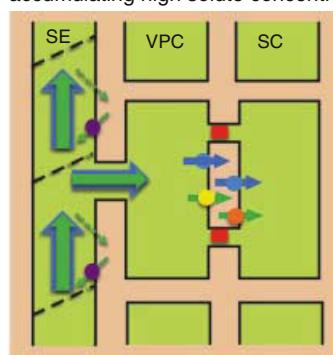


Plate 3.3 Lateral exchange of sucrose (green arrows) and water (blue arrows) along transport phloem. Irrespective of unloading pathway (A–D), large transmembrane differences in sucrose concentration between sieve element (SE) lumens and their surrounding apoplasts drive substantial passive leaks of sucrose from SEs (green arrows with broken shafts). Aquaporins (blue circles) on SE plasma membranes facilitate bidirectional water transfer (blue double-headed arrows) according to prevailing water potential gradients between SE lumens and their surrounding apoplasts. (A) Sucrose exit from protophloem SEs in meristematic zones of stems is entirely by passive leak across their plasma membranes. Cell wall invertases (Inv) hydrolyze released sucrose. The resulting glucose (Glu) and fructose (Fru) moieties are transported into sink cells (SC) by hexose transporters (orange circle). (B) In elongating stem zones, unloading occurs from metaphloem SE–CCs (companion cell complexes) through apoplastic and symplasmic pathways operating in parallel. Symplasmic unloading likely occurs by bulk flow (sucrose green arrow superimposed on blue arrow representing water flow) to adjoining vascular parenchyma cells (VPC). Thereafter onward movement to sink cells may occur symplasmically or apoplasmically with sugar recovery from sink apoplasts by carrier-mediated transport (green arrows through orange circles). In mature stems (C, D), irrespective of unloading pathway, sucrose leaked from SEs to phloem apoplasm, is retrieved by turgor (P)-regulated transporters (purple circles) located on SE–CC plasma membranes. (C) Under high source/sink ratios, a net unloading of sucrose occurs predominately through a symplasmic pathway. (D) Under low source/sink ratios, plasmodesmata linking SE–CCs to VPCs are gated closed (red rectangle) and unloading/reloading of SE–CCs follows an apoplastic route. Sucrose transport into VPCs is carrier mediated (orange circles) and thereafter sucrose accumulation by sink cells can occur through apo- and symplasmic routes operating in parallel. If low source/sink ratios persist over protracted periods, stored carbon is remobilized from sink cells, released to sink/phloem apoplasts (green L-shaped arrows) and reloaded into SE–CCs to buffer sucrose flows to terminal sinks. (E) Solute exchange from xylem transpiration streams is mediated by transporters located on xylem (XPC) and phloem parenchyma (PPC) cells (orange arrows) participate in xylem (XE)-to-phloem exchange of solutes. XPC/PPCs, located at nodal regions, often are modified to a transfer cell morphology (wall ingrowths—indentations in periclinal walls).

(A) Symplasmic unloading



(B) Apoplastic unloading in sinks accumulating high solute concentrations



(C) Apoplastic unloading in developing seeds

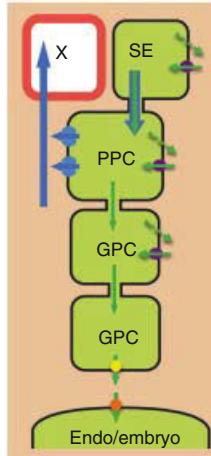


Plate 3.4 Cellular pathways of phloem unloading of sucrose (green) and water (blue) in terminal sinks from sieve elements (SE) alone or SE–companion cell (CC) complexes. Irrespective of unloading pathway (A–C), a passive leak of sucrose (green arrow with broken shaft) occurs from SEs to their surrounding apoplasts. Leaked sucrose is retrieved by transporters (green arrow through purple circle) located on plasma membranes of SEs or SE–CCs. (A) Symplasmic unloading by bulk flow (sucrose—green arrow; water—blue arrow) from SE or SE–CC via vascular parenchyma (VPC) to sink (SC) cells. (B) Plasmodesmata are gated closed (red rectangle) at SE/VPC or VPC/SC interfaces of terminal sinks accumulating sugars to high concentrations. Transporter-mediated efflux of sucrose (green arrow through yellow circle) and water (blue arrow through blue circle) occurs to sink apoplasts. Sink cells recover apoplastic sucrose (green arrow through orange circle) and water through transporters located in their plasma membranes. (C) Phloem unloading in developing seeds follows symplasmic pathways from SEs to maternal/filial interfaces. Transfer from SE or SE–CC to VPC is likely to be by bulk flow (green arrow overlying blue arrow) with diffusion of sugars (green arrow) thereafter. Excess water is recycled back to the parent plant body by aquaporin-mediated exchange (blue arrows through blue circles) to the seed apoplasm and exiting through the xylem (X). Along the symplasmic route, transporters (purple circles) in phloem parenchyma (PPC) and ground parenchyma (GPC) cells retrieve sucrose leaked (green arrows with broken shafts) to seed apoplasts. At maternal/filial interfaces, sucrose transport across cell membranes is carrier-mediated. Sucrose efflux (green arrow through yellow circle) to seed apoplasts occurs from specialized maternal GPCs. Thereafter sucrose, or its hexose moieties are taken up (green arrow through orange circle) into filial tissues comprising endosperm and/or embryo.

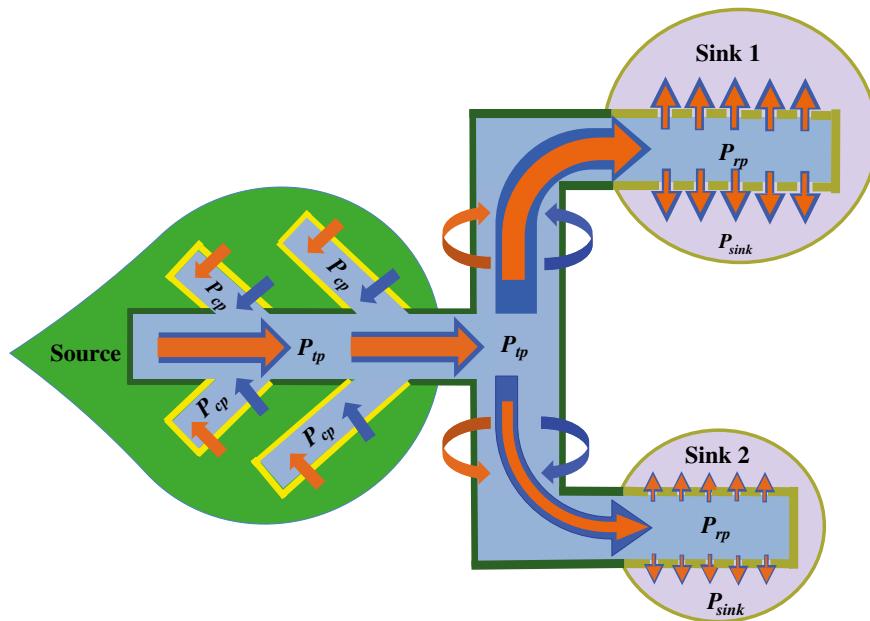


Plate 3.5 High-pressure manifold model of phloem transport and resource partitioning between two competing sinks. Loading of nutrients (orange arrows) and water (blue arrows) in collection phloem (cp; yellow border) sets nutrient concentrations (Equation 3.1) and hydrostatic pressure (P_{cp}) to drive bulk flow through sieve tubes linking source and sink (orange arrows superimposed on blue arrows; Equation 3.2). Nutrient concentrations (C) and hydrostatic pressures are maintained homeostatically throughout transport (tp; green border) and release (rp; khaki border) phloem by turgor-regulated retrieval of nutrients and hence water leaked to phloem apoplasm (curved arrows). Hence, $C_{cp} \sim C_{tp} \sim > C_{rp}$ and $P_{cp} \sim P_{tp} \sim > P_{rp}$ with a slight hydrostatic pressure gradient from transport/release phloem interface onward into release phloem. Hydraulic conductivities (L_p) or resistances (R where $R = 1/L_p$) of plasmodesmata (radii of plasmodesmata depicted by width of spaces in release phloem border and see Equation 3.2) regulate symplasmic unloading by bulk flow from SEs or SE-CCs. Their low hydraulic conductivities account for large drops in hydrostatic pressures from release phloem SEs (P_{rp}) to vascular parenchyma and sink cells (P_{sink} and see Table 3.2). Thus, overall bulk flow rates of nutrients (R_f) from collection phloem to sinks are determined by:

$$R_f = (P_{cp} - P_{sink} * C) / (R_{cp} + R_{tp} + R_{rp} + R_{sink})$$

Since $R_{sink} \gg R_{cp} + R_{tp} + R_{rp}$ and R_{sink} is regulated, nutrient partitioning to, and between sinks, is largely determined by relative magnitudes of current R_{sink} values of competing sinks.

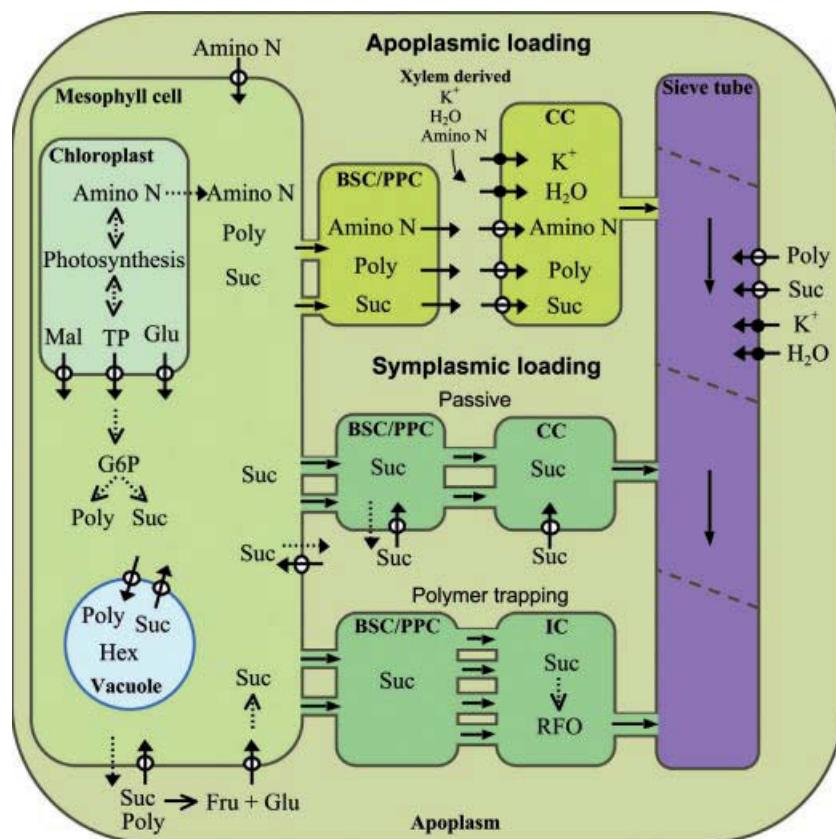


Plate 4.1 Cellular localization of resource membrane transporters located along phloem-loading pathways from mesophyll cells to sieve tubes of leaf minor veins in relation to mechanisms of apoplastic and symplasmic (passive and polymer trapping) phloem loading. Resources arising from photosynthesis (sugars and amino N compounds) in mesophyll chloroplasts are released to mesophyll cell cytosols through carriers located in their chloroplast envelopes (arrow through open circle). Carrier-mediated exchange of sugars, to and from vacuolar storage, buffers cytosolic pools of sugars available for loading. Irrespective of the phloem-loading mechanism, resources flow symplasmically from mesophyll to bundle sheath/phloem parenchyma cells (BSC/PPC). Sugars passively leaked to the leaf apoplasm (arrow with dashed line), during symplasmic passage, are retrieved by energy-coupled carriers (arrow through open circle). Upon reaching BSC/PPC, and depending upon the loading mechanism, resources either are exchanged via minor vein apoplasts through release by putative efflux carriers (black arrow with wide head), and retrieval by energy-coupled carriers located on sieve element (SE)-companion cell complexes (SE-CCs) (apoplastic loading) or continue to move through the minor vein symplasm to reach sieve tubes (symplasmic loading). Symplasmic loading may occur passively or by active symplasmic entrapment of Raffinose family oligosaccharides (RFO) synthesized from sucrose in modified companion cells (polymer trap), intermediary cells (ICs), with subsequent movement through large-diameter plasmodesmata interconnecting ICs to SEs. Irrespective of the loading mechanism, xylem delivered potassium (K⁺) and water (H₂O) are loaded into SE/CC through channels (arrow through filled circle) along with energy-coupled carriers mediating uptake of amino N compounds. Flu, fructose; Glu, glucose; Hex, hexose; Mal, maltose; Poly, polyol; K⁺, potassium; Suc, sucrose; TP, triose phosphate.

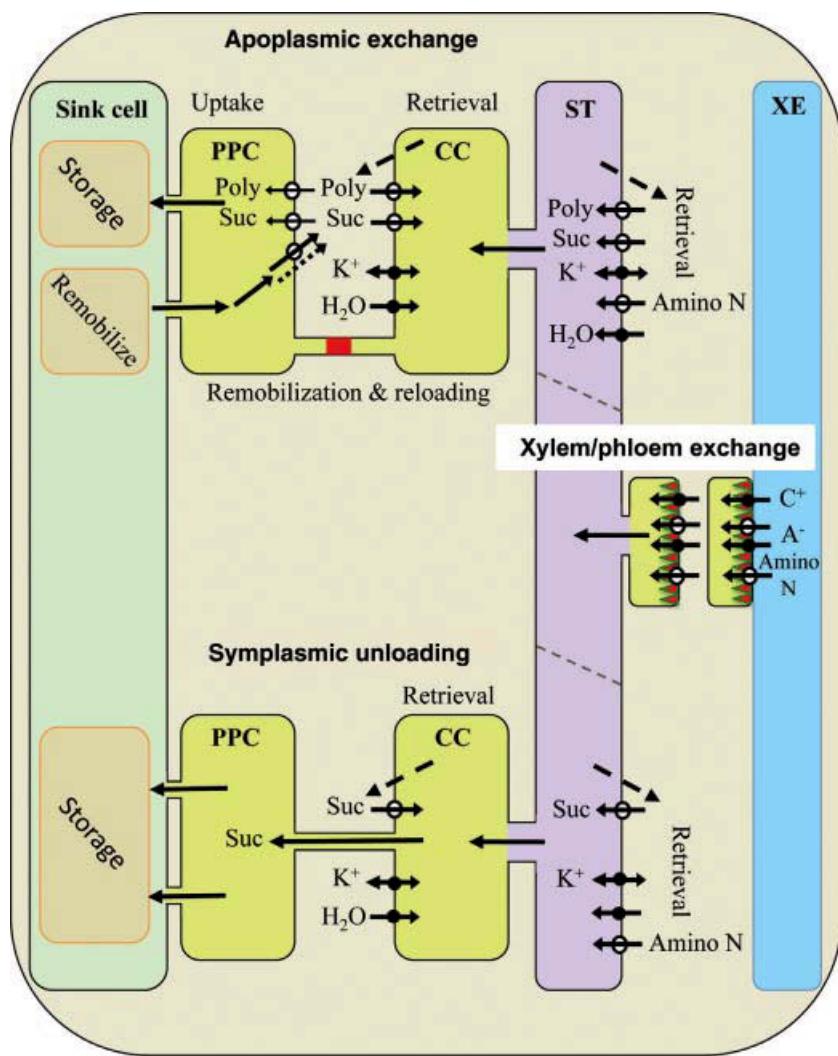


Plate 4.2 Resource membrane transporters located in cells of the transport phloem and postphloem pathways supporting apoplastic exchange, xylem-phloem exchange and symplasmic unloading. Apoplastic exchange predominates resource flows between transport phloem and surrounding sink tissues when plasmodesmata interconnecting companion cell (CC) and phloem parenchyma cell (PPC) are gated closed (red rectangle). Apoplastic exchange comprises three primary membrane transporter functions. These are retrieval of resources by SE-CCs leaked from the transport phloem, nutrient uptake into PPCs with subsequent symplasmic delivery to sink cells for storage/growth and net loading of SE-CCs with nutrients remobilized from storage in sink cells and released to the leaf apoplasm. Reloading nutrients into SE-CCs is accompanied by an osmotic uptake of water. Nutrient xylem-phloem exchange, particularly pronounced at nodal regions, involves nutrient (ions and amino N compounds) captured from the transpiration stream and delivery into SE-CCs with probable engagement of at least two consecutive apo-/symplasmic exchanges. Symplasmic unloading occurs under high source/sink ratios when excess nutrients are stored in sink cells. Retrieval of resources, leaked from SE-CCs, occurs in parallel with symplasmic unloading. Transporters include sugar and anion carriers ± energy-coupled (arrow through open circle) and sugar symporters functioning in reverse (double arrow through open circle) as well as channels supporting water (single arrow through closed circle) and two way-exchange of potassium (double arrow through closed circle). Sugar leakage to the apoplasm occurs by passive movement through plasma membranes (arrow with dashed line). A⁻, anions; C⁺, cations; H₂O, water; K⁺, potassium; SE, sieve element; Poly, polyol; ST, sieve tube; Suc, sucrose; XE, xylem element.

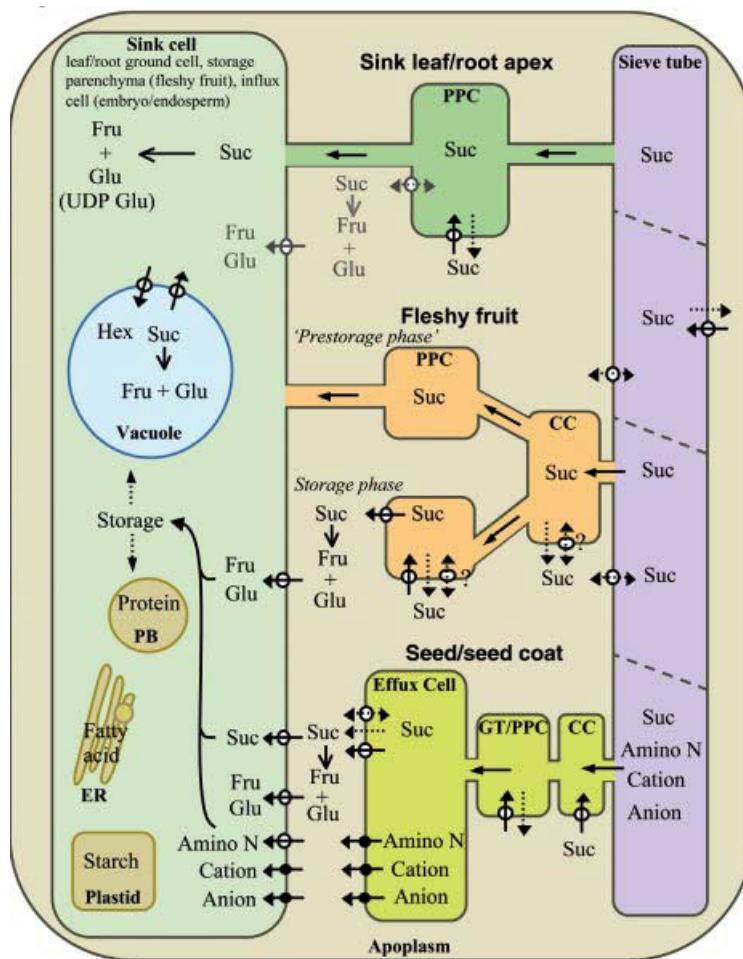


Plate 4.3 Cellular localization of resource membrane transporters in cells of release phloem and postphloem-unloading pathways in sink leaf/root apices, fleshy fruit, and developing seeds. In sink leaves and root apices, unloading primarily follows symplasmic routes possibly supplemented by release to the sink apoplasm. To optimize delivery of sucrose for symplasmic unloading, sucrose symporters localized to release phloem function in retrieving leaked sucrose. For apoplastic unloading, sucrose symporters could be reversed to support efflux to the sink apoplasm. Here, an extracellular invertase hydrolyzes sucrose and the resulting fructose and glucose are taken up into sink cells by hexose symporters. During the prestorage phase of fleshy fruit development, phloem unloading follows a symplasmic route switching to one including an apoplastic step once sugar storage commences. Reversal of sucrose symporters could be a primary route for release to the fruit apoplasm supplemented by passive release. Hydrolysis of released sucrose by extracellular invertases is followed by the resulting glucose and fructose being retrieved by hexose symporters into storage parenchyma cells for vacuolar storage facilitated by tonoplast transporters. For developing seeds, transporters located on SE-CCs, PPC, and GT cells of seed coats likely function to retrieve leaked resources from a symplasmic-unloading route delivering resources to specialized efflux cells. Efflux of resources from seed coats might include passage through efflux carriers, symporter reversal or channels accompanied by passive leakage, Influx into filial tissues, via seed apoplasmic spaces where sucrose may or may not be cleaved by an extracellular invertase, is mediated by transporters localized to specialized influx cells proximal to the maternal/filial interface. Thereafter, sugars and amino N compounds enter biosynthetic pathways, leading to carbon and nitrogen capture into protein, fatty acid and starch storage compartments. Transporters include carriers ± energy-coupled (arrow through an open circle), including sucrose symporters that may function in reverse (doubled-headed arrow through open circle), as well as channels (arrow through closed circle). Sugars leaked to seed apoplasts occur by passive movement (arrow with broken line). CC, companion cell; SE, sieve element; Fru, fructose; Glu, glucose; GT, ground tissue; Hex, hexose; PB, protein body; PPC, phloem parenchyma cell; Suc, sucrose.

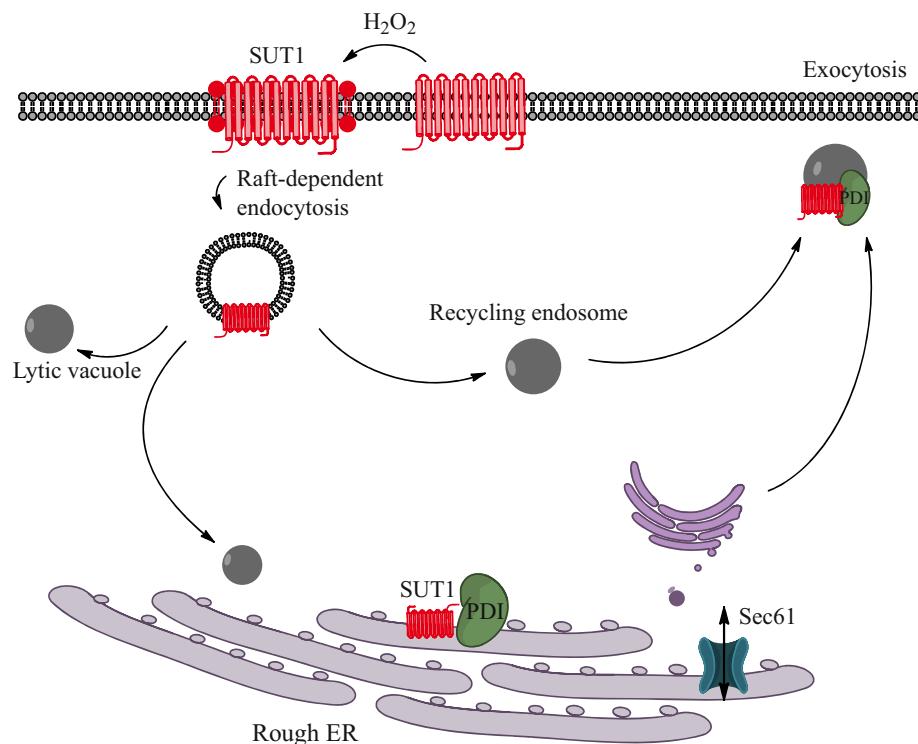


Plate 5.1 Model for plasma membrane recycling of the potato sucrose transporter StSUT1. The model is based on the biochemical investigation of the protein, the analysis of its targeting behavior in living cells and the systematic screen for SUT1-interacting proteins (Krügel et al., 2011; Krügel et al., 2008; Liesche et al., 2010). StSUT1 protein is synthesized in the ER (of companion cells?), the escort of the protein to the plasma membrane (of sieve elements?) might occur by a raft-associated protein disulfide isomerase (PDI), which was localized in the ER, in vesicles and in plasma membrane microdomains (Krügel et al., 2011). The lateral movement from companion cells into the sieve elements is assumed to occur via the desmotubules of PPUs connecting the ER cisternae of both cells. Recycling or endocytosis of the SUT1 protein is discussed to occur in a raft-mediated manner. Dimerization of the SUT1 protein under oxidizing conditions might be either the consequence of its concentration in membrane microdomains or alternatively facilitate raft association.

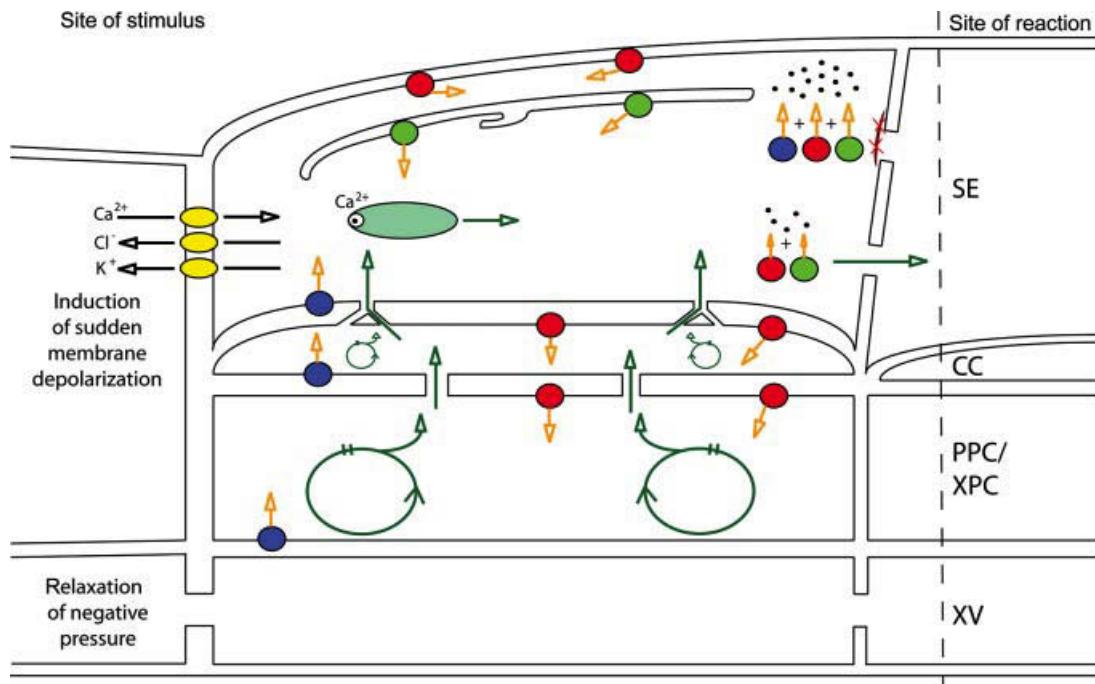


Plate 6.1 Ca^{2+} as the key link between electrical long-distance signaling and local chemical signaling. The events are presented for action and variation potentials in one diagram and actually occur simultaneously as in responses to burning [Dziubinska et al., 2001; Hlavackova et al., 2006; Furch et al., 2009]. Action potentials—brought about by Ca^{2+} influx, Cl^- efflux, K^+ efflux in succession—are triggered by several stimuli (upper left) and are transmitted by the phloem of which sieve elements (SEs) are the conducting modules. High amounts of Ca^{2+} influx (orange arrows), mediated by voltage-gated plasma membrane bound channels (red circles), enhance Ca^{2+} influx by stimulation of ER-bound Ca^{2+} -activated Ca^{2+} channels (green circles). Propagation of action potentials via plasmodesmata in lateral directions induces Ca^{2+} influx by voltage-activated channels (red circles) in other vascular cells such as companion cells (CC) and phloem (PPC) and xylem parenchyma (XPC) cells with outputs varying with the structure/function organization of each cell type. Variation potentials are triggered (e.g., by wounding) by a sudden relaxation of the negative pressures that then propagate as a wave along xylem vessels (XVs). Disturbance of the water potential equilibrium results in water uptake and increased turgor of adjacent vascular cells located along the XVs. Their enhanced turgors activate mechanosensitive Ca^{2+} channels in xylem parenchyma and adjacent cells (blue circles) leading to Ca^{2+} influx and subsequent potential waves. Variation potentials may also propagate symplasmically to SEs and induce Ca^{2+} influx including that from the ER cisternae in the sieve-element microplasma. It is unclear if Ca^{2+} influx in SEs is also elicited by substances produced in the vascular cells (green arrows) due to Ca^{2+} -triggered cascades (arrowed circles). These compounds may also play a role in long-distance signaling (green arrows in SEs). Furthermore, Ca^{2+} ions released into SEs may activate Ca^{2+} -binding proteins (green ovals) involved in long-distance signaling [Stahlberg and Cosgrove, 1997]. When the Ca^{2+} concentration in SEs (black dots) surpasses a threshold value, callose deposition is induced. Action potentials are usually unable to increase Ca^{2+} concentration to such an extent (green + red circles) that the Ca^{2+} threshold is reached. However, in the case of burning, combinatory effects of action and variation potentials conferred by the aggregate contribution of the respective Ca^{2+} channels (red + green + blue circles) lead to blockage of long-distance and short-distance symplasmic transport. (Modified after van Bel et al., 2011).

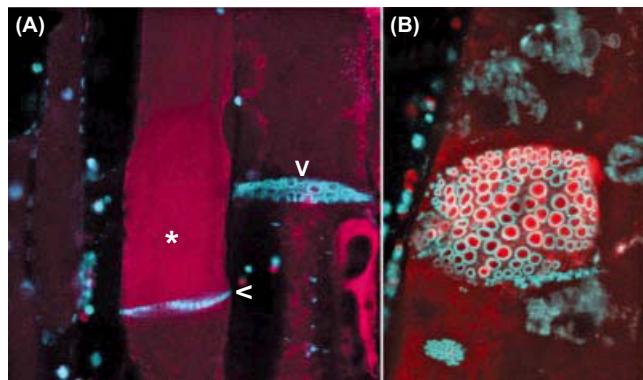


Plate 7.1 Confocal images of squash sieve elements (SEs) and sieve plates after SE injury. (A) Two sieve tubes are present in longitudinal view. The one on the left shows protein precipitation (asterisk) in front of a sieve plate (arrow heads). Cross-sectional view of a sieve plate in an injured SE shows that P-proteins fill the pores (red) and callose is deposited around these proteins (blue rings).

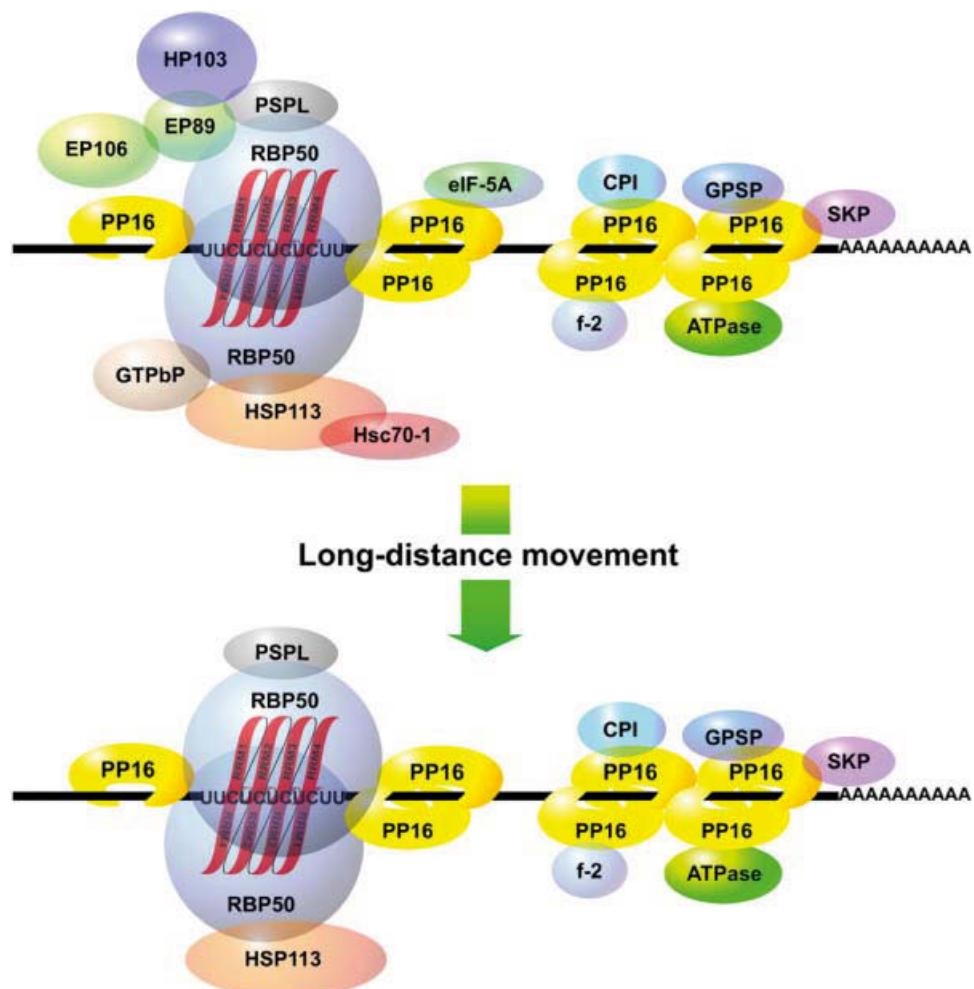
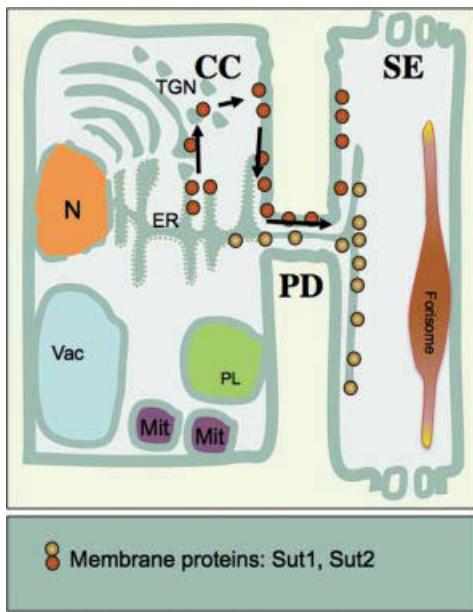


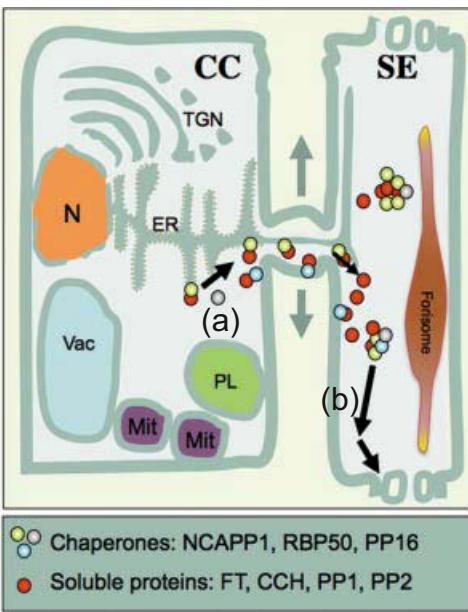
Plate 8.1 Model of a phloem *Cucurbita maxima* RNA-binding protein 50 (CmRBP50)-based ribonucleoprotein (RNP) complex that delivers mRNA species transcribed within source leaves into sink tissues. The pumpkin RBP50 binds to polypyrimidine-tract-binding (PTB) motifs located within a set of six phloem-mobile, polyadenylated transcripts. CmPP16-1/-2 binds both to RBP50 and the target mRNA, forming the core of the RNP complex. In addition, GTP-binding protein GTPbP, heat shock-related protein HSP113 and cognate heat shock protein Hsc70-1 bind to RBP50; these proteins might function to chaperone the RNP complex to and through the companion cell (CC)-sieve element (SE) plasmodesmata (PD). Another set of four proteins, composed of the 89-kD expressed protein (EP89), the 103-kD hypothetical protein (HP103), the 106-kD expressed protein, and the phosphoinositide-specific phospholipase-like protein (PSPL) are shown interacting with RBP50. Eukaryotic initiation factor 5A (eIF-5A) is an abundant protein in the pumpkin phloem sap (Ma et al., 2010) that binds transiently to PP16-1/-2, but the function of eIF-5A in RBP50-RNP complex formation remains to be elucidated. Regions outside the PTB motifs are bound by PP16-1/-2 along with five additional proteins: (1) Cys proteinase inhibitor (CPI), (2) the Csf-2-related protein (Cmf-2), (3) the 44-kD putative ATP-binding protein (ATPase), (4) the glutathione-regulated potassium-efflux system protein (GPSP), and (5) the shikimate kinase precursor (SKP). The lower image shows the composition of the phloem-mobile RBP50-based RNP complex based on co-immunoprecipitation results obtained using phloem sap collected from cucumber scions grafted onto pumpkin stock plants. (Reproduced from Ham et al. (2009) with permission from the American Society of Plant Biologists.)

(A) Membrane proteins: transport via the endomembrane system



● Membrane proteins: Sut1, Sut2

(B) Soluble proteins: (a) cell-to-cell and (b) long-distance transport, by molecular chaperones and large complexes



○ Chaperones: NCAPP1, RBP50, PP16

● Soluble proteins: FT, CCH, PP1, PP2

Plate 8.2 Models of noncell-autonomous protein (NCAP) entry into the enucleate sieve-tube system *via* the companion cell (CC)-sieve element (SE) plasmodesmata (PD). (A) Membrane proteins play an essential role in mediating the trafficking of proteins and RNA species to various intracellular and intercellular destinations. In the context of the CC-SE complex (CC-SE), it has been proposed that the plasma membrane (PM) and endoplasmic reticulum (ER) function as major routes for the delivery of proteins synthesized in CCs and translocated through the PD to SE endomembranes (Martens et al., 2006). Examples of such proteins are the PM-located sucrose transporters (e.g., SUT2) that are present both on the PM of the CC and SE (Barker et al., 2000). (B) Soluble proteins present in sieve-tube sap gain entry into the SE from the CC *via* targeted or nontargeted trafficking through PD. Selective trafficking involves the action of molecular chaperones and PD receptors that function to guide proteins along the cytoskeleton or the ER to and through the PD. Examples of such proteins are NCAPP1 (receptor) and CmPP16/PP2 (chaperones). NCAPs trafficked along this pathway are released into the lumen of the SE and move long distance by bulk flow in the translocation stream. Most soluble proteins and protein complexes present in phloem exudates are thought to belong to this class and participate in both (a) cell-to-cell transport and (b) long-distance transport. In addition to (A) and (B) scenarios, sieve-tube proteins can also be synthesized and accumulate within immature SE, then persist through SE maturation, and function in fully differentiated SE, such as the SE-ENOD proteins (Khan et al., 2007) (not shown in the figure). CC, companion cell; SE, sieve element; PD, plasmodesmata; PL, plastids; Mit, mitochondria; Vac, vacuole; N, nucleus; ER, endoplasmic reticulum; TGN, trans Golgi network.

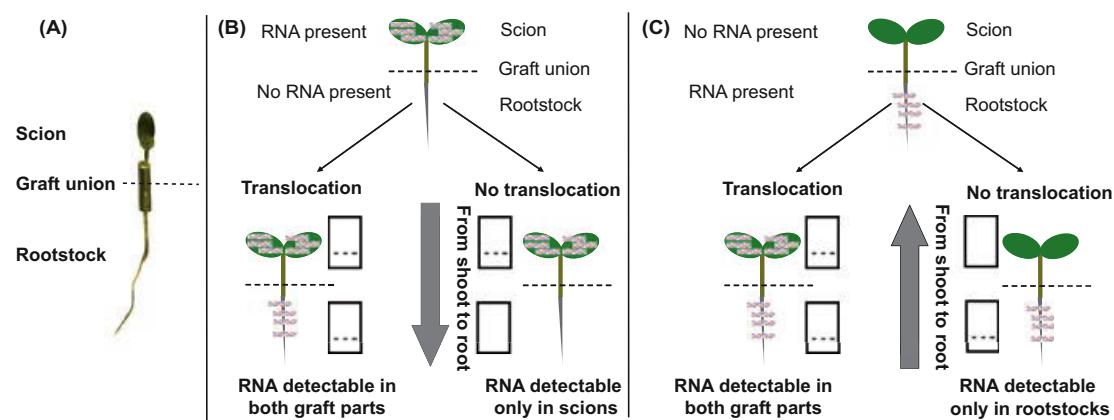


Plate 9.1 General scheme of reciprocal grafting experiments to study RNA long-distance translocation in plants. (A) Shows a grafted *Arabidopsis* seedling, the graft unit is stabilized by a plastic tube. (B) Setup for studying RNA transport from shoot to root. A scion that contains a specific RNA is grafted onto a rootstock that does not contain this particular RNA. After formation of successful graft units, it is analyzed whether this RNA is detectable in the rootstock or not, for example by RNA blots or PCR-based methods. If the RNA is found in both parts of the graft it is a strong indication that this RNA is phloem-mobile. (C) Reciprocal grafting to examine RNA transport from root to shoot. A scion that is devoid of a specific RNA is grafted onto a rootstock that does contain this particular RNA. After formation of successful graft units, it is analyzed whether this RNA is detectable in the scion or not, for example by RNA blots or PCR-based methods. If the RNA is found in both parts of the graft it is a strong indication that this RNA is phloem-mobile. Performing both reciprocal grafts yields information about the general mobility of a particular RNA and also about the direction of RNA transport.

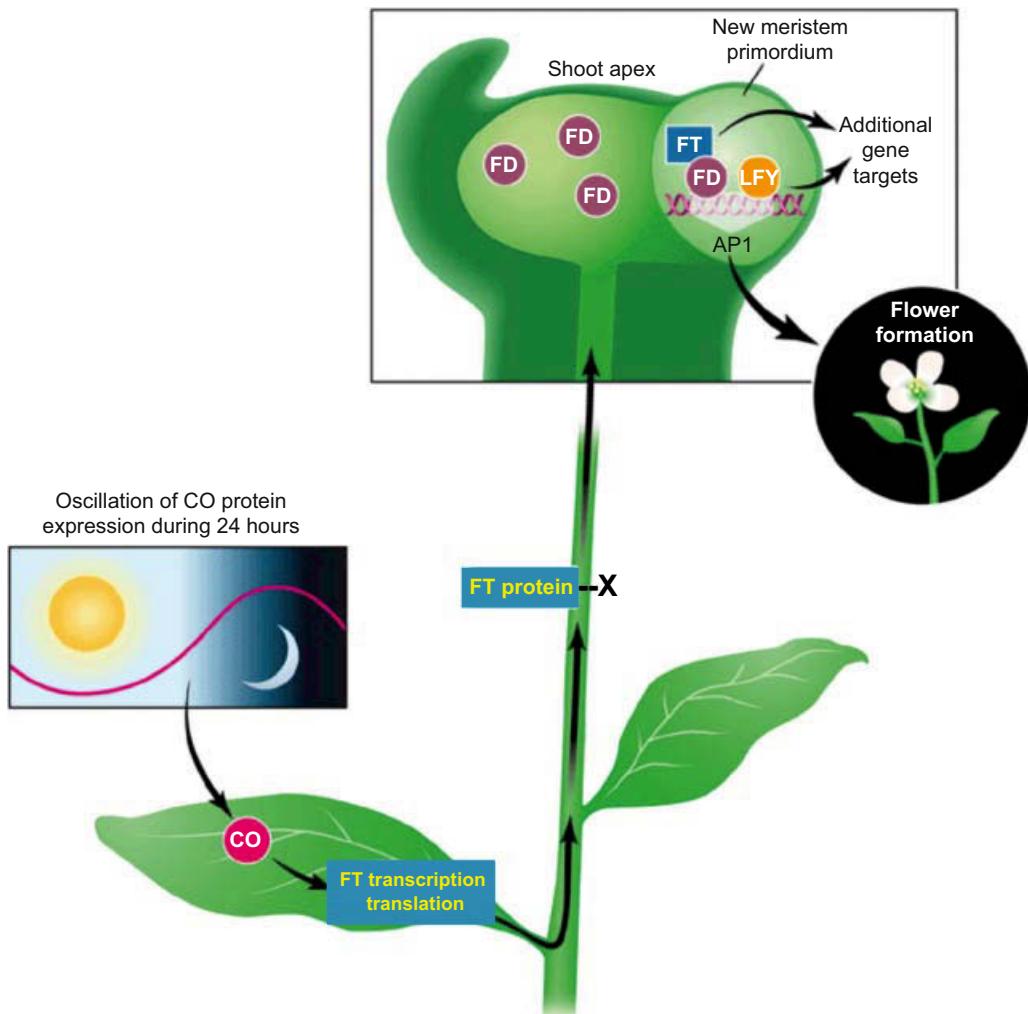


Plate 10.1 The FT protein signals activation of the floral pathway. In response to the appropriate day length (inset), accumulation of the transcription factor CONSTANS (CO) activates transcription of FT in the leaf. In the leaf, FT mRNA is translated, and FT protein moves through the phloem upward with putative unknown chaperone proteins (X) to the shoot apex. In the SAM, the FT protein interacts with the transcription factor FD. This tandem complex then activates floral pathway genes such as *AP1* to initiate floral development. The FT–FD complex acts redundantly with the transcription factor LEAFY (LFY) to activate *AP1*. FD, FLOWERING LOCUS D; FT, FLOWERING LOCUS T. (Modified from Blázquez, 2005.)

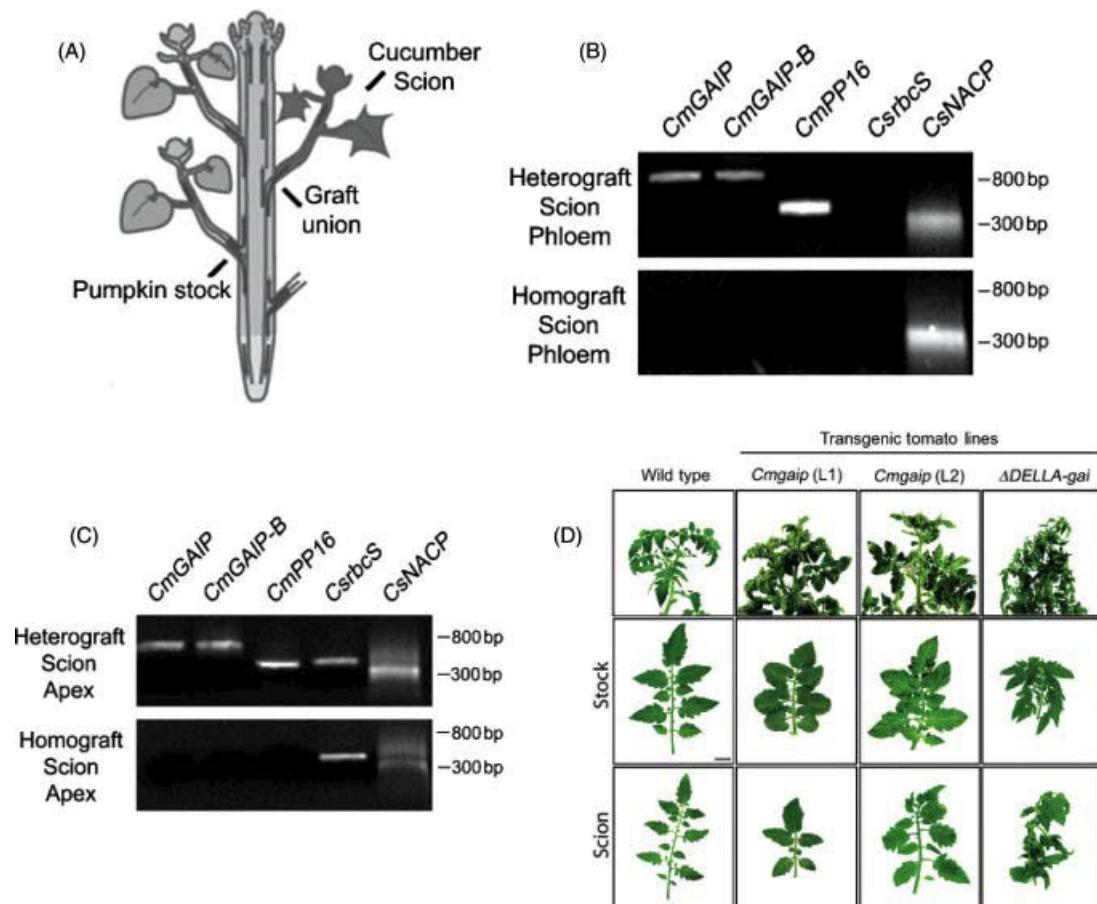


Plate 10.2 Long-distance translocation of CmGAIP and CmGAIP-B transcripts into the vegetative apex of cucumber scions grafted onto pumpkin stocks. (A) Diagram illustrating the arrangement of the grafting system used to assay for phloem long-distance transport of macromolecules. A graft-compatible but genetically distinguishable receiver shoot apex (termed the scion) is attached (graft union) onto the main axis of the donor plant (termed the stock); this system is referred to as a heterograft. (B) RT-PCR analysis, using gene-specific primers directed against RNA collected from phloem sap of cucumber scions (upper panel), amplified both CmGAIP and CmGAIP-B transcripts. Controls: CmPP16 RNA formed a positive control for phloem-mobile transcripts derived from the pumpkin stock. Absence of a CsrbcS product confirmed the lack of wound-induced contaminating RNA from surrounding tissues. Lower panel: RT-PCR failed to amplify the pumpkin transcripts using RNA collected from the phloem sap of cucumber scions grafted onto cucumber stocks (termed a homograft). A phloem-mobile cucumber RNA, CsNACP, was included as a positive control in both hetero- and homograft assays. (C) RT-PCR analysis, using gene-specific primers directed against RNA collected from vegetative apices of cucumber scions grafted onto pumpkin stocks; note that both CmGAIP and CmGAIP-B transcripts were detected in these assays (upper panel). Parallel RT-PCR analysis performed on homografted cucumber scions (control) failed to amplify the pumpkin transcripts (lower panel). Cucumber CsrbcS and CsNACP primer pairs were used to amplify these transcripts as positive controls. (D) Phloem-mediated delivery of Cmgaip and DDELLA-gai RNA induces developmental phenotypes in a tomato graft system. Phenotypic analysis of tomato leaves that develop after grafting of wild-type scions onto the indicated stocks. Upper panel: general morphology of wild-type tomato and transgenic lines expressing P35S:Cmgaip (L1 and L2; two representative transgenic lines) and P35S:DDELLA-gai. Middle panel: Leaf morphology of stock leaves. Lower panel: Scion leaf phenotype represented by the third leaf that developed postgrafting. Scale bars 1/4 2.5 cm; common to stock and scion leaves. (From Haywood et al., 2005.)

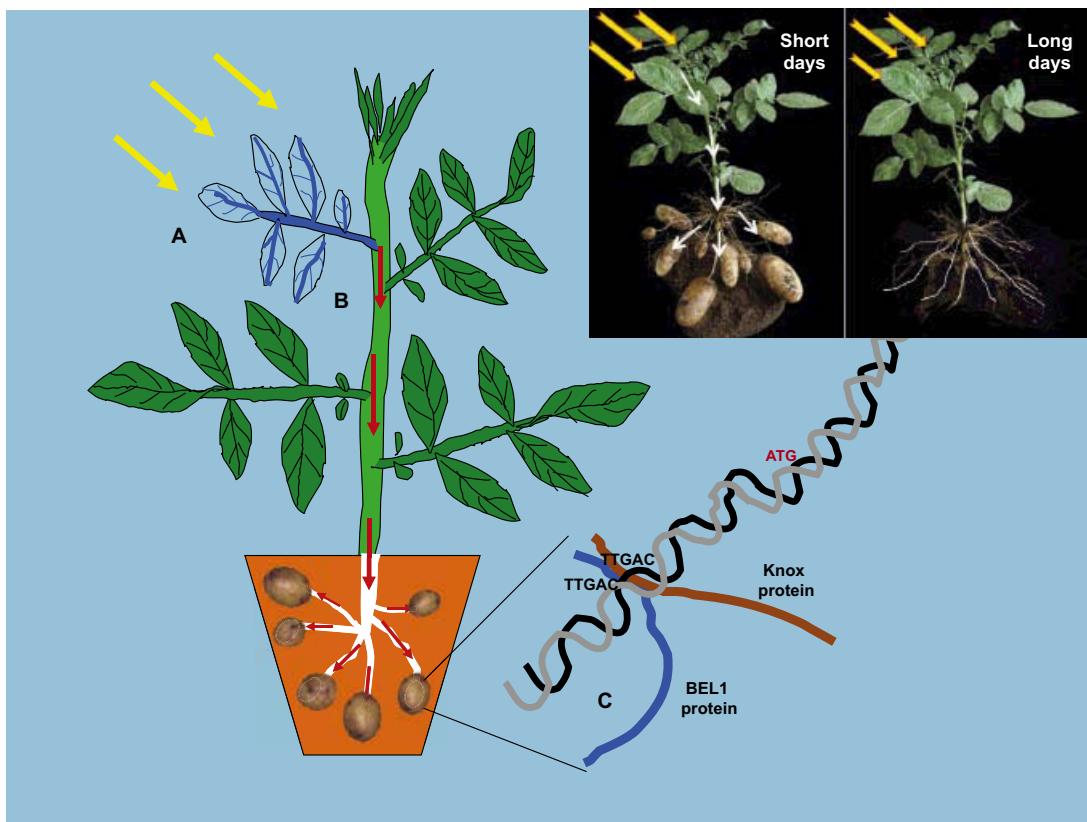


Plate 10.3 The *StBEL5* RNA signaling pathway for tuber formation. Short-day photoperiod conditions are inductive for tuber formation, whereas long days are noninductive (inset). This signaling pathway is based on the initial transcriptional activation by light (A, yellow arrows) of the *StBEL5* gene in the veins of leaves and petioles (blue). A short-day photoperiod facilitates movement of the *StBEL5* RNA through the petiole junction into the stem via the phloem (B, red arrows) by mediating the activation or expression of appropriate RNA-binding proteins. Under these conditions, RNA may then be escorted to site-specific targets, like stolon tips, via protein chaperones. Enhanced translation of BEL5 (blue line) then occurs in the stolon tip leading to binding to a Knox protein partner (brown line) and subsequent activation of transcription and regulation of select target genes by interaction with the tandem TTGAC motif (Chen et al., 2004) of the promoter (C).

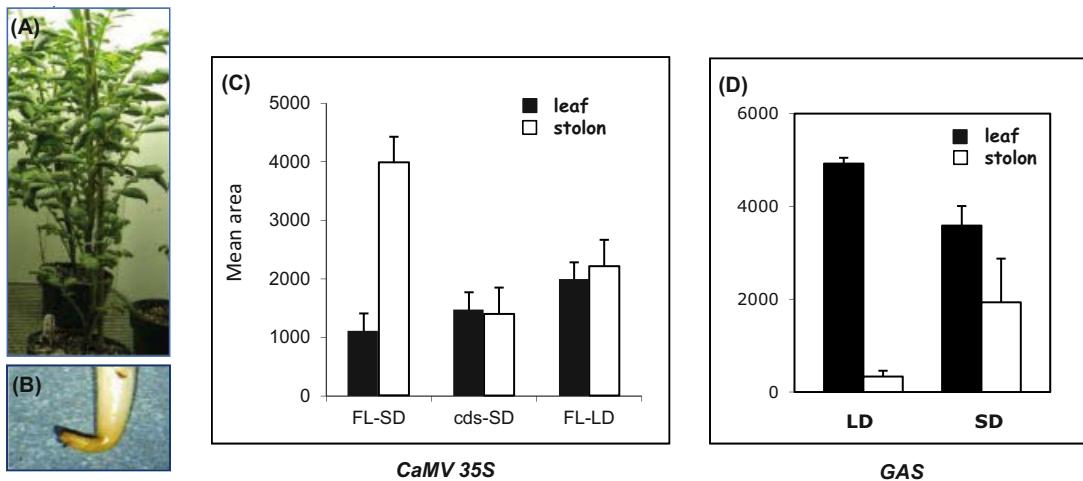


Plate 10.4 Effect of *StBEL5* UTRs and photoperiod on long-distance transcript movement into stolon tips. (A) Movement of transcripts with and without the UTRs (FL and cds, respectively) was assayed in three transgenic plants (for each construct) grown under short-day (SD) conditions for 14 days or long days (LD). RNA was extracted from recently matured source leaves. (B) Stolon tips (0.5 cm in length) on plants described in A were used to extract RNA. (C) RNA levels measured by one-step RT-PCR in leaf and stolon tissues obtained from transgenic potato lines described in A; constructs driven by the cauliflower mosaic virus (*CaMV*) 35S promoter. (D) As in C, except that full-length *StBEL5* transcripts were driven by the leaf-abundant galactinol synthase (GAS) promoter that in potato is active only in the minor veins. Values represent Mean \pm SEM for three biological replicates. (Reprinted from Banerjee et al. (2006); Copyright American Society of Plant Biologists, www.plantcell.org.)

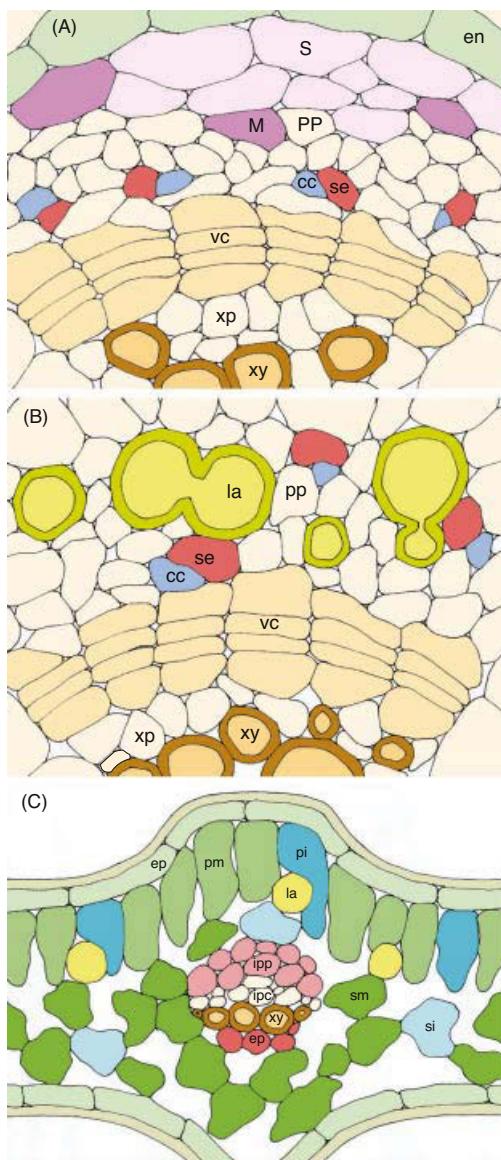


Plate 12.1 Schematic representations of multicell models for the biosynthesis and accumulation of defense compounds. (A) In *Arabidopsis thaliana* flower stalks, glucosinolate-rich S-cells (S) are located at the distal periphery of the phloem of vascular bundles. Expression of thioglucoside glucohydrolase 1 (myrosinase) was shown to occur in specialized phloem myrosinase cells (M) (Husebye et al., 2002). Although glucosinolates and myrosinase enzymes are normally stored in different compartments, tissue disruption by herbivores mixes these two components releasing defensive, bioactive hydrolysis products. Other abbreviations: companion cells (cc), endodermis (en), phloem parenchyma (pp), sieve elements (se), vascular cambium (vc), xylem parenchyma (xp), xylem tracheids (xy). (B) In opium poppy (*Papaver somniferum*) the production of benzylisoquinoline alkaloids such as morphine requires the coordinated participation of companion cells (cc), sieve elements (se) and laticifers (la). Biosynthetic enzymes are synthesized in companion cells and transferred to sieve elements, where alkaloid biosynthesis occurs. These alkaloids are then stored in neighboring laticifer networks. Other abbreviations: phloem parenchyma (pp), vascular cambium (vc), xylem parenchyma (xp), xylem vessels (xy). (C) The production of monoterpenoid indole alkaloids in Madagascar periwinkle (*Catharanthus roseus*) leaves involves numerous cell types, including adaxial internal phloem parenchyma (ipp), laticifers (la) within the palisade mesophyll (pm), idioblasts of palisade and spongy mesophyll (pi and si, respectively) and upper epidermis (ep). Isoprenoid precursors are synthesized in internal phloem parenchyma, and translocation of intermediates to epidermal cells allows the complete biosynthesis of antifungal catharanthine, which is then secreted into the waxy cuticle. In contrast, vindoline biosynthesis requires the additional participation of laticifer and idioblast cells. Other abbreviations: adaxial internal phloem conducting elements (ipc), xylem vessels (xy), abaxial external phloem (ep), spongy mesophyll (sm).



Plate 12.2 Examples of plant-insect interactions involving plant defensive metabolites. (A) Green peach aphids align along leaf vascular tissue to feed on sieve-tube sap. Sap of cruciferous plants often contain glucosinolates, the hydrolysis products of which are toxic or possess antifeedent qualities. However, the careful feeding habit of aphids maintains glucosinolate hydrolysis at a minimum, since hydrolyzing enzymes are compartmentalized separately in the phloem and are only released upon tissue damage (see the text). (B) A pine beetle burrows into the bark of a conifer stem. The secondary phloem of conifers harbors a minefield of anatomical structures containing chemical defenses such as polyphenolics and oleoresin. (C) A cabbage looper engages in trenching behavior while feeding on the leaf of prickly lettuce. The larva chews furrows across leaf vascular tissue, releasing latex containing toxic metabolites such as sesquiterpene lactones. Later, the larva will feed beyond the cut sites where latex outflow is minimal. (Photo courtesy of Dr. David Dussourd (copyright).)

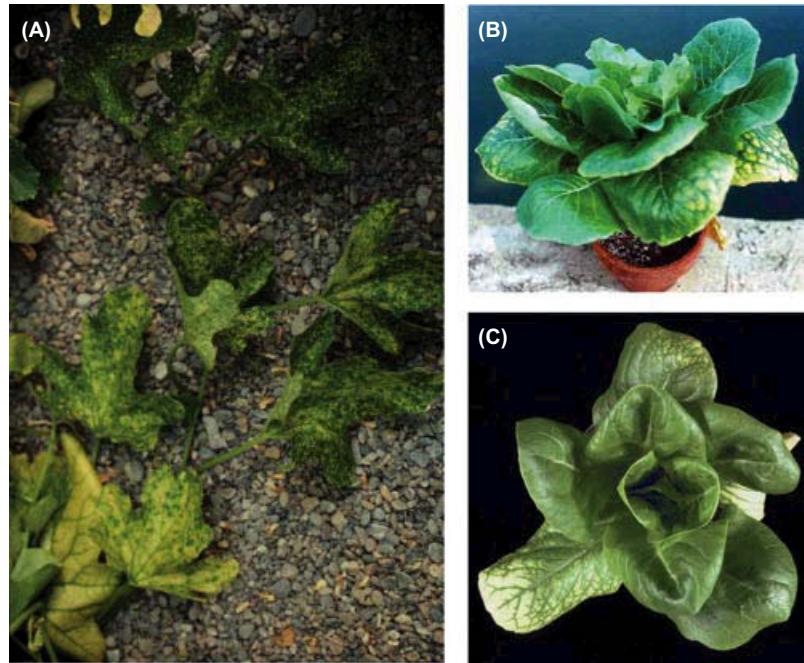


Plate 13.1 Symptoms of phloem-limited viruses. (A) A melon plant infected by *Cucurbit yellow stunting disorder virus* (CYSDV), a *Crinivirus*. (B) A lettuce “Summer Bibb” plant infected by *Beet western yellows virus* (BWYV). (C) A lettuce plant infected by *Lettuce infectious yellows virus* (LIVY). Note yellowing in brittle older leaves with bright green veins, while symptoms are lacking in young leaves.

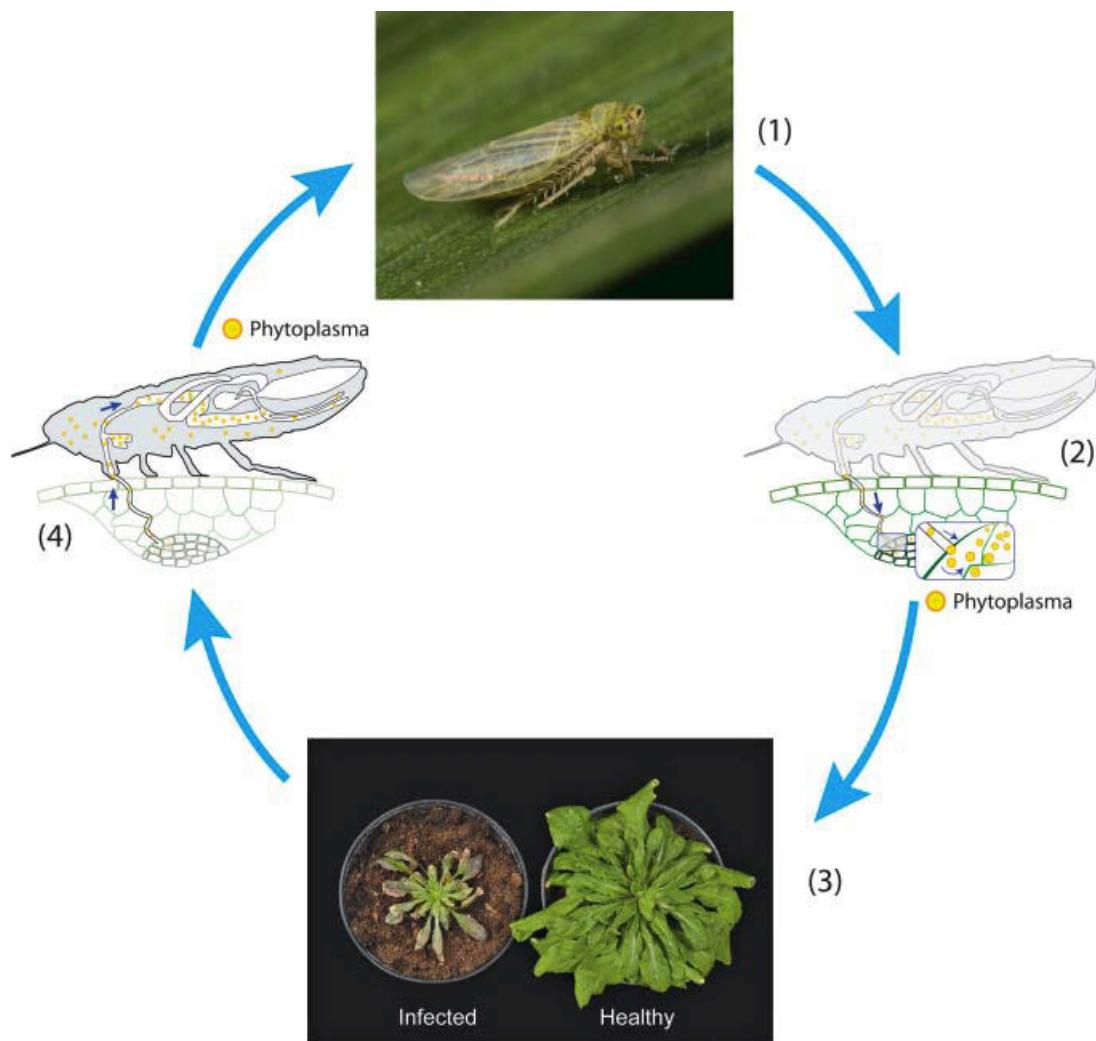


Plate 14.1 Phytoplasmas and spiroplasmas have a unique dual host life cycle: (1) Phytoplasmas and spiroplasmas are plant pathogenic bacteria that can be transmitted to plants by a phloem-feeding insect vector such as the aster leafhopper (*Macrostelus quadrilineatus*); (2) A phytoplasma-infected leafhopper injects the mollicutes into the phloem of a healthy plant, a process referred to as inoculation feeding; (3) The bacteria colonize the new host, inducing a variety of symptoms such as yellowing and stunting; and (4) An uninfected leafhopper acquires the plant pathogenic bacteria while feeding from the sieve tube of an infected plant, a process referred to as acquisition feeding. The bacteria are ingested into the midgut of the insect, and must cross the epithelial cell layer to access the hemolymph of the host. Following replication in the hemolymph, phytoplasmas and spiroplasmas migrate to the salivary glands of the insect, which subsequently becomes competent to transmit the infectious bacteria to a plant while feeding from the phloem.



Plate 14.2 Phytoplasmas induce symptoms consistent with alterations in plant development when infected with AY-WB. (A) *Arabidopsis* plant with the witches' broom phenotype. (B) Infected plants form green flowers as compared to the white flowers of a healthy plant. (C) The witches' broom phenotype produces an increased number of axillary stems in infected as compared with healthy plants.



Plate 14.3 Expression of a phytoplasma effector protein induces the formation of atypical flowers in *Arabidopsis*. (A and B) Flowers produced by transgenic *Arabidopsis* plants expressing the phytoplasma effector gene *SAP54* have leaf-like petals, enlarged sepals, and produce secondary flowers from the center of the flower. (C) Wild-type *Arabidopsis* flower.

Section A

Introduction

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2	Cell Biology of Sieve Element–Companion Cell Complexes	8
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1 Phloem, the Integrative Avenue

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By the end of the nineteenth century, plant biologists recognized the paramount importance of phloem transport for plant growth. They suspected that plant growth strongly relies on the phloem-mediated supply of photosynthates and other organic compounds. These initial studies culminated in 1930 with the pressure flow hypothesis proposed by Ernst Münch, which offered a solid theoretical and unifying platform to understand the fundamental mechanism of phloem translocation. For decades following the general acceptance of Münch's concept, phloem research predominantly focused on the movement and distribution of photoassimilates. Source supply and sink demand combined with the concepts of donor and receiver organs were seen as key factors in determining plant productivity and, hence, the agricultural yield.

The field of phloem physiology became well established as new tools were developed that allowed researchers to quantifiably measure translocation and to visualize the phloem tissue at high resolution. Many studies of photoassimilate movement throughout the plant were conducted using ¹⁴C-labeled carbohydrates. These approaches were widely used in the 1970s and early 1980s to learn about carbohydrate metabolism and sugar carrier activities in source and sink tissues. From the 1960s, transmission electron microscopy provided views unparalleled at the time into the ultrastructure of phloem cells. Great strides were made in detailing the variation and development of sieve element–companion cell complexes and other phloem cell types in different plant taxa. However, the challenges associated with cellular preservation were recognized as limiting factors in obtaining a reliable view of this dynamic tissue.

New tools associated with molecular biology and genomics combined with significant advances in real-time microscopy rejuvenated phloem physiology. Identifying and manipulating the genes encoding phloem-specific proteins were only initial steps leading to comprehensive cataloging of genes, proteins, and metabolic components of the phloem. Advances in cell biology, such as development of molecular makers combined with new fluorescent tagging technologies, micromanipulation, and confocal microscopy, have provided new levels of resolution that continue to contribute to our understanding of this tissue.

Abbreviations: ¹⁴C, carbon-14; Ca²⁺, calcium; CC, companion cell; Cl⁻, chloride; K⁺, potassium; miRNA, micro RNA; Na⁺, sodium; PD, plasmodesmata; PPUs, pore-plasmodesma units; R, resistance; rRNA, ribosomal RNA; SE, sieve element; SE-CC, sieve element-companion cell complex; siRNA, short-interfering RNA; smRNA, small RNA; tRNA, transfer RNA

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The discoveries enabled by molecular approaches are now being combined with the tools of physics and chemistry to address the complex physiological questions that have been posed by investigators for many decades. Understanding physical forces such as the thermodynamics of membrane transport and quantification of parameters such as the transporter density and their turnover rates relies on integrated approaches. Unraveling complex signaling and metabolic networks within the phloem during plant development, and as plants interact with the environment, will only be resolved by using all available tools and continually developing new analytical approaches.

This book is intended to showcase the advances in our understanding of phloem biochemistry, molecular biology, physiology, and interactions with other living organisms as we continue in the second decade of the twenty-first century. One notable feature of the book is the considerable and intended overlap that occurs among the chapters, which is further demonstration of the integrated nature of the topics and the work that is ongoing at this point in time.

The text is divided into four sections: the first section is an introductory overview composed of three chapters designed to provide a contextual framework for chapters in the subsequent sections. Chapter 2 (White) focuses on the intimate relationships that occur between sieve elements and companion cells. Sieve element–companion cell (SE–CC) complexes are the modular components of sieve tubes that are symplasmically connected to one another, yet relatively isolated from surrounding cells along most of the transport path. Plasmodesmata (PD) in the nascent sieve plates located between successive SEs transform into sieve pores with large functional diameters, establishing a contiguous, living conducting sieve-tube conduit. Simultaneously, SEs detach fully from the surrounding cells with exception of the companion cells, to which they become linked by pore–plasmodesma units (PPUs). CCs have a reduced connectivity to phloem parenchyma cells by a low number of optically conventional plasmodesmata. This plasmodesmal configuration is thought to create an exclusive trafficking system for a diversity of substances between SEs and CCs. In the relatively short stretches of collection and release phloem, plasmodesmal connectivity between CCs and phloem parenchyma cells strongly varies among species. In collection phloem, the symplasmic connectivity varies by a factor of 1000; in release phloem, there is abundant symplasmic connection between SEs and surrounding cells.

Chapter 3 (Patrick) reflects on the diverse structural frameworks in which SE–CCs are embedded and, in which, SE–CC specialization gives rise to different functions in the successive collection, transport, and release phloem sections. In sources (mostly green leaves), the collection phloem accumulates an arsenal of biochemical substances among which carbohydrates predominate. In main veins of stems and roots, balanced accumulation and release by SE–CCs in transport phloem facilitates development of cambial tissues, maintenance of mature cells in transport organs, and exchange of compounds with the surrounding cells. In terminal sinks (e.g., shoot and root apices, flowers, fruits, seeds), release phloem delivers materials to the target organs. This general structure and organization of the phloem is responsible for mass flow and concerted action among the organs.

The second section explores the structural and functional relationships of SE components. Revealing the diversity and nature of associated and integral membrane proteins along with mapping their location has tremendously contributed to the fundamental understanding of phloem physiology. Chapter 4 (Tegeder, Ruan, and Patrick) provides an overview of the progress in understanding membrane transporters. A wealth of carriers facilitates the transfer of sucrose, the primary carbohydrate transported in many plant species, in particular fast-growing herbaceous plants of the temperate zones. Moreover, membrane-bound translocators for hexoses, raffinose-related sugars and sugar alcohols are responsible for distribution of carbohydrates and are part of a plant-wide carbohydrate-controlled communication network. Because sucrose is the principal energy-carrying compound for long-distance transport, the control mechanisms behind sucrose processing could be

more elaborate than those for other compounds. Chapter 5 (Kühn) discusses the machinery behind the membrane transfer of sucrose and the complicated regulatory mechanisms, of which details are beginning to emerge, that appear to be responsible for fine-tuning of sucrose carrier activities.

Plasma membrane ion channels also play a pivotal role in phloem function. Chapter 6 (Hafke and van Bel) shows that a large variety of ion channels are involved in ion uptake and release as well as counterbalancing the electrical consequences of carbohydrate uptake and in propagating electrical signals. Electrical signaling in plants largely diverges from that in animals. In plants, the ions involved are K^+ , Cl^- , and Ca^{2+} rather than K^+ and Na^+ , and energy for ion exchange is provided by proton pumps rather than Na^+/K^+ pumps. In contrast to animals, moreover, where minor amounts of ions are exchanged along the path to influence targets at the end of the propagation pathway, electrical propagation in plants displaces large amounts of ions along the pathway. In particular, Ca^{2+} ions are presumed to trigger a variety of intracellular cascades.

The cellular basis of sieve pore occlusion and its effect on mass flow is discussed in Chapter 7 (Knoblauch and Mullendore). Mass flow calculations are still not entirely conclusive, in particular for low-concentration solutes. One reason for the inaccuracies could be the exchange of solutes between sieve tubes and adjacent cells. Their exchange rates determine the amount of each individual solute in the solvent flow. Partial occlusion of sieve pores in intact plants as well as the nonuniform diameters of sieve tubes and sieve pores could also invalidate mass flow calculations. Furthermore, inconsistencies in the calculations could be linked to lateral exchange between parallel sieve tubes, possibly via lateral sieve plates, transporting in opposite directions.

The third section of the book focuses on long-distance signaling via the phloem. Work in the past decade revealed the phloem as the key integrator of genetic, developmental, and physiological responses that are conveyed over long distances throughout the plant. Signaling molecules, including proteins and RNAs, transported in the sieve-tube sap appear to be distributed over long distances. Chapter 8 (Dinant and Lucas) presents a comprehensive overview of the soluble proteins identified in sieve tubes and their potential functions. A surprisingly large proteome composed of hundreds of proteins has been identified in sieve-tube exudates. Important classes of proteins appear to assist in PPU-trafficking of both proteins and RNAs and have roles in maintaining protein stability as well as degradation. Proteins impact a variety of putative signaling pathways and regulate the oxidative status of the phloem. Sieve-tube sap appears to be replete with proteins involved in responses to biotic and abiotic stresses. Classical structural phloem proteins are joined by structural components of the translational machinery that perform puzzling functions in the highly modified conducting elements that by all accounts seem to lack ribosomes.

The transformative discovery of RNA in sieve-tube exudates along with putative large protein–RNA complexes that could bind and convey RNA species over long distances emphasize the integrated nature of macromolecules in the phloem. Chapter 9 (Kehr and Buhtz) critically reviews recent developments in the rapidly expanding area of RNA biology within the solute stream. Several RNA species have been detected, each with a specific spectrum of tasks. Messenger RNAs (mRNA) in sieve-tube sap could intervene in metabolism and protein synthesis in distant cells. Nonprotein coding RNAs including ribosomal RNAs (rRNA) and transfer RNAs (tRNAs) also have been identified in sieve-tube exudates. Regulatory small RNAs (smRNA), including short-interfering RNAs (siRNA) and micro RNAs (miRNA) appear to be common and can have diverse roles in affecting plant development and responses to biotic and abiotic stresses.

The elaborate signaling system, composed of proteins and RNAs translocated from source organs via the phloem, impacts differentiation of growing zones to mediate developmental processes. Several case studies are presented in Chapter 10 (Hannapel) describing how phloem transport of macromolecules affects development in remote meristems. Flower induction has been a prominent

and long-standing example of this mechanism. The identity of the enigmatic floral activator, florigen, was discovered to be a protein expressed by *FLOWERING LOCUS T*. A second case study examines the evidence for a phloem–mobile ribonucleoprotein complex that mobilizes mRNAs, affecting the gibberellic acid signaling pathway. The final case study reveals that mRNA encoding the *BEL5* transcription factor is transported from leaves to the tips of stolons to activate the formation of potato tubers.

Chapter 11 (Gaupels and Vlot) provides an in-depth view into the challenging world of unraveling stress responses that are perceived locally, yet enhance systemic resistance in distant tissues by transmitting signaling compounds through the phloem. Topics such as the systemic wound response, systemic acquired resistance, and systemic acquired acclimation are coupled with an analysis of their associated systemic signals in response to biotic or abiotic stresses. Given the enormous diversity of candidates, the quest to identify more than a few stress signaling compounds continues to challenge researchers. Signaling can depend upon cell-specific information cascades operating in parallel or antagonistically that can be intertwined by reciprocal amplification and weakening along the phloem pathway.

The fourth and final section of the book demonstrates that sieve tubes not only provide avenues for integrative signaling but also offer rich resources and a transport system that is often exploited by other living organisms. The spectrum of organisms that successfully interact with plant vascular systems have evolved complex biochemical, structural, and in some cases behavioral mechanisms to exploit this nutrient-rich resource while coping, often unsuccessfully, with plant defense responses. Chapter 12 (Hagel, Onoyovwi, Yeung, and Facchini) sheds light on the secondary metabolism of phloem, which is a largely unexplored yet intriguing field in plant biology. In many plant species, sieve tubes contain repellents or toxic substances to combat animal predators. The cooperation between various cell types in phloem and intercellular trafficking among associated tissues is often required to synthesize these chemical deterrents. Specialized phloem structures such as latex-exuding laticifers and resin ducts provide physicochemical barriers as a significant line of defense against herbivores.

Many of the interactions that occur among phloem cells and associated tissues were initially revealed by studying phloem-mediated virus movement during systemic infections. Chapter 13 (Stewart, Ding, and Falk) focuses on the interrelations of viruses and viroids with the phloem in higher plants. Plant viruses utilize sieve tubes for systemic movement. Viruses that replicate in parenchyma cells encode specialized viral movement proteins that modify plasmodesmata to facilitate their intercellular movement into sieve tubes. In contrast, phloem-limited viruses are injected directly into sieve tubes or companion cells by phloem-feeding insects and multiply exclusively in phloem cells. Why these viruses remain confined to the phloem is not understood but indicates that PPUs and PDs between SE–CC and phloem parenchyma are of a different molecular nature. Specialization of these cellular connections is further demonstrated by viroids that also interact with the phloem but are able to pass this barrier during systemic infections.

Phytoplasms and spiroplasms are two fascinating groups of microbes that were recently discovered in sieve tubes. Chapter 14 (MacLean and Hogenhout) is one of the first reviews on the relationships between these fascinating bacterial organisms and the phloem. These unusual prokaryotes are inserted by phloem-feeding insect vectors directly into sieve tubes where they are transported into sink tissues to establish systemic infections. Several key metabolic pathways are lacking in these organisms and as a consequence, they rely heavily on the assimilate stream in the phloem to provide adequate nutrition. While effector proteins secreted directly into sieve tubes by these minuscule bacteria have significant effects in altering plant development and morphology, host plants appear to have defense mechanisms that can limit the development of the disease.

Phloem-feeding insects are a spectacular example of structural, biochemical, and physiological adaptation to parasitize the vascular tissues of plants. Most of these insect taxa utilize their highly modified mouthparts, called stylets, to penetrate through the weak pectin lamellae inside cell walls, puncturing and ultimately feeding from the sieve tubes. Chapter 15 (Will, Carolan, and Wilkinson) discusses the integral role of aphid saliva as the molecular interface between the insect and plant. Two types of saliva are involved aphid probing: gel saliva forms a flexible, lubricating, protective tube around the stylet tip during cell wall penetration, whereas aqueous saliva is secreted after cell puncture. Both saliva types are likely responsible for molecular interactions with host plants. Components of the aqueous saliva are only now becoming fully characterized; some molecules aid in establishing an effective feeding environment, while others could serve as a likely source of molecular effectors that trigger plant resistance.

The coevolution of plants and phloem-feeding insects has resulted in sophisticated biochemical and genetic mechanisms that govern their interactions. Genetic mechanisms that confer resistance to phloem-feeding insects are reviewed in Chapter 16 (Walling and Thompson). Insects inject virulence factors contained within their saliva that overcome the plant's innate immune response to establish a compatible interaction. Plant resistance (*R*) proteins are able to perceive and counteract the virulence factors allowing the perception of the insect and activating defenses that confer resistance to the phloem-feeding insect. Significant advances have been made in understanding *R* gene-mediated resistance against phloem-feeding insects and the deployment of signaling cascades to induce defense molecules.

In conclusion, phloem research has made a quantum leap forward since the publication of the classic phloem textbooks. While some of the questions in phloem physiology have been solved, new challenges continually emerge. Novel developments in research show that the phloem provides a plant-wide communication system that unites the capabilities of nervous, hormonal, and blood systems in animals.

2 Cell Biology of Sieve Element–Companion Cell Complexes

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The distinct structure and central role of the phloem in long-distance transport have intrigued scientists ever since Malpighi (1686) observed continued growth in tissue immediately above a stem girdle and suggested that food transported downward from shoot to roots accumulated above the girdle and stimulated growth there (cited in Esau, 1969). Hartig (1837) was the first to define sieve tubes (Siebröhren) as the transporting cells in phloem (reviewed in Esau, 1939, 1969), and a century later, electron microscopy revealed their intricate ultrastructural details (Esau, 1969; Evert, 1990; Figure 2.1). Subsequent studies also revealed the diversity of phloem types, their interconnections, and their integration with surrounding tissues in a number of species (Esau, 1969; Gamalei, 1989; 1991; Kempers et al., 1998; Haritatos et al., 2000). Phloem ultrastructural and functional analysis slowed until recently (Thompson, 2006; Mullendore et al., 2010; Barratt et al., 2011), when advances in light and fluorescence microscopy combined with molecular approaches to study phloem function led to a renaissance in phloem cell biology (Martens et al., 2006; Thompson and Wolniak, 2008; Truernit et al., 2008; Fitzgibbon et al., 2010; Barratt et al., 2011; Xie et al., 2011). The study of phloem dynamics has been particularly challenging because this deeply buried tissue is predisposed to shut down transport with any perturbation (Knoblauch and van Bel, 1998; Imlau et al., 1999; Knoblauch et al., 2001; van Bel et al., 2002; Lalonde et al., 2003; Stadler et al., 2005; Knoblauch and Peters, 2010). This chapter reviews the fundamentals of phloem development and structure with particular focus on their intercellular connections.

Abbreviations: ATP, adenosine triphosphate; BS, bundle sheath; C, callose; CALS7, CALLOSE SYNTHASE 7; Ch, chloroplast; CC, companion cell; CLSM, confocal laser scanning microscopy/microscope; CW, cell wall; D, dictyosome; DT, desmotubule; DW, desmotubule wall; ER, endoplasmic reticulum; GFP, green fluorescent protein; GSL7, GLUCAN SYNTHASE-LIKE 7; IPM, plasma membrane inner leaflet; kDa, kiloDaltons; *knotted1*, mutant/nonfunctional knotted1; M, mitochondrion (pl. mitochondria); ML, middle lamella; MP, movement protein; mRNA, messenger RNA; PD, plasmodesma (pl. plasmodesmata); PL, plastid; PM, plasma membrane; PP, phloem parenchyma; PPC, phloem parenchyma cell; PPU, pore–plasmodesma unit; RNA, ribonucleic acid; RPP13-1, rice phloem protein 13-1; SE, sieve element; SEL, size exclusion limit; SER, sieve element reticulum; SP, sieve plate; SUC2, sucrose/proton transporter 2; SUT1, sucrose/proton transporter 1; TEM, transmission electron microscopy/microscope; UDP, uridine diphosphate; V, vacuole; VP, vascular parenchyma; XP, xylem parenchyma

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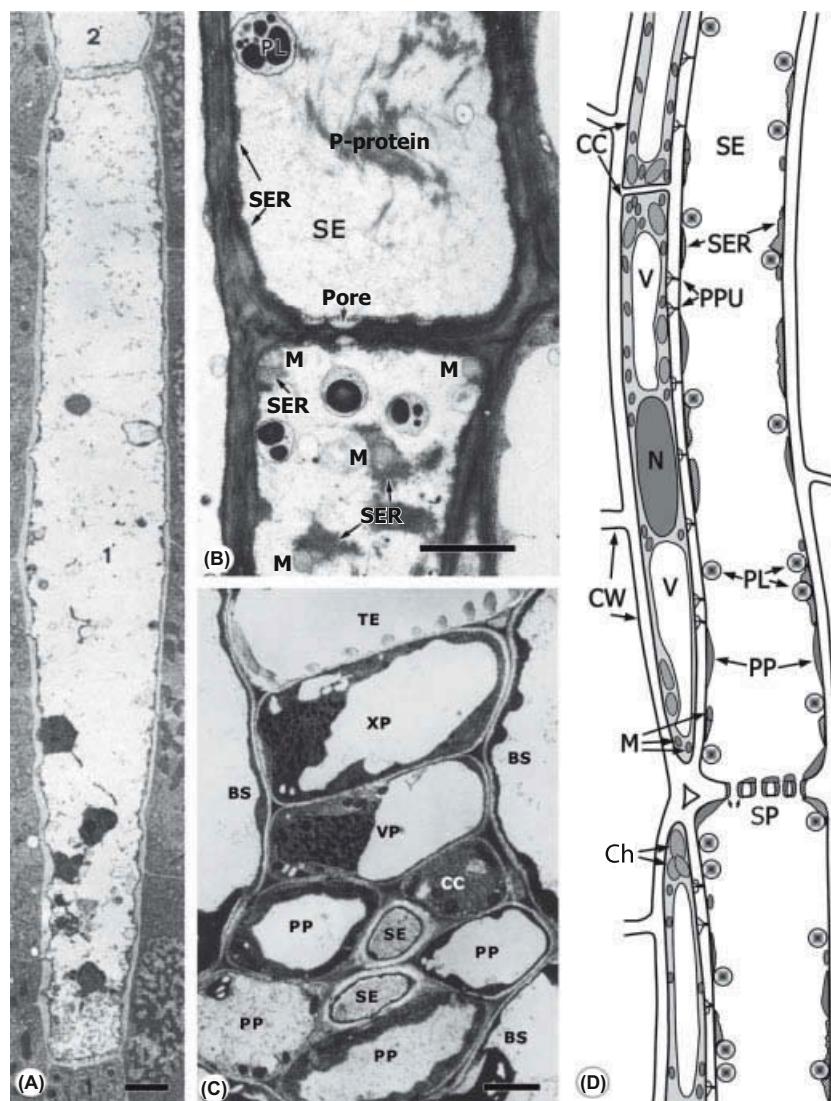


Figure 2.1 Ultrastructure of developing phloem sieve elements (SEs) and their organelles as seen in transmission electron microscopy (TEM). (A) Longitudinal section through an immature protophloem SE approximately 0.5 mm from the root tip of goatgrass (*Aegilops comosa* var. *thessalica*). Most of the cell contents have been degraded, with the exception of dark remnants of the nucleus near the base of the cell. Bar = 2 μm (Eleftheriou and Tsekos, 1982). (B) Longitudinal section through maturing SEs in a young stem of shieldleaf (*Streptanthus tortuosus*) showing remaining plastids (PL), sieve element reticulum (SER), and phloem protein (P-protein) inside the SE. A maturing sieve pore and remnant mitochondria (M) are visible in the lower SE. Bar = 1 μm (Sjölund, 1997). (C) Cross section of a minor vein from non-importing leaf tissue of tobacco (*Nicotiana tabacum*) showing the arrangement of SE and companion cell (CC) surrounded by phloem parenchyma (PP) and vascular parenchyma (VP). Above the phloem is a tracheary element (TE) of the xylem and adjacent xylem parenchyma (XP) cell, all enclosed in bundle sheath (BS) cells. Bar = 2 μm (Ding et al., 1988). (D) Diagram of a longitudinal section of phloem CC and SE showing their relationship and typical components, including SER, pore-plasmodesma units (PPUs), PL, parietal phloem protein (P-protein), a few M, and sieve plate (SP) lined with callose (small arrows) in the SE. The CCs are shown with vacuoles (V), a nucleus (N), chloroplasts (Ch) and many more M in their cytoplasm. (Adapted from Knoblauch and van Bel, 1998.)

Development of the Sieve Element–Companion Cell Complex

One of the most intriguing aspects of the phloem is the connection between the living sieve tubes formed from interconnected sieve elements (SEs) and the sieve-tube control system provided by the intimately associated companion cells (CCs). In angiosperms, these two tightly linked but very different cell types derive from an unequal division of a fusiform mother cell (Esau, 1969; Behnke and Sjölund, 1990). One daughter cell develops into one or several metabolically active CCs possessing a dense cytoplasm and numerous mitochondria with well-developed cristae (Esau, 1969; van der Schoot and van Bel, 1989; van Bel, 1993; Sjölund, 1997; van Bel and Knoblauch, 2000; Evert, 2006; Figure 2.1). The other daughter cell goes through a controlled loss of most cellular components but survives as a living cell, sometimes for many years, in a dependent relationship with the CC (Esau, 1969; Wergin et al., 1975; Behnke, 1981; Evert, 1990; Raven, 1991; van Bel, 2003a, 2003b).

During SE maturation, the SE nucleus swells before gradually disintegrating, while the vacuolar membrane together with cytoskeletal elements, ribosomes, and Golgi bodies becomes reduced in number and eventually disappears (Esau, 1969; Behnke and Sjölund, 1990). At maturity, a highly modified cell remains that is composed of the plasma membrane (PM) and a thin layer of parietal cytoplasm with a few often dilated mitochondria (Evert, 1990), phloem-specific plastids (Behnke, 1991a), and, in all species except the grasses, phloem proteins (P-proteins; Cronshaw and Sabnis, 1990; Evert, 1990; Behnke, 1991b; Iqbal, 1995; van Bel 2003a). Depending on the species, the SE plastids contain protein filaments or crystals or amylopectin-rich starch grains (Behnke, 1991a). In angiosperms, the only structures within the lumen of a SE are the phloem-specific proteins arranged as filaments, tubules, or crystalline bodies. The major component of the remaining cytoplasm is the sieve element reticulum (SER); an elaborate peripheral meshwork of stacked or fenestrated endoplasmic reticulum (ER) that is free of ribosomes (Thorsch and Esau, 1981a, 1981b; Sjölund and Shih, 1983).

Symplastic Connections to and within the Phloem

Early in development, the SE and CC have similar plasmodesmal contacts at the various interfaces with adjoining meristematic cells (Esau, 1939, 1969). During differentiation, all plasmodesmata (PDs) shut off in the developing SE–CC complexes (SE–CCs; van Bel and van Rijen, 1994; Ehlers and van Bel, 2010), which appears to be common in differentiating meristematic cells (Ehlers et al., 1999; Ruan et al., 2001, 2004; Kim 2007) as a prerequisite for autonomous development without interference by neighboring cells (Pfluger and Zambryski 2001; Kim et al., 2005). At maturity, the SE–CCs are linked by highly specialized PD connections to surrounding tissues, recognizable by their different ultrastructural modifications, detailed in Section “Connections between SE–CCs and Surrounding Cells”.

Origins and Development of Plasmodesmata

Plasmodesmata, the symplastic connections between plant cells, are inserted into the new cell wall that forms between daughter nuclei during the final stage of cell division. Nascent PDs are initiated when strands of ER become trapped among the coalescing vesicles of the forming cell plate (Hepler, 1982; Overall et al., 1982). As the new cell wall thickens and solidifies, each captured ER strand becomes compressed into a tight tube known as the desmotubule that links the ER networks of the two adjacent cells (Figures 2.2A and B). Each desmotubule is surrounded by a thin layer of cytoplasm,

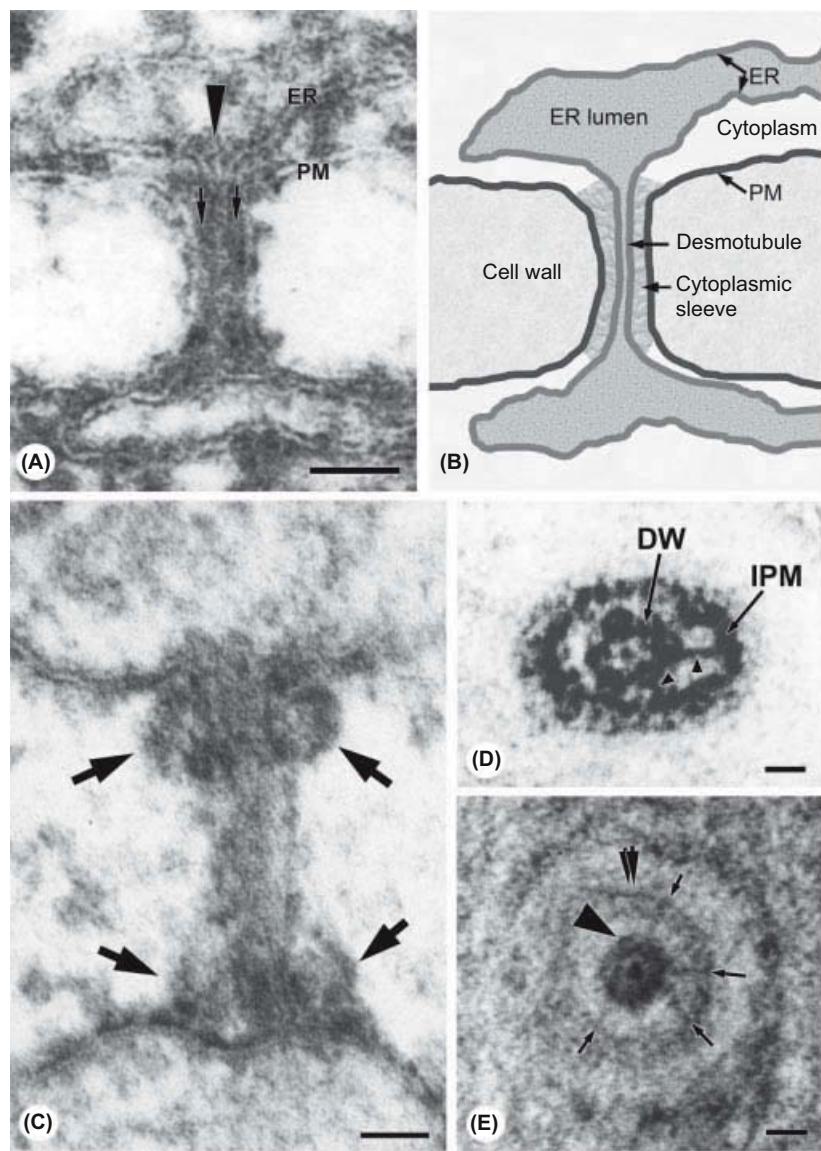


Figure 2.2 Ultrastructure of plant plasmodesmata (PDs) revealed by transmission electron microscopy (TEM) analysis of intact tissues. (A) Longitudinal section of a single mosquito fern (*Azolla pinnata*) PD, showing cytoplasmic endoplasmic reticulum (ER) narrowing into the desmotubule (DT; large arrowhead), which links the ER of two adjacent cells. The plasma membrane (PM) and cytoplasmic sleeve (small arrows) are also continuous from cell to cell. Bar = 50 nm (Overall et al., 1982). (B) Diagram highlighting PD components, showing how the ER narrows into the DT within the cytoplasmic sleeve of the PD, all enclosed in the PM. (Adapted from White and Barton, 2011.) (C) Longitudinal section showing a young onion (*Allium cepa*) root PD with electron-dense material (arrows) in the cell wall around the neck region. Bar = 50 nm (Radford et al., 1998). (D) Transverse section of tobacco leaf PD, showing particles possibly attached to or embedded in both desmotubule wall (DW) and PM (IPM, inner plasma membrane) and connected by strands (arrowheads) across the cytoplasmic sleeve. Bar = 10 nm (Ding et al., 1992). (E) Transverse section of a PD in Brazilian waterweed (*Egeria densa*) in which particles (e.g., large arrowhead) are mainly associated with the DT, and strands (arrows) appear to link the DT and PM (double arrow). The dark central core of the DT is prominent. Bar = 10 nm (Overall and Blackman, 1996). (Continued)

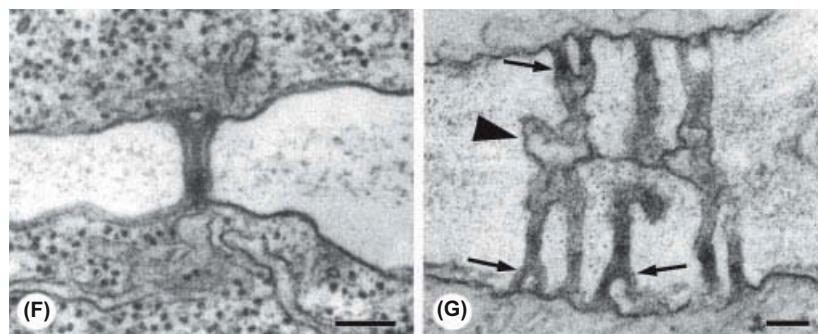


Figure 2.2 (Continued) (F) A branched PD from black nightshade (*Solanum nigrum*) showing branching in the younger layers of the plant cell wall. Bar = 100 nm (Ehlers and Kollmann, 1996). (G) A complex branched PD from broad bean (*Vicia faba*) pith cells with several plasmodesmal strands, some showing recent branching (arrows), which merge in a median branching plane, which is dilated to form a central cavity (large arrowhead). Bar = 100 nm (Ehlers and Kollmann, 2001).

and the desmotubule and its cytoplasmic sleeve are enclosed in PM (Hepler, 1982; Overall et al., 1982; reviewed in Maule, 2008), which is derived from the phragmoplast vesicle membranes. In this way, the ER, cytoplasm, and PM are continuous from cell to cell. In young PDs, the membranes form straight concentric cylinders, and either at this stage or later in development extracellular proteinaceous structures may be detected at each end of the PD, termed the “neck,” where the PD cytoplasmic sleeve joins the cytoplasm of the connected cells (Robards, 1976; Mollenhauer and Morré, 1987; Olesen and Robards, 1990; Tilney et al., 1991; Badelt et al., 1994; Waigmann et al., 1997; Overall, 1999; Heinlein and Epel, 2004; Figure 2.2C). As PDs mature, they usually gain an extracellular collar of callose, a β -1,3-linked glucan polymer. Older PDs often become branched to varying degrees and may develop a median cavity within the interconnecting cell wall, in which the desmotubule and cytoplasmic sleeve are enlarged (Lucas et al., 1993; Oparka et al., 1999; Roberts et al., 2001; Figures 2.2F and G).

These ultrastructural changes usually coincide with changes in the size and type of molecules that will readily traffic from cell to cell (Oparka et al., 1999; Overall, 1999). Young PDs may allow passage of proteins up to 54 kDa (kilo-Daltons) in size (Kim et al., 2005), and the youngest phloem elements in *Arabidopsis* roots certainly allow efflux of green fluorescent protein (GFP) (27 kDa) into surrounding tissues (Imlau et al., 1999; Stadler et al., 2005). As they mature and become more complex in structure, PDs generally close to allow only fairly small molecules, approximately 0.6–1.0 kDa in size, free passage from cell to cell (reviewed in Lucas et al., 2009). Some signaling molecules, such as transcription factors (e.g., KNOTTED1) (Jackson et al., 1994) or SHORTROOT, Nakajima et al., 2001), move across one or more cell boundaries in more mature tissue, and certain cell boundaries allow one-way traffic of proteins as large as GFP (Christensen et al., 2009). The largest molecule that can move through PDs defines their size exclusion limit (SEL), and although molecular weight does not correlate exactly with molecular size, this is usually how PD SEL is characterized.

Ultrastructural analyses show that either or both of the PM and ER of PDs may be lined with electron-dense structures, interpreted to be macromolecular components (Overall et al., 1982; Ding et al., 1992; Overall, 1999; Oparka, 2004; Roberts, 2005; Benitez-Alfonso et al., 2010; Faulkner and Maule, 2011; Radford and White, 2011; White and Barton, 2011; Figures 2.2D and E). Although none of these structures have been unequivocally resolved, they are likely to comprise one or more

cytoskeletal proteins, adenosine triphosphatases (ATPases) and ribonucleic acid (RNA) unwinding proteins, plus a range of PD-specific proteins that are slowly being identified (reviewed in Faulkner and Maule, 2011). As yet unidentified PD proteins are proposed to actively regulate PD permeability and may be involved in loading and unloading cargo at each end of the PD. The PDs of phloem CCs and SEs undergo specific structural modifications and changes in their SEL that are likely to involve insertion and/or deletion of regulatory proteins, depending on the cell types they are connecting.

Connections between SE-CCs and Surrounding Cells

Symplastic connections between the SE-CCs and surrounding phloem parenchyma cells (PPCs) are generally absent or very restricted (Fisher, 1986; van Bel and Kempers, 1991; Wimmers and Turgeon, 1991; Botha, 1992; Botha and van Bel, 1992; van Bel and van Rijen, 1994; Botha et al., 2000; van Bel, 2003a). Isolation of the SE-CC in transport phloem presumably limits symplastic leakage of solutes into surrounding tissue, which is reflected in changes in PD structure, distribution, and SEL (Kempers et al., 1998; Ehlers and van Bel, 2010). PDs are very rare at the SE-PPC interface and tend to be single and unbranched, while PDs at the CC-PPC interface are sparse and occasionally branch at the CC side (Botha and van Bel, 1992; Kempers et al., 1998).

Even those PDs present appear less permeable than other, ultrastructurally similar PDs connecting, for example, mesophyll tissues (Kempers et al., 1998). Certain proteins normally found exclusively within the phloem, such as P-proteins (Balachandran et al., 1997; Clark et al., 1997) and thioredoxin h (Ishiwatari et al., 1995, 1998), can increase the SEL of PDs between tobacco (*Nicotiana tabacum*) mesophyll cells and themselves move from cell to cell. However, they appear unable to either increase the SEL or move through PDs connecting CCs to surrounding cells, except to their associated SEs. This suggests that the PDs around the SE-CC may not be modified readily by endogenous movement factors, preventing protein escape from the SE-CC, and this may also prevent, or at least restrict, invasion of the phloem by viruses in the mesophyll (Leisner and Turgeon, 1993; Oparka and Turgeon, 1999).

Sieve Pores—Connecting Sieve Elements into Sieve Tubes

SEs in primary phloem are usually in vertical files with mostly transverse connecting walls, whereas SEs in later-formed secondary phloem are often more fusiform with oblique abutting walls that provide a larger contact area (Esau, 1939, 1969). PDs in the transverse walls connecting two SEs enlarge to form pores of up to 1 μm in diameter, and this porous wall is termed a sieve plate (Figures 2.3A, B, C, and D; see Chapter 7). The SE PM is continuous through the sieve pores with a net-like ER residing at the pore margins (Esau, 1969; Evert, 1990). Pore width must accommodate the rapid mass transit of photosynthates through SE files as well as numerous large and small proteins, ribonucleoprotein (RNP) complexes, RNAs, and other compounds that have been identified in sieve-tube sap (see Chapters 8 and 9). The wide pores in sieve plates facilitate rapid transport, but their very interconnectivity presents a potential point of vulnerability to the vascular system. Elaborate protective mechanisms have evolved to occlude the sieve pores to prevent the loss of energy expensive metabolites when sieve tubes are damaged (see Chapter 7).

One such mechanism is callose deposition, which plays a central part in sealing of damaged SEs (for details and information on the role of P-proteins, see Chapter 7). Callose is especially prominent at the sieve plates of wounded sieve tubes where it forms callose collars around the

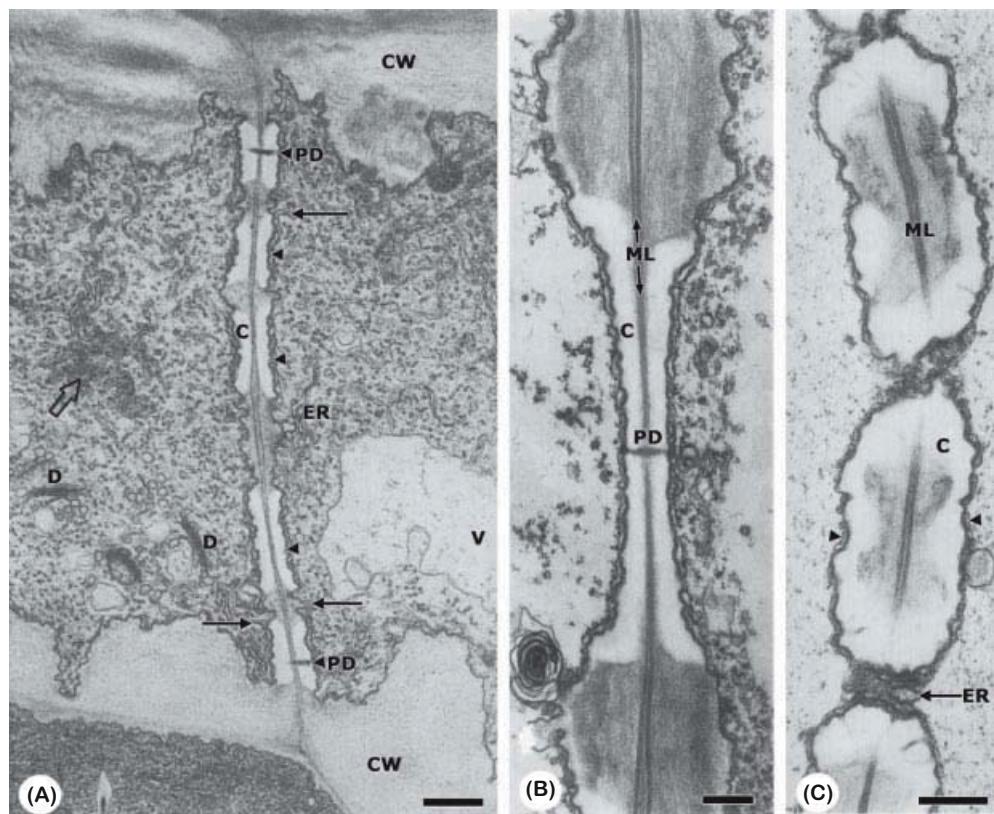


Figure 2.3 Developing and mature sieve plates in wild-type and mutant phloem, showing the importance of callose (C) in sieve pore development, and a mature pore-plasmodesma unit (PPU), seen in transmission electron microscopy (TEM) (A, B, C, D, G, and J) and confocal laser scanning microscopy (CLSM). (A) Longitudinal section of portions of two immature sieve elements (SEs) of pumpkin (*Cucurbita maxima*) with a differentiating sieve plate between them. Future sieve pores contain abundant C in bright sieve-plate regions and are traversed by plasmodesmata (PDs). The endoplasmatic reticulum (ER) is associated with ribosomes (ER), and also localized along the plasma membrane (PM; arrowheads) and leads away from the PM at or near noncallosic sites (long arrows). Dictyosomes (D) are seen in cross section and ER cisternae (open arrow) in glancing section in these still active cells, in which vacuoles (V) are beginning to develop. CW = longitudinal wall of the SE. Bar = 1 μm (Esau and Cronshaw, 1968). (B) A section through a young pumpkin sieve plate shows a single sieve-plate pore site with C deposits and a central plasmodesma (PD), which is somewhat enlarged in the middle lamella (ML) region. Bar = 0.5 μm (Esau and Cronshaw, 1968). (C) Sieve pores in a mature sieve plate of pumpkin have a C lining, with ER close to the PM (arrowheads) and continuity of ER through one of the pores (arrow). ML = middle lamella. Bar = 0.5 μm (Esau and Cronshaw, 1968). (*Continued*)

pores, which become plugged with P-proteins (Esau and Cheadle, 1961; Evert and Derr, 1964; Eschrich, 1975; Furch et al., 2007; Mullendore et al., 2010). Early experiments showed that phloem transport is reduced by callose-inducing treatments (Scott et al., 1967; McNairn and Currier, 1968), and SE damage can block phloem transport within seconds (Currier, 1957; Eschrich, 1965). More recent evidence shows that callose synthesis is sufficiently fast for full occlusion of the sieve plates almost instantly (Nakashima et al., 2003) or within minutes (Furch et al., 2010; Mullendore et al., 2010). Furthermore, phloem transport can resume within less than 1 hour with rapid degradation of wound-induced callose (Currier and Webster, 1964; Meier et al., 1981; Iglesias and Meins, 2000; Bucher et al., 2001; Nakashima et al., 2003; Furch et al., 2007).

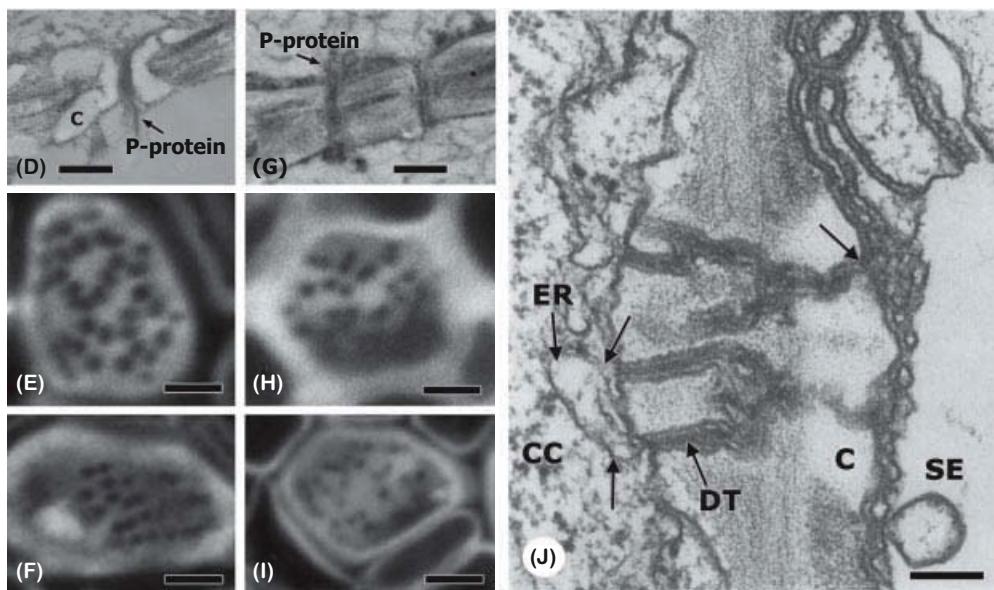


Figure 2.3 (Continued) (D) Single pore in a sieve plate of *Arabidopsis* phloem, with C lining and P-protein trapped within. Bar = 0.5 μm (Barratt et al., 2011). (E), (F) In two representative sieve plates from *Arabidopsis*, the pores can be seen by observing cellulose fluorescence using a CLSM. Bars = 2 μm (Barratt et al., 2011). (G) Two pores in a sieve plate from *Arabidopsis* lacking a phloem-specific callose synthase (GSL7) showing absence of C but containing P-protein. Bar = 0.5 μm (Barratt et al., 2011). (H), (I) Two representative sieve plates from the *gsl7* mutant of *Arabidopsis* showing smaller sieve pore diameters, detected by cellulose fluorescence on a CLSM. Bars = 2 μm (Barratt et al., 2011). (J) Highly modified PDs form the PPUs between a companion cell (CC) and mature SE in pumpkin. Arrows indicate association of ER membranes with the desmotubule (DT) within each branch of the PPU. C is deposited around the much less-branched SE side of the PPU strands. Bar = 0.2 μm (Esau and Cronshaw, 1968).

The synthesis and degradation of callose also plays a prominent role in sieve pore development, but its precise function and the details of its appearance in SE maturation are not well understood. Callose in young SEs is deposited on the nascent sieve plates (Esau, 1969; Evert, 1990) and accumulates along the PM in the wall around the PDs (Figures 2.3A, B, and C). Some researchers speculate that callose deposition replaces cellulose around the developing pore to facilitate pore maturation through subsequent callose degradation (Esau and Thorsch, 1985; Thorsch and Esau, 1988). An alternate view is that pore formation requires degradation of the original cellulosic cell wall together with some of the nascent callose. In this case, callose deposition could prevent further deposition of the cellulosic wall around the pore (Evert et al., 1966; Deshpande, 1974, 1975).

Recent analyses of a mutant *Arabidopsis* line lacking the phloem-specific CALLOSE SYNTHASE 7 (CALS7; Xie et al., 2011) (or GLUCAN SYNTHASE-LIKE 7, GSL7; Barratt et al., 2011) have convincingly demonstrated that callose synthesis is essential for correct pore formation. The transport channels in *cals7* mutant and wild-type sieve plates appear similar in dimension, but the mutants have cellulosic material around the channels rather than the usual callose lining (Barratt et al., 2011; Xie et al., 2011; Figures 2.3D and G). Phloem transport in the *cals7* mutants is reduced and plants are dwarfed (Barratt et al., 2011; Xie et al., 2011), and fluorescence staining for cellulose showed fewer and narrower sieve pores in the *cals7* mutant (Figures 2.3E, F, H, and I). This suggests that the cellulose-lined, callose-deficient pores in the *cals7* mutants cannot be modified sufficiently to accommodate normal phloem flow rates. Moreover, the *Arabidopsis cals7* mutants lacking sieve

pore callose also show very reduced callose production in response to phloem wounding (Barratt et al., 2011; Xie et al., 2011). *Arabidopsis* mutants lacking the phloem-specific sucrose synthases SUS5 and SUS6, which are tightly coexpressed with CALS7 (Barratt et al., 2009), also have reduced callose linings around sieve pores, although double mutants show no phenotypic effects (Bieniawska et al., 2007; Barratt et al., 2009).

Mature sieve pores usually have a callose lining (Bouck and Cronshaw, 1965; Deshpande, 1974, 1975; Thorsch and Esau, 1988; Eleftheriou, 1990; Sjölund, 1997; Ehlers et al., 2000; van Bel et al., 2002; Figures 2.3C and D), and the *cals7* mutant phenotype suggests that as in other PDs (Iglesias and Meins, 2000; Bucher et al., 2001; Nakashima et al., 2003) the thickness of this callose lining could regulate sieve pore permeability. It is clear that the size of sieve plate pores and the effects on phloem transport of pore narrowing by callose have a major impact on plant growth and development, although callose regulation *in vivo* is still not well understood.

Modified Plasmodesmata between Sieve Elements and Companion Cells: The Pore–Plasmodesma Units

Molecules enter and exit sieve tubes through the highly specialized PDs that join SEs and CCs. During differentiation and maturation of the SE–CC, the simple PD connections between CCs and SEs become modified to form pore–plasmodesma units (PPUs) (van Bel and Kempers, 1997; van Bel, 2003a; Figure 2.3J). The PDs become elaborated on the CC side with up to 100 branches linking to a central lacuna (Esau, 1969; Evert, 1990), while on the SE side, the PDs widen to form a single or, at most, a few simple pores (Esau, 1969; Kempers et al., 1998). Tubules of ER appear to traverse the individual branches of PPUs (Ding et al., 1993), but details of the central cavity have not been resolved (van Bel and Kempers, 1997; van Bel et al., 2011a).

It is assumed that the PPU branches are similar to other PDs in their basic structure, but how the initially symmetric PDs develop into the highly asymmetric PPUs is unknown. The CC-side elaboration may be similar to the PD “twinning” seen at the base of trichomes as PDs are added to enlarging cell walls (Faulkner et al., 2008) and in cambial tissues (Ehlers and van Bel, 2010) or to the increased branching and central cavity formation seen during elaboration of other types of secondary PDs (Oparka et al., 1999; Ehlers and Kollmann, 2001; Burch-Smith et al., 2011).

The wider pore on the SE side might arise from a process similar to the final pore widening in the sieve plate, with a thicker callose sleeve in the wall and slightly enlarged central strand of ER. It would be interesting to determine whether the PPUs are widened as usual on the SE side in the *cals7* mutants; if phloem loading is symplastic, the narrower PPU pores may further restrict phloem transport capacity. In any event, the highly asymmetric ultrastructure of PPUs presumably reflects a compositional and functional asymmetry in molecular exchange between CCs and SEs, whose implications are considered in detail in Section “Transport between Sieve Element and Companion Cell.”

Other Specialized Features of Sieve Elements

The Sieve Element Reticulum

The SER is a specialized system of smooth ER in mature SEs (Hébant, 1977; Oparka and Johnson, 1978; Thorsch and Esau, 1981a, 1981b; Behnke, 1983; Evert, 1984; Figures 2.4A, B, and C). Confocal laser scanning microscope images (Knoblauch and van Bel, 1998; Thompson and Wolniak,

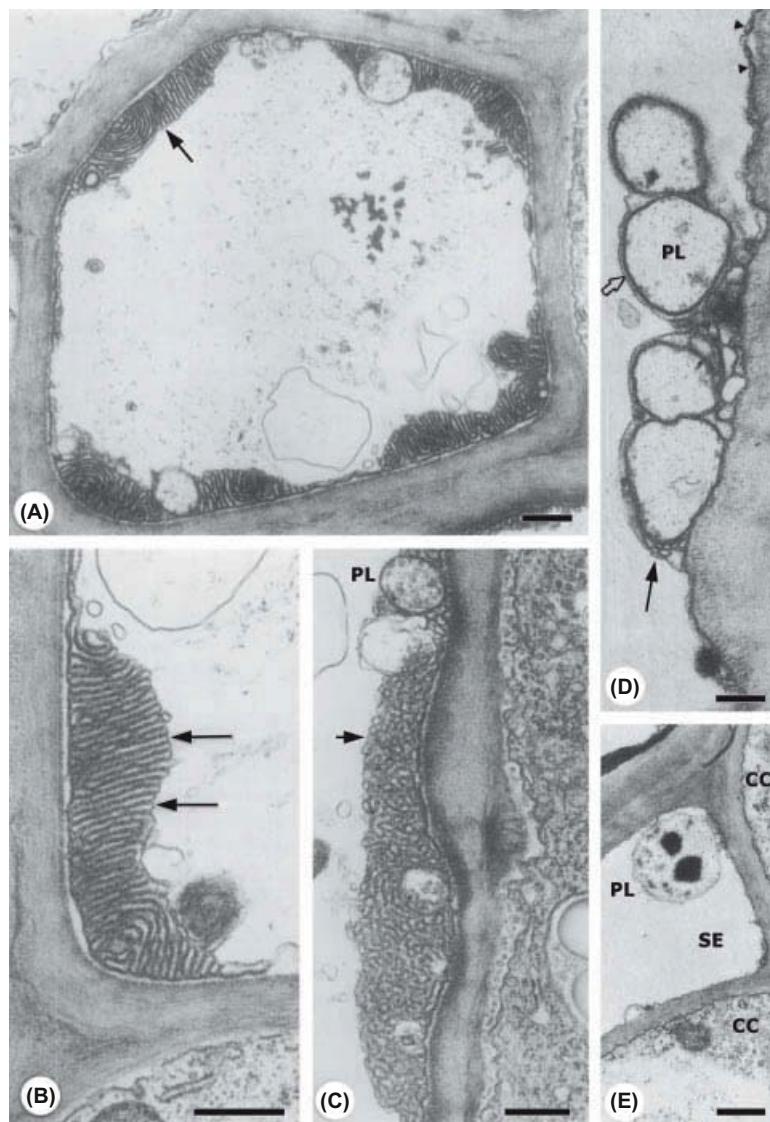


Figure 2.4 Sieve element reticulum (SER) and plastids (PL) in mature phloem, examined by TEM. (A) A mature sieve element (SE) of cotton (*Gossypium hirsutum*) in cross section showing stacked SER oriented perpendicular to the cell wall (arrow). The SER covers much of the PM. Bar = 0.5 μm (Thorsch and Esau, 1981a). (B) Higher magnification of a portion of the SE shown in (A). Arrows indicate individual cisternae of the SER. Bar = 0.5 μm (Thorsch and Esau, 1981a). (C) Convoluted SER (arrow) in a longitudinal section of a mature SE of cotton. The innermost cell wall layer has the dense fibrillar appearance characteristic of nearly mature and mature SE. Bar = 0.5 μm (Thorsch and Esau, 1981a). (D) PL next to the plasma membrane of a mature SE of pumpkin (*Cucurbita maxima*). They may be enclosed by a membrane (open arrow) and have adjacent SER (long arrow). Bar = 0.5 μm (Esau and Cronshaw, 1968). (E) A PL with crystalline inclusions in a mature SE of goatgrass (*Aegilops comosa* var. *thessalica*). CC = companion cell. Bar = 0.5 μm (Eleftheriou and Tsekos, 1982).

2008; Fitzgibbon et al., 2010) and electron micrographs (Esau, 1969; Evert, 1990) show a strictly parietal position of the SE components. In translocating SEs, the SER appears to be held in place by 7-nm-long macromolecular extensions that also anchor mitochondria and plastids (Figures 2.4D and E) to each other and to the PM (Ehlers et al., 2000).

Within mature SEs, the SER forms a layer of cisternae that covers almost the entire PM with deep stacks of ER (Esau and Gill, 1971; Thorsch and Esau, 1981a; Sjölund and Shih, 1983) or circular openings (fenestrations) of approximately 80 nm in diameter (Esau and Cronshaw, 1968; Sjölund and Shih, 1983). The space between the PM and the SER may allow retention of molecules that would otherwise be carried along in mass flow. Most of the stacked SER is closely associated with SE plastids and mitochondria, some of which are completely enclosed by the ER (Thorsch and Esau, 1981b; Ehlers et al., 2000). Unusual SER is seen in waterlily (*Nymphaea* species), which have ER-derived tubules with diameters of 100–200 nm in mature SEs (Behnke 1996). Other unusual ER-derived structures in mature SEs include short curved tubules in weeping fig (*Ficus benjamina*) (Behnke, 1989) and short straight tubules of 35–40 nm diameter in various palms (Parthasarathy, 1974a, 1974b). The ER is a remarkably dynamic and adaptable membrane system, with different conformations achieved by rather subtle modifications in membrane protein or lipid composition and surface charge (Borgese et al., 2006; Shibata et al., 2006), and similar modifications may underlie SER conformation.

Although some investigators have considered the SER to be inactive (Wooding 1967), its retention in mature SEs and elaborate structure speak to some essential function in sieve tubes. In some species, the SER has acid phosphatase activity, suggesting a role in the final autolysis of the SE cytoplasm (Zee, 1969; Esau and Charvat, 1975; Bentwood and Cronshaw, 1976; Oparka et al., 1981). In other species, nucleoside phosphatase activity (Bentwood and Cronshaw, 1978; Cronshaw, 1980; Sjölund and Shih, 1983; Evert, 1984; Arsanto, 1986) has led some investigators to suggest a role in phloem loading. Other suggested functions include calcium storage (Sjölund, 1990) and regulation of SE Ca^{2+} concentration, since very high concentrations of calcium can be maintained near the SE PM compared with calcium levels in the SE lumen (Furch et al., 2009; van Bel et al., 2011b). Calcium release may play an important role in wound signaling, and is known to induce, and be essential for, synthesis of wound-induced callose in SE (King and Zeevart, 1974), and around PD in general (Botha et al., 2000; Iglesias and Meins, 2000; Roberts and Oparka, 2003) (further details in Chapters 6 and 7).

The SER organization as a highly layered or fenestrated membrane system appressed to the PM suggests retention of sufficient surface area for physiological activities, similar to layered Golgi membranes or stacks of thylakoid membranes in chloroplast grana. Clearly, the physiological functions of the SER system remain to be fully elucidated.

Cytoskeletal Proteins in Mature Sieve Elements

Differentiating SEs do contain filamentous structures resembling actin-like filaments or filament bundles (Cronshaw and Esau, 1967; Evert, 1990; Parthasarathy and Pesacreta, 1980; Pesacreta and Parthasarathy, 1984; Figures 2.1B and D), and at one time, it was suggested that these filaments may actively participate in long-distance transport (Thaine, 1969; MacRobbie, 1971), but this was subsequently shown not to be the case (Williamson, 1972). In the widely accepted mass-flow theory of phloem transport, outlined in Chapters 1 and 7, there is no direct role for the cytoskeleton, although recent evidence points to some role in regulating transport. Both actin and myosin were detected at the sieve pores in phloem of hybrid aspen (*Populus tremula × Populus tremuloides*),

horse chestnut (*Aesculus hippocastanum*) and stone pine (*Pinus pinea*), and suggested to participate in gating of the pores (Chaffey and Barlow, 2002). Application of actin or microtubule disrupters to the isolated root vascular cylinder (Guo et al., 1998) or to isolated phloem strands (Yang et al., 2007) reduced the flux of radiolabeled sucrose into pea roots (Guo et al., 1998) or radish roots (Yang et al., 2007). It is unclear whether the SEs themselves or the function of the CCs was affected, but these results suggest that an intact cytoskeleton is essential for phloem function.

Immediately after division, both young CCs and SEs contain a full complement of cytoskeletal proteins, and even at maturity, sieve-tube exudate from several species (wheat, *Triticum aestivum*; rice, *Oryza sativa*; Adam's needle, *Yucca filamentosa*; castor bean, *Ricinus communis*; pumpkin, *Cucurbita maxima*; black locust, *Robinia pseudoacacia*; large-leaved linden, *Tilia platyphyllos*) contains readily detectable actin and actin-binding proteins (Schobert et al., 1998; Kulikova and Puryaseva, 2002; Lin et al., 2009). In some cases, filamentous components of exudate have been shown to bind to myosin (Kulikova and Puryaseva, 2002), suggesting the possibility of active actin–myosin motility, but in other cases, this was not observed (Williamson, 1972).

The actin-sequestering monomer, profilin, has also been identified in sieve-tube exudates from the same species listed previously (Schobert et al., 1998; 2000). Profilin could regulate actin dynamics in sieve tubes and perhaps attenuate wound-induced actin polymerization (La Claire, 1989; Foissner et al., 1996; Foissner and Wasteneys, 1997). The absence of readily identified actin microfilaments in transmission electron microscopy (TEM) or fluorescence micrographs of mature SEs could be a result of unconventional assemblage similar to what has been observed in the nuclear cytoskeleton, which is now known to include actin, profilin, and myosin (Cruz and Moreno Díaz de la Espina, 2009). There have been few immunological studies of phloem to identify cytoskeletal elements, so this remains an avenue for future analysis.

Microtubules are generally absent from mature SEs (Toth et al., 1994), but can be associated with the SER stacks in a highly ordered fashion even at later stages of SE maturation (Esau and Hoefert, 1980; Thorsch and Esau, 1981b) and have been observed in mature SEs of horse chestnut (Chaffey et al., 2000). Transcriptomic analyses of isolated celery (*Apium graveolens*) phloem strands (Vilaine et al., 2003), phloem exudates from castor bean (Doering-Saad et al., 2006) and pumpkin (Ruiz-Medrano et al., 2011), and phloem plugs from several ash (*Fraxinus*) species (Bai et al., 2011) have also identified transcripts encoding microtubule-associated proteins, α - and β -tubulins, myosin, and the actin-binding protein actin depolymerizing factor. Further work is required to confirm the presence of intact cytoskeletal elements within SEs and to determine their function, particularly any role in cell-to-cell transport.

Transport between Sieve Element and Companion Cell

The entry of larger molecules into SEs occurs mainly via the PPUs from the CCs. This exchange, and the very active membrane-based loading and unloading of solutes, discussed in Chapters 3, 4, and 5, requires energy to drive the membrane transporters as well as to provide sustained proton export for energizing of carrier-mediated uptake and retrieval of metabolites. Blocking sugar catabolism in CCs inhibits sugar uptake or retrieval by the SE–CC, greatly impairing phloem loading and causing considerable assimilate loss along the translocation pathway (Lerchl et al., 1995; Geigenberger et al., 1996). Also essential is a constant supply of substrates for callose synthesis and turnover. Sucrose synthase specifically located to CCs is required to provide sufficient uridine diphosphate (UDP)-glucose for callose biosynthesis (Martin et al., 1993; Nolte and Koch, 1993). The mode of energy supply is less clear. Since the interface between SEs and CCs may be only

25% of the SE surface in transport phloem, and sugars may escape from areas not covered by CCs, ATP produced by CCs may supply the SEs to support membrane ATPases (Lehmann, 1979; van Bel, 2003a).

Long-term maintenance of the differentiated SE after it has lost the capacity for protein synthesis requires that essential macromolecules, especially proteins, be continually supplied from the CC. Turnover of phloem-specific proteins was demonstrated in CCs using ^{35}S -methionine labeling (Fisher et al., 1992; Sakuth et al., 1993), and many P-proteins are produced in the CCs that accumulate in SEs (Bostwick et al., 1992; Dannenhoffer et al., 1997; Thompson, 1999), indicating that the PPUs allow passage of larger molecules than are generally trafficked through mature PD in other tissues. More recently, most of the components essential for both protein assembly and breakdown have been detected in sieve-tube exudates, suggesting that the SEs are not simply passive carriers of photosynthates and signals, but play an active role in protein turnover to regulate their protein complement (Lin et al., 2009).

Of further interest is the asymmetry of protein components that could regulate transport between the CC and SE. For example, the presence of cytoskeletal proteins such as actin and myosin in PDs (reviewed in White and Barton, 2011) raises interesting questions. If the PPUs contain cytoskeletal elements within the tubules on the CC side, does the actin terminate in the central cavity or does it continue through to the SE? If cytoskeletal elements are present throughout the PPU, does the cytoskeleton then play a role in regulating transport between CC and SE?

Certain SE- and CC-specific proteins do show asymmetries in distribution through the PPU that could reflect function. The leaf sucrose transporter sucrose/proton transporter 1 (SUT1) is a high-turnover protein that is essential for phloem loading and long-distance assimilate transport and is found primarily in the SE PM of tobacco (*N. tabacum*), potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*). In contrast, SUT1 messenger RNA (mRNA) synthesized in CCs is detected in both SEs and CCs and is most abundant at the orifices of the PPUs between the two cell types (Kühn et al., 1997; Kühn, 2003; see also Chapters 5 and 8).

Cytosolic Transport through PPUs

Most discussions of trafficking through PPUs focus on macromolecules with the assumption that small solutes are exchanged by diffusion through the cytoplasmic channels and then transported indiscriminately in the translocation stream. However, an analysis of several endogenous and exotic solutes synthesized in CCs of minor veins showed that while small metabolites enter SEs by diffusion, their selective loss via carrier-mediated membrane transport together with nonselective diffusion out of the SE could either retain a compound for long-distance transport or rapidly clear it from the translocation stream (Ayre et al., 2003; van Bel et al., 2011a).

As well as photosynthates, many of the proteins synthesized in the CCs appear able to move into SEs by passive diffusion even though some are larger than 100 kDa, and they can be specifically targeted to the SE (Bostwick et al., 1992; Fisher et al., 1992; Sakuth et al., 1993; Clark et al., 1997; Dannenhoffer et al., 1997; Kempers and van Bel, 1997; Imlau et al., 1999; Oparka et al., 1999; Fisher and Cash-Clark, 2000; Lin et al., 2009; see also Chapters 8 and 9). Indeed, considering the size of macromolecules exchanged from CC to SE, PPUs were predicted to have a large diameter (Fisher et al., 1992). In living phloem, fluorescently tagged macromolecules in the order of 20–30 kDa were seen to transfer across the PPUs (Balachandran et al., 1997; Kempers and van Bel, 1997), and the 27 kDa GFP, expressed in CCs of tobacco and *Arabidopsis*, could move into the SEs and migrate along the sieve tubes (Imlau et al., 1999). Furthermore, when expressed under the CC-specific

sucrose/proton transporter 2 (SUC2) promoter, GFP fusion proteins as large as 67 kDa can move into the SEs via the PPUs and then translocate within the phloem (Stadler et al., 2005).

Selective unloading at specific cell boundaries suggests tighter control of P-protein exit in sink tissues (Balachandran et al., 1997; Ishiwatari et al., 1998; Lee et al., 2003; Aoki et al., 2005). Regulation of the PPUs is likely since phloem-specific proteins from pumpkin and castor bean microinjected into mesophyll tissue enabled mature PDs to transport macromolecules of up to 30 kDa, where their usual limit is approximately 1 kDa (Balachandran et al., 1997). In addition, a thioredoxin h protein, rice phloem protein 13-1 (RPP13-1), from rice (*Oryza sativa*) sieve-tube exudate enabled PDs between tobacco mesophyll cells to transport molecules of 9–20 kDa (Ishiwatari et al., 1998). Thus, despite their superficial similarity to the many-branched secondary PDs in other tissues, which generally have reduced transport capacity (Oparka et al., 1999), specific gating effectors in the phloem enable much larger macromolecules to be loaded and unloaded through the PPUs.

There is some evidence for an endogenous facilitated system in which certain proteins are recognized to allow trafficking across specific symplastic boundaries. For example, heat-shock proteins detected in sieve-tube sap could unfold proteins for trafficking from CC to SE (Schobert et al., 1995), and peptides that interact with PD proteins and block cell–cell transfer of normally motile transcription factors could interfere with PPU dilation (Kragler et al., 2000). Together, these observations suggest that a protein complex, composed of the transferred protein, a chaperone, and a docking protein, might bind to receptors at the PPU then transfer through the PPU (Lucas et al., 2001; Oparka, 2004).

It is unclear whether protein trafficking through PPUs between CCs and SEs is directional. For example, the defense-related protein serpin (43 kDa) does not move from SEs into CCs (la Cour Peterson et al., 2005), while the P-proteins PP1 and PP2 move bidirectionally between CCs and SEs within the transport phloem (Golecki et al., 1999; la Cour Petersen et al., 2005). This suggests that PPUs can regulate protein exchange to retain certain proteins in the CC. Indeed, there is growing evidence for one-way transport through other types of PDs in different tissues, and that their transport capacity and major direction of transport changes during PD development (Kim et al., 2005; Christensen et al., 2009).

The presence of abundant RNA, especially mRNAs, in sieve-tube sap was initially a surprise and a puzzle, since the SEs contain no nuclei as an immediate source of RNA, but the physiological roles for translocated mRNAs as long-distance signaling molecules are now starting to be revealed (see Chapters 9 and 10; Lucas et al., 2001; van Bel et al., 2002; Kehr and Buhtz, 2008; Turgeon and Wolf, 2009; Kragler, 2010). Although phloem sap contains no detectable RNase activity (Sasaki et al., 1998; Doering-Saad et al., 2006), the current perception, based on virus movement studies and the identification of RNA-binding proteins in phloem sap (Gómez et al., 2005; Ham et al., 2009), is that mRNA molecules traffic long distances in SEs as RNP complexes and not as naked molecules. The chaperoning mechanism to load them into the SEs and unload them in their target sinks may be similar to that regulating protein trafficking via PPUs (Ruiz-Medrano et al., 1999; Ham et al., 2009). A number of P-proteins have been identified with RNA binding and transport properties, and several of these RNA-binding proteins have been shown to open PD for RNA movement (see Chapters 8 and 9; Balachandran et al., 1997; Dannenhoffer et al., 1997; Golecki et al., 1999; Dinant et al., 2003; Gómez et al., 2005).

Virus movement can also provide insights into the regulation of protein trafficking through PDs, including the modified PDs of the PPUs. For example, the 3a movement protein (MP) of cucumber mosaic virus fused to GFP can traffic out of the SE–CC in tissue where there is no movement of solute through the same PDs (Itaya et al., 2002), and virus infection also enables the movement

of GFP from the mesophyll into SEs (Peleg et al., 2007). The MP of potato leaf roll virus, a phloem-limited virus, directs its genome through PPUs (Prüfer et al., 1997; Sokolova et al., 1997). Phosphorylation of the acidic terminus of this protein by a membrane-bound kinase located near PPUs could change the conformation of the viral RNA prior to loading into the PPU (Sokolova et al., 1997). In this way, the virus MPs might be functional analogues of plasmodesmal proteins needed to allow cell-to-cell protein transfer where the PPU, ER, and SER play roles in protein sorting.

Membrane-Associated Transport through PPUs

In addition to the cytosolic pathway through PPUs, the ER membrane or lumen could serve as an alternative transport pathway in trafficking molecules through PPUs. ER membrane lipids move readily between soybean (*Glycine max*) suspension cells (Grabski et al., 1993) and between tobacco epidermal cells with the exception of symplastically isolated guard cells and certain trichomes (Martens et al., 2006). The rapid exchange of ER membrane lipids through PPUs has been shown in experiments using fluorescence recovery after photobleaching demonstrating that the ER membrane extending through PPUs is a dynamic transport system that could facilitate trafficking of molecules between the CC and SE (Martens et al., 2006).

The ER lumen may provide another pathway for symplastic transport. The transfer of fluorescent dextrans or peptides of up to 10 kDa through the lumen of the ER was shown for PDs in trichomes of both wishbone flower (*Torenia fournieri*; Cantrill et al., 1999) and tobacco (Barton et al., 2011). Furthermore, a transiently expressed 10.4 kDa luminal reporter protein moved from cell-to-cell in epidermal cells of Virginia spiderwort (*Tradescantia virginiana*; Barton et al., 2011), but 27 kDa GFP did not, providing a model for similar modes of trafficking between CCs and SEs. However, GFP containing an ER-retention signal that was targeted to the ER of CCs or to the SER in transgenic plants did not move between the CCs and SEs (Stadler et al., 2005; Martens et al., 2006). The failure to transfer GFP was interpreted to indicate that movement through the ER lumen is restricted to molecules of less than 27 kDa, similar to PDs in wishbone flower and tobacco trichomes.

The ER surface in SEs has also been proposed to function as a rail system for the trafficking of proteins released from CCs into SEs, in which phloem-specific proteins could bind to and enable transport of other proteins by docking to receptors in the ER tubules traversing the PPU (Sjölund, 1997; Oparka and Turgeon, 1999; Oparka and Santa Cruz, 2000). This system may also be a means for protein sorting (van Bel and Knoblauch, 2000; van Bel et al., 2011a). Although plant proteins have not yet been identified that utilize this mechanism in PDs, many viral MPs are integral ER membrane proteins that function in opening PD for viral protein and RNA trafficking (Wu et al., 2011). It has been speculated that specific PPU chaperones facilitate transport of ER-targeted proteins or RNA between CCs and SEs for retention in the SER rather than for rapid transit via mass flow (Oparka and Turgeon, 1999; van Bel and Knoblauch, 2000).

Future Directions

Many aspects of the intimate connections between SEs and CCs are only superficially understood with regard to the physiology and biochemistry of the phloem. Much of the information available is incomplete and is derived from different developmental stages, organs, and species. There is a tendency to generalize about the function of SE–CCs without considering specific adaptations that occur in many cases. A major hindrance is our rudimentary understanding of PD structure and

function. Concepts such as directional transport, involvement of the contiguous PM and ER, or that PDs in the different walls of a single cell can have different composition and function have only recently been considered.

Key gaps in our knowledge require a more sophisticated understanding of the basic cell biology of SEs. The roles of the SE plastids, mitochondria, and the stacked ER require elucidation, and the presence, nature, and role of the cytoskeleton remains obscure. The subtleties underlying regulation of macromolecular trafficking via the PPUs are just beginning to be realized. The protein and lipid composition of PPUs is virtually unknown, as is the significance of the alternative transport routes through the PPU cytosol, ER membrane, and ER lumen. A detailed knowledge of the molecules that are exchanged between CC and SE is essential to clarify long-distance signaling. The exciting new evidence for protein assembly and turnover in SEs requires further exploration, since it suggests more independence from CCs and new roles for phloem in plant growth and development.

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3 Fundamentals of Phloem Transport Physiology

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Sieve element–companion cell complexes (SE–CCs), organized into repeating units (sieve tubes), are structurally and functionally designed to support large fluxes of resources (nutrients and water) transported over long distances throughout the plant body to meet metabolic demands of remote heterotrophic sink organs. Solvent flow through sieve tubes also systemically distributes signals and defense response molecules to integrate overall plant development, homeostasis, and responses to pathogens/mechanical injury.

Phloem systems of vascular plants comprise three sequential sectors, each of which serves a specific transport function (van Bel, 1996 and see Figure 3.1). Broadly, photosynthetic products (photoassimilates primarily composed of sugars) generated as products of photosynthesis by mesophyll cells, and nutrients arriving in transpiration streams from roots, are loaded into *collection phloem* (phloem loading) located within minor veins of fully expanded leaves (sources—net nutrient exporters). Loaded nutrients are translocated by *transport phloem* through the main veins of source leaves, petioles and ultimately enter stem and root axes to be distributed between sinks (net nutrient importers). Delivered nutrients are unloaded (phloem unloading) into expanding or nutrient storage cells of sink organs from *release phloem*.

This chapter briefly reviews the physiological processes considered to underpin flow to, through, and from, the phloem. We start with an analysis of chemical species translocated. This information is then built into an overall appraisal of the principles governing flows from sources to sinks before dissecting the key physiological processes regulating these flows within each phloem sector. Finally, phloem translocation is explored in a whole plant context.

Abbreviations: A, cross-sectional area; Akt, *Arabidopsis* potassium transporter; C, concentration; C, photosynthetically reduced; CC, companion cell; cm, centimeter; D, Dalton; η , solution viscosity; g, gram; GSH, glutathione; h, hour; IC, intermediary cell; J_v , volume flux; kDa, kiloDalton; l, length; L, liter; L, hydraulic conductivity; MPa, megaPascal; m, meter; μM , micrometer; mM, millimolar; N, nitrogen; π , osmotic pressure; π_i , cell osmotic pressure; π_o , apoplastic osmotic pressure; ΔP , hydrostatic pressure difference; P_i , cell hydrostatic pressure; P_o , apoplastic pressure potential; P_{source} , hydrostatic pressure at source; P_{sink} , hydrostatic pressure at sink; r, radius; RFO, raffinose family oligosaccharide; R_f , bulk flow rate; s, second; S, sulfur; SE, sieve element; SE–CC, sieve element–companion cell complex; *sdx1*, sucrose export defective mutant; V, velocity

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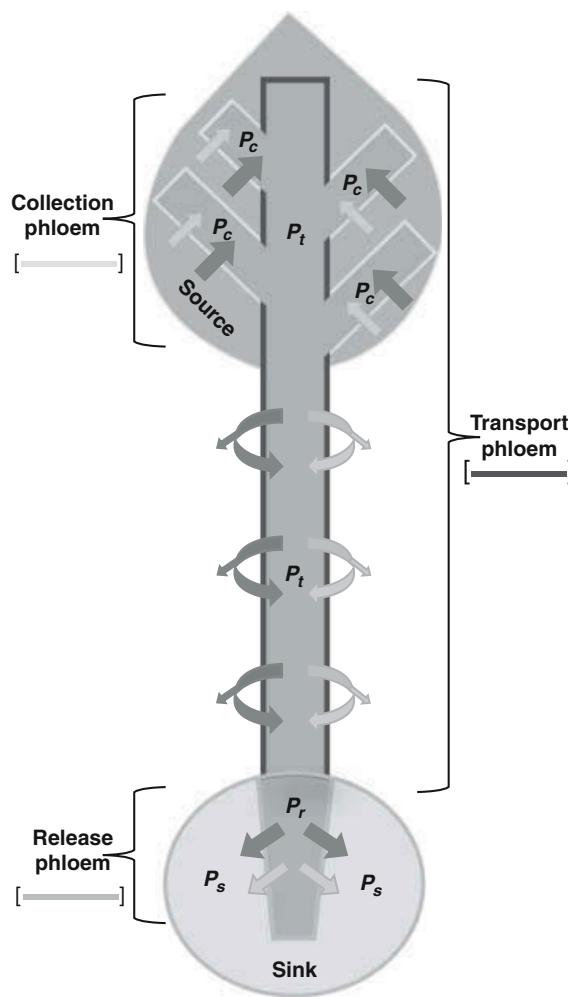


Figure 3.1 Münch pressure flow through three functional phloem zones: collection, transport, and release phloem. Uptake and release of nutrients (dark-grey arrows) into/from sieve elements are accompanied by concurrent fluxes of water (light-grey arrows). In collection phloem, major osmotic species are loaded into sieve tubes to high concentrations. As a result, their osmotic pressures (π_c) offset transpiration-induced tensions in the leaf apoplasm (P_o) to drive water uptake and hence generate hydrostatic pressure heads (P_c) in collection phloem (see Equation 3.3). Transport phloem retains a high hydrostatic pressure (P_t) by retrieval of leaked osmotica accompanied by water influx. At sinks, exit of phloem sap from sieve elements occurs by bulk flow through plasmodesmata and, as a consequence, hydrostatic pressure at the sink-end of the bulk-flow pathway (P_s) is located in the post sieve-element-element unloading pathway. Since sinks are hydraulically isolated from the remaining plant body, P_s is predominantly determined by $\pi_s - \pi_o$ with P_o exerting little influence (see Equation 3.3). The grey gradient represents the presumptive large hydrostatic pressure drop from release phloem (P_r) to sink cells (P_s). Resource loading/reloading and unloading set the magnitude of $P_c/P_t - P_s$ to drive volume flux ($\text{m}^3 \text{ m}^{-2} \text{ s}^{-1}$) through sieve tubes modulated by pathway geometry (largely dominated by plasmodesmal radii of post sieve-element unloading pathways—see Equation 3.2). Phloem sap concentration (C) of a nutrient species is determined by loading/reloading and, together with volume flux, is a major regulator of bulk flow rates (R_f —see Equation 3.1). (For a color version of the figure, please see Plate 3.1.)

What's In?—Constituents and Their Concentrations in Sieve-Tube Sap

Obtaining representative samples of sieve-tube sap free of contamination from surrounding cell types presents considerable technical challenges. Aphid stylectomy offers the least compromised sap samples (for a review, see Turgeon and Wolf, 2009). Our survey is restricted to chemical species comprising translocated resources, noting that defense and signaling molecules in phloem saps are discussed in Chapters 9–13 inclusive.

Water is the principal constituent of sieve-tube sap at around 80–90% on a weight-for-weight basis (Milburn and Baker, 1989). Profiles of nutrient species found in sieve-tube and xylem-vessel saps are illustrated by chemical analyses of exudates collected from stems of castor bean (*Ricinus communis*; see Table 3.1). Residual dry mass of phloem sap largely comprises transported nutrients generating osmotic potentials ranging from −1.4 to −2.6 MPa. Sap osmotic potentials in xylem vessels are an order of more positive magnitude (Table 3.1).

As found for many other species (see Ziegler, 1975), the most abundant nutrient species present in sieve-tube exudates are a soluble sugar species and potassium, which, respectively, account for 72% and 11% of phloem sap osmolality (Table 3.1). Depending upon plant species, sugar composition of sieve-tube saps falls into three main groupings. Many plant species translocate sucrose as their principal sugar (Table 3.1). Two smaller groupings of plant species translocate raffinose family oligosaccharide (RFO) members (predominantly raffinose, stachyose, and verbascose—e.g., *Cucurbitaceae*) or polyols (mannitol, sorbitol, dulcitol—e.g., *Rosacea*) in addition to smaller quantities of sucrose and RFOs (Ziegler, 1975). More recently, van Bel and Hess (2008) found that hexoses formed the major translocated carbohydrates in representative members of *Lythraceae*, *Papaveraceae*, *Ranunculaceae*, and *Valerianaceae*. Interestingly, absence of hexoses in sieve-tube saps could result from these sugars not being moved symplasmically from mesophyll cells to be available for apoplastic loading into SE-CCs. For instance, if made available to leaf apoplasms by

Table 3.1 Concentrations (mM) of chemical constituents detected in phloem and xylem exudates collected from hypocotyls or stems of *Ricinus communis* seedlings or plants, respectively

Chemical	Phloem exudate	Xylem exudate
Sucrose	433	5 ^a
Amino-N	68	5 ^a
Potassium	67	5 ^a
Sodium	7	0.7 ^a
Magnesium	3.7	1.1 ^a
Calcium (total)	1.2	1.8 ^a
Orthophosphate	6.6	0.7 ^a
Chloride	12	1.3 ^a
Nitrate	0.6	7.2 ^a
Sulfate	1.3	0.6 ^a
Copper	20×10^{-3} ^b	ND
Iron	50×10^{-3} ^b	ND
Manganese	10×10^{-3} ^b	ND
Zinc	50×10^{-3} ^b	ND

^aPeuke, 2010.

^bSchmidke and Stephan, 1995.

ND, no data available.

irrigating leaves with a hexose solution, SE-CCs are capable loading and transporting these sugars (see Kallarackal and Komor, 1989).

Sieve-tube sap concentrations of potassium are two to six times lower than those of the principal translocated sugars (see Table 3.1). Other nutrients accumulated by SE-CCs and accounting for approximately 15% of sap osmolality, include amino-N and -S compounds (amino acids, amides and peptides), and inorganic cations and anions (Table 3.1).

For a number of plant species amino-N composition of sieve-tube saps approximate osmotic concentrations generated by potassium (see Table 3.1). Although all protein amino acids can be found in sieve tubes, glutamate, glutamine, aspartate, and asparagine are often present at the highest concentrations. These vary with plant species. For example, most abundant organic N compounds in temperate legumes are asparagine and homoserine (Tan et al., 2010). Small peptides also contribute to long-distance N transport. For instance, glutathione (γ -Glu-Cys-Gly, GSH) has been detected in sieve-tube saps at much higher concentrations than methionine and cysteine. In rice, concentrations can be as high as 5 mM suggesting that GSH is an important form for transporting organic S (Kuzuhara et al., 2000). In addition, and dependent on plant species, S-methylmethionine and sulfate are main S forms in sieve-tube saps (Kuzuhara et al., 2000; Tan et al., 2010).

Excluding potassium, other inorganic cations are sodium, magnesium, and calcium. These cations are present in sieve tube saps at concentrations an order of magnitude or more lower than those of potassium. However all, except calcium (total of bound and free, with the latter in the low nM range—Furch et al., 2009), exhibit higher concentration values than their respective counterparts in xylem sap (see Table 3.1).

Inorganic anions are dominated by orthophosphate and chloride at concentrations commensurate with magnesium and sodium. In contrast both nitrate and sulfate are present at much lower concentrations (Table 3.1). A cohort of essential microelements, in the μ M concentration range, also is found in sieve-tube saps. Included amongst these elements are copper, iron, manganese, zinc, and boron (Table 3.1; Takano et al., 2008).

Propulsion of a Nutrient Enriched Sap through Sieve Tubes—Pressure Flow and Its Physiological Implications

Any proposed mechanism of phloem translocation mechanism must account for driving a nutrient enriched sap (Table 3.1) at extraordinary high fluxes of around $555 \text{ g biomass m}^{-2}$ sieve tube (ST) cross-sectional area s^{-1} (Canny, 1975). A flux sustained over exceptionally long distances (100 m) in tall trees. Diffusion theory irrefutably demonstrates that diffusion is unable to account for nutrient fluxes of such magnitudes over distances exceeding several millimeters (Fisher, 2000). This leaves bulk flow as the principal mechanism propelling resource translocation through transport phloem. A conclusion verified by elegant biomimicry studies of phloem translocation using a microfluidic system (e.g., Jensen et al., 2009, 2011) and experimental observations showing that solutes and water move at similar velocities (e.g., van Bel and Hafke, 2005; Windt et al., 2006) consistent with a bulk flow phenomenon. High nutrient fluxes through transport phloem are accompanied by even higher water fluxes. For instance, biomass concentrations of phloem sap of 10–20% (weight per volume—Milburn and Baker, 1989) estimate water fluxes of $440\text{--}500 \text{ L m}^{-2}$ sieve tube cross-sectional area s^{-1} . Thus, phloem transport serves not only as a long-distance pathway for nutrient movement but also as a significant conduit for water distribution from source to sink. This especially applies to those sink organs with low transpiratory fluxes such as shoot apices and developing fruits

and seeds. Under steady state conditions, resource fluxes into collection and from release phloem match those of the transport phloem (see Figure 3.1).

Mechanistic aspects of bulk flow through transport phloem are encapsulated in the now widely accepted pressure flow hypothesis put forward by Münch (1930 and for a translation into English, see Milburn and Kreeb, 2003) and summarized in Equations 3.1 and 3.2. Deduced from bulk flow theory, phloem transport rate (R_f) of a nutrient species is the product of transport velocity (V), sieve tube cross-sectional area (A), and phloem sap concentration (C) of a specified nutrient species whereby

$$R_f = V \cdot A \cdot C \quad (3.1)$$

Thus, high concentrations of nutrients in sieve tube saps, and especially those of sugars (Table 3.1), contribute to generating high rates of phloem translocation. This brings focus to the underlying mechanisms responsible for accumulating and retaining high nutrients concentrations in collection and transport phloem, respectively (see Figure 3.1).

Factors determining transport velocity (see Equation 3.1) or solvent volume flux (J_v – $\text{m}^3 \text{ m}^{-2} \text{ s}^{-1}$ or m s^{-1}) can be identified on the presumption that flow through sieve tubes approximates laminar flow through capillaries encapsulated by the Hagen–Poiseuille law as follows:

$$J_v = \Delta P \pi r^2 / 8\eta l \quad (3.2)$$

where ΔP is the hydrostatic pressure difference between source (P_{source}) and sink (P_{sink} and see Figure 3.1), across length (l) of the sieve tube conductive pathway, driving a solution of known viscosity (η) through radii (r) of sieve tubes most limiting to transport (i.e., sieve pores and see Mullendore et al., 2010).

Hydrostatic pressures (P_i) of any cell (e.g., SE–CCs) are determined by differences in osmotic pressures (π) between their protoplasts (π_i) and water potentials of their surrounding apoplasms. Apoplasmic water potentials are the sum of osmotic (π_o) and pressure (P_o) potentials. The pressure potential component can be a negative tension or positive hydrostatic pressure. Thus, assuming quasi water potential equilibrium, hydrostatic pressure at any point in a phloem pathway, including nonphloem cells supporting bulk flow to, or from, the phloem, is given by:

$$P_i = \pi_i - (\pi_o - P_o) \quad (3.3)$$

Phloem Loading in Collection Phloem

Equation 3.3 demonstrates that sieve tube hydrostatic pressures are in particular influenced by their sap concentrations of major osmotic species. These are soluble carbohydrate (sucrose, RFO or polyol), potassium, and total cohort of amino-N compounds (see section entitled “What’s In?—Constituents and Their Concentrations in Sieve-Tube Sap” and Table 3.1). Thus phloem loading of major osmotic species regulates rates of phloem translocation through setting their concentrations (Equation 3.1) as well as influencing hydrostatic pressures (Equation 3.3) driving bulk flow (Equation 3.2). Since sugars account for at least 50% of the osmotic content of sieve tube sap (Table 3.1), their loading is crucial in generating hydrostatic pressure heads in collection phloem to drive bulk flow. Regulating translocation rates of minor osmotic species (Table 3.1) is exerted solely through phloem loading altering their sieve-tube concentrations (Equation 3.1).

Phloem Loading Mechanisms and Pathways

Given that sugars represent the major osmotic species in sieve-tube saps (Table 3.1), we choose to define phloem loading as all transport events contributing to intercellular transport of photosynthetically reduced carbon (C) from chloroplast stromas to SE lumens (van Bel, 1993 and see Figure 3.2). In thermodynamic terms, phloem-loading mechanisms fall into two broad groupings—passive and active. Emerging evidence shows that phloem loading of sugars (sucrose and polyols) in woody plant species can be passive down chemical or osmotic gradients from mesophyll cell cytosols to SE lumens (Rennie and Turgeon, 2009 and see Figure 3.2A). Alternatively, phloem loading in monocots and herbaceous eudicots occurs against a chemical potential gradient of the major translocated sugar species into SE-CCs and thus depends upon expenditure of metabolic energy (Turgeon, 2010a). Active phloem loading is achieved by two disparate mechanisms (Figures 3.2B and C). One depends upon energy-coupled sugar (sucrose and polyols) transport across plasma membranes of SE-CCs and is wide spread through monocots and herbaceous eudicots (Figure 3.2B). The other is confined to herbaceous eudicots and depends upon sucrose being converted to RFOs in specialized CCs (intermediary cells—ICs). IC-synthesized RFOs are conjectured to exceed size exclusion limits of plasmodesmata present in the symplasmic path linking mesophyll cells with ICs. Thus, these RFOs are trapped and accumulate to high concentrations in SE-IC complexes of minor veins (polymer trap mechanism—Turgeon, 2010a and see Figure 3.2C).

Passive loading and polymer trap mechanisms load SE-CCs of collection phloem with sugars delivered through symplasmic routes extending from mesophyll cells to SE-CCs—symplasmic loaders (Figures 3.2A and C). Active loading across plasma membranes of SE-CCs of necessity involves sugar accumulation from the phloem apoplasm released from bundle sheath/phloem parenchyma cells following symplasmic passage from mesophyll cells—apoplasmic loaders (Figure 3.2B). Various combinations of two or all phloem loading mechanisms have been detected in one plant species (Turgeon, 2010a).

Pre-SE-CC Loading Transport Processes Are Common to All Phloem Loading Mechanisms and Pathways

Compartmental analysis of ^{14}C -photoassimilate dynamics reveals carbon (C) export, and hence SE-CC loading of sugars occurs from two kinetic pools distinguishable by their exchange rates differing by an order of magnitude (e. g., Rocher et al., 1994). The more rapidly turning-over pool represents cytosolic sucrose alone or in combination with polyols, from which minor vein SE-CCs are loaded (Grodzinski et al., 1998). It is derived from C captured in photosynthetically produced triose phosphates released into mesophyll cytosols across chloroplast envelopes during the day (Figure 3.2). The more slowly turning over pool represents C released from intracellular storage compartments flowing into the cytosolic sucrose/polyol pool. The storage compartments comprise starch located in mesophyll (C3 plants) or bundle sheath (C4 plants) chloroplasts and sucrose/hexoses stored in vacuoles of cells located along the symplasmic component of the phloem-loading pathway (Figure 3.2). Collectively remobilization of sugars from chloroplast starch (Zeeman et al., 2007) and vacuolar storage (Martinoia et al., 2000) buffer sucrose and polyol levels in mesophyll cytosols available for loading into SE-CCs against alterations in leaf photosynthetic rates and are the dominant pool supporting leaf export during the night.

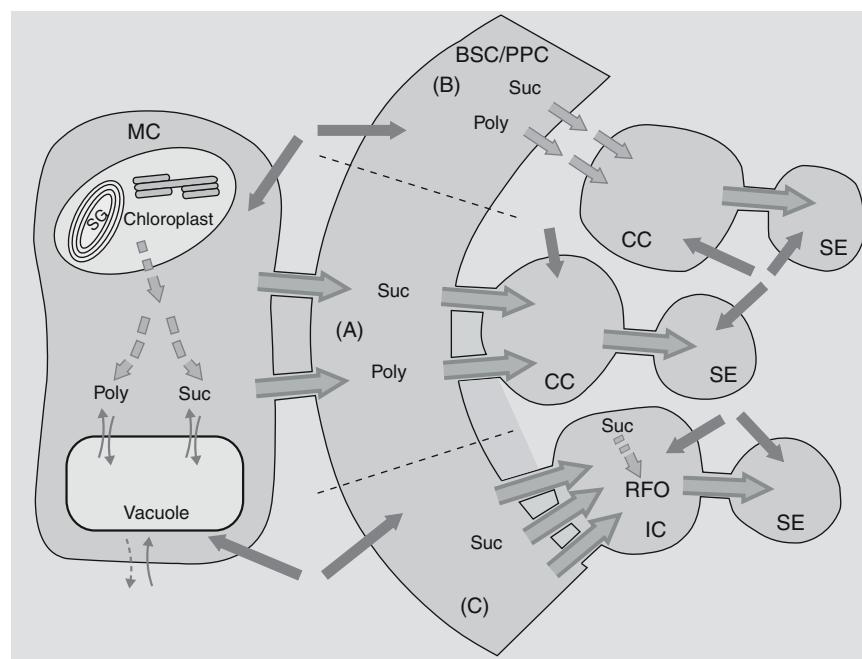


Figure 3.2 Phloem loading pathways for sugars (light-grey arrows) and water (dark-grey arrows) in source leaves. Cytoplasmic pool sizes of sucrose (Suc) or polyols (Poly) in mesophyll cells (MC) determine amounts available for loading. Metabolic transfers into these pools (light-grey arrows with broken shafts) is primarily from current photosynthetically reduced carbon (C) crossing chloroplast envelopes with excess C stored as chloroplastic starch grains (SG) or as sugars sequestered in mesophyll cell vacuoles by transport across their tonoplast membranes (curved light-grey arrows). When C flows from current photosynthesis (e.g., during the night) do not meet demand for C, cytosolic sugar pools are buffered by remobilization of C from chloroplastic starch or sugars stored in vacuoles (light-grey arrows). Water flows (dark-grey arrows) into MCs, down osmotically generated water potential gradients, create hydrostatic pressure gradients to drive bulk flow of cytosolic sugars (light-grey arrows for sugar; water dark grey border) through interconnecting plasmodesmata to reach bundle sheath/phloem parenchyma cells (BSC/PPC). During this passage, sugars passively leaked curved light-grey arrow with broken shaft from the MC/BSC/PPC symplasmic domain are retrieved by sugar symporters (curved light-grey arrows). Sugar movement from BSC/PPC to sieve element–companion cell complexes (SE–CCs) fall into three categories that are species specific. (A) Continued passive movement through a symplasmic route by bulk flow down hydrostatic pressure gradients is prevalent in woody plants transporting sucrose or polyols. (B) Sucrose or polyols released to the phloem apoplasm from BSC/PPCs are retrieved by energy-coupled transporters (light-grey arrows) to accumulate in SE–CCs to concentrations a magnitude higher than those in MC cytosolic pools. Osmotic water uptake generates hydrostatic pressure heads to drive bulk flow through sieve tubes to exit source leaves and flow onto sinks. (C) Sucrose symplasmically enters specialized CCs, intermediary cells (IC), where it is metabolized (light-grey arrow with broken shaft) to raffinose family oligosaccharides (RFOs). RFO molecular dimensions exceed size exclusion limits of plasmodesmata interconnecting BSC/PPC with ICs effectively trapping RFOs in ICs. RFOs accumulate to high concentrations in IC/SEs to drive osmotic water uptake that create hydrostatic pressure heads to drive bulk through sieve tubes. (For a color version of the figure, please see Plate 3.2.)

Shared by all phloem-loading mechanisms is symplasmic transfer of cytosolic sucrose and polyols, possibly by bulk flow (Voitsekhoukaja et al., 2009), and certainly so for passive loaders (Rennie and Turgeon, 2009), toward minor veins through three to seven mesophyll cells (Beebe and Russin, 1999 and see Figure 3.2).

Symplasmic passage of sugars from mesophyll cells to minor veins raises the question of their cellular retention in this compartment. Based on sugar concentration estimates in mesophyll cell apoplasms and cytosols, differences in sugar concentrations across mesophyll plasma membranes

range from 52 to 266 mM for species translocating sucrose alone or in combination with polyols (e.g., Lohaus et al., 1995; Nadwodnik and Lohaus, 2008). In plant species that are purported to load passively (Rennie and Turgeon, 2009), these sugar concentration differences would be expected to be particularly large (around 500 mM). These large transmembrane concentrations differences drive a significant passive leak of sugars from mesophyll cells into their surrounding apoplasts. Using an estimated membrane permeability coefficient for sugars of 10^{-10} m s⁻¹ (Cram, 1984), predicted fluxes of sugars passively leaked to leaf apoplasts are in the range of 2–5 mol m⁻² s⁻¹. For comparative purposes, these fluxes are 4–10 times less than those of sugars loaded apoplasmically into SE-CCs of minor veins (Giaquinta, 1983). Nevertheless, their magnitude represents a considerable potential loss of sugars from the symplasmic loading route and highlights a significant role for retrieval mechanisms (Figure 3.2 and see Maynard and Lucas, 1982). The most direct evidence for sugar retrieval comes from electrophysiological studies of detached leaves perfused with sugar solutions. Consistent with proton symport retrieval systems for sucrose, hexoses and raffinose, concentration-dependent, and transitory membrane depolarizations of mesophyll cells were elicited upon introducing these sugar solutions into leaf apoplasts (Franz and Tattar, 1981; van Bel et al., 1996).

Apoplasmic Loaders—Probable Cellular Site of Apoplasmic Exchange and Membrane Transport Mechanisms

Sucrose and polyol transit across plasma membranes to accumulate in SE-CCs is peculiar to apoplasmic phloem loading species (Figure 3.2B). Low densities of plasmodesmata interconnecting SE-CCs with adjacent phloem parenchyma and/or bundle sheath cells (Gamalei, 1989), combined with steep intercellular sugar concentration and osmotic differences at this interface (Nadwodnik and Lohaus, 2008), infers active sugar influx occurs across plasma membranes of SE-CCs. Less certainty applies to cellular site(s) of sugar release to leaf apoplasts. Based on their location as well as anatomical and physiological evidence, bundle sheath and phloem parenchyma cells are favored candidates (see Figure 3.2B). For instance, blockage of phloem loading by closure of plasmodesmata interconnecting mesophyll and bundle sheath cells in the *sdx1* maize mutant (Botha et al., 2000) points to bundle sheath and phloem parenchyma cells as likely sites for sucrose efflux. Phloem parenchyma cells, that are modified to a transfer cell morphology, contain wall labyrinths characteristically polarized to regions abutting SE-CCs (Offler et al., 2003) suggest a transport function by this region.

Estimates of sugar effluxes across plasma membranes of bundle sheath/phloem parenchyma cells are of a magnitude commensurate with a facilitated membrane transport mechanism (Giaquinta, 1983). However, the precise mechanism of sugar release is yet to be identified.

Attempts to identify membrane transport mechanisms responsible for sugar uptake into SE-CCs using intact leaves and leaf discs infused with ¹⁴C-labeled sugar solutions are confounded by simultaneous sugar uptake by both mesophyll and phloem cells (Giaquinta, 1983; van Bel et al., 1986). Protoplasts derived from CCs (Ivashikina et al., 2003) and SEs (Hafke et al., 2007) offer a powerful opportunity to determine presence or absence of functional transporters in these two cell types (Ivashikina et al., 2003; Hafke et al., 2007).

A system to study *in planta* functioning of sugar transporters in SE-CCs has made use of the apoplasmic barrier in bundle sheath cells separating mesophyll from vascular cells of maize leaves that load SE-CCs apoplasmically (Ma et al., 2009). A set of elegant experiments by Heyser (1980) was based around this premise. Xylem of leaf strips was infused separately with solutions

containing an array of sugars as well as metabolic inhibitors. Consistent with a sucrose/proton symport mechanism, a transient alkalinization of perfusate only was detected when a sucrose solution was perfused through leaf veins, the magnitude of which was sucrose concentration dependent and was abolished by metabolic inhibitors (Heyser, 1980). That SE-CCs of maize leaves perform sucrose symport was verified by a similar experimental set up recording SE-CC membrane potentials by aphid stylectomy (Carpaneto et al., 2005). There is no matching study in leaf veins of eudicots for sucrose or polyol membrane transport.

Phloem Loading of Nutrients Other Than Sugars

To our knowledge phloem-loading studies of amino N compounds and ions have been restricted to species that load sugars apoplasmically (Figure 3.2B). Prior to the onset of leaf senescence, most amino N compounds and ions are delivered in the transpiration to minor vein sites of phloem loading. Therefore, it follows these chemicals will be taken up into SE-CCs of collection phloem from their apoplasts, irrespective of mechanism and pathway of phloem loading (see Figure 3.2).

In the case of amino N compounds synthesized within leaves, their phloem loading may not be highly selective since combinations and concentrations of amino acids in leaf tissues and sieve-tube saps are similar (e.g., Lohaus et al., 1994, 1995). This particularly raises questions about existence of trapping mechanisms in symplasmic loading species. However, consistent with an apoplasmic phloem loading mechanism, concentrations of some amino acids are higher in sieve-tube saps than in leaf apoplasts (Lohaus et al., 1994, 1995).

Root-assimilated ions and amino N compounds, delivered in the transpiration stream to source leaves, are partitioned between leaf ground tissues and collection phloem for immediate re-export (Atkins, 2000; Karley and White, 2009). Partitioning appears to occur within the minor vein apoplasmic compartment formed by a barrier located in anticlinal walls of their bundle sheath cells in both mono- and eudicots (Canny, 1995; Keunecke et al., 2001). How potassium channel activity on plasma membranes of bundle sheath cells (Keunecke et al., 2001) and SE-CCs (Ivashikina et al., 2003; Hafke et al., 2007) coordinates potassium partitioning between these two pathways is unknown.

Phloem Loading of Water and SE-CC Water Relations

Phloem loading of nutrients is coupled with an osmotic uptake of water that generates high hydrostatic pressures to drive bulk flow from source to sink (see Figures 3.1 and 3.2; Equation 3.3). Thus, cellular sites of water uptake depend on loading pathways followed by sugars, major osmotica of phloem saps (van Bel, 2003). For passive symplasmic loading, mesophyll cells are predicted as major sites for water uptake as bulk flow commences from these cells (van Bel, 2003; Turgeon, 2010a and see Figure 3.2A). In contrast, for active phloem loading (Figures 3.2B and C), high osmotic contents of SE-CC or IC complexes could render these cells as principal sites for water uptake (van Bel, 2003). The symplasmic discontinuity in apoplasmic loading species, combined with the apoplasmic barrier located in bundle sheath cell walls, renders hydraulic properties of SE-CCs independent from those of surrounding mesophyll cells. In contrast, active symplasmic loaders are hydraulically coupled with their surrounding mesophyll cells through interconnecting plasmodesmata. Therefore, in order to prevent pressure flow from SE/ICs to mesophyll cells, SE/ICs must be able to regulate their hydrostatic pressures independent of mesophyll water relations. This condition would be satisfied through an apoplasmic compartmentation of their water relations by

the barrier deposited in anticlinal walls of bundle sheath cells (Canny, 1995). Alternatively plasmodesmata interconnecting ICs with bundle sheath cells might impose a polarized transport of both solute (Christensen et al., 2009) and somehow solvent. Irrespective of the mechanism, water transfer against an intracellular osmotic gradient takes place as indicated by the finding that symplasmic movement of sugars from mesophyll to bundle sheath/phloem parenchyma cells is by bulk flow irrespective of loading mechanism (Voitsekhoukaja et al., 2009). This observation suggests hydraulic conductivities of plasmodesmata forming this symplasmic route must be exceptionally high, and particularly so for passive phloem loaders where the entire solution flow arises from water entry into mesophyll cells (Turgeon, 2010a and Figure 3.2A). Less equivocation rests with water fluxes across plasma membranes into SE-CCs and mesophyll cells. For instance, water fluxes across plasma membranes of soybean leaf SE-CCs and mesophyll cells are estimated to be 45×10^{-9} and $4 \times 10^{-9} \text{ m}^3 \text{ m}^{-2} \text{ s}^{-1}$, respectively (Giaquinta, 1983). Assuming water potential differences of 0.1 MPa, these water fluxes yield estimates of osmotic water permeability values consistent with being aquaporin facilitated for passage into SE-CCs but not necessarily mesophyll cells (Alleva et al., 2006).

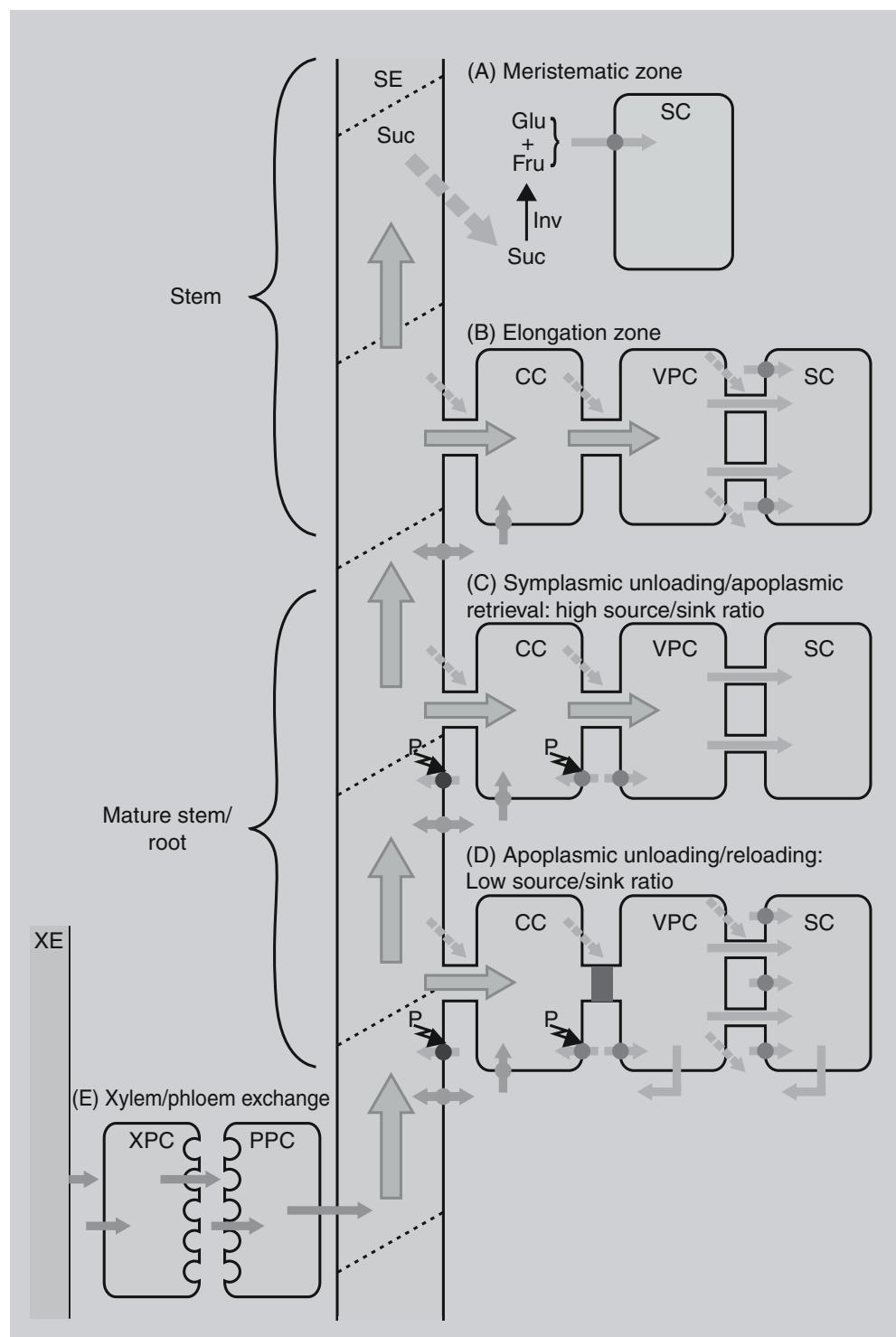
In source leaves, osmoregulatory activities of SE-CCs offset diurnal variations in xylem tensions ($-P_o$ and see Equation 3.3), created by transpiration, to homeostatically maintain their hydrostatic pressures (Lalonde et al., 2003) and hence phloem transport rates (Windt et al., 2006). Hydrostatic pressure homeostasis in SE-CCs is achieved through their turgor-regulated loading of sucrose (Smith and Milburn, 1980b).

Transport Phloem—the Multitasker

Transport phloem extends from minor veins in leaves to release phloem in sinks and over distances of 0.1–3 m (small to large herbs) and up to 100 m (tall trees). These lengths contrast with those of several hundred microns for collection and release phloem. In source leaves of monocots, transverse, intermediate, and large veins function as transport phloem. For eudicots, resources flow from minor veins into lower order veins that collectively function as transport phloem exporting resources from each source leaf. Thereafter, flow continues through primary vascular bundles in stems and roots to enter release phloem in sinks (Figure 3.1). For eudicots, with progressive development of secondary phloem arising from vascular cambia, primary phloem is ultimately crushed in stems and roots leaving secondary phloem as transport phloem.

Transport Phloem Functions

First and foremost, transport phloem forms a conduit to support high axial fluxes of nutrients and water translocated to terminal sinks (see section entitled “Propulsion of a Nutrient Enriched Sap through Sieve Tubes—Pressure Flow and its Physiological Implications”). Key to this function is maintenance of elevated nutrient concentrations in phloem saps (Table 3.1) that underpin high rates of axial flow (see Equations 3.1 and 3.2). Maintenance of high nutrient concentrations in phloem saps face continual depletion by *passive leakage* across SE-CC plasma membranes driven by large concentration differences between SE-CC lumens and their surrounding apoplasms. Passive solute leakage is countered by solute *retrieval* by SE-CCs. *Partitioning of resources* between transport phloem and surrounding ground tissues may depend upon relative activities of transporters located in SE-CCs and opposing phloem parenchyma cells. *Net solute retrieval* serves as a homeostatic mechanism to counter short- and long-term downward shifts in resource loading in collection phloem



to sustain phloem sap concentrations and sieve-tube turgors. In addition, *xylem–phloem exchange* of mineral ions and amino N compounds, traveling in the transpiration stream, occurs along the transport phloem. Exchange can be magnified at nodal regions by vascular parenchyma transfer cells (Offler et al., 2003). In the following review, lateral nutrient exchanges between transport phloem and neighboring ground and xylem tissues will be addressed (Figure 3.3). Some of this information then will be drawn upon to discuss homeostasis of longitudinal fluxes through the transport phloem (see section entitled “Pressure Flow—Integrating Collection, Transport and Release Phloem Function”).

Sugar and Amino N Leak and Retrieval Functions

Large transmembrane concentration differences of sucrose or polyols between SE–CCs lumens and their surrounding apoplasms drive a passive leak by simple diffusion through their plasma membranes (Figures 3.3A, B, C, and D). Phloem apoplasmic concentrations of sucrose in stem tissues are relatively low (1–12 mM—see Table 3.1) and hence, concentration differences between SE–CC lumen and their surrounding apoplasms are primarily determined by variations in sieve-tube sap concentrations. These range from lower values of 163 mM in an *akt2/akt3* *Arabidopsis* mutant (Deeken et al., 2002) to high-end values of 1030 mM in spinach (Lohaus et al., 1995). Using an estimated membrane permeability coefficient for sugars of 10^{-10} ms $^{-1}$ (Cram, 1984), passive membrane fluxes of sucrose are predicted to range from 1.63×10^{-6} to 10.3×10^{-6} mol m $^{-2}$ s $^{-1}$. These values approximate maximal values for facilitated membrane transport by sucrose symporters (Fisher, 2000). Estimates of relative magnitudes of sucrose leakage from transport phloem vary considerably. For instance, mature *Phaseolus* stems sieve tubes loose 6% of their photoassimilate per cm, of which, 4% is retrieved (Minchin and Thorpe, 1987). In contrast, *Cyclamen* stems loose

←
Figure 3.3 Lateral exchange of sucrose (light-grey arrows) and water (dark-grey bordered arrows) along transport phloem. Irrespective of unloading pathway (A–D), large transmembrane differences in sucrose concentration between sieve element (SE) lumens and their surrounding apoplasms drive substantial passive leaks of sucrose from SEs (light-grey arrows with broken shafts). Aquaporins (light-grey circles) on SE plasma membranes facilitate bidirectional water transfer (light-grey double-headed arrows) according to prevailing water potential gradients between SE lumens and their surrounding apoplasms. (A) Sucrose exit from protophloem SEs in meristematic zones of stems is entirely by passive leak across their plasma membranes. Cell wall invertases (Inv) hydrolyze released sucrose. The resulting glucose (Glu) and fructose (Fru) moieties are transported into sink cells (SC) by hexose transporters (dark-grey circle). (B) In elongating stem zones, unloading occurs from metaphloem SE–CCs (companion cell complexes) through apoplasmic and symplasmic pathways operating in parallel. Symplasmic unloading likely occurs by bulk flow (sucrose light-grey arrow superimposed on dark-grey arrow representing water flow) to adjoining vascular parenchyma cells (VPC). Thereafter onward movement to sink cells may occur symplasmically or apoplasmically with sugar recovery from sink apoplasms by carrier-mediated transport (light-grey arrows through dark-grey circles). In mature stems (C, D), irrespective of unloading pathway, sucrose leaked from SEs to phloem apoplasm, is retrieved by turgor (P)-regulated transporters (dark-grey circles) located on SE–CC plasma membranes. (C) Under high source/sink ratios, a net unloading of sucrose occurs predominately through a symplasmic pathway. (D) Under low source/sink ratios, plasmodesmata linking SE–CCs to VPCs are gated closed (dark-grey rectangle) and unloading/reloading of SE–CCs follows an apoplasmic route. Sucrose transport into VPCs is carrier mediated (dark-grey circles) and thereafter sucrose accumulation by sink cells can occur through apo- and symplasmic routes operating in parallel. If low source/sink ratios persist over protracted periods, stored carbon is remobilized from sink cells, released to sink/phloem apoplasms (light-grey L-shaped arrows) and reloaded into SE–CCs to buffer sucrose flows to terminal sinks. (E) Solute exchange from xylem transpiration streams is mediated by transporters located on xylem (XPC) and phloem parenchyma (PPC) cells (mid-grey arrows) participate in xylem (XE)-to-phloem exchange of solutes. XPC/PPCs, located at nodal regions, often are modified to a transfer cell morphology (wall ingrowths—indentations in periclinal walls). (For a color version of the figure, please see Plate 3.3.)

0.6% of photoassimilate per cm (Grimm et al., 1997). These differences are likely to reflect relative activities of sugar retrieval mechanisms.

Use of isolated vascular bundles (e.g., Grimm et al., 1990), isolated phloem segments (Bielecki, 1966a; Daie and Wilusz, 1987) or plasma-membrane vesicles prepared from phloem segments (Salmon et al., 1995), in ^{14}C -sugar transport studies, demonstrate that retrieval of sucrose and linear polyols by transport phloem is facilitated and exhibits biochemical properties consistent with proton symport (Figures 3.3C and D). A conclusion supported by biophysical evidence derived from intact phloem using aphid stylets (Wright and Fisher, 1981; Carpaneto et al., 2005).

Net Resource Efflux from Transport Phloem

Transport phloem undergoes considerable developmental change from proto- to metaphloem of primary vascular bundles and, in eudicots, ultimately secondary phloem that arises as cell lineages from meristematic activities of vascular cambia. Linked with these developmental changes are shifts in stem function from elongation growth to maturity in which excess resources are stored to be remobilized during periods when resource demand exceeds their rates of assimilation. Below net resource efflux from stems tissues is considered at differing development states—meristematic, cell elongation, and fully elongated.

Stem Meristematic Zones

Primary vascular bundles traverse stem meristematic zones with sieve tubes comprised of symplasmically isolated protophloem SEs lacking CCs (Patrick, 1972; Wood et al., 1997). As a consequence, resource unloading from protophloem SEs occurs directly into meristem apoplasms (see Figure 3.3A). Absence of any functional sucrose retrieval mechanisms (Wood et al., 1998; Eisenbarth and Weig, 2005), combined with high activities of extracellular invertases hydrolyzing released sucrose (e.g., Godt and Roitsch, 2006), provide conditions to optimize apoplasmic unloading of sucrose from protophloem SEs by simple diffusion (see section entitled “Transport Phloem Functions” and Figure 3.3A). Surrounding meristematic cells recover apoplasmic hexoses by high affinity (μM) hexose uptake mechanisms (e.g., Komor et al., 1981 and see Figure 3.3A).

Cell Elongation Zones

Cell elongation zones are located between meristematic and fully elongated portions of each internode and represent a key transition step in phloem unloading pathway and mechanism. Metaphloem SEs, CCs, and vascular parenchyma cells are interconnected by relatively high frequencies of plasmodesmata. However, lower plasmodesmal frequencies are present between vascular parenchyma and adjoining nonvascular cells in elongating bean stems (Wood et al., 1997). Recruitment of this expanded metaphloem symplasmic domain increases plasma membrane surface areas available for apoplasmic unloading (Wood et al., 1997 and see Figure 3.3B). An apoplasmic pathway of phloem unloading in elongating stems (Minchin and Thorpe, 1984) does not exclude a symplasmic route operating in parallel (see Figure 3.3B). For instance, physiological properties of phloem unloading in extending pea hypocotyls conforms with photoassimilates moving through a symplasmic route (Schmalstig and Cosgrove, 1990). Indeed a 40% randomization of asymmetrically ^{14}C -labeled sucrose translocated from a source leaf and accumulated in immature internodes of sugarcane (Hatch and Glasziou, 1964) is consistent with apoplasmic and symplasmic phloem unloading operating in parallel. Whether unloading is predominately apo- or symplasmic may depend upon the prevailing source/sink ratio (Patrick and Offler, 1996).

In stem elongation zones, solute retention by phloem is weak (cf. Patrick and Turvey, 1981; Bielecki, 1966a, 1966b; Deeken et al., 2002) with only 14% and 20%, respectively, of photoassimilates and potassium retained by phloem (Wood et al., 1994). This compares with 98% and 99.4% retention per cm, respectively, by mature stems of *Phaseolus* (Minchin and Thorpe, 1987) and *Cyclamen* (Grimm et al., 1997).

Mature Stems

Once internodes are fully elongated, differentiation of metaphloem reaches completion and, in eudicots, vascular cambia give rise to secondary phloem that ultimately crushes the metaphloem. An apoplastic route of sucrose unloading across plasma membranes of SE-CCs by simple diffusion (see section entitled “Transport Phloem Functions”) is invariably available (see Figures 3.3C and D). Indeed, symplasmic isolation of metaphloem SE-CCs is suggested by low plasmodesmal connectivity with adjoining phloem parenchyma cells, absence of membrane-impermeant dye movement and differing membrane potentials between the two cell types (e.g., Hayes et al., 1985; van Bel and van Rijen, 1994; Kempers et al., 1998). Apoplastic solutes are partitioned between SE-CCs, remaining vascular cells and surrounding ground tissues (e.g., Hafke et al., 2005) with SE-CC retrieval dominating accumulation over any other cell type by more than fivefold (Patrick and Turvey, 1981; Bielecki, 1966a, 1966b; Deeken et al., 2002 and see Figure 3.3D).

Under high source/sink ratios, ground tissues, radial to transport phloem, store excess nutrients and nutrient unloading may switch to a symplasmic route (Grignon et al., 1989; Patrick and Offler, 1996 and see Figure 3.3C). Under these conditions, competition between SE-CCs and phloem parenchyma cells (Kempers et al., 1998) is circumvented. Moreover in grass stems, radial flows of nutrients to ground tissues are restricted to their symplasms as suberin bands, laid down in anticlinal walls of bundle sheath cells, block radial movement through their stem apoplasts (e.g., sugarcane—Jacobsen et al., 1992; wheat—Aoki et al., 2004; rice—Scofield et al., 2007). Depending upon the relative potential energies contained in concentration and turgor gradients, diffusion or bulk flow may dominate nutrient transport through symplasmic unloading pathways (Murphy, 1989; Schmalstig and Cosgrove, 1990; Patrick and Offler, 1996).

Net Resource Influx into Transport Phloem

Short-term adjustments to a decrease in resource loading into collection phloem are mediated through transporter-facilitated retrieval of nutrients by transport phloem of herbaceous (Minchin and Thorpe, 1984, 1987; Gould et al., 2004a) and woody eudicots (McQueen et al., 2005 and see Figures 3.3C and D). Decreased solute loading by collection phloem could be sensed as a decrease in sieve-tube turgor, resulting from a lowering of phloem sap osmolality (see Equation 3.3). A claim supported by findings that sucrose (e.g., Grimm et al., 1990) and mannitol (Daie and Wilusz, 1987) symporter activities, located in transport phloem, are inversely related to sieve-tube turgors (Figures 3.3C and D).

More extended periods of reduced resource loading by collection phloem, elicit remobilization of stored resources located in ground tissues surrounding transport phloem to buffer resource supply to sink organs (e.g., Yang and Zhang, 2006). Here remobilized solutes are presumably released to axial apoplasts for ultimate retrieval by SE-CCs through an apoplastic route of phloem loading formed under low source/sink ratios by plasmodesmal closure linking metaphloem SE-CCs with surrounding vascular parenchyma cells (Patrick and Offler, 1996 and see Figure 3.3D).

Xylem-to-Phloem Exchange

Growth and storage sinks characteristically have low transpiration rates. Consequently, nutrient import by most sinks relies primarily on phloem transport. Thus, to reach these organs, root acquired minerals, and assimilated N must be transferred from the xylem transpiration stream followed by apoplastic phloem loading (Figure 3.3E). These transport events occur within transport as well as collection phloem. Here we shall focus on the transport phloem; xylem-to-phloem exchange in collection phloem has been described in section entitled “Phloem Loading of Nutrients Other Than Sugars.”

Modeling of partitioning points to an intense exchange of organic N compounds between xylem and phloem localized to nodal regions of monocots and herbaceous eudicots (e.g., Jeschke and Pate, 1991). The phenomenon involves a sequence of transport processes beginning with solute retrieval from the transpiration stream by xylem parenchyma cells, symplasmic transfer to phloem parenchyma cells, and ultimately an apoplastic loading step into SE-CCs. Thus, three plasma membrane transport steps may contribute to xylem–phloem exchange and, in some species, these steps could be facilitated by xylem and phloem transfer cells (Offler et al., 2003 and see Figure 3.3E).

Secondary vascular tissue organization of woody species dictates that resource movement from xylem to phloem occurs radially through parenchyma rays in their apoplasm or symplasms (van Bel, 1990). Uptake of amino acids into the symplasmic route from the xylem transpiration stream is mediated by proton symport (van Bel, 1990). Mechanism(s) of resource release from ray parenchyma cells into phloem apoplasts is unknown. Loading SE-CCs likely occurs from phloem apoplasts by proton symport (e.g., McQueen et al., 2005).

In addition to nutrient transfer, there is also a tight hydraulic coupling between phloem and xylem resulting in sieve tubes being close to water potential equilibrium with their surrounding apoplasts including xylem elements (Sevanto et al., 2011). Radial water movement from xylem to phloem exhibits radial conductances (10^{-14} to $10^{-13} \text{ m}^3 \text{ m}^{-2} \text{ Pa s}^{-1}$) consistent with involvement of aquaporins (Sevanto et al., 2011).

Release Phloem—Letting Go of the Translocation Stream

Phloem unloading is defined as a continuum encompassing all transport processes participating in transferring resources from SE lumens of release phloem to cellular sites of storage/utilization in adjoining nonvascular tissues (sink cells and see Figure 3.4). Thus, similar to phloem loading, vascular and nonvascular cells participate in phloem unloading, the precise cellular nature of which are organ and development specific. To tease out some commonalities in phloem unloading pathways shared by all sink types, we first map cellular pathways of phloem unloading in vegetative apices and terminal storage sinks. Pathway commonalities are then used as a framework on which to draw some generalizations about physiological mechanisms contributing to resource unloading.

Mapping Cellular Pathways of Phloem Unloading

Root and Shoot Apices—An Increasingly Constrained Symplasmic Route Linked with Organ Development

SEs with/without CCs are absent from meristematic regions of root and shoot apices with any vasculature present as provascular strands (Esau, 1965). Protophloem SEs with/without CCs

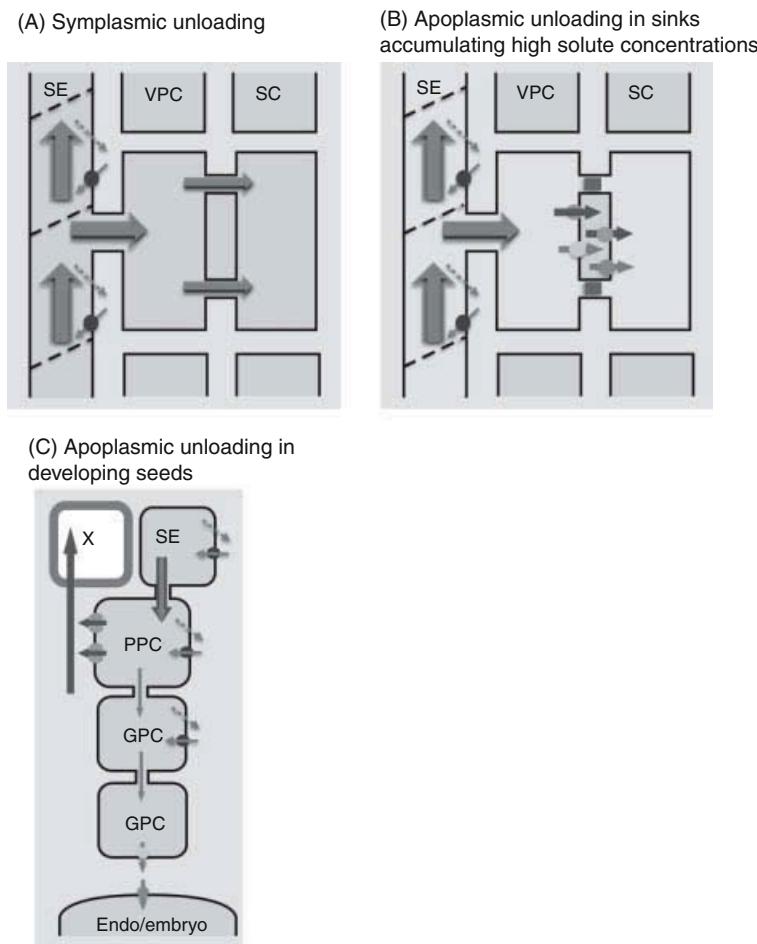


Figure 3.4 Cellular pathways of phloem unloading of sucrose (light grey) and water (dark grey) in terminal sinks from sieve elements (SE) alone or SE–companion cell (CC) complexes. Irrespective of unloading pathway (A–C), a passive leak of sucrose (light grey arrow with broken shaft) occurs from SEs to their surrounding apoplasts. Leaked sucrose is retrieved by transporters (light-grey arrow through dark-grey circle) located on plasma membranes of SEs or SE–CCs. (A) Symplasmic unloading by bulk flow (sucrose—light-grey arrow; water—dark grey arrow) from SE or SE–CC via vascular parenchyma (VPC) to sink (SC) cells. (B) Plasmodesmata are gated closed (mid-grey rectangle) at SE/VPC or VPC/SC interfaces of terminal sinks accumulating sugars to high concentrations. Transporter-mediated efflux of sucrose (light-grey arrow through white circle) and water (dark-grey arrow through mid-grey circle) occurs to sink apoplasts. Sink cells recover apoplastic sucrose (light-grey arrow through dark-grey circle) and water through transporters located in their plasma membranes. (C) Phloem unloading in developing seeds follows symplasmic pathways from SEs to maternal/filial interfaces. Transfer from SE or SE–CC to VPC is likely to be by bulk flow (light-grey arrow overlying dark-grey arrow) with diffusion of sugars (light-grey arrow) thereafter. Excess water is recycled back to the parent plant body by aquaporin-mediated exchange (dark-grey arrows through mid-grey circles) to the seed apoplasm and exiting through the xylem (X). Along the symplasmic route, transporters (dark-grey circles) in phloem parenchyma (PPC) and ground parenchyma (GPC) cells retrieve sucrose leaked (light-grey arrows with broken shafts) to seed apoplasts. At maternal/filial interfaces, sucrose transport across cell membranes is carrier-mediated. Sucrose efflux (light-grey arrow through mid-grey circle) to seed apoplasts occurs from specialized maternal GPCs. Thereafter sucrose, or its hexose moieties are taken up (light-grey arrow through mid-grey circle) into filial tissues comprising endosperm and/or embryo. (For a color version of the figure, please see Plate 3.4.)

differentiate toward apical meristems from provascular cells in distal zones of apices dominated by cell expansion. Protophloem SEs are likely to form a continuum with metaphloem SEs via shared lateral sieve plates in mature root/stem regions through which resources are delivered from proximal source leaves.

Distribution of membrane-impermeant fluorochromes, imported through the phloem, have identified putative symplasmic unloading pathways extending from terminal ends of protophloem SEs ± CCs through provascular stands to reach meristematic cells in root apices of monocots (e.g., Hukin et al., 2002) and eudicots (e.g., Stadler et al., 2005a). Further back from root tips, in root elongation zones, the symplasmic domain is constrained to protophloem SE-CCs and vascular parenchyma cells (Hukin et al., 2002; Stadler et al., 2005a and see Figure 3.4B). However, some symplasmic connectivity between protophloem and cortical cells is retained (Stadler et al., 2005a) indicating that symplasmic and apoplastic-unloading routes may operate in parallel (Figure 3.4A).

In contrast to roots, shoot apical meristems are comprised of a number of developmentally dynamic symplasmic domains (e.g., Gisel et al., 2002). These domains likely have more significance in regulating distribution of signals controlling tissue developmental patterns rather than influencing resource flows. Most attention has been paid to phloem unloading in developing leaves in which an acropetal-directed wave of protophloem differentiation occurs in major veins (Esau, 1965). These vascular bundles are responsible for phloem import of resources into sink leaves and their subsequent unloading to support leaf expansion (Turgeon, 1987; Robinson-Beers et al., 1990). Phloem-imported membrane-impermeant fluorochromes exit from major veins into surrounding nonvascular tissues in sink leaves of monocots (Haupt et al., 2001) and eudicots (Opalka et al., 1999; Stadler et al., 2005a) demonstrating a putative symplasmic pathway for phloem unloading from their protophloem SEs (Figure 3.4A). As leaf development progresses, a second wave of differentiation occurs basipetally, the so-called sink-source transition (Turgeon, 1989). This phenomenon is linked with minor vein networks completing differentiation from provascular strands to commence resource loading and major vein metaphloem rendering their SE-CCs symplasmically isolated to function as transport phloem (Figure 3.3D).

Phloem Unloading Pathways in Tubers and Fleshy Fruits—Pathway Switching between Apo- and Symplasmic Routes Accommodates Changes in Sink Function

Phloem unloading in developing potato tubers switches from an apoplastic route (Figure 3.4B) in stolons to a symplasmic pathway (Figure 3.4A) once starch accumulation commences within tubers (Viola et al., 2001). In contrast phloem unloading pathways in fleshy fruits, including tomato (Ruan and Patrick, 1995; Patrick and Offler, 1996), grape (Zhang et al., 2006), and Chinese jujube (Nie et al., 2010), switch from symplasmic during their prestorage phase (low intracellular osmotic concentrations and see Figure 3.4A) to ones in which an apoplastic step appears at the onset of sugar storage (high intracellular osmotic concentrations and see Figure 3.4B). The link between intracellular osmotic concentration and phloem unloading pathway is further highlighted in fruits of walnut and apple. In walnut fruit, resources are unloaded symplasmically to the seed pericarp (seed coat) but apoplasically into the fleshy pericarp (Wu et al., 2004). Across fruit development, unloading in apple fruit exhibits an apoplastic step at the SE-CCs boundary (Zhang et al., 2004) accompanied by high intracellular concentrations of soluble sugars in fruit flesh that reach 930 mM (Beruter et al., 1997). These observations suggest that imposition of an apoplastic unloading step is linked with sinks accumulating high osmolyte concentrations. In this context, apoplastic unloading might function to hydraulically isolate sink cells from SE-CCs and vascular parenchyma cells to avoid their cell turgors compromising those of the importing SE-CCs.

Phloem Unloading Pathways in Developing Seeds Include an Obligatory Apoplastic Step

Absence of symplasmic continuity between maternal (integuments modified to a seed coat) and filial (endosperm and embryo that exist as separate symplasmic domains) tissues of developing seeds results in two obligatory transport steps across cell plasma membranes. These are efflux of resources from coats to seed apoplasmic spaces and their subsequent influx into endosperm and embryo (Figure 3.4C). Specialized transport cells, located at maternal/filial interfaces, are responsible for resource efflux into, and influx from, seed apoplasmic spaces (Zhang et al., 2007).

Resources imported into developing seeds are delivered by vasculatures that terminate at funicular/coat boundaries of small seeds (e.g., *Arabidopsis*—Stadler et al., 2005b) or permeate coats of larger seeds (e.g., cereals and grain legumes—Zhang et al., 2007). SE unloading of resources and their subsequent movement to specialized efflux cells follows symplasmic routes (Stadler et al., 2005b; Zhang et al., 2007). Released to seed apoplasts, resources are distributed between a series of separate symplasmic domains (Morley-Smith et al., 2008) located in endosperm and embryo (Stadler et al., 2005b; Zhang et al., 2007 and see Figure 3.4C), the boundaries of which may alter during seed development (Stadler et al., 2005b). For larger seeds, postphloem transport through symplasmic pathways in maternal and filial tissues can extend up to several centimeters (Zhang et al., 2007) and hence resource retrieval mechanisms, analogous to those of transport phloem, need to be considered (Figure 3.4C).

Some General Physiological Principles of Phloem Unloading

Mapping phloem-unloading pathways shows that, irrespective of sink type, unloading from SEs or SE–CCs follow symplasmic routes (Figure 3.4). As a consequence, release phloem may retain a retrieval function to retain nutrients within these symplasmic compartments; a function that might extend into post-SE component of the unloading pathway (Figure 3.4C). In certain storage sinks, a symplasmic discontinuity may be present or form in the post-SE apoplasmic-unloading path (Figures 3.4B and C). This invokes two mandatory transfers across plasma membranes of pathway cells of nutrient efflux arranged in series with nutrient influx. We start by exploring implications of a release phloem pathway of putative low hydraulic conductivity in vegetative apices.

Root and Shoot Apices Are Served by Release Phloem of Low Hydraulic Conductivity

Protophloem SEs complete their differentiation behind apical meristems and, in roots, depending upon their elongation rate, this event can be located upward of 1 cm from the root tip (Esau, 1965). Thereafter, onward symplasmic flow is through a symplasmic pathway of increasingly lower hydraulic conductance imposed by partially differentiated protophloem SEs merging into provascular strands and ultimately meristematic cells. The lowered hydraulic conductance imposes a restriction on sucrose supply supporting root growth (e.g., Walter et al., 2009), leaf primordial initiation (Wardlaw, 1990), and is a key factor in determining floral abortion (e.g., Ghiglione et al., 2008).

Symplasmic Unloading from SEs—Implication for Resource Flows

In the few cases where measurements have been undertaken, large osmotic differences of 0.7–0.9 MPa exist between SEs of release phloem and downstream cells in symplasmic unloading pathways of root tips and developing seeds (Table 3.2). Apoplasmic hydrostatic pressures in sinks approach zero as they are hydraulically isolated from the remainder of the plant body and their transpiratory losses are minimal (Lalonde et al., 2003). Thus, these osmotic differences translate into equally large decreases in hydrostatic pressures. In some cases, SE hydrostatic pressures have been measured

Table 3.2 Osmotic or hydrostatic pressures of sieve tubes and adjoining specified cells in root tips and developing wheat grains

Root-tip cell type					
Pressure potential (MPa)	Sieve tube	Elongating epi/cortical cell	Pressure difference (MPa)	Plant species	Reference
Osmotic	-1.62	-0.98	-0.64	Maize	Warmbrodt, 1987
Osmotic	-1.42	-0.71	-0.71	Barley	Pritchard, 1996
Osmotic				Barley	Gould et al., 2004a
<i>High-K plants</i>	-1.92	-0.65	-1.27		
<i>Low-K plants</i>	-1.42	-0.62	-0.70		
Turgor					
<i>High-K plants</i>	1.62	0.33	1.29		
<i>Low-K plants</i>	1.32	0.32	1.0		
Grain cell type					
Pressure potential (MPa)	Sieve tube	Vascular parenchyma	Pressure difference (MPa)	Plant species	Reference
Osmotic				Wheat	Fisher and Cash-Clark, 2000b
<i>Normal watered</i>	-2.21	-1.30	-0.91		
<i>Water stressed</i>	-2.97	-1.73	-1.24		
Turgor					
<i>Normal watered</i>	1.11	0.12	1.0		
<i>Water stressed</i>	1.30	0.08	1.12		

directly (Table 3.2 and see Equation 3.3). In developing wheat grains, large hydrostatic pressure differences occur across one cell wall width separating SE-CCs from adjacent vascular parenchyma cells (Table 3.2). Furthermore, there is a growing body of evidence that pressure differentials drive bulk flow through symplasmic unloading routes (see Figure 3.4). Among this evidence is that phloem water contributes to root extension growth (Hukin et al., 2002; Wiegers et al., 2009) and model-based predictions of hydrostatic pressure potentials required to account for symplasmic nutrient transport by bulk flow (Bret-Harte and Silk, 1994) are of similar magnitude to those detected in barley roots (Table 3.2). Moreover, attenuating pressure differentials in barley roots by a potassium deficiency decreasing sieve-tube turgors (Gould et al., 2004b and see Table 3.2) or by galactose increasing cortical cell turgors (Pritchard et al., 2004) caused a proportionate decrease in import rates consistent with bulk flow. Similar conclusions have been drawn for unloading from SEs in developing wheat grains (Fisher and Cash-Clark, 2000b and see Table 3.2).

Bulk flow from SE-CCs “simplifies” unloading than if it occurred by diffusion. In the latter case, each solute would exit SE-CCs at a rate that depended upon its diffusion coefficient and concentration difference between SE-CCs and vascular parenchyma cells. Phloem-imported water would diffuse at rates dictated by the water potential differences between SE-CCs and vascular parenchyma cells. Under these circumstances, it would be very problematical for rates of individual resource inflows into release phloem to match those of their exit from SE-CCs. As a consequence, regulation of sieve-tube hydrostatic pressures of release phloem could be compromised. A similar problematical issue applies to exclusive apoplastic unloading across SE-CC plasma membranes mediated by transporters (e.g., Zhang et al., 2004; Carpaneto et al., 2005 and see Figure 3.3A). The latter mechanism would need a complex regulatory system to ensure activities of each substrate-specific transporter (including aquaporins)-supported membrane transport of each solute/solvent at

rates that matched their delivery rates by bulk flow through sieve tubes combined with a capacity to respond to alterations in sink demand.

Large differentials in osmotic and hydrostatic pressures between SE-CCs and vascular parenchyma cells (Table 3.2) are the result rather than cause of bulk flow through a pathway of low hydraulic conductivity. Thus, control of unloading is likely exercised by conductivities of plasmodesmata linking SE-CC with vascular parenchyma cells as this is the site where the largest decrease in cell osmotic pressures are detected (Table 3.2). Significantly, size exclusion limits of plasmodesmata at these cellular sites are unusually large in roots (60 kDa—Stadler et al., 2005a), sink leaves (50 kDa—Opalka et al., 1999; Stadler et al., 2005a), and developing seeds (400 kDa—Fisher and Cash-Clark, 2000a; Stadler et al., 2005b) compared to the frequently reported 800 D for plasmodesmata linking various cell types found in ground tissues (Fisher, 2000). As discussed by Fisher and Cash-Clark (2000a), size exclusion limits based on molecular weight can be misleading and Stokes radius is a preferable measure. Plasmodesmal hydraulic conductivities computed on their Stokes radii are sufficient to accommodate observed bulk flows from SE-CCs (Fisher and Cash-Clark, 2000b).

Large plasmodesmal conductivities offer scope for a considerable range of control to be exercised over symplasmic bulk flow across SE-CC and adjoining vascular parenchyma interfaces. That such a system is operative is illustrated by finding that a pharmacological block of sucrose uptake into endosperm of attached wheat grains was not accompanied by any change in sucrose concentrations located in cells forming the unloading pathway of maternal grain tissues (Fisher and Wang, 1995). This points to a direct link between sucrose uptake by endosperm and plasmodesmal conductivities at SE-CC-vascular parenchyma interfaces. The nature of the regulatory mechanism is yet to be determined, but has significant implications for phloem translocation as discussed in the section entitled “Pressure Flow—Integrating Collection, Transport and Release Phloem Functions.”

Symplasmic Unloading Routes and Nutrient Retrieval

In order to account for observed canalized symplasmic flows, and particularly for those observed for large-seeded grain legumes, a case can be made for retrieval of nutrients by release phloem and cells located along post-SE unloading pathways (Zhang et al., 2007). Evidence for retrieval mechanisms operating in release phloem and post-SE unloading pathway is presented in Chapter 4. Canalized flows ensure that nutrients are delivered to cells responsible for their efflux into seed apoplasmic spaces (Figure 3.4C).

Symplasmic Discontinuities and Plasma Membrane Transport

Some broad generalization can be drawn about plasma membrane transport operating to bridge symplasmic discontinuities located in post-SE unloading pathways of some sink types (see sections entitled “Phloem Unloading Pathways in Tubers and Fleshy Fruits—Pathway Switching between Apo- and Symplasmic Routes Accommodates Changes in Sink Function” and “Phloem Unloading Pathways in Developing Seeds Include an Obligatory Apoplastic Step”). Sucrose membrane fluxes are consistent with some form of carrier-mediated transport (Patrick, 1997). In coats of developing seeds, anion and cation ion channels mediate efflux of phloem-delivered mineral ions (Zhang et al., 2007). Uptake of sugars and amino acids from sink apoplasts rely on energy-coupled transporters to concentrate these nutrients into storage cells (Patrick, 1997). A turgor homeostat mechanism forms a central plank in coordinating plasma membrane transport fluxes into, and from, apoplasmic spaces of developing seeds with demand by storage cells and phloem import (Zhang et al., 2007).

Absence of cell wall invertase activity during the storage phase of seed development (Weber et al., 1997) reduces the capacity for sucrose symporters and facilitators to function as effluxers (e.g., see Zhou et al., 2007a). In contrast, favorable transmembrane differences in sucrose concentration could

drive release by facilitated diffusion from seed coat efflux cells of wheat (Bagnall et al., 2000) and grain legumes (Ritchie et al., 2003; Zhou et al., 2007a and see Figure 3.4C). However, for efflux cells of developing grain legume seeds, minimal (several mM) differences in sucrose concentration across their plasma membranes raise doubts about contributions sucrose facilitators could make to *in planta* sucrose fluxes (Zhou et al., 2007a). For these seeds, physiological observations are consistent with efflux being mediated by a sucrose/H⁺ antiport mechanism (Zhang et al., 2007).

Aquaporins and Phloem Unloading of Water

In the majority of sinks, water is unloaded from SE–CCs symplasmically by bulk flow thus avoiding any build-up of hydrostatic pressures within SEs in release phloem (Lalonde et al., 2003 and see Figure 3.4). For growth sinks, continued symplasmic water flow of phloem-imported water can drive cell expansion (e.g., root apices—Hukin et al., 2002; Wiegers et al., 2009 and see Figure 3.4A). In postphloem unloading pathways with an apoplastic step, such as fleshy fruit and developing seeds (Figures 3.4B and C), water must exit cells of the unloading path at rates commensurate with those of phloem import (Zhang et al., 2007). These water fluxes are commensurate with being facilitated by aquaporins (Zhou et al., 2007b). In storage sinks, excess phloem water is returned to the parent plant down apoplastic water potential gradients (Pate et al., 1985; Choat et al., 2009 and see Figure 3.4C).

Pressure Flow—Integrating Collection, Transport, and Release Phloem Functions

An overview of phloem translocation on a whole plant context is presented to explore resource flow through collection, transport, and release phloem. A model of one source leaf servicing two sinks is used to provide a framework for this analysis (see Figure 3.5).

Phloem loading by collection phloem is responsible for generating high intracellular osmotic contents of sieve-tube saps (Tables 3.1 and 3.2). The resulting osmotically driven water uptake (Equation 3.3) develops sieve tube hydrostatic pressures of 1.0–1.5 MPa (e.g., Gould et al., 2005). Source-sink differentials in sieve-tube hydrostatic pressures are temporally invariant as reflected by constant translocation fluxes (transport velocities and see Equation 3.2) across the diurnal period (Windt et al., 2006). During the day, transpiration tensions (P_o in Equation 3.3) increase to their largest negative magnitudes in xylem abutting collection phloem (Sack and Tyree, 2005). These are countered by osmoregulatory loading of solutes by SE–CCs (Smith and Milburn, 1980b). During the night, when transpiration tensions approach zero (Equation 3.3), sugar-loading rates, and hence hydrostatic pressures, are buffered by sucrose remobilized from temporary storage reserves located in mesophyll cell chloroplasts and vacuoles (Figure 3.2) along with a greater proportion of potassium loaded into the sieve-tube sap (Smith and Milburn, 1980b). Collectively these activities account for homeostasis of sieve-tube turgors in collection phloem across the photoperiod (Windt et al., 2006). In addition, pressure-concentration waves (e.g., Smith and Milburn, 1980a; Mencuccini and Hölttä, 2010) communicate sink demand to processes regulating loading SE–CCs in collection and transport phloem (Smith and Milburn, 1980b; Ransom-Hodgkins et al., 2003) to sustain near-constant sucrose concentrations of sieve tube saps as water fluxes flowing into and through sieve tubes vary (Smith and Milburn, 1980a).

As illustrated in Figure 3.5, source leaves do not exert any discriminatory influence over partitioning of resources loaded into sieve tubes between competing sinks. Rather collection phloem functions to establish the major component of the hydrostatic pressure head driving bulk flow as shown by direct effects of manipulating phloem loading on translocation fluxes (e.g., Thorpe and

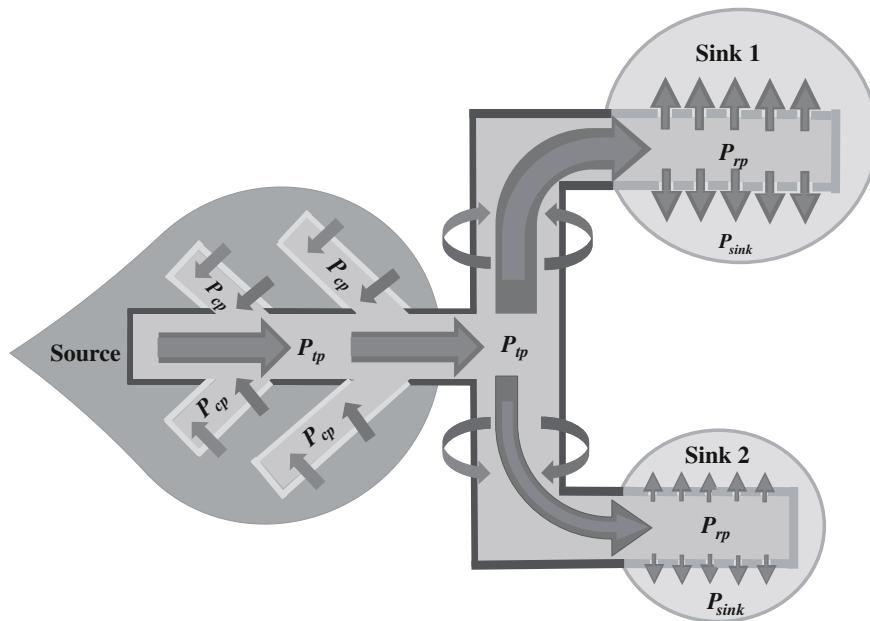


Figure 3.5 High-pressure manifold model of phloem transport and resource partitioning between two competing sinks. Loading of nutrients (mid-grey arrows) and water (dark-grey arrows) in collection phloem (cp ; white border) sets nutrient concentrations (Equation 3.1) and hydrostatic pressure (P_{cp}) to drive bulk flow through sieve tubes linking source and sink (mid-grey arrows superimposed on dark-grey arrows) Equation 3.2. Nutrient concentrations (C) and hydrostatic pressures are maintained homeostatically throughout transport (tp ; dark-grey border) and release (rp ; light-grey border) phloem by turgor-regulated retrieval of nutrients and hence water leaked to phloem apoplasm (curved arrows). Hence, $C_{cp} \sim C_{tp} \sim C_{rp}$ and $P_{cp} \sim P_{tp} \sim P_{rp}$ with a slight hydrostatic pressure gradient from transport/release phloem interface outward into release phloem. Hydraulic conductivities (L_p) or resistances (R where $R = 1/L_p$) of plasmodesmata (radii of plasmodesmata depicted by width of spaces in release phloem border and see Equation 3.2) regulate symplasmic unloading by bulk flow from SEs or SE-CCs. Their low hydraulic conductivities account for large drops in hydrostatic pressures from release phloem SEs (P_{rp}) to vascular parenchyma and sink cells (P_{sink} and see Table 3.2). Thus, overall bulk flow rates of nutrients (R_f) from collection phloem to sinks are determined by:

$$R_f = (P_{cp} - P_{sink} * C) / (R_{cp} + R_{tp} + R_{rp} + R_{sink})$$

Since $R_{sink} \gg R_{cp} + R_{tp} + R_{rp}$ and R_{sink} is regulated, nutrient partitioning to, and between sinks, is largely determined by relative magnitudes of current R_{sink} values of competing sinks. (For a color version of the figure, please see Plate 3.5.)

Minchin, 1987; Gould et al., 2005). In addition, phloem-loading activities of collection phloem set sieve-tube sap concentrations of each transported nutrient species that in turn contributes to their individual rates of translocation (see Equation 3.1). For minor osmotic species, regulation of their concentrations in sieve-tube saps by phloem loading (Table 3.1) is the only direct means by which their rates of transport are controlled (Equation 3.1). This feature is illustrated by the positive correlation between concentrations of amino N compounds in sieve-tube saps and seed protein contents found across three *Brassica* genotypes (Lohaus and Moellers, 2000).

Following export from a source leaf, resources are partitioned between sinks in characteristic patterns that reflect phyllotactic linkages between source leaves and sinks. However, resource allocation patterns are highly flexible as shown by their responses to experimentally imposed alterations in source/sink ratios (Wardlaw, 1990). These findings demonstrate that transport phloem pathways are interconnected throughout plant bodies so as to effectively form a common pool of resources available to all sinks (e.g., Thorpe et al., 2011).

In this context, resource interchange between sieve tubes likely occurs symplasmically through lateral sieve areas, phloem anastomoses (Esau, 1965) or through intervening phloem parenchyma cells (Oross and Lucas, 1985) linking adjacent vascular bundles. In monocots, lateral transfer between primary vascular bundles is restricted to nodal regions (e.g., Patrick and Wardlaw, 1984). Complete interconnectivity of all vascular pipelines in woody plants, and particularly in trees, is undoubtedly more problematic. With this caveat in mind, allocation of resources from a source leaf between competing sinks is determined primarily by relative conductivities of phloem pathways interconnecting a source leaf with a cohort of competing sinks and relative magnitudes of hydrostatic pressures developed at sink ends of phloem pathways (see Figure 3.5).

Bulk flow from source to sink is considered to extend to symplasmic unloading from SE-CCs and possibly some of the post-SE phloem-unloading route (see section entitled “Symplasmic Unloading from SEs—Implication for Resource Flows”). Thus, in evaluating regulatory influences of pathway hydraulic conductivities on translocation flows, consideration needs to be given not only to sieve-tube hydraulic conductivities (e.g., Mullendore et al., 2010) but also to hydraulic conductivities of phloem unloading routes supporting bulk flow. Transport/release phloem are arranged in series with phloem unloading so that their hydraulic resistances (inverse of conductivities) are additive to determine overall rates of bulk flow from collection phloem to sink cells for a given pressure differential (Figures 3.1 and 3.5).

Most attention has focused on estimating hydraulic conductivities of transport phloem sieve tubes (Thompson and Wolniak, 2008; Mullendore et al., 2010). Each sieve tube can be conceptualized as two hydraulic components comprised of an SE lumen and its downstream sieve pores arranged in series. Derived from the Hagen–Poiseuilli law (see Equation 3.2), hydraulic conductivity L is given by

$$L = \pi r^2 / 8\eta l \quad (3.4)$$

For most sieve tubes found in transport phloem, sieve pore radii are predicted to impose the greatest limitation on overall sieve-tube hydraulic conductivities (Thompson and Wolniak, 2008; Mullendore et al., 2010). Their influence diminishes as SE length increases for a given pore geometry (see Equation 3.4).

Highlighting the significance of sieve tube conductivity in phloem transport biology has spawned a renewed interest in phloem anatomy (Thompson, 2006). This has been accompanied by development of innovative tools to preserve *in planta* geometries of delicate sieve-tube substructures from which more valid estimates of sieve-tube hydraulic conductivities can be obtained (Thompson and Wolniak, 2008; Mullendore et al., 2010). Using these tools, a quantitative structural analysis of sieve-tube geometries, located in transport phloem, of eudicot herbaceous life forms yielded estimates of sieve tube hydraulic conductivities that related inversely with phloem transport velocities. Clearly, a finding in contradiction of the Hagen–Poiseuilli law (see Equations 3.2 and 3.4) and, which for some of the life forms examined, predicted nonphysiologically high hydrostatic pressure differentials (Mullendore et al., 2010). These outcomes suggest other, yet to be accounted for, factors exerting significant influence in setting overall hydraulic conductivities of the phloem pathway. Subtle structural factors may exert profound and unanticipated effects on sieve pore hydraulic conductivities. For instance, an *Arabidopsis* mutant of callose synthase (*gsl7*), a gene specifically expressed in SE-CCs, prevents callose deposition lining sieve pores. An absence of sieve pore callose causes a substantial slowing of phloem transport (i.e., decreased sieve tube hydraulic conductivity) in stems to support inflorescence development (Barratt et al., 2011).

Circumspect consideration of a number of physiological observations collectively point to hydraulic conductivities of transport phloem exerting little influence over rates of translocation.

A simple but telling observation is that removal of substantial proportions of transport phloem cross-sectional areas from stems of eudicots (e.g., 66% in cotton—Mason and Maskell, 1928) and monocots (e.g., 50% in wheat—Wardlaw and Moncur, 1976) have little impact on rates of translocation through the narrowed phloem zones. Moreover, indirect but compelling evidence demonstrates that components contributing to hydraulic conductivity of sieve tubes (see Equation 3.4) impose minimal impacts on translocation rates. For instance, sieve-tube sap viscosity varies approximately 2.5 fold across the range of sieve-tube sucrose concentrations (see section entitled “What’s In?—Constituents and Their Concentrations in Sieve-tube Sap”) and hence influences hydraulic conductivity accordingly (see Equation 3.4). Within this physiological range, variations in sieve-tube sap viscosity unlikely impact translocation rates as shown by localized temperature effects on translocation rates. For example, increasing sap viscosity twofold in a localized region of transport phloem by gradually decreasing stem temperatures from 25°C to below 10°C but above freezing point (see Hölttä et al., 2006), results in no, or a transitory, decrease in transport rates through cooled stem zones (e.g., Wardlaw, 1974; Minchin and Thorpe, 1983; Wardlaw and Bagnall, 1981; Peuke et al., 2006). A differing conclusion might apply to RFO translocating species as RFO viscosities are much higher than sucrose (Lang, 1978) and could account for their lower sieve-tube sap concentrations compared to sucrose (Ziegler, 1975). Consistent with sieve-tube conductivity readily accommodating translocation fluxes is the finding that Münch pressure flow, modeled for optimization of translocation speed (i.e., bulk flow flux—see Equation 3.2), predicted sieve tube radius scaled with lengths of collection and transport phloem (Jensen et al., 2011). Experimental measurements of a microfluidic system mimicking phloem pressure flow as well as transport properties of “real” plants, including tall trees, were found to conform with the model prediction (Jensen et al., 2011). Collectively these studies provide some evidence supporting the contention that sieve-tube conductivities do not impose a significant limitation on transport fluxes along phloem pathways, even over considerable lengths of sieve tubes.

Sieve tubes of high hydraulic conductivity are predicted to support rapid propagation of pressure-concentration waves generated by resource unloading in sinks. Pressure-concentration waves move at velocities an order magnitude higher than phloem translocation over considerable distances (m) of phloem pathways (Smith and Milburn, 1980a; Mencuccini and Hölttä, 2010). Such a signaling system is envisioned to underpin unified responses by all SE-CCs, comprising phloem paths from release to collection phloem, to altered resource demand by sinks (Thompson, 2006). These responses are mediated by turgor-regulated membrane transport of sugars into SE-CCs of transport phloem sourced from axial pools (see section entitled “Net Resource Efflux from Transport Phloem”) and into SE-CCs of collection phloem from mesophyll pools (see section entitled “Phloem Loading of Water and SE-CC Water Relations”). The mechanism results in hydrostatic pressure homeostasis in sieve tubes along phloem pathways (Gould et al., 2004b) and could account for differentials in hydrostatic pressures between collection and release phloem not scaling with transport distance, particularly in tall trees (Turgeon, 2010b). Indeed, for the few reported estimates of sieve-tube hydrostatic pressures of monocots and herbaceous eudicot species, those located in collection phloem (e.g., Gould et al., 2005) are of similar magnitude to those found in their transport (Gould et al., 2004b) and release phloem (Pritchard, 1996; Fisher and Cash-Clark, 2000b; Gould et al., 2004a).

By omission, symplasmic bulk flow from SE-CCs of release phloem into surrounding vascular parenchyma cells (Figures 3.4 and 3.5) likely exerts significant regulation over phloem translocation. Direct evidence for this assertion is lacking but a number of indirect experimental observations support it. A spectacular example is a study of translocation rates through pedicels supporting apical fruits in racemes of developing fruits of *Ricinus*. Upon removal of apical fruits, and allowing exudation from their severed petiole stumps to proceed, pedicel translocation rates increased from 166 to 3111 g biomass m⁻² sieve-tube area s⁻¹. This finding suggests that phloem transport rates

are sink not phloem pathway controlled (Kallarackal and Milburn, 1984 and also see Smith and Milburn, 1980a). Hydraulic conductivities of plasmodesmata interconnecting SE-CCs and vascular parenchyma cells appear to control bulk flow to, and into, sinks as inferred from substantial osmotic or hydrostatic pressure differentials found across these interfaces (see Table 3.2 and associated text; Figure 3.5). Dissipation of hydrostatic pressure heads results from sieve-tube sap flowing through plasmodesmal pathways of low hydraulic conductivities (see Equation 3.2) as well as by diluting unloaded phloem saps into larger cell volumes presented by phloem parenchyma cells.

Unloading by bulk flow regulated primarily by hydraulic conductivities of interconnecting plasmodesmata, combined with high sieve-tube turgors being sustained along entire lengths of phloem pathways, led Don Fisher to formulate a high-pressure manifold analogue of phloem transport (Fisher, 2000 and see Figure 3.5). Fisher's model provides valuable mechanistic insights into partitioning of resources between competing sinks. In particular, on the central role played by relative hydraulic conductivities of plasmodesmata linking SE-CCs with adjacent vascular parenchyma cells (see Figures 3.4 and 3.5) determining flow rates through phloem transport pathways to, and into, competing sinks. Direct evidence for such a regulatory role for these plasmodesmata is yet to be demonstrated. However, some insight is obtained as to how the remaining phloem system responds to an increased path hydraulic conductivity by removing the SE-CC to phloem parenchyma cell step by excision to allow direct sap flow from severed sieve-tubes in *Ricinus* stems (Smith and Milburn, 1980a) and pedicels (Kallarackal and Milburn, 1984). At least in the short term (h), the order of magnitude increase in transport fluxes was accompanied by maintenance of sucrose and potassium concentrations in sieve-tube exudates (Smith and Milburn, 1980b). This finding demonstrates that, as implied by the high-pressure manifold model (Fisher, 2000), resource loading along the entire transport pathway, from collection to release phloem, has an excess capacity to meet increases in sink demand. An unaccounted for, but significant component of the model, is whether such a capacity is matched by recycling of phloem-imported water, destined for transpiratory loss from leaves, across sink cell membranes in those organs in which volume growth has ceased (see Figure 3.4C).

Future Directions

The high-pressure manifold model of phloem translocation is far from proven. However, as indicated by findings presented in this review, it provides compelling explanations for many current challenges confronting phloem transport biologists. For instance, it offers a resolution to the observed absence of phloem turgors scaling with transport length (Turgeon, 2010b). We contend the model has sufficient merit to be tested through meeting the challenge of designing and fabricating a microfluidic system in which solution exit from "sieve-tube" equivalents occurs through fine pores (plasmodesmata equivalents) rather than across a membrane (cf. Jensen et al., 2009). Finally, the high-pressure manifold model provides a fresh perspective as to how resource partitioning between competing sinks could be regulated through sink-controlled modulation of plasmodesmal conductivities linking release phloem SE-CC with their surrounding vascular parenchyma cells. This phenomenon deserves further experimental scrutiny to build a more complete understanding of phloem translocation. In working toward this goal, the somewhat neglected area of the regulatory influence aquaporins may exert over phloem water flows is considered to be a fertile area for reaching a holistic understanding of phloem transport.

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Section B

Functional Aspects of Structural SE Components

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4 Roles of Plasma Membrane Transporters in Phloem Functions

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Phloem functions as a supracellular highway for long-distance transport of resources (nutrients and water), toxic elements/compounds and signals (macromolecules, phytohormones, redox reagents and electrical potential waves) coordinating growth, development, plant homeostasis, and defense responses. Apart from macromolecules, phloem transport function includes membrane passage of the above constituents at some point(s) along the phloem transport pathway. These membrane transport events are mediated by transporters localized to plasma membrane (primarily) or tonoplast with the latter functioning in buffering transport fluxes by exchange to, and from, cell vacuoles. Transporter proteins function as carriers (\pm energy coupled) or channels. For each transporter, dependent upon published information, consideration will be given to their intra- and intercellular localization, transport properties (biochemical and biophysical) related to perceived cellular function, and physiological significance (deduced from knockout/knockdown approaches) to achieve an overall phloem transport flux.

Abbreviations: AHA, *Arabidopsis* H⁺-ATPase; AAP, amino acid permease; AKT, *Arabidopsis* potassium transporter; ANT, aromatic-neutral amino acid transporter; AUX, influx efflux carrier; AQP, aquaporin; BAT, bidirectional amino acid transporter; C, organic carbon; CaMV, cauliflower mosaic virus; CAT, cationic amino acid transporter; CAX, cation exchanger; CC, companion cell; CmGAS, *Cucurbita melo* GALACTINOL SYNTHASE; CoYMV, *Commelina* yellow mottle virus; ERD, early-responsive to dehydration; GABA, gamma-aminobutyric acid; GAS, GALACTINOL SYNTHASE; GDU, glutamine dumper; H⁺-ATPase, P-type proton-pumping adenosine triphosphatase; HMA, P-type heavy metal ATPase; HKT, K⁺/Na⁺ transporter; HT, hexose transporter; IC, intermediary cell; INT, myoinositol transporter; IRT, iron transporter; K, potassium channel; KAT, K⁺ transporter; K_m, Michealis-Menten constant; LHT, lysine-histidine-like transporter; MaT, mannitol transporter; MITR, myoinositol transporter; mM, millimolar; MST, monosaccharide transporter; N, nitrogen; NAX, Na-exclusion transporter; NIP, NOD26-like intrinsic protein; NRAMP, natural resistance-associated macrophage protein; NTR, nitrate transporter; OPT, oligopeptide transporter; P1B-ATPases, P-type ATPases; PIN, PIN-FORMED; PIP, plasma membrane intrinsic protein; Pht, phosphate transporter; PLT, polyol transporter; pma, plasma membrane H⁺-ATPase; pmf, proton-motive force; ProT, proline transporter; PTR, tripeptide transporter; PUP, purine permease; RFO, raffinose family oligosaccharide; RNAi, RNA interference; rolC, *Agrobacterium rhizogene* oncogene; SBP, sucrose-binding protein; SE, sieve element; SE-CC, sieve element-companion cell complex; SMM, S-methylmethionine; SOT, sorbitol transporter; STP, sugar transporter; SUC, sucrose carrier; SUF, sucrose facilitator; Sultr, sulfate transporter; SUT, sucrose transporter; T-DNA, transferred DNA; TIP, tonoplast intrinsic protein; TMT, tonoplast monosaccharide transporter; YMV, yellow mottle virus; YSL, yellow stripe-like; VGT, vacuolar glucose transporter; VFK, *Vicia faba* K⁺ transporter; ZIP, Zinc-regulated transporters, iron-regulated transporter-like protein; ZMK, *Zea mays* K⁺ transporter

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The chapter addresses separately membrane transport in relation to phloem transport of resources, toxic elements/compounds, water, and signals but excluding macromolecules. Most attention is focused on resource transport that includes consideration of pre- and postphloem routes of phloem loading and unloading, respectively (for more details, see Chapter 3). Our analysis commences with an evaluation of membrane transporter function in phloem loading of resources.

Membrane Transporters and Loading of Resources in Collection Phloem

Irrespective of the pathway of phloem loading (see Chapter 3), membrane transporters play vital roles in mediating flows of resources into sieve element–companion cell complexes (SE–CCs) of collection phloem. The headwaters of these flows are shaped by transporters located on chloroplast envelopes and tonoplasts of mesophyll cells, regulating flows of organic carbon (C) into cytosolic sugar pools of mesophyll cells.

Membrane Transporters Regulate Cytosolic Pool Sizes of Sugars in Mesophyll Cells Destined for Phloem Loading

Release of photosynthetically produced triosophosphate across chloroplast envelopes is the predominant flux transferring C into the cytosolic sucrose pool of mesophyll cells (Figure 4.1). This membrane transport event occurs as facilitated diffusion mediated by the triose phosphate/phosphate translocator exchanging triose phosphates from chloroplast stromas with orthophosphate derived from cytosolic sucrose biosynthesis (Weber et al., 2005). During the night, the triose phosphate flux is replaced by maltose, and to a lesser extent glucose. These sugars are derived from amyloytic hydrolysis of chloroplast starch and are released to mesophyll cytosols through a chloroplast-envelope-localized maltose MEX1 (maltose excess 1) and glucose transporters, respectively (Zeeman et al., 2007).

Throughout the diurnal cycle, the cytosolic sucrose pool size is buffered by sugar exchange between cytosol and vacuolar pools (Figure 4.1). Participating tonoplast-localized hexose transporters (HTs) include members from three subfamilies of monosaccharide transporters (MSTs) comprising tonoplast monosaccharide transporters (TMTs—Wormit et al., 2006; Cho et al., 2010), vacuolar glucose transporters (VGTs; Aluri and Büttner, 2007), and early-responsive to dehydration-like transporters (ERD6s; Yamada et al., 2010). Excess cytosolic sugars are loaded into vacuoles by energy-coupled transport mediated by TMTs (Wormit et al., 2006; Cho et al., 2010) and VGTs (Aluri and Büttner, 2007). Patch clamp studies in *Arabidopsis* show that TMTs in native membranes function as proton antiporters capable of transporting both hexose (Wingenter et al., 2010) and sucrose (Schulz Beyhl et al., 2011). When C exchange from chloroplasts slows, vacuolar sugars are released to augment cytosolic sucrose pools (Figure 4.1). ERD6 family members, functioning as hexose facilitators (Antony et al., 2008; Yamada et al., 2010), could fulfill such a role for glucose and fructose. Clade 4 family members of sucrose transporters (SUTs or SUCs; see Chapter 5 for phylogenetic details) of *Arabidopsis*, barley (*Hordeum vulgare*; Endler et al., 2006), and poplar (*Populus tremula × alba*; Payyavula et al., 2011) are localized to tonoplasts of mesophyll cells. These function as sucrose symporters (Weise et al., 2000; Weschke et al., 2000; Payyavula et al., 2011). As demonstrated by knockdown of poplar SUT4, *PtaSUT4*, tonoplast-localized SUT4s play a key physiological role in transfer sucrose from vacuole to cytosol to sustain sucrose levels available for phloem loading (Payyavula et al., 2011).

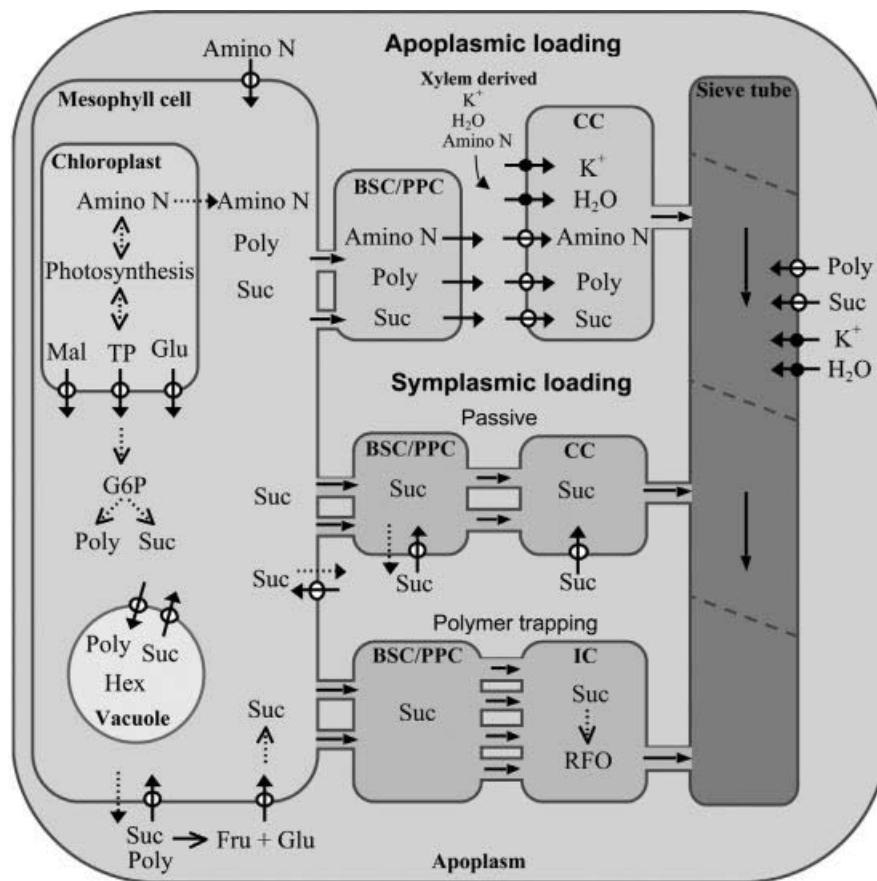


Figure 4.1 Cellular localization of resource membrane transporters located along phloem-loading pathways from mesophyll cells to sieve tubes of leaf minor veins in relation to mechanisms of apoplastic and symplasmic (passive and polymer trapping) phloem loading. Resources arising from photosynthesis (sugars and amino N compounds) in mesophyll chloroplasts are released to mesophyll cell cytosols through carriers located in their chloroplast envelopes (arrow through open circle). Carrier-mediated exchange of sugars, to and from vacuolar storage, buffers cytosolic pools of sugars available for loading. Irrespective of the phloem-loading mechanism, resources flow symplasmically from mesophyll to bundle sheath/phloem parenchyma cells (BSC/PPC). Sugars passively leaked to the leaf apoplasm (arrow with dashed line), during symplasmic passage, are retrieved by energy-coupled carriers (arrow through open circle). Upon reaching BSC/PPC, and depending upon the loading mechanism, resources either are exchanged via minor vein apoplasts through release by putative efflux carriers (black arrow with wide head), and retrieval by energy-coupled carriers located on sieve element (SE)-companion cell complexes (SE-CCs) (apoplastic loading) or continue to move through the minor vein symplasm to reach sieve tubes (symplasmic loading). Symplasmic loading may occur passively or by active symplasmic entrainment of Raffinose family oligosaccharides (RFO) synthesized from sucrose in modified companion cells (polymer trap), intermediary cells (ICs), with subsequent movement through large-diameter plasmodesmata interconnecting ICs to SEs. Irrespective of the loading mechanism, xylem delivered potassium (K^+) and water (H_2O) are loaded into SE/CC through channels (arrow through filled circle) along with energy-coupled carriers mediating uptake of amino N compounds. Flu, fructose; Glu, glucose; Hex, hexose; Mal, maltose; Poly, polyol; K^+ , potassium; Suc, sucrose; TP, triose phosphate. (For a color version of the figure, please see Plate 4.1.)

Sugars Are Retrieved into Mesophyll Cells during Their Symplasmic Transit to Collection Phloem

High symplasmic concentrations of sugars in mesophyll cells probably drive a passive diffusional leak to their surrounding apoplasm (see Chapter 3 and Figure 4.1). Leaked sucrose can be hydrolyzed by cell wall invertases and as a consequence regulate levels of sucrose available for export (Kocal et al., 2008). Apoplastic hexoses are retrieved (Figure 4.1) by sugar transporter (STP) subfamily members expressed in mesophyll cells of *Arabidopsis* (*AtSTP3*—Büttner et al., 2000; *AtSTP13*—Schofield et al., 2009) and rice (*Oryza sativa*—*OsMST6*; Wang et al., 2008). These transporters encode functional hexose/proton symporters. *Atstp13* knockout mutant (Nørholm et al., 2006) or an overexpression line (Schofield et al., 2009) did not produce an observable phenotype, suggesting functional redundancy within the STP subfamily.

Currently, there is no direct molecular evidence for expression of SUT or polyol transporters (PLT) in leaf mesophyll cells. This is especially surprising for species that passively load sucrose and polyols that rely on an aggressive retrieval of leaked sugars from their high concentrations in mesophyll cytosols (see Chapter 3). For instance, apple (*Malus domestica*) appears to load sorbitol passively (Reidel et al., 2009). However, all three isoforms of sorbitol transporters (SOTs) were detected exclusively in minor vein phloem (Watari et al., 2004). Some insights have been obtained for SUTs (Figure 4.1). Comparison of StSUT1 protein levels detected in, and transporter activities of, plasma membrane vesicles isolated from leaves of *StSUT1* antisense lines under control of 3SS (constitutive) and *rolC* (*Agrobacterium rhizogene* oncogene; CC specific) promoters indicated that mesophyll cells had StSUT1 activity (Lemoine et al., 1996). However, since whole plant phenotypes were identical for the two transgenic lines, this finding suggests that StSUT1s expressed in leaf CCs are crucial for phloem loading with retrieval by mesophyll-expressed StSUT1 serving a subsidiary role (Kühn et al., 1996). Immunocytochemical localization of a sucrose-binding protein (SBP) to paraveinal mesophyll cells suggested these cells engage in sucrose retrieval from the leaf apoplasm during passage to SE–CCs (Lansing and Franceschi, 2000).

Sugar Release in Apoplastic Phloem Loading Species

One outstanding omission from current knowledge of STP function in apoplastic phloem loading species (see Chapter 3 and Figure 4.1) is the molecular identity of sucrose and PLTs responsible for sugar release from bundle sheath/phloem parenchyma cells prior to their loading into SE–CCs. One possible scenario is reversal of sugar/proton symporters under conditions where potential energies of transmembrane differences in sugar concentrations exceed that of the opposing proton motive force (*pmf*; Carpaneto et al., 2005). In this context, it is tantalizing to consider *Arabidopsis* AtSUC3/SUT2 (Meyer et al., 2000) and sugarcane (*Saccharum officinarum*) ShSUT1 (Rae et al., 2005) as sucrose effluxer candidates (Carpaneto et al., 2005) as both proteins localize to bundle sheath/phloem parenchyma cells. Significantly, ShSUT1 shares similar kinetic properties with HvSUT1 (Sivitz et al., 2005) that is expressed in nucellar projection transfer cells of developing barley caryopses committed to sucrose efflux (Weschke et al., 2000). No equivalent cellular localization in minor veins has been reported for PLTs.

H^+ -ATPases: Membrane Potential Difference, ΔpH , and Alkalinization of Phloem Sap

Except for calcium, sieve-tube sap is characterized by each solute being held at concentrations exceeding those in the surrounding vascular apoplasm by one or more orders of magnitude (see

Chapter 3, Table 3.1). High solute concentrations in SE–CCs are linked with their large negative membrane potentials ranging from –120 to –180 mV (e.g., Wright and Fisher, 1981; Deeken et al., 2002; Carpaneto et al., 2005; Hafke et al., 2005). These membrane potentials equate with cells capable of supporting large solute fluxes across their plasma membranes moving down electrochemical gradients (positively charged solutes) or negatively charged solutes and nonelectrolytes moving in symport with protons returning down their electrochemical gradients (*pmf*—Duby and Boutry, 2009).

The active component of membrane potentials in all plant cells is generated by electrochemical extrusion of protons by plasma membrane H⁺-ATPases (Duby and Boutry, 2009). Therefore, H⁺-ATPases are likely to play significant roles in solute loading by collection phloem and in retaining high solute concentrations in transport phloem. Surprisingly, there is a dearth of information on phloem-localized H⁺-ATPases and particularly so for collection phloem. Using lead precipitation to detect ATPase activity, Cronshaw (1980) reported evidence of plasma membrane localized ATPases in SEs of tobacco (*Nicotiana tabacum*) and CCs of pea (*Pisum sativum*). An immunocytochemical study discovered H⁺-ATPases localized to CCs, modified to transfer cell morphology, in collection phloem of another apoplastic loading species, broad bean (*Vicia faba*; Bouché-Pillon et al., 1994). Interestingly, higher densities of H⁺-ATPases decorated wall ingrowths through which solutes likely were loaded (Bouché-Pillon et al., 1994). Substantial plasmodesmal connectivity between CCs and SEs is anticipated to support comparable levels of electrical connectivity (van Bel, 1996). Thus, large negative membrane potentials recorded for SEs (e.g., Wright and Fisher, 1981; Deeken et al., 2002; Carpaneto et al., 2005; Hafke et al., 2005) indicate that SEs and CCs share equal potentials to support high fluxes of solutes across their plasma membranes.

Of the eleven P-type H⁺-ATPases encoded by *Arabidopsis*, four are expressed in leaves (*AHA1*, *AHA2*, *AHA3*, and *AHA11*—Alsterfjord et al., 2004), with *AHA3* showing expression in CCs of collection phloem (DeWitt et al., 1991; DeWitt and Sussman, 1995). For *Nicotiana plumbaginifolia* (Tex-Mex tobacco) leaves, the two most highly expressed P-type H⁺-ATPase genes, *PMA2* and *PMA4*, exhibit overlapping expression patterns in vascular parenchyma cells as well as SE–CCs of minor and major veins. In addition, their expression in a number of vascular cell types may serve several transport functions such as energizing apoplastic loading/retrieval into SE–CCs as well as xylem–phloem solute transfer (Morlau et al., 1999 and see Figure 4.1). H⁺-ATPase isoforms exhibit differing kinetic properties adding to the array of specific functions their coexpression may confer to SE–CCs (Arango et al., 2003). Consistent with a function in driving apoplastic loading of sucrose by proton symport (Lalonde et al., 2003), cosuppression of *PMA4* caused a significant reduction in sucrose translocation accompanied by sugars accumulating in mature leaves of transgenic plants (Zhao et al., 2000).

Membrane Transporters Loading Sucrose, the Ubiquitous Phloem Sugar, into SE–CCs

SUTs/SUCs have been cloned and functionally characterized from a cohort of species that function as putative apoplastic phloem loaders (Kühn and Grof, 2010). All SUT family members have been functionally characterized as sucrose/proton symporters except several members from pea and French bean (*Phaseolus vulgaris*) that function as sucrose facilitators (SUFs; Zhou et al., 2007a).

Eudicot Sucrose Transporters

The SUT1 clade is eudicot specific (for further information, see Chapter 5) and includes functionally characterized sucrose symporters from *Arabidopsis* (AtSUC1; AtSUC2; AtSUC5; AtSUC9), plantain (*Plantago major*; PmSUC1; PmSUC2), potato (*Solanum tuberosum*; StSUT1), tobacco

(NtSUT1) and tomato (*Solanum lycopersicum*; LeSUT1) (Braun and Slewinski, 2009; Kühn and Grof, 2010). *AtSUC2*, *PmSUC2*, and *SUT1s* from solanaceous species are strongly expressed in leaves with high transcripts levels localized to minor veins (*StSUT1*—Riesmeier et al., 1993) and specifically to CCs (*AtSUC2*—Truernit and Sauer, 1995; Schulze et al., 2003; *PmSUC2*—Stadler et al., 1995; *NtSUT1* and *StSUT1*—Kühn et al., 1997). Immunocytochemical findings suggest that *PmSUC2* and *AtSUC2* proteins are localized to minor vein CCs (Stadler et al., 1995; Stadler and Sauer, 1996) in contrast to solanaceous SUTs that localize to SEs (Kühn et al., 1997; Barker et al., 2000; Reinders et al., 2002 and see Figure 4.1). Differences in SUT immunolocalization may arise from technical discrepancies of sera properties and preparation along with choice of fixation chemicals altering protein antigenicity of SUT1 proteins (Schmitt et al., 2008). Alternatively, different plant species might have developed different strategies to maintain high sucrose concentrations in the phloem. Transport studies using CC and SE protoplasts may help to resolve this question (Ivashikina et al., 2003; Hafke et al., 2007).

Sucrose fluxes across plasma membranes of SE–CCs of sugar beet minor veins approach maximal values for membrane transport (Geiger and Cataldo, 1969). On these grounds, it would not be surprising to find the entire SE–CC, rather than only one of the cell partners, participates in sucrose uptake. In this context, AmSUT1 in the mixed loader (sucrose and Raffinose family oligosaccharides (RFO)) maskflower (*Alonsoa meridionalis*) localizes to both SEs and CCs (Knop et al., 2004). In plantain and *Arabidopsis*, members of the SUT2 clade, *PmSUC3* (Barth et al., 2003), and *AtSUC3* (Meyer et al., 2004) localize to SEs of their transport phloem, thus complementing CC localization of their SUT1 counterparts. On the basis of similar functions of sugar retrieval by collection and transport phloem, it is likely that this localization pattern also applies to minor veins.

In addition, sucrose affinities of SUT clade members are complementary to ensure optimal rates of sucrose uptake by SE–CCs from apoplasmic sucrose concentrations that vary diurnally across their range of apparent K_m (Michalealis-Menton constant) values. The same physiological outcome applies to colocalization of SUT1 with SUT2 and SUT4 paralogs of solanaceous species in minor vein SEs (Reinders et al., 2002). However, the universality of a model with three SUTs being present in SE–CC plasma membranes is contested by findings from nonsolanaceous species. These data show that, in comparison to GFP-tagged *StSUT4* localizing to plasma membranes (Chincinska et al., 2008), GFP-tagged *AtSUT4* (Endler et al., 2006), *LjSUT4* (Reinders et al., 2008), and *PtaSUT4* (Payyavula et al., 2011) are tonoplast targeted.

Physiological significance of each SUT clade member in phloem loading has been explored using molecular genetic approaches of knockdowns by antisense or RNA interference (RNAi) technologies or by knockout mutants. Consistent with a blockade of apoplasmic phloem loading, antisense *StSUT1* transformants of potato (Riesmeier et al., 1994; Kühn et al., 1996; Lemoine et al., 1996), *NtSUT1* of tobacco (Bürkle et al., 1998; Zhang and Turgeon, 2009) and *LeSUT1* of tomato (Hackel et al., 2006) exhibit a characteristic leaf phenotype of elevated levels of soluble sugars and starch. Transferred DNA (T-DNA) insertional mutants of *AtSUC2* evince phenotypes of perturbed phloem loading comparable to those of solanaceous species (Gottwald et al., 2000).

Driving antisense expression by the CC-specific *rolC* promoter elicited an identical phenotype to transgenic potato lines in which antisense *StSUT1* expression was under control of the constitutive *CaMV 35S* promoter (Kühn et al., 1996). Together with the finding that the phenotype arose from a shoot effect, deduced from reciprocal grafts between wild-type and antisense *rolC* lines, these observations point to *StSUT1* activity playing a key role in apoplasmic phloem loading of sucrose in potato (Kühn et al., 1996) and in other solanaceous species (Bürkle et al., 1998; Hackel et al., 2006). This conclusion is consistent with partial reversal of the *StSUT1* antisense phenotype by overexpressing spinach (*Spinacea oleracea*) *SUT1* (*SoSUT1*—Leggewie et al., 2003).

An issue further refined in an elegant study where a homozygous *atsuc2* mutant background was complemented specifically in collection phloem by expressing *AtSUC2* under control of a collection-phloem specific melon (*Cucumis melo*) *GALACTINOL SYNTHASE* promoter (*CmGAS1p*). Complementation was demonstrated by a partial restoration to a wild-type phenotype providing irrefutable evidence for *AtSUC2* facilitating loading of collection phloem (Srivastava et al., 2008). Instructive however was the finding that, despite knocking out the functional *AtSUC2* protein (Srivastava et al., 2009a), *atsuc2* mutants exhibited some capacity for phloem transport of sucrose (Gottwald et al., 2000; Srivastava et al., 2008) including completion of their life cycle (Srivastava et al., 2009a). Although this residual transport is yet to be accounted for (Srivastava et al., 2009a), contributions by SUTs from other clades are a possibility.

SUT1 clade members share expression in SE-CCs with members from SUT2 and SUT4 clades in solanaceous species (e.g., Reinders et al., 2002) and the SUT2 clade in *Arabidopsis* (*AtSUC3*—Meyer et al., 2004). An absence of a phloem-loading phenotype when SUT2 expression levels were downregulated by antisense knockdown in tomato (Hackel et al., 2006) or by T-DNA insertional knockout in *Arabidopsis* (Meyer et al., 2004) indicates that SUT2s play no direct role in phloem loading. In contrast, knockdown of *StSUT4* expression resulted in enhanced rates of sucrose export from transgenic leaves possibly mediated through posttranslational regulation by heterodimerization of *StSUT1* with *StSUT4* (Chincinska et al., 2008 and for further information, see Chapter 5). Overall, SUT1 clade members appear to exert primary control over phloem loading with solanaceous SUT4 clade members modulating SUT1 activities.

A number of overexpression studies have explored whether maximal activities of SE-CC localized SUT1s exert coarse control of phloem loading. Overexpressing *SoSUT1* in potato had no impact on rates of phloem loading despite elevated symporter activity detected in plasma membrane vesicles prepared from transgenic leaves (Leggewie et al., 2003). Similarly, enhanced phloem-loading potential through overexpressing *AtSUC2*, under control of the CC-specific *Commelin yellow mottle virus* (*CoYMV*) promoter, did not elicit a growth phenotype nor elevated rates of phloem export from source leaves (Srivastava et al., 2009b). An absence of any impact on phloem loading to overexpressing SUT1s is suggestive that maximum activities of endogenous SUT1s offer spare transport capacity in contrast to predictions made by top-down analyses of photoassimilate flows from source to sink (cf. Sweetlove et al., 1998).

Sucrose is a component of the translocated sugar profile of species that symplasmically load RFOs (Turgeon and Wolf, 2009 and see Figure 4.1). Many of these species show a mixed collection-phloem anatomy with two types of CCs present in their collection phloem—intermediary cells (ICs) responsible for RFO synthesis and ordinary CCs (e.g., Knop et al., 2004). Ordinary CCs and accompanying SEs contain SUTs in their plasma membranes (Knop et al., 2004). Knockdown of *SUT1* expression and activity in such a species, mullein (*Verbascum phoeniceum*), did not result in the characteristic leaf phenotype linked with blocking phloem loading (Zhang and Turgeon, 2009). Such an outcome is consistent with an absence of an apoplastic phloem loading step (Zhang and Turgeon, 2009) or possibly a compensatory increase in sucrose flow through the RFO polymer trap mechanism (see Knop et al., 2004).

Monocot Sucrose Transporters

Except for early work with palms (van Die and Tammes, 1975), most studies of phloem translocation in monocots have focused on grasses of agronomic importance and particularly cereals (rice, wheat, barley, and maize) and sugarcane that are considered to load their phloem via an apoplastic route (Braun and Slewinski, 2009).

Clade 3 SUT members from barley (*HvSUT1*—Weschke et al., 2000), rice (*OsSUT1* and *OsSUT3*—Aoki et al., 2003), maize (*Zea mays*; *ZmSUT1*—Aoki et al., 1999), sugarcane (*ShSUT1*—Rae et al., 2005) and wheat (*Triticum aestivum*; *TaSUT1*—Aoki et al., 2002) are expressed in leaf blades. Cellular localization of these SUT proteins appears to be species specific. For instance, and in contrast to all other monocot SUTs, *ShSUT1* is most strongly linked with mestome sheath and vascular parenchyma cells and is absent from SE–CCs (Rae et al., 2005). The remaining monocot SUT3s are phloem localized with *TaSUT1* restricted to SEs alone (Aoki et al., 2004) in comparison to *OsSUT1* that is present in SEs and CCs (Scofield et al., 2007). For sugarcane, a SUT homolog(s) was detected in SE–CCs (Rae et al., 2005). Cellular localization of SUT3 members for maize and barley remains to be determined.

SUT3 clade members exhibit a strong preference for sucrose over other sugars and their apparent K_m values for sucrose range from 2 to 10.6 mM. *In planta*, SUT3 clade members could operate below their maximal transport velocities since leaf apoplasmic sucrose concentrations for barley and maize of 0.9–2.6 mM (Heyser et al., 1978; Lohaus et al., 2001) are at the lower end of their K_m range. For rice, *OsSUT1* (Sun et al., 2010) with an apparent K_m value of 7.5 mM sucrose is complemented by all other SUTs that are expressed to varying degrees in source leaves (Aoki et al., 2003). For instance, *OsSUT5* has an estimated apparent K_m value of 2.3 mM (Sun et al., 2010). Whether additional SUTs in other grass species function in this way is unknown.

In contrast to more extensive studies in eudicots, testing physiological roles of SUT3 clade members as contributors to apoplasmic phloem loading in grass leaves have received less attention. Antisense knockdown of *OsSUT1* expression in rice did not elicit the classic phloem-loading phenotype, suggesting *OsSUT1* played no role in phloem loading (Ishimaru et al., 2001; Scofield et al., 2002) despite being present in SE–CCs of all classes of leaf vein (Scofield et al., 2007). The reason for this outcome is unknown. In contrast, a knockout mutant of the homologous *ZmSUT1* of maize exhibits a phloem-loading phenotype (Slewinski et al., 2009). Similar to observations with *atsuc2* knockouts (Srivastava et al., 2009a), the maize mutant was capable of completing its life cycle, indicating other auxillary phloem-loading mechanism(s) operate in parallel with *ZmSUT1* (Slewinski et al., 2009).

Polyol and Inositol Transporters and Their Role(s) in Phloem Loading

Membrane transport of linear polyols likely serves a retrieval role in woody species, while in herbaceous species it plays a more direct role in apoplasmic phloem loading (Figure 4.1, and for more details, see Chapter 3). Current evidence at this scale is lacking for cyclic polyols (e.g., myoinositol).

PLT homologs have been identified in sequenced genomes of both eudicots (Büttner, 2007) and rice (Johnson and Thomas, 2007). Surveys of sieve-tube saps have not detected appreciable levels of linear polyols in monocots (grasses and palms) possibly indicating their transporters unlikely play a significant role in phloem translocation. However, such a conclusion needs to be treated with caution. For example, although linear polyols are not detected in sieve-tube saps of *Arabidopsis* (Haritatos et al., 2000), the *Arabidopsis* genome contains six putative linear PLTs (Büttner, 2007) of which one, *AtPLT5*, is strongly expressed within leaf veins (Klepek et al., 2005). A hint that PLTs could serve a more generalized monosaccharide transport role is indicated by their low substrate specificities for monosaccharides (Noiraud et al., 2001; Gao et al., 2003; Ramsperger-Gleixner et al., 2004; Watari et al., 2004; Klepek et al., 2005; Conde et al., 2007; Juchaux-Cachua et al., 2007; Klepek et al., 2010). However, as found for other STP subfamily members (Sherson et al., 2000; Leterrier et al., 2003), a T-DNA knockout of *AtPLT5* did not evince a phenotype (Klepek et al., 2005).

Cloned linear PLTs function as polyol/proton symporters for a number of species known to translocate significant levels of polyols. This cohort includes celery (*Apium graveolens*; *AgMaT1*, Noiraud et al., 2001; *AgMaT2*, Juchaux-Cachua et al., 2007), sour cherry (*Prunus cerasus*; *PcSOT1*, *PcSOT2*, Gao et al., 2003), apple (*MdSOT3*, *MdSOT5*, Watari et al., 2004), common plantain (*PmPLT1*, *PmPLT2*, Ramsperger-Gleixner et al., 2004) and olive (*Olea europaea*; *MT1*, Conde et al., 2007). Of these transporters, *AgMaT1* (Noiraud et al., 2001), *AgMaT2* (Juchaux-Cachua et al., 2007), *MdSOT3*, *MdSOT5* (Watari et al., 2004), *PmPLT1* and *PmPLT2* (Ramsperger-Gleixner et al., 2004; Pommerenig et al., 2007) are strongly expressed in veins of source leaves. For celery and common plantain, a vascular localization of PLTs (Noiraud et al., 2001; Ramsperger-Gleixner et al., 2004; Juchaux-Cachua et al., 2007) is indicative of a role in apoplastic loading (Reidel et al., 2009). Furthermore, both *PmPLT1* and *PmPLT2* colocalize to CCs with *PmSUC2* (Ramsperger-Gleixner et al., 2004; Pommerenig et al., 2007). In contrast, in collection phloem of celery source leaves, *AgMaT2* was detected in SEs, CCs and vascular parenchyma cells (Juchaux-Cachua et al., 2007). CC and SE-CC localizations suggest a role of these transporters in phloem loading. Presence of *AgMaT2* in vascular parenchyma cells (Juchaux-Cachua et al., 2007) may indicate a role in facilitating efflux of mannitol to the phloem apoplasm by carrier reversal (Carpaneto et al., 2005) or a subsidiary storage function.

The apparent K_m value for common plantain *PmPLT1* of 12.3 mM (*PmPLT1* nonsaturable up to 20 mM—Ramsperger-Gleixner et al., 2004) matches an apoplastic sorbitol concentration in their leaves of 5.5 mM (Nadwodnik and Lohaus, 2008). Such concurrence points to *PmPLT1*, but not *PmLT2*, functioning near its maximal velocity. Apparent K_m values for *AgMaT1* (340 μ M—Noiraud et al., 2001) and *AgMaT2* (1.8 mM—Juchaux-Cachua et al., 2007) are complementary and cover a tenfold range of apoplastic mannitol concentrations. Comparing these values with estimated mannitol apoplastic concentrations of 6.7 mM (Nadwodnik and Lohaus, 2008) in celery leaves suggests that mannitol carriers also function at their maximal velocities *in planta* to actively load collection phloem.

Cyclic polyols, and in particular myoinositol, are phloem mobile (Chapter 3). The *Arabidopsis* genome contains four putative myoinositol transporters (INT) of which one is a pseudogene (*AtINT3*) and the remaining three encode functional inositol/proton symporters (Schneider et al., 2006, 2007, 2008). *AtINT2* (Schneider et al., 2007) and *AtINT4* (Schneider et al., 2006) target to plasma membranes, while *AtINT1* is tonoplast associated and regulates inositol release from vacuolar storage (Schneider et al., 2008). Both *AtINT2* and *AtINT4* are expressed in leaves with *AtINT2* expressed in all cell types including vascular cells (Schneider et al., 2007). In contrast, *AtINT4* expression is phloem localized being most strongly expressed in CCs (Schneider et al., 2006). Absence of a clear phenotype for single or double *atint* mutants (Schneider et al., 2006, 2007) leaves the functional significance of *AtINT2* and *AtINT4* unknown. Their cellular localization is consistent with possible roles in phloem loading and/or retrieval of mesophyll- and CC-synthesized inositol (Nelson et al., 1998).

Potassium Channels: Working in Harmony with Sugar Transporters to Drive Phloem Translocation

Potassium is the most abundant ionic species present in sieve-tube sap (see Chapter 3) and, similar to sodium, is held close to its electrochemical equilibrium. Indeed potassium channels play a key role in conducting ion currents across plasma membranes of SE-CCs as shown by manipulating apoplastic concentrations of potassium to cause Nernst predicted depolarizations of their membrane potentials (Wright and Fisher, 1981; Ache et al., 2001).

Members of the shaker family of potassium channels are expressed in leaves and are phloem-localized (Lebaudy et al., 2007). Amongst these, membrane proteins are a subfamily of weakly rectifying potassium channels, inhibited by extracellular acidification and encoded by *AKT2/AKT3* genes in *Arabidopsis* (Marten et al., 1999; Lacombe et al., 2000), maize (*ZMK2*; Philipp et al., 1999; Bauer et al., 2000) and broad bean (*Vicia faba*; *VFK1*; Ache et al., 2001). In broad bean, *VK1* exerts a major influence on electrical conductances of SE-CC plasma membranes (Ache et al., 2001 and also see Deeken et al., 2002). An *Arabidopsis*, T-TDNA insertional mutant of *AKT2/AKT3* elicits an impaired growth phenotype demonstrating a regulatory role of *AKT2/AKT3* in phloem loading and retrieval of photoassimilates (Deeken et al., 2002). An inward-rectifying potassium channel, *KAT2*, selectively coexpresses with *AKT2/AKT3* in leaf phloem of *Arabidopsis* (Pilot et al., 2001). A similar relationship exists for the At*KAT2* homolog in maize *KZM1* (Philipp et al., 2003).

Phloem Loading of Water and Aquaporins

On the basis of their sequence homology and to a less extent, their subcellular localization, aquaporins (AQPs) are divided into plasma membrane *intrinsic proteins* (PIP_s), tonoplast intrinsic proteins (TIP_s), *NOD26*-like *intrinsic proteins* (NIP_s), and small basic *intrinsic proteins* (SIP_s) (Maurel et al., 2009).

Relevant to phloem loading, PIP_s and TIP_s facilitate water flows across plasma and tonoplast membranes, respectively (Maurel et al., 2009). Consistent with this function, in mature maize leaves, ZmPIP_s are highly expressed in mesophyll, bundle sheath, and CCs with their abundance elevated during the light period (Hachez et al., 2008) coincident with maximal phloem-loading rates. SoPIP1;2 is localized to plasma membranes of source leaf SEs of the apoplasmic loader, spinach (Fraysse et al., 2005). These cellular localizations suggest that water influx for active phloem loaders may be dissipated across both CCs and SEs, thus dampening plasmodesmal water fluxes from CC to SE that might otherwise excessively deplete CC soluble content (Figure 4.1). TIP_s are expressed in CCs (e.g., Barrieu et al., 1998) as well as mesophyll and bundle sheath cells (Heinen et al., 2009). Thus, TIP_s could act to maintain osmotic equilibrium between cytosols and vacuoles of these cells, supporting high-symplasmic water fluxes associated with phloem loading (Chapter 3). Mature SEs lack vacuoles and hence, not surprisingly, TIP_s.

Nitrogen (and in Some Circumstances Sulfur) Are Primarily Accumulated into the Phloem by Amino Acid Transporters

Amino acids are the major N form transported via the phloem to developing sinks (see Chapter 3). Depending on plant species and environmental conditions, amino-N compounds are synthesized in roots and/or shoots (Coruzzi, 2003). Root-synthesized amino acids are transported in the xylem mainly to fully expanded leaves due to their high transpiration rates. Here, they are imported into mesophyll cells by lysine-histidine-like transporters (LHT1; Hirner et al., 2006) or immediately loaded into phloem for source-sink transport (Figure 4.1). This phloem-loading step primarily occurs in leaf major veins mediated by amino acid permeases (e.g., AAP2; Zhang et al., 2010). However, some root-derived amino acids may be transferred from xylem to phloem along the translocation pathway (Figure 4.2; see Chapter 3).

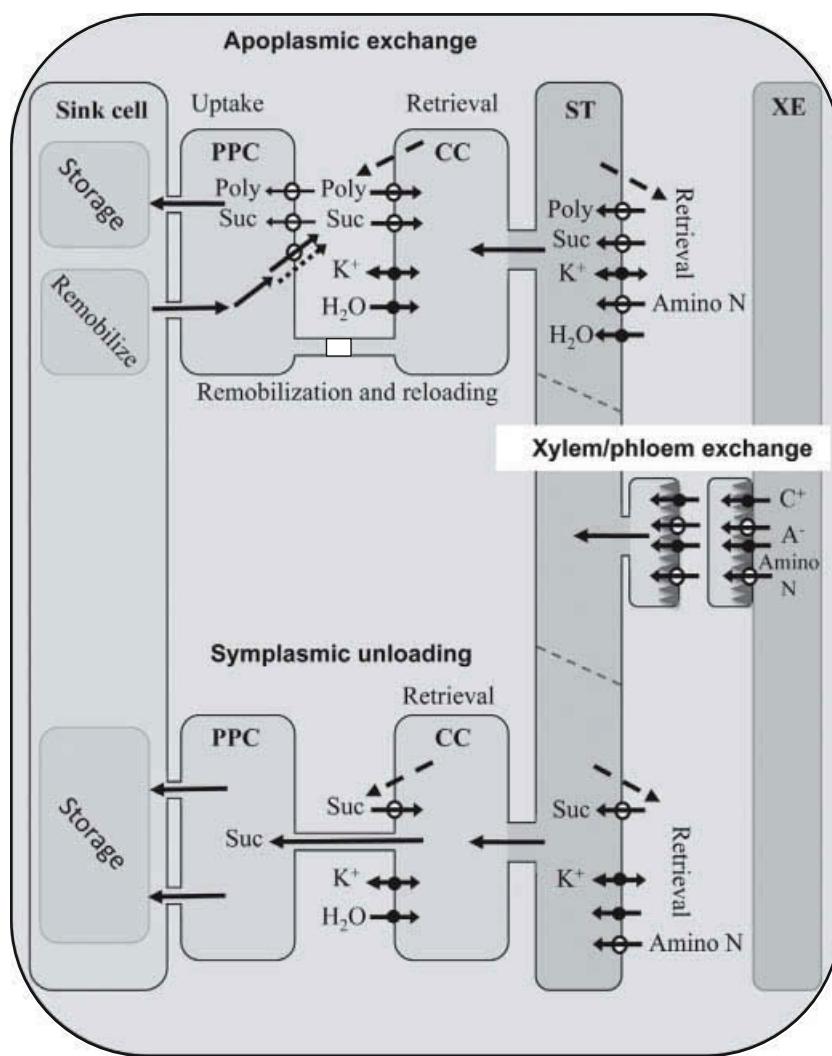


Figure 4.2 Resource membrane transporters located in cells of the transport phloem and postphloem pathways supporting apoplastic exchange, xylem-phloem exchange and symplasmic unloading. Apoplastic exchange predominates resource flows between transport phloem and surrounding sink tissues when plasmodesmata interconnecting companion cell (CC) and phloem parenchyma cell (PPC) are gated/closed (white rectangle). Apoplastic exchange comprises three primary membrane transporter functions. These are retrieval of resources by SE-CCs leaked from the transport phloem, nutrient uptake into PPCs with subsequent symplasmic delivery to sink cells for storage/growth and net loading of SE-CCs with nutrients remobilized from storage in sink cells and released to the leaf apoplasm. Reloading nutrients into SE-CCs is accompanied by an osmotic uptake of water. Nutrient xylem-phloem exchange, particularly pronounced at nodal regions, involves nutrient (ions and amino N compounds) captured from the transpiration stream and delivery into SE-CCs with probable engagement of at least two consecutive apo-/symplasmic exchanges. Symplasmic unloading occurs under high source/sink ratios when excess nutrients are stored in sink cells. Retrieval of resources, leaked from SE-CCs, occurs in parallel with symplasmic unloading. Transporters include sugar and anion carriers ± energy-coupled (arrow through open circle) and sugar symporters functioning in reverse (double arrow through open circle) as well as channels supporting water (single arrow through closed circle) and two way-exchange of potassium (double arrow through closed circle). Sugar leakage to the apoplasm occurs by passive movement through plasma membranes (arrow with dashed line). A⁻, anions; C⁺, cations; H₂O, water; K⁺, potassium; SE, sieve element; Poly, polyol; ST, sieve tube; Suc, sucrose; XE, xylem element. (For a color version of the figure, please see Plate 4.2.)

Amino acids synthesized in leaves, or derived from photorespiration and leaf protein hydrolysis (Buchanan-Wollaston, 1997; Rachmilevitch et al., 2004), are imported into SE-CCs of minor veins (Tegeder and Rentsch, 2010). Depending on the plant species and the frequency of plasmodesmata, amino acid phloem loading follows the apoplastic or symplasmic pathway (Chapter 3). In the symplasmic route, amino acids move to the phloem down their concentration gradient. In *Arabidopsis*, and most temperate-zone crop plants, amino acid import into SE-CCs occurs via an apoplastic-loading route (Delrot et al., 2001; Lalonde et al., 2003). First, amino acids are released into the leaf apoplasm (Figure 4.1) by an unknown, presumably passive mechanism (Lohaus et al., 1994, 1995) that might involve bidirectional amino acid transporters (BATs) such as AtBAT1 (Dündar and Bush, 2009), GDU (glutamine dumper) proteins regulating amino acid export by activating nonselective amino acid carriers (Pilot et al., 2004; Pratelli and Pilot, 2006; Pratelli et al., 2010) or yet to be characterized transporters with homologies to mammalian, bacterial or yeast export systems (see Tegeder and Rentsch, 2010; Okumoto and Pilot, 2011). Similarly, transporters functioning in amino acid phloem loading of leaf minor veins have not been identified (Figure 4.1). However, in legumes, phloem localization of amino acid permeases (*PsAAP1* and *PvAAP1*; Tegeder et al., 2007; Tan et al., 2008), transporting neutral and acidic amino acids with moderate or low affinity (Fischer et al., 1995, 2002), suggest that these transporters could serve this function. In *Arabidopsis*, AtAAP5 may be important for phloem loading as it is expressed in leaves (Fischer et al., 1995) and more significantly, localizes to CCs (Brady et al., 2007; Zhang et al., 2008). Some evidence of the importance of AAP-mediated amino acid transport in leaves for N distribution between source and sink is illustrated by downregulating a leaf-expressed AAP that leads to decreased amino acid levels of potato tubers (Koch et al., 2003). Further, overexpressing an AAP transporter in pea phloem, increased N transport to, and protein accumulation, by cotyledons (Zhang and Tegeder, unpublished data).

On the basis of leaf expression and localization to CCs (Toufighi et al., 2005; Brady et al., 2007), other transporters, besides AAPs, could contribute to phloem uptake of amino acids. This cohort includes neutral amino acid transporters such as *Arabidopsis* AtCAT6 and AtCAT9 (Su et al., 2004; Hammes et al., 2006) and AtProT1, a transporter for compatible solutes such as proline and glycine betaine (Rentsch et al., 1996; Grallath et al., 2005). However, their function in phloem loading remains to be demonstrated. On the other hand, mutants of the aromatic-neutral amino acid transporter, *AtANT1*, display increased sieve-tube amino acid content. While localization of ANT1 needs to be resolved, it might move amino acids out of SE/CCs (Hunt et al., 2006).

In addition to amino acids, peptides may be exported from leaves (Higgins and Payne, 1980, 1982). However, their role in N phloem transport might be more important in senescing compared to green leaves (and during seed germination) when breakdown of proteins is high (e.g., Lim et al., 2007). Phloem loading by peptide transporters has not been demonstrated but their phloem localization infers such a function. For instance, phloem localization applies to the *Arabidopsis* di- and tripeptide transporter, AtPTR1 (Dietrich et al., 2004) and the pitcher plant (*Nepenthes alata*) NaNTR1 potentially transporting peptides, but more likely nitrates (Tsay et al., 2007). Similar conclusions are drawn for oligopeptide transporters (OPT) of rice OsGT1 (OsOPT3) and *Arabidopsis* AtOPT6 that mediate transport of glutathione or glutathione derivatives and hence phloem translocation of S (Cagnac et al., 2004; Zhang et al., 2004b; Stacey et al., 2006; Pike et al., 2009; see also Lubkowitz, 2011). Transporters of S-methylmethionine (SMM), another important S compound in phloem saps (see Chapter 3), have yet to be identified. However, phloem-specific overexpression of a yeast S-methylmethionine transporter in pea resulted in an increase of sieve-tube SMM levels, S supply of sinks and overall sieve tube protein amino acid levels as well as in an increase of seed protein content and yield (Tan et al., 2010).

Phloem Loading of Macroelements Other Than Potassium

Influxes of calcium and magnesium into SE-CCs are likely to occur through channels down their electrochemical gradients (Chapter 3, Table 3.1). In this context, calcium channels have been detected in SE plasma membranes of major leaf veins (Volk and Franceschi, 2000; Furch et al., 2009). In order to account for the low sieve-element sap concentrations of calcium (see Chapter 3, Table 3.1), this cation must be effluxed actively against its inward-directed electrochemical gradient. To our knowledge, presence of calcium ATPases or calcium proton antiporters on plasma membranes of SE-CCs are yet to be determined.

Nernst predictions demonstrate that major anions (orthophosphate, nitrate, and sulfate) are loaded against their electrochemical gradients into SE-CCs and, therefore, depend upon energy-coupled carriers present in their plasma membranes. It is important to note that some members of the *Pht1* family of phosphate transporters, encoding proton-coupled symporters (Chen et al., 2008), are expressed in leaf phloem. Included amongst these are *AtPht1;5* (Mudge et al., 2002), *HORvu;Pht1;6* (Rae et al., 2003) and *OsPT2* (Ai et al., 2009). All *ht1* family members are expressed most strongly in senescing leaves where phosphorus remobilization peaks. Significantly, these transporters function as low-affinity symporters (Rae et al., 2003; Ai et al., 2009) to retrieve orthophosphate released to the leaf apoplasm.

A member of a high-affinity sulfate transporter family, *AtSultr1;3* (Buchner et al., 2004), is phloem-expressed throughout the seedling vasculature. Here, it functions in phloem loading/retrieval as demonstrated by sulfate export from cotyledons being blocked in a T-DNA knockout mutant of *AtSultr1;3* (Yoshimoto et al., 2003). In poplar, expression of *PtaSULTR1;1* increased in phloem of both minor and major veins as leaves enter senescence consistent with a role in phloem loading/retrieval of remobilized sulfate (Dürr et al., 2010). High affinity SULTR1 transporters are complemented by low affinity SULTR2 isoforms in leaf vasculatures (e.g., Takahashi et al., 2000; Liang et al., 2010). Nitrogen (N) remobilization from leaves occurs as amino N (see Chapter 3 and Section “Nitrogen (and in Some Circumstances Sulfur) are Primarily Accumulated into the Phloem by Amino Acid Transporters”) in combination with nitrate released from vacuolar storage (Richard-Molard et al., 2008). A member of the *Arabidopsis* NTR family of low-affinity nitrate transporters (NRT1.7) is expressed in SE-CCs of leaf minor veins (Fan et al., 2009). Nitrate retention in older leaves and enhanced sensitivity to nitrate starvation of a T-DNA insertion mutant demonstrated that NRT1.7 serves a key function in nitrate remobilization from leaves by loading SE-CCs (Fan et al., 2009).

Microelement Phloem Loading: An Emerging Key Field in Biofortification

Iron, copper, manganese, and zinc are rendered soluble in sieve-tube sap, and hence phloem-mobile, by forming metal complexes with nicotianamine (Takahashi et al., 2003). Maize *yellow stripe 1* (*YS1*), the founding member of yellow stripe-like (*YSL*) cluster OPT, encodes a proton-coupled nicotianamine (NA)-metal transporter capable of transporting iron, nickel, zinc, copper, manganese, and cadmium (Schaaf et al., 2004). Large gene families of *YS1*-like (*YSL*) homologs have been identified in genomes of rice (18 members—Koike et al., 2004) and *Arabidopsis* (8 members—Curie et al., 2001). In *Arabidopsis*, *AtYSL1* and *AtYSL2* are expressed in vascular parenchyma cells of leaf veins (Waters et al., 2006) and their roles in phloem loading of microelements was demonstrated by reduced seed levels in the knockout mutant *atysl1* (Le Jean et al., 2005) and the double knockout, *Atysl1/Atysl3* (Fe, zinc and copper—Waters et al., 2006). A plasma membrane localized YSL,

capable of NA-Fe and -Mn transport, *OsYSL2* and phylogenetically closely related to AtYSL1-3 subclass, is expressed strongly in CCs of leaf veins (Koike et al., 2004).

The central significance of OsYSL2 in phloem loading of microelements was demonstrated by its RNAi knockdown resulting in reduced zinc and manganese levels accumulated in grains and by overexpressing *OsYSL1*, under control of CC-specific *OsSUT1* promoter, causing increased metal accumulation by grains (Ishimaru et al., 2010). Members of the zinc-regulated transporters, iron-regulated transporter-like protein (ZIP) family, highly selective for zinc, *OsZIP1*, *OsZIP3* (Ramesh et al., 2004) and *OsZIP4* (Ishimaru et al., 2005), colocalize with OsYSL in the phloem suggestive of a phloem-loading function.

Transport Phloem: A Plurality of Membrane Transport Functions

The primary function of transport phloem, as the term implies, is to transport resources from source to sink. To achieve this goal, leaked solutes are continuously retrieved. Depending on the prevailing source/sink ratio, this may result in a net release into temporary storage pools surrounding the phloem (high source/sink ratio) or a net solute loading through remobilization from storage reserves (low source/sink ratio). In addition, transport phloem serves as a key site for xylem–phloem exchange of solutes. Contributions to these functions are reviewed following identifying those transporters expressed in transport phloem (also see Chapter 3).

Transporters Detected in the Transport Phloem

As a generalization, transporters located in collection phloem are also present in transport phloem (see Figure 4.2). This characteristic is highlighted for potassium channels as well as sugar and amino N symporters. Where data are available, their expression profiles in transport compared to collection phloem are evaluated in the following text.

Transport activities of symporters and potassium channels, respectively, depend upon *pmfs* or membrane potentials being of sufficient magnitudes to sustain high phloem sap concentrations (see Chapter 3, Table 3.1). These biophysical properties are generated by H⁺-ATPases such as AHA3 (*Arabidopsis* H⁺-ATPase isoform 3) localized to CCs in *Arabidopsis* stems (DeWitt and Sussman, 1995). Interestingly, differing isoforms of H⁺-ATPases are present in SEs and CCs located in stems of castor bean (*Ricinus communis*) and pumpkin (*Cucurbita maxima*) (Langhans et al., 2001).

In several eudicot species, SUT1 clade members are less strongly expressed in transport compared to collection phloem (e.g., *PmSUC2*—Stadler et al., 1995; *LeSUT1* and *StSUT1*—Barker et al., 2000). In contrast, *LeSUT2* (Barker et al., 2000) and *LeSUT4* (Weise et al., 2000) are expressed more strongly in transport compared to collection phloem but, similar to collection phloem, colocalize with SUT1 in SEs (Kühn et al., 1997; Barker et al., 2000; Weise et al., 2000). *PmSUC2* expression is enriched in CCs of collection compared to transport phloem (Stadler et al., 1995), but *AtSUC2* is equally expressed between collection and transport phloem (Truernit and Sauer, 1995; Martens et al., 2006). In plantain and *Arabidopsis*, SUT2 clade members, *PmSUC3* (Barth et al., 2003) and *AtSUC3* (Meyer et al., 2004), localize to transport phloem SEs thus complementing CC localization of their SUT1 counterparts (Stadler et al., 1995; Truernit and Sauer, 1995). Expression levels of monocot SUT3 clade members appear similar in collection and transport phloem of wheat (Aoki et al., 2004) and rice (Scofield et al., 2007).

Linear PLTs are expressed in transport phloem. For instance in celery, mannitol transporters *AgMaT1* (Noiraud et al., 2001) and *AgMaT2* (Juchaux-Cachua et al., 2007) are expressed at lower levels in celery petioles compared to laminae (*AgMaT1*—Noiraud et al., 2001). AgMAT2 localizes to SEs, CCs, and vascular parenchyma cells (*AgMaT2*—Juchaux-Cachua et al., 2007). One of three SOTs in apple collection phloem, *MdSOT4*, is expressed in stem phloem (Watari et al., 2004).

Recently, a breakthrough was achieved for phloem uptake of amino acids along the translocation pathway. A knockout of *Arabidopsis AtAAP2*, a broad specific amino acid transporter localized to plasma membranes of CCs throughout the plant led to decreased amino acid transport to sinks including reduced total elemental N and protein levels in seeds (Zhang et al., 2010). Further, *Arabidopsis AtAAP3* was localized to the root phloem suggesting a function as retrieval system of the transport phloem *Arabidopsis* (Okumoto et al., 2002, 2004). However, *aap3* mutants failed to exhibit a phenotype. Although not directly involved in phloem uptake, expression of *AtAAP6* in xylem parenchyma cells (Okumoto et al., 2002) and reduced amino acid levels in SEs of *ataap6* plants (Hunt et al., 2010) further underlines importance of transporters in amino acid transfer from xylem to phloem.

Potassium channels, detected in leaf veins, are also expressed in stem phloem such as AKT2/AKT3 in *Arabidopsis* (Marten et al., 1999; Lacombe et al., 2000), maize (ZMK2—Bauer et al., 2000) and broad bean (*VFK1*—Ache et al., 2001). Expression levels of *VFK1* are higher in stems than in leaf veins and increase with stem maturity (Ache et al., 2001). The AK2/AKT3 channel sustains hyperpolarized membrane potentials for symporter retrieval of sucrose (Deeken et al., 2002). In contrast, KAT2 (Pilot et al., 2001) and its maize homolog KZM1 (Philipp et al., 2003), responsible for potassium loading in collection phloem, are not expressed in transport phloem (Pilot et al., 2001; Philipp et al., 2003). Another, yet to be detected, channel may facilitate retrieval of potassium by transport phloem.

Membrane Transporters Act in Retrieval

Given sucrose and linear polyol symporters are present in SE–CCs of transport phloem (see Section “Transporters Detected in the Transport Phloem”), sugar release from SE–CCs could occur by reversing sugar/proton symport when outward-directed potential energies of sugar concentration gradients exceed those of inward-directed *pmf's* (Carpaneto et al., 2005). However, an elegant evaluation of *AtSUC2* function in transport phloem did not support reversal of sucrose symporters (Srivastava et al., 2008). Here, an *atsuc2* knockout mutant was complemented with *AtSUC2* under control of a collection phloem specific promoter from melon *CmGAS1p*. Elevated levels of soluble sugars and starch in nonphloem petiolar tissues of these transgenic plants demonstrated loss of *AtSUC2* activity from transport phloem exerted a much stronger negative impact on sucrose retrieval by, compared to sucrose release from, SE–CCs (Srivastava et al., 2008 and see Figure 4.2). Similar elevated leakage of photoassimilates was observed in the *akt2/3* *Arabidopsis* mutant in which sucrose symporter activity of transport phloem was stalled, but clearly not reversed, by a depolarized membrane potential (Deeken et al., 2002).

Sucrose retrieval is likely to be mediated by sucrose/H⁺ symporters localized to transport metaphloem SE–CCs in monocots (Aoki et al., 2004; Rae et al., 2005; Scofield et al., 2007) and eudicots (Stadler et al., 1995; Truernit and Sauer, 1995; Kühn et al., 1997; Barker et al., 2000; Noiraud et al., 2000; Weise et al., 2000; Barth et al., 2003; Meyer et al., 2004). In this context, *OssUT1*, *OssUT2*, and *OssUT4* were expressed in enclosed parts of rice flag internodes, with *OssUT1* localizing to SEs and CCs (Scofield et al., 2007). Located in this position, *OssUT1* likely

functions in retrieval and exerts no control over sucrose unloading as these parts of flag internodes are committed to starch storage, and phloem unloading is symplasmic (Scofield et al., 2007 and see Figure 4.2). Similarly, during fructan storage in flag internodes of wheat, membrane-impermeant fluorescent dyes move symplasmically from phloem to storage parenchyma cells leaving TaASUT1, localized to SEs, to retrieve leaked sucrose (Aoki et al., 2004).

The role of sucrose symporter activity in sustaining axial transport has been tested unambiguously by complementing an *atsuc2* mutant of *Arabidopsis* with *AtSUC2* under control of its own promoter to ensure expression along the entire phloem transport pathway or, with *CmGAS1p* that is specific to collection phloem of melon. Using this approach, a significant role in whole-plant photoassimilate distribution for retrieval by transport phloem was demonstrated by a 45% reduction in leaf growth when *AtSUC2* expression was restricted to collection phloem (Srivastava et al., 2008).

A retrieval function (see Figure 4.2) can be ascribed to linear polyol symporters expressed in petiole (Noiraud et al., 2001; Juchaux-Cachua et al., 2007) or stem (Watari et al., 2004) vascular bundles with AgMAT2 localizing to SEs and CCs (Juchaux-Cachua et al., 2007). High metabolic activity of transport phloem (e.g., van Dongen et al., 2003) could well rely on retrieval of hexoses leaked from CCs; a role possibly fulfilled by a HT expressed in CCs and vascular parenchyma cells of sugarcane stems (Casu et al., 2003). Indeed autoradiographs of discs of sugarcane stems incubated in a [¹⁴C]glucose solution demonstrated that accumulated radiolabel was largely confined to stem vascular bundles (Bielecki, 2000).

Expression and localization studies in *Arabidopsis* and legumes indicate that AAP family members are involved in reloading leaked amino acids as well as playing a role in xylem–phloem transfer (see Chapter 3, Section “Phloem Loading of Water and SE-CC Water Relations”). This includes *Arabidopsis* AtAAP2 (Kwart et al., 1993; Hirner et al., 1998), pea PsAAP1 (Tegeder et al., 2007), and common bean (*P. vulgaris*) PvAAP1 (Tan et al., 2008). Other AAPs, like *Arabidopsis* AtAAP8 (Schmidt et al., 2007) and AtAAP3 (Okumoto et al., 2004), are expressed in transport phloem of young flowers, peduncles, siliques or roots for amino acid retrieval (or xylem–phloem transfer).

Retrieval function of transport phloem also extends to ions such as phosphate and sulfate in which STs are the strongest cellular sites for accumulation (Bielecki, 1966a, 1966b). In this context, selective expression of a sulfate symporter, SULTR2;1, in rachides of developing *Arabidopsis* siliques, combined with a reduced sulfur seed content by RNAi knockdown of its expression, demonstrated that SULTR2;1 plays a key role in sulfate retrieval and consequently phloem import into developing seeds (Awazuhara et al., 2005).

Net Resource Efflux from Transport Phloem

In relation to withdrawal by nonconducting cells, sucrose (AtSUC3—Meyer et al., 2000; ShSUT1—Rae et al., 2005) and mannitol (AgMaT2—Juchaux-Cachua et al., 2007) symporters have been detected in vascular parenchyma/bundle sheath cells of primary vascular bundles of *Arabidopsis*, sugarcane, and celery, respectively.

For apoplastic phloem unloading, net storage of resources in stems depends upon a coordinated regulation of transporter activities located in SE-CCs and phloem parenchyma cells. Affinities for sugars by transporters expressed in SE-CCs and their phloem parenchyma cell counterparts do not appear to offer a point of control as these are essentially identical (see AgMaT1 vs. AgMaT2—Noiraud et al., 2001; Juchaux-Cachua et al., 2007; AtSUC2 vs. AtSUC3—Sauer and Stolz, 1994; Meyer et al., 2000). More flexible control could be mediated by differentially regulating symporter activity. In this context, SE-CCs were shown to exhibit more negative membrane potentials in

a cohort of known apoplastic phloem-loading species, thus favoring retrieval by SE-CCs over sink cell withdrawal of solutes (Hafke et al., 2005). Consistent with this model, depression of sucrose retrieval by SE-CCs in the *akt2/akt3* mutant enhanced photoassimilates distribution into nonphloem stem cells (Deeken et al., 2002). Competition between SE-CCs and phloem parenchyma cells are rendered redundant when resource storage switches to a symplasmic pathway (Figure 4.2). However, in those instances when soluble resources are stored (e.g., sucrose in sugarcane stems), radial symplasmic transport will depend upon activities of membrane transporters located in storage cells to sustain favorable concentration (diffusion) or turgor (bulk flow) gradients (Patrick and Offler, 1996). In this context, it is interesting that *OsSUT2*, the homolog of vacuolar localized HvSUT2 (Endler et al., 2006), was most strongly expressed in sheath-enclosed portions of rice flag internodes where the highest concentrations of sucrose were found to accumulate and the phloem-unloading pathway is symplasmic (Scofield et al., 2007).

Net Resource Influx into Transport Phloem

In exposed portions of rice flag internodes, remobilization of stored starch is linked with a decreased capacity for symplasmic-unloading and an upregulated expression of *OsSUT1* accompanied by selective expression of *OsSUT3* (Scofield et al., 2007). Both transporters likely underpin net phloem loading of sucrose remobilized from stem stored starch to supplement a decreased supply of exported photosynthetically reduced C to fuel grain development (Scofield et al., 2007). Similarly, remobilization of stored photoassimilates from sheaths, upon defoliation of ryegrass (*Lolium perenne*) to support regrowth, was accompanied by apoplastic phloem loading mediated by an upregulated expression of *LpSUT1* (Berthier et al., 2009).

Xylem-to-Phloem Exchange of Solutes

A number of transporters are selectively expressed at nodal regions. These include a boron-specific AQP, NIP6;1, that localizes to SE-CCs and phloem parenchyma cells in nodes of *Arabidopsis* stems (Tanaka et al., 2008). The mutant *nip6;1* phenotype of reduced leaf growth accompanied by a decreased boron supply to emerging leaves demonstrates that NIP6;1 plays a key role in boron partitioning by facilitating xylem–phloem exchange (Tanaka et al., 2008).

Similarly, a rice iron transporter, OsYSL18, is selectively expressed in CCs and phloem parenchyma cells at lamina joints (laminae/sheath interfaces) where it is considered to facilitate xylem–phloem exchange (Aoyama et al., 2009). However, transporters facilitating xylem-to-phloem transfer need not be localized to nodal regions. For instance, most sucrose symporters occur in phloem throughout the plant body as do zinc (e.g., OsZIP4—Ishimaru et al., 2005) and amino acid (e.g., AAP2—Zhang et al., 2010) transporters.

Secondary vascular tissue organization of woody species dictates that resource movement from xylem to phloem occurs radially through parenchyma rays in their apoplasm or symplasms (van Bel, 1990). Uptake of amino acids into the symplasmic route from the xylem transpiration stream is mediated by proton symport (van Bel, 1990). Mechanism(s) of resource release from ray parenchyma cells into phloem apoplasts is unknown. Amino acid release could involve recently discovered facilitators (Dündar and Bush, 2009; Pratelli et al., 2010). Loading phosphate and sulfate into SE-CCs of young secondary phloem is energy dependent (Bielecki, 1966b). In this context, a sulfate symporter, *PtaSULTR3;3a* (Dürr et al., 2010), is coexpressed with a plasma membrane H⁺-ATPase (Arend et al., 2002) in SE-CCs and ray initials of poplar stems.

Letting Go: Membrane Transporters Participate in Phloem Unloading of Resources

Phloem unloading is a continuum of resource transport from SE lumens of release phloem to cellular sites of storage/utilization in adjoining nonvascular tissues (sink cells; see Figure 4.3 and for further information, see Chapter 3). Roles of membrane transporters in phloem unloading are reviewed in Sections “Membrane Transporters Contributing to Phloem Unloading in Growth Sinks” and “Phloem Unloading in Storage Sinks: Fleshy Fruits and Tubers.”

Membrane Transporters Contributing to Phloem Unloading in Growth Sinks

Root Apices

In root tips of *Arabidopsis* CC-localized AtSUC2 (Truernit and Sauer, 1995) and SE-localized AtSUC3 (Meyer et al., 2004) might function in facilitating sucrose release to the root apoplasm. Operation of such an unloading route depends upon whether an inward-directed *pmf*, generated by AHA3 localized to protophloem CCs (DeWitt et al., 1991), is less than potential energies contained in sucrose concentration differences across plasma membranes of protophloem SE-CCs (Carpaneto et al., 2005). This is a condition that is also relevant to monocot species where H⁺-ATPases localize to protophloem at root tips (e.g., MHA2 in root tips of maize—Frias et al., 1996). An alternative model is that SUTs function as influxers to sustain optimal SE sucrose concentrations and cell turgor to drive symplasmic phloem unloading (e.g., see Figure 4.3 and Chapter 3).

In contrast to *Arabidopsis*, protophloem in root tips of common plantain lack PmSUC2 (Stadler et al., 1995) and PmSUC3 (Barth et al., 2003) in their CCs and SES, respectively. Similar findings were made for the tobacco SUT1 homolog (Martens et al., 2006). Under these conditions, sucrose unloading across plasma membranes of SE-CCs and/or phloem parenchyma cells within the root elongation zone may occur by simple diffusion alone or be mediated by a sucrose facilitator (e.g., SBP, Warmbrodt et al., 1991; Contim et al., 2003 and see Figure 4.3).

As the Caspary strip develops to form an apoplastic barrier to radial movement through endodermal cell walls, outward radial movement of phloem-unloaded solutes through root apoplasts becomes increasingly problematical. Moreover, radial apoplastic movement of sucrose is not consistent with an absence of its extracellular hydrolysis in maize roots (Giaquinta et al., 1983). Thus, a strong imperative exists for sucrose, released from protophloem SE-CCs, to be reloaded within the stele energized by say AHA4 present in endodermal cells of *Arabidopsis* roots (Vivart et al., 2001). In this context, AtSUC2 (Thompson and Wolniak, 2008) and PmSUC3 (Barth et al., 2003), located in epidermal and several layers of underlying cortical cells, could function in retrieval of sucrose leaked to the root apoplasm. However, the level of this symporter activity is brought into question for *Arabidopsis* plants by finding that outer cell layers of their root tips are dominated by facilitated diffusion rather than by proton-coupled transport mechanisms (Chaudhuri et al., 2008).

Predominance of hexose over sucrose symporter activities in root tips of maize (Lin et al., 1984) may be related to retrieval of hexoses leaked to the root apoplasm resulting from high cleavage rates of symplasmically unloaded sucrose by vacuolar invertases (Blee and Anderson, 2002 and see Figure 4.3). Significantly, MSTs are strongly expressed in root tips of *Arabidopsis* (AtSTP1—Sherson et al., 2000; Chaudhuri et al., 2008; AtSTP4—Truernit et al., 1996; Chaudhuri et al., 2008; AtPLT5 selectively in the root elongation zone—Klepik et al., 2005; Reinders et al., 2005) and rice (*OsMST3* localized to xylem parenchyma—Toyofuka et al., 2000).

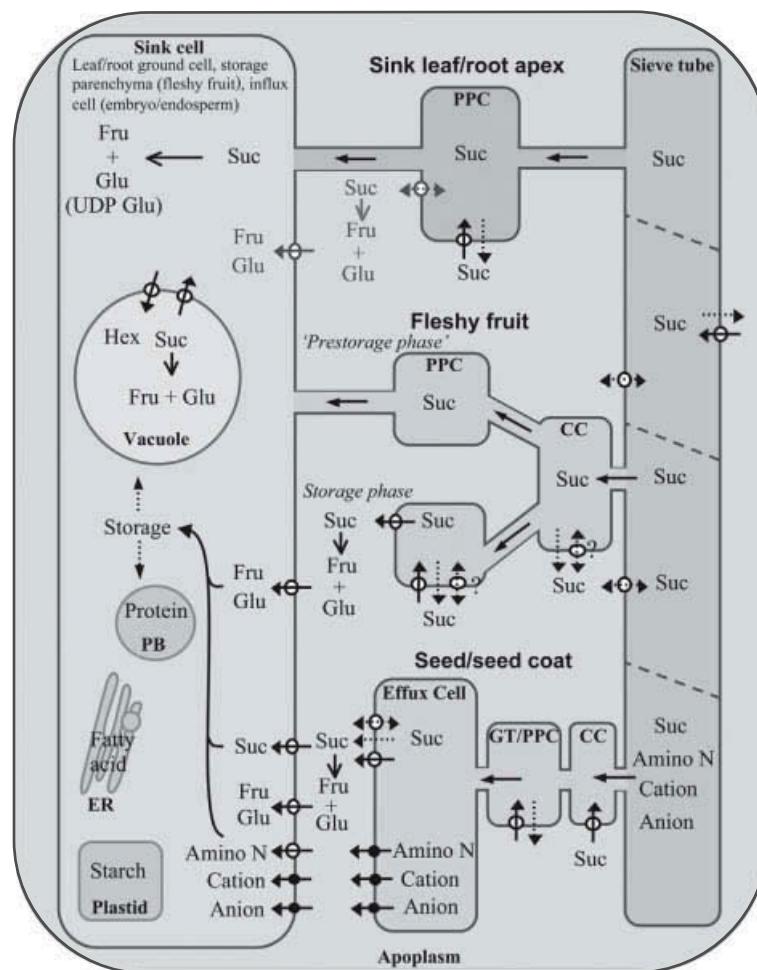


Figure 4.3 Cellular localization of resource membrane transporters in cells of release phloem and postphloem-unloading pathways in sink leaf/root apices, fleshy fruit, and developing seeds. In sink leaves and root apices, unloading primarily follows symplasmic routes possibly supplemented by release to the sink apoplasm. To optimize delivery of sucrose for symplasmic unloading, sucrose symporters localized to release phloem function in retrieving leaked sucrose. For apoplasmic unloading, sucrose symporters could be reversed to support efflux to the sink apoplasm. Here, an extracellular invertase hydrolyzes sucrose and the resulting fructose and glucose are taken up into sink cells by hexose symporters. During the prestorage phase of fleshy fruit development, phloem unloading follows a symplasmic route switching to one including an apoplasmic step once sugar storage commences. Reversal of sucrose symporters could be a primary route for release to the fruit apoplasm supplemented by passive release. Hydrolysis of released sucrose by extracellular invertases is followed by the resulting glucose and fructose being retrieved by hexose symporters into storage parenchyma cells for vacuolar storage facilitated by tonoplast transporters. For developing seeds, transporters located on SE-CCs, PPC, and GT cells of seed coats likely function to retrieve leaked resources from a symplasmic-unloading route delivering resources to specialized efflux cells. Efflux of resources from seed coats might include passage through efflux carriers, symporter reversal or channels accompanied by passive leakage. Influx into filial tissues, via seed apoplasmic spaces where sucrose may or may not be cleaved by an extracellular invertase, is mediated by transporters localized to specialized influx cells proximal to the maternal/filial interface. Thereafter, sugars and amino N compounds enter biosynthetic pathways, leading to carbon and nitrogen capture into protein, fatty acid and starch storage compartments. Transporters include carriers ± energy-coupled (arrow through an open circle), including sucrose symporters that may function in reverse (doubled-headed arrow through open circle), as well as channels (arrow through closed circle). Sugar leakage to the seed apoplasms occurs by passive movement (arrow with broken line). CC, companion cell; SE, sieve element; Fru, fructose; Glu, glucose; GT, ground tissue; Hex, hexose; PB, protein body; PPC, phloem parenchyma cell; Suc, sucrose. (For a color version of the figure, please see Plate 4.3.)

Shoot Apices: Developing Leaves

For developing leaves of *Arabidopsis* (Truernit and Sauer, 1995) and tobacco (Wright et al., 2003; Martens et al., 2006), expression of SUC2 occurs concomitantly with the basipetal wave of differentiation and appears restricted to CCs of minor veins and metaphloem of major veins. Similar conclusions apply to PmSUC2 (Barth et al., 2003). Thus, SUT1 clade sucrose symporters are absent from release phloem of developing leaves of *Arabidopsis*, tobacco, and common plantain. However, AtSUC9, and its homologs, expressed in mesophyll cells of sink leaves (Sivitz et al., 2007) could function in retrieving symplasmically-transported sucrose leaked to the leaf apoplasm (Figure 4.3).

In contrast, SE-localized LeSUT2 (Barker et al., 2000) and PmSUC3 (Barth et al., 2003) are expressed in major veins of sink and source leaves. Given that AHA3 is expressed in sink phloem (DeWitt et al., 1991), the degree to which sucrose symporters contribute to apoplastic unloading (see Figure 4.3) depends upon the activity of this proton pump along the lines outlined for root apices (see Section “Root Apices”).

A GmSBP2, a putative sucrose facilitator (Overvoorde et al., 1996), is present in sink and source leaf phloem (Contim et al., 2003). Sucrose release facilitated by SBPs (Contim et al., 2003), extracellular hydrolysis by cell wall invertases (Kocal et al., 2008), and recovery by MSTs (e.g., AtSTP4—Fotopoulos et al., 2003; MST1—Sauer and Stadler, 1993) may form an apoplastic-unloading pathway operating in parallel to the symplasmic route (see Figure 4.3 and Chapter 3). Similarly, expression of a potassium channel, VFK1, in phloem of broad bean sink leaves, could function to facilitate apoplastic potassium unloading (Ache et al., 2001). One of two sour-cherry PLTs (*PcSOT1*) is most strongly expressed in young expanding leaves but its cellular localization was not determined (Gao et al., 2003).

Phloem Unloading in Storage Sinks: Fleshy Fruits and Tubers

Fleshy Fruits

Early in development unloading could occur symplasmically, switching to an apoplastic route once sugar accumulation commences (see Figure 4.3 and Chapter 3). At the later stage in tomato fruit, absence of expression (Dibley et al., 2005) and activity (Ruan and Patrick, 1995) of a plasma membrane H⁺-ATPase in vascular bundles may drive reversal (Carpaneto et al., 2005) of LeSUT1 and LeSUT2 localized to SEs (Hackel et al., 2006 and, for more information, see Chapter 3). Apoplastic hexoses, resulting from extracellular hydrolysis of unloaded sucrose, are taken up by hexose symporters (LeHT1, LeHT2, and LeHT3—Gear et al., 2000; McCurdy et al., 2010) energized by H⁺-ATPases colocalized to fruit storage parenchyma cells (Dibley et al., 2005).

A similar apoplastic phloem-unloading mechanism may operate in grape berries (*Vitis vinifera*). At the onset of berry hexose storage, expression of an extracellular invertase, localized to phloem (Zhang et al., 2006), and an H⁺-ATPase, localized to mesocarp storage cells (Yu et al., 2006), are upregulated along with two sucrose symporters (*VvSUC11* and *VvSUC12*—Davies et al., 1999; Ageorges et al., 2000; Manning et al., 2001) and two hexose symporters (*VvHT2* and *VvHT3*; Hayes et al., 2007). Cellular localization of these STPs is yet to be determined, but it is likely that the sucrose symporters will be phloem localized for retrieval and that hexose symporters are located in berry storage cells. Of interest, during the prestorage phase, a hexose symporter (*VvHT1*) localizes to SE-CCs and storage parenchyma cells (Vignault et al., 2005). *VvHT1* expression is accompanied by that of *VvSUC11* and *VvSUC12* (Davies et al., 1999; Ageorges et al., 2000; Manning et al., 2001), indicating apoplastic and symplasmic unloading may operate in parallel during the prestorage phase of berry development (Zhang et al., 2006).

A different apoplastic-unloading strategy may apply to apple fruits, in which sorbitol is a major phloem-transported sugar (Beruter et al., 1997). Indeed, in contrast to tomato and grape (see previous two paragraphs), a H⁺-ATPase is present in SE-CCs, vascular parenchyma and storage parenchyma cells (Peng et al., 2003; Zhang et al., 2004a). An MST, possibly a sorbitol symporter homolog (Watari et al., 2004 and for reference to fruit, see Gao et al., 2003), colocalizes with H⁺-ATPases to phloem and nonphloem cells (Zhang et al., 2004a). Given H⁺-ATPase activity in SE-CCs (Peng et al., 2003; Zhang et al., 2004a), from which apoplastic unloading is thought to occur (Zhang et al., 2004a), an energized sorbitol-unloading mechanism is favored over reversal of a sorbitol symporter.

Potato Tubers

StSUT1 localizes to SEs of potato tubers and tuber-selective downregulation of its expression results in lowered rates of tuber biomass gain during early tuber development (Kühn et al., 2003) when phloem unloading follows an apoplastic route (Viola et al., 2001). Colocalization of StSUT1 (Kühn et al., 2003) with H⁺-ATPase activity (Oparka, 1986) in SE-CCs across tuber development brings into question whether reversal of StSUT1 contributes to sucrose unloading. However, downregulated StSUT1 expression more likely regulates, directly or indirectly, sucrose retrieval mechanisms by tuber storage parenchyma cells (Wright and Oparka, 1989).

Resource Unloading in Developing Seeds: A Cacophony of Membrane Transporters in a Sink System Committed to an Apoplastic Step in the Postphloem Unloading Pathway

Resources move from importing SEs of maternal integuments to ultimate sequestration in filial storage cells with an obligatory exchange across an intervening apoplastic step separating maternal and filial seed tissues (for further information, see Chapter 3).

Sugar Transporters

Given that SE-CC unloading occurs symplasmically (see Chapter 3), transporters localized to SE-CC in seed coat vasculatures function primarily to retrieve leaked resources to optimize turgor and solute concentration gradients to drive resource flows through postphloem symplasmic routes (Figure 4.3). Solute retrieval could also extend along postphloem part of the unloading pathway. Solute retrieval depends upon proton pumping activities of H⁺-ATPases to generate favorable membrane potentials for retrieval of cations or pmfs for retrieval of anions and nonelectrolytes. Consistent with this proposal, in developing *Arabidopsis* seeds, AHA3 localizes to SE-CCs located in the phloem terminus at the funiculus/seed coat interface (DeWitt et al., 1991) and AHA10 is expressed in outer integument cells (Harper et al., 1994) forming the postphloem pathway (Stadler et al., 2005a, 2005b).

Colocalizing with these H⁺-ATPases at the phloem termini are AtSUC2 in CCs (Stadler et al., 2005b) and possibly AtSUC3 in SEs (Meyer et al., 2004). AtSUC3 is also expressed in cells forming the postphloem-unloading pathway (Meyer et al., 2004). Comparable colocalization of H⁺-ATPases and SUTs occur along maternal phloem-unloading pathways in developing grains of wheat (Bagnall et al., 2000) and rice (Furbank et al., 2001). In coats of developing grain legume seeds, sucrose symporters (VfSUT1—Weber et al., 1997; PvSUT1 and PsSUT1—Zhou et al., 2007a) and facilitators (PvSUF1, PsSUF1, and PsSUF4—Zhou et al., 2007a) are expressed in cells comprising their symplasmic-unloading pathways. *In planta* retrieval activities of sucrose

symporters and facilitators have been demonstrated for seed coats of pea (de Jong and Borstlap, 2000) and broad and French bean (Ritchie et al., 2003).

In rice and *Arabidopsis* seed coats, MSTs are coexpressed with cell wall invertases that hydrolyze sucrose leaked to their apoplasts (Sherson et al., 2003; Wang et al., 2008, respectively). During prestorage and storage phases of rice grain development, a hexose symporter, OsMST4, expressed in seed-coat cells supporting symplasmic transport from SEs to specialized efflux cells, presumptively functions to retrieve leaked hexoses (Wang et al., 2007). Three MSTs are generically expressed in coats of developing *Arabidopsis* seeds—AtSTP1 during early seed development and AtSTP12 and AtSTP14 across the entire course of seed development all of which could contribute to hexose retrieval (Büttner, 2010). Two tonoplast-localized hexose/H⁺ antiporters, OsTMT1 and OsTMT2, expressed during the prestorage phase of seed fill, contribute to buffering sugar supply destined to support filial tissues (Cho et al., 2010). A comparable function can be assigned to a TMT homolog, HvSTP1, expressed in maternal tissues of developing barley grains (Weschke et al., 2003).

Of the species examined thus far, H⁺-ATPases and STPs, present in cells forming seed coat phloem-unloading pathways, are also expressed in specialized efflux cells (Figure 4.3). Added to this cohort of transporters are VfSTP1 in prestorage broad beans (Weber et al., 1997) and PmSUC1 and AtSUC1 expressed transiently in inner integuments following anthesis (Gahrtz et al., 1996; Lauterbach et al., 2007). In prestorage phase seeds of cereals (Weschke et al., 2003) and grain legumes (Weber et al., 1997), cell wall invertases may create favorable energetic conditions to reverse their SUTs to efflux (Carpaneto et al., 2005). For storage phase seeds, additional transporters include H⁺-ATPases in broad and French bean (Wang et al., 1995; Harrington et al., 1997) and pea (Tegeder et al., 1999) along with HvSUT1 in barley grains (Weschke et al., 2000).

Filial tissues retrieve sugars released to seed apoplasmic spaces proximal to their maternal–filial interfaces (Zhang et al., 2007 and see Figure 4.3). During the prestorage phase of seed fill in pea, *PsSUT1* and *PsSUF1* expression in the endosperm precedes their expression in embryos (Melkus et al., 2009). In *Arabidopsis*, AtSUC5 appears to perform a similar role in filling the endosperm and a T-DNA knockdown phenotype shows that AtSUC5-mediated sucrose accumulation plays an important regulatory role in early embryo development (Baud et al., 2005).

Hexose symporters dominate sugar transport in developing embryos of prestorage phase seeds of cereals (Weschke et al., 2003; Wang et al., 2007, 2008) and grain legumes (Weber et al., 1997) and are retained during their storage phase in tropical grasses (Patrick and Offler, 2001) and oil-storing endospermic seeds of eudicots (Tomlinson et al., 2004). In contrast, hexose symporters are replaced by sucrose symporters, colocalized with H⁺-ATPases to specialized influx cells, in storage phase seeds of temperate cereals (Bagnall et al., 2000; Weschke et al., 2000; Furbank et al., 2001) and nonendospermic eudicots such as grain legumes (Harrington et al., 1997; Weber et al., 1997; Tegeder et al., 1999). These sucrose symporters play a central role in regulating C flows into developing seeds as demonstrated by their selective overexpression causing increased seed biomass gains in pea (Rosche et al., 2002) and wheat (Weichert et al., 2010) and by knockdown resulting in a decline in grain biomass without affecting sucrose loading in flag leaves of rice (Ishimaru et al., 2001; Scofield et al., 2002).

Amino Acid Transporters

Transport of amino acids to, and into, growing embryos/cotyledons is developmentally regulated (Figure 4.3). During early embryogenesis, *Arabidopsis* AtAAP8 functions in importing amino acids into endosperm. This uptake step is essential for embryo development as around 50% of fertilized ovules abort in *aap8* siliques (Schmidt et al., 2007). Expression and localization during later seed development suggest that amino acid import into embryos/cotyledons is mediated by AtAAP1, and

the neutral and cationic amino acid transporter AtCAT6 (Hirner et al., 1998; Hammes et al., 2006; Sanders et al., 2009) and in pea and common bean via PsAAP1 and PvAAP1 (Tegeder et al., 2000; Tegeder et al., 2007; Tan et al., 2008). While the mechanism of amino acid release from the seed coat is unknown, reduced amino acid levels in *ataap1* embryos and decreased N and protein levels in *ataap1* mutant seeds provide direct evidence for the importance of AAP transporters in N uptake by the embryo (Sanders et al., 2009). Nevertheless other embryo-expressed amino acid and peptide transporters, such as AtCAT6 (Hammes et al., 2006) and AtPTR1, respectively (Dietrich et al., 2004; Komarova et al., 2008), may partly compensate for AAP1 loss of function in *Ataab1* mutants (Sanders et al., 2009). Additional transporters, expressed during seed development and putatively involved in N uptake, are peptide transporters AtPTR2 and AtPTR5 in *Arabidopsis* (Rentsch et al., 1995; Song et al., 1997; Dietrich et al., 2004; Komarova et al., 2008) and VfPTR1 in broad bean (Miranda et al., 2003). Cotyledon storage parenchyma cells also express AAP transporters. These include *Arabidopsis* AtAAP1, pea PsAAP1 and broad bean VfAAP1 (Tegeder et al., 2000; Miranda et al., 2001; Tan et al., 2008; Sanders et al., 2009) that might function in amino acid retrieval or N redistribution within cotyledons crucial for storage protein accumulation (Miranda et al., 2003). These functions are supported by an observed elevated amino acid uptake, leading to increases in seed weight, protein content, and size by overexpressing broad bean VfAAP1 in storage parenchyma cells of purple broad vetch (*Vicia narbonensis*) cotyledons (Rolletschek et al., 2005).

Minerals

For major elements, sulfate transporters (SULTR) have been localized to seed coats of chickpea (*Cicer arietinum*; *CaSultr3-1*- Tabe and Droux, 2002) and *Arabidopsis* (SULTR3;1, SULTR3;2, SULTR3;3, and SULTR3;4—Zuber et al., 2010). These transporters function as proton symporters (Zuber et al., 2010) and could act to retrieve sulfate leaked from symplasmic unloading pathways.

Patch clamp studies, using protoplasts derived from efflux cells of grain legume seeds, have identified two types of nonselective channels and one pulsing anion channel. The nonselective channels are permeable to univalent inorganic cations and large organic cations, TEA⁺ and choline⁺ (Zhang et al., 2000, 2002). The fast-activating nonselective channel likely plays a role in mediating *in planta* K⁺ efflux while the slowly-activating nonselective channel may provide a route for efflux of divalent cations such as Mg²⁺, Zn²⁺, and Mn²⁺. The pulsing Cl⁻ channel is likely to act as a route for Cl⁻ release (Zhang et al., 2004c). A cotyledon channel, relatively nonselective for univalent cations, retrieves cations from seed apoplasms (Zhang et al., 2004d). Genes encoding these channels remain to be cloned.

Plasma membrane transport of iron and manganese is mediated by OPTs and YSLs that form two subfamilies of OPT (Curie et al., 2009). A T-DNA knockout mutant (*opt3-2*) demonstrated that AtOPT3 is essential for iron chelate loading into developing seeds (Stacey et al., 2008), where it is expressed in integuments, chalazal endosperm, and embryos (Stacey et al., 2002). AtYSL1 is expressed in the funiculus vasculature and chalazal endosperm of *Arabidopsis* seeds. A knockout mutant (*yssl1-1*) of AtYSL1 caused a seed-specific reduction in seed iron levels, indicating that AtYSL1 plays a key role in seed loading of iron (Le Jean et al., 2005).

OsYSL2 is expressed in outer cell layers of endosperm where minerals accumulate (Koike et al., 2004) and, based on knockdown studies, regulates iron and manganese accumulation by developing rice grains (Ishimaru et al., 2010). In developing barley grains, a P-type heavy metal ATPase (HMA2) is key candidate mediating zinc efflux from nucellar transfer cells to the endosperm cavity, while subsequent zinc uptake into aleurone cells is primarily mediated by four ZIP (ZRT/IRT-like protein) transporters (Tauris et al., 2009).

Aquaporins and Phloem Unloading of Water in Growth and Storage Sinks

In expanding postveraison grape berries, water flows into, and from, the berry apoplasm are likely facilitated by strong PIP expression (Picaud et al., 2003). Upon cessation of expansion but continued biomass gain, phloem-imported water transported to storage sink apoplasms is recycled back to the parent plant body through a xylem route. In this context, AQPs may play important roles in water recycling in developing seeds. For instance, transcript levels of *PsPIP2;1*, expressed in coats, significantly increased after developing pea seeds reached their maximal size (Schuurmans et al., 2003). A comparable relationship was found for coats of developing seeds of French bean (Zhou et al., 2007b). Here, strong expression of *PvPIP2;3* in their coat vascular parenchyma cells probably mediates water backflow to the xylem and hence avoids sweeping away nutrients released further downstream along the unloading pathway from ground parenchyma cells (Zhou et al., 2007b).

The Dark Side: Ferrymen Transporting Toxic Elements or Compounds

Phloem Transporters Confer Adaptive Responses to Salt Stress

Plants osmotically adjust to salt and water stress by changing ion transport and compartmentation, and by synthesizing and accumulating osmotically active compounds such as polyols, glycine betaine and proline. Developing organs may rely on source-sink translocation of these solutes for osmotic adjustment. For example, when alfalfa plants were exposed to a water-deficit, their leaf water potential decreased from -1.0 to -2.0 MPa and sieve-tube proline concentrations dramatically increased by 60 times (Girousse et al., 1996).

ProT (proline transporter) expression is strongly induced by salt and water stress (Rentsch et al., 1996; Ueda et al., 2001). AtProT1 is phloem-localized and hence may function in source-sink translocation of proline in salt stressed *Arabidopsis* plants (Grallath et al., 2005). However, *atprot1* mutants failed to display a phloem-related phenotype. Functional redundancy may explain this outcome (Lehmann et al., 2011) since AAPs and LHTs also transport proline and GABA (gamma-aminobutyric acid; Fischer et al., 1995, 2002; Hirner et al., 2006; Lee et al., 2007; Svennerstam et al., 2007), and AAPs seem to be involved in phloem loading (see Section “Nitrogen (and in Some Circumstances Sulfur) are Primarily Accumulated into the Phloem by Amino Acid Transporters”). However, expression of some *AtAAPs* decreases under high salt conditions (Rentsch et al., 1996).

PLTs, including MATs, SOTs, and INTs, may function in phloem loading (see Section “Polyol and Inositol Transporters and Their Role(s) in Phloem Loading”). In this context, salt stress causes upregulated expression of phloem or phloem-associated *PLTs* including myoinositol transporters from ice plant (*Mesembryanthemum crystallinum*) and *Arabidopsis* (*McMITR1*, Chauhan et al., 2000; *AtINT4*, Schneider et al., 2006), mannitol transporters from celery (*AgMaT3*, Landouar-Arsivaud et al., 2011), and SOTs of plantain (*PmPLT1* and *PmPLT2*, Pommerrenig et al., 2007). Stress-regulated changes in phloem-localized expression of *PmPLT1*, *PmPLT2* are accompanied by a dramatic increase in the sorbitol–sucrose ratio in sieve-tube sap, supporting an adaptation of carbohydrate metabolism and source-sink translocation in response to environmental challenge (Pommerrenig et al., 2007).

Stable ion concentrations in sieve-tube sap and shoot apices suggest that salt-stressed plants are able to control translocation of Na^+ and Cl^- to developing shoot tissues (e.g., Munns and Rawson, 1999). However, the underlying mechanism(s) controlling Cl^- and Na^+ phloem transport under salt or drought stress have not been resolved (Conn and Gillham, 2010). In *Arabidopsis*, the Na^+

selective uniporter, *AtHKT1;1*, is expressed in leaf phloem, and *athkt1;1* mutants exhibit decreased root but elevated shoot Na⁺ levels and display a salt hypersensitive phenotype (Berthomieu et al., 2003). Thus, it was concluded that, under salt stress, AtHKT1;1 mediates Na⁺ phloem loading and retranslocation to roots to protect leaf physiology. However, more recent radioactive tracer studies suggest that AtHKT1;1 functions in Na⁺ retrieval from the xylem (Davenport et al., 2007).

A similar function has been demonstrated for the isoform from rice, OsHKT1;5 that is localized throughout the plant and specifically to parenchyma cells surrounding xylem vessels (Ren et al., 2005). In addition, allelic variation of OsHKT1;5 accounted for cultivar differences in salt tolerance (Ren et al., 2005). Nevertheless, recirculating Na⁺ under salt stress is supported by the expression of metal-binding proteins, NaKR1/NPCC6, in CCs of leaves contributing to long-distance movement of metals (i.e., Na⁺, K⁺, and Rb⁺) to *Arabidopsis* roots (Tian et al., 2010). Other proteins might be directly or indirectly function in phloem transport of Na⁺ and salt tolerance including NAX (Na⁺ exclusion) transporters and members of the CAX cation exchanger family (see Mian et al., 2011).

Transporter Infidelity Opens Gates to Heavy Metals

Toxic heavy metals such as arsenic (As), cadmium (Cd), and cobalt (Co) are taken up and transported within plants, although their phloem mobility is species dependent (Riesen and Feller, 2005). Very little is known about transporters involved in long-distance transport of heavy metals, and studies have mainly addressed Cd. Phloem transport of Cd to tubers or seeds has been demonstrated in a range of species including potato (Dunbar et al., 2003), soybeans (*Glycine max*; Yada et al., 2004), wheat (Riesen and Feller, 2005), and rice (Tanaka et al., 2007). Leaf to root recirculation of Cd in *Arabidopsis* putatively avoids toxic effects on shoot tissues (van Belleghem et al., 2007).

Work on oilseed rape (*Brassica napus*) resolved that Cd is transported in the phloem as thiol–Cd complexes (Mendoza-Cózatl et al., 2008). In rice, iron transporters (OsIRTs), specifically OsIRT1 that is expressed in the CCs of roots, stems and leaves, have been suggested to function in phloem-loading and source-sink translocation of Cd (Ishimaru et al., 2005; Nakanishi et al., 2006). Other Cd-transporting proteins that might play a role in phloem loading include P1B-ATPases (P-type ATPases; Williams and Mills, 2005), cation transporters (CAX; Hirschi et al., 2000), NRAMPs (natural resistance-associated macrophage proteins, Lanquar et al., 2005) and ZRT/IRT-like proteins (ZIP), specifically OsZIP4 expressed in leaf phloem of rice (Ishimaru et al., 2005).

Phloem Transporters Engaged in Signal Transmission

Phloem Loading/Unloading of Plant Hormones

Phloem transport of IAA from source leaves to sinks plays essential roles in coordinating plant development (Robert and Friml, 2009). The mechanism of phloem loading of auxin is unknown but could occur by passive diffusion of the protonated acid across SE–CC plasma membranes possibly facilitated by the auxin influx carrier (AUX; Robert and Friml, 2009). In *Arabidopsis* root apices, unloading of auxin is directed to the apical dome by AUX and PIN1 transporters organized at opposite ends of consecutive protophloem SEs (Kleine-Vehn et al., 2006). Phloem loading of transpiration delivered cytokinins to source leaves could be mediated by purine permease (PUP) transporters as illustrated by AtPUP2 expressed in phloem of *Arabidopsis* leaves (Bürkle et al., 2003). Methyl jasmonate is loaded into leaf phloem by a proton-coupled mechanism (Thorpe et al.,

2007). Phloem loading of ABA may occur by passive diffusion of the protonated acid through plasma membranes of SE-CCs (Seo and Koshiba, 2011).

Sieve Tube Ion Channels Involved in Electrical Signaling

Electrical potential waves are transmitted rapidly along sieve tubes in response to a number of environmental stimuli. On the basis of studies with nonphloem plant cells, electrical potentials are created by an initial calcium influx through calcium channels triggering anion channels to release chloride causing a rapid depolarization of membrane potential. Repolarization is achieved by potassium exit through potassium channels (Fromm and Lautner, 2007). Transmission of electrical potentials in sieve tubes is triggered by calcium fluxes through calcium channels (Furch et al., 2009). A role for potassium in sieve-tube propagation of electrical potentials is inferred from the dominant influence of potassium channels on SE-CC membrane pds (Ache et al., 2001; Deeken et al., 2002; Philipp et al., 2003). Elevated cytosolic calcium levels in SEs, initiated by injury-mediated electrical potentials, likely result from activating both plasma membrane and ER-localized calcium channels (Furch et al., 2009). The calcium signal induces sieve pore occlusion through rapid forisome swelling (Furch et al., 2007) or coagulation of soluble SE proteins (Furch et al., 2010) followed by a slower deposition of callose (Furch et al., 2007, 2010). Reversal of sieve pore occlusion by lowered cytosolic calcium levels (Furch et al., 2010) probably depends upon calcium expulsion mediated by calcium ATPases.

Phloem Loading of Plant Defense Compounds

Ascorbic acid and glutathione are major players in plant defense against oxidative stress as well as regulatory elements controlling plant development (Foyer and Noctor, 2011). SE-CCs contain enzymes required for biosynthesis of these redox agents (e.g., Rodriguez-Medina et al., 2011). This source of redox biosynthesis is supplemented by loading these compounds produced in mesophyll cells of mature leaves (Franceschi and Tarlyn, 2002; Herschbach et al., 2010). Physiological evidence demonstrates dehydroascorbate-specific transporters are present in plasma membranes of plant cells (Horemans et al., 2008); however, whether these are responsible for phloem loading remains to be determined. In contrast, phloem-localized OPT likely function to load glutathione (Lubkowitz, 2011).

Salicylic acid, and in some cases methyl salicylate, function as systemic signals transported via the phloem from a localized infected region to activate defense responses in distal uninfected tissues (Shah, 2009). Salicyclic acid is selectively loaded into castor bean phloem by a membrane carrier that discriminates against methyl salicylate that transits membranes by diffusion through their phospholipid bilayers (Rocher et al., 2009).

Future Directions

Membrane transporters, facilitating movement of resources, toxic elements and signals, have been identified in certain cell types comprising components of the phloem translocation pathway mediating flows of these constituents from source to sink. This cellular mapping of membrane transporters, together with determining their functional properties and physiological significance, is most

advanced for sugar and microelement transporters responsible for plasma membrane influx and to a lesser extent tonoplast efflux. However, yet to be determined are the molecular identities of effluxers supporting high sugar fluxes in sinks containing an apoplastic step in their postphloem unloading pathways. Furthermore, considerable scope remains for discovering the molecular identities, cellular localization and transport functions of membrane proteins facilitating movement of water and small molecular weight systemic signals coordinating developmental patterns and wound responses. Indeed, current preoccupation with nutrient transporters has overshadowed progressing understanding of AQP function that may prove to be of equal or greater importance in regulating flows of sieve-tube sap from source to sink and hence biomass crop yields. The emerging fields of microelement and toxic element/compound transport are progressing rapidly driven by the urgent need to secure food nutritional quality.

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5 Regulation of Sucrose Carrier Activities

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Sucrose is the most prominent transport carbohydrate in the majority of plant species. As the principal osmotic component of the sieve-tube sap, sucrose is mainly responsible for driving phloem translocation and, hence, for the efficiency of long-distance transport of signaling macromolecules such as proteins and RNA species. Furthermore, sucrose itself acts as an important signal for induction of flowering (King and Ben-Tal, 2001) and is also involved in concerted phytohormonal effects on gene expression (Blazquez et al., 1998).

Sucrose is actively loaded into sieve element–companion cell complexes (SE–CCs) by specific sucrose-transporting membrane proteins in collection and transport phloem (see Chapter 3). Therefore, these transporters are directly or indirectly engaged in the control of plant growth and development by fine-tuning sucrose allocation between sink organs. Behind the activities of sucrose transporters (SUTs) is a regulatory network, the complexity of which we are just beginning to unravel. New insights that will be discussed here also shed new functional light on phloem ultrastructure.

Functional Characteristics of Sucrose Transporters

Genes encoding SUTs belong to multimember gene families within different plant species (Table 5.1). Phylogenetic analysis revealed at least five different phylogenetic clades: two clades with members from monocotyledons, one clade specific to dicotyledons, and two mixed clades with members from both mono- and dicotyledons (Kühn and Grof, 2010). The nomenclature of sucrose transporting proteins originates from SUTs (Riesmeier et al., 1993), sucrose carriers or SUCs (Sauer and Stolz, 1994), and sucrose facilitators or SUFs (Zhou et al., 2007). SUTs and SUCs act as sucrose-proton cotransporters, whereas SUFs act in a proton-uncoupled manner.

Abbreviations: BFA, brefeldin A; c-myc, myelocytomatosis viral oncogene homolog; FM4-64, Fei Mao dye 4-64; GFP, green fluorescent protein; MCC, membrane compartment of Can1; PMF, proton-motive force; PPC, phloem parenchyma cell; PPU, pore–plasmodesma unit; RNP, ribonucleoprotein particle; SE–CC, sieve element–companion cell complex; SER, sieve element reticulum; SUC, sucrose carrier; SUF, sucrose facilitator; SUT, sucrose transporter

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Table 5.1 Table of characterized sucrose transporter proteins from higher plants, fungi, and animals. The classification follows the phylogenetic analysis published by Kühn and Grof, 2010

Species	Name	Km	Localization	Reference
SUT1 clade (dicotyledons):				
<i>Solanum tuberosum</i>	StSUT1	1 mM	SE, CC	Kühn et al., 1997; Schmitt et al., 2008
<i>Solanum lycopersicum</i>	SISUT1	1 mM	SE, CC	Reinders et al., 2002; Schmitt et al., 2008
<i>Nicotiana tabacum</i>	NtSUT1	Functional	SE, CC	Weise et al., 2008; Bürkle et al., 1998; Schmitt et al., 2008
<i>Nicotiana tabacum</i>	NtSUT3		Pollen	Lemoine et al., 1999
<i>Spinacia oleracea</i>	SoSUT1	1.5 mM		Riesmeier et al., 1992
<i>Plantago major</i>	PmSUC1	0.3 mM	Ovules	Gahrtz et al., 1996
<i>Plantago major</i>	PmSUC2	1 mM	CC	Stadler et al., 1995
<i>Arabidopsis thaliana</i>	AtSUC1	0.45 mM	Pollen	Sauer and Stolz, 1994; Stadler et al., 1999
<i>Arabidopsis thaliana</i>	AtSUC2	0.53 mM	CC	Stadler and Sauer, 1996
<i>Apium graveolens</i>	AgSUC1	0.14 mM		Noiraud et al., 2000
<i>Alonsoa meridionalis</i>	AmSUT1	1.8 mM	SE + CC	Knop et al., 2004
<i>Vicia faba</i>	VfSUT1	1.4 mM		Weber et al., 1997
<i>Pisum sativum</i>	PsSUT1	1.5 mM	Embryonic transfer cells	Tegeder et al., 1999 Zhou et al., 2007
<i>Pisum sativum</i>	PsSUF1	99.8 mM		Zhou et al., 2007
<i>Phaseolus vulgaris</i>	PvSUT1	8.5 mM		Zhou et al., 2007
<i>Phaseolus vulgaris</i>	PvSUF1	27.9 mM		Zhou et al., 2007
<i>Ricinus</i>	RcScr1	2 mM		Weig and Komor, 1996
<i>Hevea brasiliensis</i>	HbSUT1		Young phloem laticifers	Dusotoit-Coucaud et al., 2009
<i>Hevea brasiliensis</i>	HbSUT3	1.24 mM	Laticifers	Tang et al., 2010
<i>Rosa</i>	RhSUC2	2.99 mM	Buds, stems	Henry et al., 2011
<i>Daucus carota</i>	DcSUT2	0.5 mM	Parenchyma	Shakya and Sturm, 1998
<i>Asarina barclaiana</i>	AbSUT1		Phloem	Knop et al., 2001
SUT2 clade (mixed):				
<i>Plantago major</i>	PmSUC3	5.5 mM	SE	Barth et al., 2003
<i>Arabidopsis thaliana</i>	AtSUT2/	1.9 mM	SE	Meyer et al., 2004; Schulze et al., 2000
	AtSUC3	11.7 mM		
<i>Solanum lycopersicum</i>	SISUT2		SE, pollen	Hackel et al., 2006
<i>Solanum tuberosum</i>	StSUT2	Not functional	SE	Barker et al., 2000
<i>Hevea brasiliensis</i>	HbSUT2a		Young phloem laticifers	Dusotoit-Coucaud et al., 2009
SUT4 clade (mixed):				
<i>Solanum lycopersicum</i>	SISUT4		SE	Weise et al., 2000
<i>Solanum tuberosum</i>	StSUT4	6 mM	SE PM	Weise et al., 2000; Chincinska et al., 2008
<i>Nicotiana tabacum</i>	NtSUT4		Vacuole, PM	Okubo-Kurihara et al., 2010
<i>Arabidopsis thaliana</i>	AtSUT4	11.6 mM	Minor veins, vacuole, chloroplasts	Weise et al., 2000; Endler et al., 2006; Rolland et al., 2003
<i>Hordeum vulgare</i>	HvSUT2	5 mM	Vacuole, PM	Endler et al., 2006
<i>Lotus japonicus</i>	LjSUT4	16 mM	Vacuole, PM	Reinders et al., 2008
<i>Populus</i>	PtaSUT4		PM, Vacuole	Nilsson et al., 2010; Payyavula et al., 2010
<i>Daucus carota</i>	DcSUT1A	0.5 mM	Phloem	Shakya and Sturm, 1998
<i>Pisum sativum</i>	PsSUF4	37.9 mM		Zhou et al., 2007
<i>Malus domestica</i>	MdSUT1	630 µM	Parenchyma cells, phloem	Fan et al., 2009

(Continued)

Table 5.1 (Continued)

Species	Name	Km	Localization	Reference
SUT3 clade (monocotyledons):				
<i>Saccharum officinalis</i>	ShSUT1	2 mM, 8.26 mM	Vascular bundle	Rae et al., 2005; Reinders et al., 2006
<i>Oryza sativum</i>	OsSUT1	7.5 mM	SE + CC	Scofield et al., 2007; Sun et al., 2010
<i>Zea mays</i>	ZmSUT1	3.7 (6.5) mM	Phloem	Slewinski et al., 2009; Carpaneto et al., 2005
<i>Triticum aestivum</i>	TaSUT1		SE	Aoki et al., 2004
<i>Hordeum vulgare</i>	HvSUT1	7.5 mM		Weschke et al., 2000
<i>Lolium perenne</i>	LpSUT1	580 μ M		Berthier et al., 2009
SUT5 clade (monocotyledons):				
<i>Oryza sativum</i>	OsSUT5	2,32 mM		Sun et al., 2010
Others:				
<i>Saccharomyces pombe</i>	Sut1p	36.3 mM	PM	Reinders and Ward, 2001
<i>Ustilago maydis</i>	Srt1	26 mM	PM	Wahl et al., 2010
<i>Drosophila melanogaster</i>	SCRT	16 mM	Embryonic hindgut, ovarian follicle cells	Meyer et al., 2011

PM, plasma membrane; SE, sieve elements; CC, companion cells.

Cellular and Subcellular Localization of Sucrose Transporters Involved in Sucrose Uptake by Sieve Tubes

SUTs are engaged in sucrose uptake by SE–CCs in the collection phloem of source leaves as well as in retrieval in the transport phloem along the entire route from source to sink. However, the exact location of phloem loading in either SEs or CCs is not conclusively resolved. Anatomical features provide arguments for phloem loading by CCs with subsequent transfer of carbohydrates to SEs. Modification of CCs into transfer cells with numerous cell wall ingrowths in minor veins (i.e., in broad bean, *Vicia faba*) argue for phloem loading by CCs sustained by an intensified membrane transport (see Chapter 3). A further argument in favor of CCs being the location of phloem loading is that SEs in minor veins are often surrounded by CCs or phloem parenchyma cells (PPCs).

Localization studies are less equivocal with regard to the phloem localization of SUTs. Immunolocalization of sucrose transporting proteins in SEs and CCs suggests that direct sucrose uptake by SEs is feasible (Kühn et al., 1997; Knop et al., 2004; Aoki et al., 2004; Scofield et al., 2007). Sucrose retrieval along the phloem pathway is generally assumed to be executed by SEs (see for anatomical argumentation, van Bel, 1996). Most of the phloem-specific SUTs have been immunolocalized to SEs (Kühn et al., 1997; Barker et al., 2000; Weise et al., 2000; Reinders et al., 2002; Barth et al., 2003; Aoki et al., 2004; Meyer et al., 2004; Hackel et al., 2006; Weise et al., 2008), while others have been localized either to CCs (Stadler et al., 1995; Stadler and Sauer, 1996) or both SEs and CCs (Knop et al., 2004; Scofield et al., 2007).

Diverse experimental strategies have been employed to localize sucrose transporter 1 (SUT1) to either SEs or CCs. Analysis of SUT1 antisense plants revealed that the leaf SUT1 is essential for phloem loading and long-distance transport of assimilates (Riesmeier et al., 1994). Both SUT1 messenger RNA (mRNA) and protein were shown to be diurnally regulated and to have

high turnover rates (Kühn et al., 1997). The first indication for the phloem specificity of *SUT1* expression in potato (*Solanum tuberosum*) plants came from RNA *in situ* hybridization experiments (Riesmeier et al., 1993). In addition, antisense inhibition of *SUT1* expression under control of a CC-specific *rolC* promoter indicated synthesis of *SUT1* mRNA in CCs (Kühn et al., 1996). The expression of the tomato *sucrose transporter 1* (*SISUT1*-*uidA* (GUS) gene under control of the *SISUT1* promoter in transgenic tobacco plants (Weise et al., 2008) as well as the expression of a *SISUT1*-green fluorescent protein (GFP) fusion construct under control of the CC-specific *Agrobacterium rhizogenes* *rolC* promoter (Lalonde et al., 2003) directed the expression and accumulation of both fusion proteins in CCs. Thus, it appears that *SUT1* expression takes place in the transcriptionally active CCs. However, the *SUT1* protein was immunolocalized in plasma membranes of the enucleate SEs in tobacco, potato, and tomato. Analysis by *in situ* hybridization showed that *SUT1* mRNA localizes mainly to SEs and is preferentially associated with plasmodesmata (Kühn et al., 1997).

SUT mRNAs have been identified in sieve-tube exudates and are not restricted to solanaceous plants (Ruiz-Medrano et al., 1999; Knop et al., 2001; Doering-Saad et al., 2006). Phloem mobility of SUT mRNAs was confirmed by grafting experiments (He et al., 2008) as well as by symplasmic transfer of the transcripts from host plants to the parasitic plant *Cuscuta reflexa* (Deeken et al., 2008). The ability of SUT mRNAs to move via plasmodesmata was shown by microinjection experiments (Xoconostle-Cázares et al., 1999). These results provide evidence for trafficking of this endogenous mRNA and potentially also *SUT1* protein through specialized plasmodesmata (pore-plasmodesma units (PPUs)) between CCs and SEs in order to function in sucrose loading at the SE plasma membrane.

Contradictory immunolocalization data regarding the cell-specific localization of the *SUT1* protein in solanaceous plants (Schmitt et al., 2008) are most likely due to differences in the procedures used for antibody purification (Kühn, 2010) and do not fully correlate with *SUT1*-promoter activity as revealed by promoter reporter gene studies (Weise et al., 2008). The reason for doubts on the SE-specific localization of *SUT1* proteins certainly originates from incertitude about the synthesis and targeting of SE-specific proteins.

Here, substantial progress was made in the past few years. The mobility of GFP constructs expressed in transgenic plants under the control of a CC-specific *SUC*-promoter might have implications for understanding the deployment of SUTs. When GFP alone is expressed under control of the CC-specific *Arabidopsis sucrose transporter 2* (*AtSUC2*) promoter, the 27 kDa fluorescent reporter protein moves into the SEs and is unloaded in various sink organs (Imlau et al., 1999). The mobile GFP is thought to be unloaded symplasmically from the SEs into sink tissues. Soluble proteins like sporamin or patatin fused to GFP with sizes up to 67 kDa expressed under control of the CC-specific *AtSUC2* promoter revealed a large size exclusion limit for plasmodesmata of protophloem SEs (Stadler et al., 2005). All soluble GFP-fusions expressed under control of the CC-specific *AtSUC2*-promotor moved from CCs into the SEs and were translocated through the sieve tubes (Stadler et al., 2005).

Cytosolic trafficking through PPUs was not observed when GFP fusion proteins were membrane-anchored or retained in the endoplasmic reticulum (ER) by additional N-terminal signal sequence and C-terminal HDEL-retention signals. However, these observations contradict more recent studies using the small membrane-anchored *AtRCI2A-mCitrine* fusion protein of only 6 kDa expressed under control of the *AtSUC2*-promoter and containing two transmembrane spanning domains (Thompson and Wolniak, 2008). *AtRCI2a* is a small hydrophobic membrane protein, which is known to be cold inducible and to be targeted to the plasma membrane (Medina et al., 2001). This membrane-anchored fluorescent protein trafficked from the CCs into the SEs in *Arabidopsis* and tobacco

(*Nicotiana tabacum*). The fusion protein was detected in the plasma membrane of SEs as well as in vesicular structures that appear within the lumen of SEs.

Electron micrographs revealed the presence of vesicle-like structures in SEs from California shield leaf (*Streptanthus tortuosus*; Sjölund, 1997), English ivy (*Hedera helix*), carpetweed (*Ajuga reptans*; Hoffmann-Thoma et al., 2001), and broad bean (*V. faba*; Ehlers et al., 2000). Likewise, light-level microscopic analyses revealed the presence of vesicle-like structures in the SEs of tobacco (Noll et al., 2009), tomato (*Solanum lycopersicum*; Weise et al., 2000), potato (*S. tuberosum*; Liesche et al., 2010), and *Arabidopsis* (Thompson and Wolniak, 2008).

This raises the possibility that SEs have capacity to carry out vesicle-mediated intracellular trafficking and that SEs and CCs are coupled via ER-derived membranes within the PPUs. PPU desmotubules connecting the ER cisternae of CCs with the sieve element reticulum (SER) of SEs have been hypothesized to facilitate cell-to-cell movement of membrane-anchored proteins (Martens et al., 2006). ER coupling through desmotubules has been demonstrated using ER-specific fluorochromes and by fluorescence recovery after photobleaching (FRAP) (Martens et al., 2006). However, the size of the membrane-anchored protein appears to be a decisive factor for the ability of membrane proteins to traffic through PPUs, because the AtSUC2-GFP fusion protein, as well as the SISUT1-GFP and the SISUT1-GUS fusion proteins are retained in CCs (Lalonde et al., 2003; Weise et al., 2008)

Subcellular Localization of Sucrose Transporters

A Model for the Endocytosis of Sucrose Transporters

The half-life of SUT mRNA and protein was shown to be very short and to undergo permanent turnover (Kühn et al., 1997; Vaughn et al., 2002; He et al., 2008). This implies that SUTs in enucleate SEs need to be constantly renewed. The association of proteins to certain membrane compartments might be related to protein turnover or endocytosis and recycling as holds for animal cells (Ikonen, 2001). In yeast, the ergosterol-rich membrane compartment of Can1 (MCC) represents an area within the plasma membrane that controls the turnover of transport proteins (Grossmann et al., 2008).

Proteomics and imaging techniques recently showed a correlation between a reduced detergent solubility and membrane microdomain association of putative raft proteins (Simon-Plas et al., 2011). Many membrane proteins including membrane receptors that are known to be internalized by endocytosis are found in the detergent-resistant membrane (DRM) fraction of plant tissues (Mongrand et al., 2004; Borner et al., 2005; Morel et al., 2006; Titapiwatanakun et al., 2009; Krügel et al., 2011). DRM proteins represent 5–10% of total plant plasma membrane proteins, and specific signaling components are enriched in the DRM fraction (Shahollari et al., 2004).

The concept of lipid rafts, membrane rafts, or raft-like membrane microdomains on plant plasma membranes has been intensively investigated over the past few years. Membrane rafts have been defined by Pike (2006) as small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Concentrating membrane proteins in specific membrane microdomains is assumed to play a role in signaling, protein activity, endocytosis, oligomerization, degradation, or transport of proteins (Opekarova et al., 2005; Grossmann et al., 2008). Recently, a connection between plant membrane microdomains and the biosynthesis of cell wall polysaccharides was established (Bessueille et al., 2009). In animal cells, raft-like signaling platforms are also known to be involved in apoptosis and redox signaling (Jin et al., 2011). It is

important to note here that the insolubility of a protein in detergents is not necessarily indicative for its presence in membrane rafts *in vivo*: detergent resistance is not synonymous to raft association (Mongrand et al., 2010; Tanner et al., 2011).

Oligomerization of presumptive raft proteins was studied in detail by biochemical methods. While studying the oligomerization behavior of SUTs, a redox-dependent ability to form homodimeric complexes at the plasma membrane was detected both in plants and in heterologous expression systems (Krügel et al., 2008). The dimeric form of the SUT1 protein is detectable under nonreducing conditions, whereas only the monomeric form of the protein is detectable in the presence of reducing agents such as dithiothreitol (DTT) or β -mercaptoethanol. Surprisingly, the redox environment does not only affect the oligomerization behavior of SUT1 from potato and tomato but also the efficiency of plasma membrane targeting in yeast. In the presence of oxidizing agents, the potato sucrose transporter StSUT1 in the dimeric form appeared in 200 nanometer lipid raft-like membrane microdomains when fused to GFP and overexpressed in yeast (*Saccharomyces cerevisiae*) (Krügel et al., 2008).

SUT1-GFP is distributed homogenously over the membrane in a yeast *erg6* mutant deficient in ergosterol biosynthesis indicating that the sterol composition of the yeast plasma membrane is important for the raft association of SUT1. In contrast, the SUT1-GFP protein is constitutively found in raft-like microdomains in the $\Delta end3$ endocytosis-mutant of yeast (Liesche et al., 2010). In yeast cells, large immobile protein assemblies at the plasma membrane mark endocytotic sites. These sites, which are termed eisosomes, were characterized as static sites for internalization of membrane proteins and lipids (Walther et al., 2006). It is assumed, that StSUT1-GFP accumulates at these internalization sites in the endocytosis-deficient yeast mutant $\Delta end3$ (Liesche et al., 2010).

In plasma membrane fractions from potato wild-type plants, the SUT1 protein was enriched in the DRM fraction after solubilization with Triton X-100 and separation by sucrose density centrifugation (Krügel et al., 2008). Similar to the observations for yeast cells, SUT1 protein likely associates to membrane subcompartments or microdomains in plants. It is still unclear whether or not the association of the SUT1 protein to membrane microdomains affects its activity, stability, localization, or oligomerization.

There is evidence in favor of raft localization of the SUT1 protein as a prerequisite for endocytosis or recycling of the SUT at the SE plasma membrane. The ability of the SUT1 protein to be internalized by endocytosis was first tested with GFP fusion proteins expressed in yeast or in epidermis cells of infiltrated tobacco leaves. Internalization of the SUT1 protein in response to brefeldin A (BFA) treatment in mature SEs was also confirmed by immunolocalization (Liesche et al., 2010). In yeast and plant cells, the StSUT1-GFP fusion protein is internalized rapidly by endocytosis in response to BFA, inhibiting exocytosis, or cycloheximide, inhibiting *de novo* translation. The SUT1-GFP fusion protein colocalizes with the fluorescent endocytosis marker FM4-64 (Liesche et al., 2010). No internalization of the SUT1 protein is observed after sterol depletion of the plasma membrane with methyl- β -cyclodextrin, which argues that endocytosis of SUT1 is raft mediated (Liesche et al., 2010). Movement of BFA-induced SUT1-GFP containing compartments is completely abolished after application of cytochalasin D or latrunculin, inhibitors of actin polymerization/depolymerization, leading to the speculation that SUT1-vesicle trafficking is actin mediated.

BFA also affects vesicle movement in mature SEs as shown by immunolabeling of the SUT1 protein with fluorescence- or gold-labeled secondary antibodies (Krügel et al., 2008; Liesche et al., 2008; Liesche et al., 2010) interfering with components of the cytoskeleton that are generally believed to be absent in SEs (van Bel, 2003). Actin and actin depolymerizing factors are present in SEs of oilseed rape (*Brassica napus*) and pumpkin (*Cucurbita maxima*) as revealed by sieve-tube

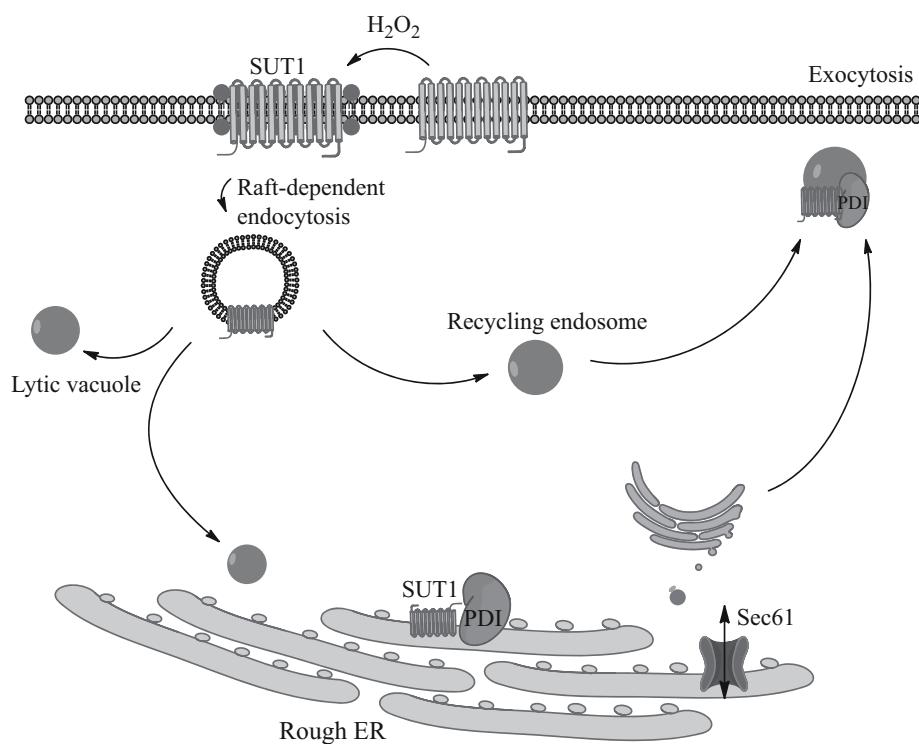


Figure 5.1 Model for plasma membrane recycling of the potato sucrose transporter StSUT1. The model is based on the biochemical investigation of the protein, the analysis of its targeting behavior in living cells and the systematic screen for SUT1-interacting proteins (Krügel et al., 2011; Krügel et al., 2008; Liesche et al., 2010). StSUT1 protein is synthesized in the ER (of companion cells?), the escort of the protein to the plasma membrane (of sieve elements?) might occur by a raft-associated protein disulfide isomerase (PDI), which was localized in the ER, in vesicles and in plasma membrane microdomains (Krügel et al., 2011). The lateral movement from companion cells into the sieve elements is assumed to occur via the desmotubules of PPUs connecting the ER cisternae of both cells. Recycling or endocytosis of the SUT1 protein is discussed to occur in a raft-mediated manner. Dimerization of the SUT1 protein under oxidizing conditions might be either the consequence of its concentration in membrane microdomains or alternatively facilitate raft association. (For a color version of the figure, please see Plate 5.1.)

sap analysis (Walz et al., 2004; Giavalisco et al., 2006; Lin et al., 2009). In squash (*Cucurbita pepo*) sieve-tube sap, high amounts of actin were identified by immunological localization and by electron microscopy (Kulikova et al., 2003).

It is assumed that raft association of the SUT1 protein does not only depend on the sterol composition and the membrane potential but is also related to SUT1 oligomerization, internalization, or degradation (Figure 5.1).

Vacuolar Sucrose Transporters

Vacuolar sucrose compartmentalization is an important prerequisite for the availability of sucrose for phloem transport (see Chapters 3 and 4). The vacuole is an important cellular storage compartment where sucrose can accumulate to high concentrations (Barbier-Bryggo et al., 1986; Willenbrink,

1987). Nevertheless, only a few vacuolar sugar transporters have been identified. Because of the highly acidic contents of vacuoles, sucrose-proton antiport has been assumed to be the mechanism for sucrose accumulation by vacuoles (Willenbrink, 1987).

SUTs that are predicted to be located in the tonoplast have been identified in *Arabidopsis* (*AtSUT4*; Endler et al., 2006), barley (*Hordeum vulgare*; *HvSUT2*; Endler et al., 2006), *Lotus japonicus* (*LjSUT4*; Reinders et al., 2008), and tobacco (*NtSUT4*; Okubo-Kurihara et al., 2010). All known vacuolar SUTs are functional for sucrose uptake when heterologously expressed in yeast or in *Xenopus laevis* oocytes (Weise et al., 2000; Weschke et al., 2000; Reinders et al., 2008; Okubo-Kurihara et al., 2010). Most of them show functional characteristics of sucrose-proton cotransporters implying a role in sucrose export from the vacuole rather than in sucrose import into the vacuole. All SUTs that so far have been identified in vacuolar membranes belong to the SUT4 clade of SUTs (Table 5.1) and act as proton cotransporters.

Although promoter-reporter gene studies revealed strongest *AtSUT4* promoter activity in minor veins of source leaves (Weise et al., 2000), expression of *AtSUT4* was also detected in mesophyll protoplasts that have been picked by microcapillaries (Endler et al., 2006). The *Arabidopsis* SUT4 protein plays only a minor role in vacuolar sucrose transport under physiological conditions (Schulz et al., 2011) and *sut4* mutant plants do not develop any phenotypical modifications.

The function of a tonoplast-localized SUT4 protein from rice, *OsSUT2*, was recently investigated. *OsSut2* mutant plants show higher sugar accumulation in leaves and reduced sucrose export from leaves (Eom et al., 2011). The increased sucrose efflux from leaves in *StSUT4*-inhibited potato plants indicates different functions for these two members of the SUT4 clade. A synthetic expression-optimized *OsSUT2* cDNA clone is able to mediate sucrose uptake at the plasma membrane of yeast cells in a proton-coupled manner with an unexpectedly high affinity (K_m of 1.9 mM at pH 4). All SUT4-members that have been localized to the tonoplast are also functional at the plasma membrane if heterologously expressed (Weise et al., 2000; Weschke et al., 2000; Okubo-Kurihara et al., 2010; Eom et al., 2011). Plasma membrane localization is claimed to be due to improper targeting of the transporter (Schulz et al., 2011).

The SUT4 protein from poplar (*Populus tremula × alba*) *PtaSUT4* is expressed in mesophyll cells and in the phloem. Silencing of *PtaSUT4* leads to the accumulation of sugars in leaves; therefore, it was assumed that *PtaSUT4* supports efflux from leaves (Pavyavula et al., 2010). Visualization of a GFP-PtaSUT4 construct clearly revealed GFP fluorescence in the interior of the protoplast, most likely at the tonoplast. *PtaSUT4* is able to complement sucrose uptake in the deficient yeast strain *SUSY7/ura3* arguing for plasma membrane localization. In a proteomic analysis of poplar plasma membrane proteins, the SUT4 sucrose transporter was detected as a transporter associated with the plasma membrane (Nilsson et al., 2010). The tobacco *NtSUT4* protein also functions at the plasma membrane when expressed in yeast, but could be targeted to the tonoplast since it colocalizes with FM4-64 in pulse-chase experiments. Dependent on its concentration and incubation time, FM4-64 is supposed to tag the plasma membrane, endocytotic vesicles, or the tonoplast (Kutsuna and Hasezawa, 2002). Overexpression of *NtSUT4-GFP* in tobacco BY-2 cells inhibits cell growth, supporting the idea that *NtSUT4* is involved in cell elongation via sucrose homoeostasis and affects cellulose production (Okubo-Kurihara et al., 2010).

In potato source leaves, a SUT4 antibody recognizes a protein of the expected size in the plasma membrane fraction and a truncated version of the protein in the endomembrane fraction, implying that the *StSUT4* protein could be degraded in vacuolar compartments (Chincinska et al., 2008). Discrepancies regarding localization of SUT4 proteins in different membrane compartment could either be due to species-specific differences (Ayre, 2011) or a dual SUT4 targeting to tonoplast as well as plasma membrane.

Regulation of Sucrose Transporters

Transcriptional Control of Sucrose Transporter Expression

In silico analysis of *cis*-regulatory elements of the promoter region of SUTs has assisted to identify essential regulatory units that are involved in the transcriptional control of SUT expression. Analysis of the 1.5 kb promoter regions of all five SUTs from rice plants, as well as all nine SUTs from *Arabidopsis* revealed the presence of one or more known *cis*-regulatory elements in each promoter sequence (A-box, RY, CAT, pyrimidine-box, sucrose-box, ABRE, ARF, ERE, GARE, Me-JA, ARE, DRE, GA-motif, GATA, GT-1, MYC, MYB, W-box, and I-box) (Ibraheem et al., 2010). The motif for sugar-mediated repression TACGTA (A-Box) was identified in the promoters of the rice *OsSUT1* and *OsSUT5* and tomato *SISUT1* genes (He et al., 2008).

Members of the *SUT1* subfamily of genes from beet (*Beta vulgaris*) *BvSUT1* or broad bean *VfSUT1* showed a negative effect of sucrose on their expression (Weber et al., 1997; Vaughn et al., 2002; Ransom-Hodgkins et al., 2003). A detailed analysis of the temporal transcriptional responses was carried out in *Arabidopsis* seedlings under sucrose depletion and resupply (Osuna et al., 2007). In these experiments, transcript levels of the sucrose transporter *AtSUC1* increased after 3 hours of sucrose resupply suggesting a very tight and coordinated regulation by sucrose at the transcriptional level.

The promoter region of the tomato *SUT1* gene contains regulatory elements for auxin responsiveness, circadian expression, ethylene responsiveness, pollen-specific expression, light regulation, guard cell-specific expression, and salicylic acid induction (He et al., 2008). Early investigations revealed the involvement of auxin in sucrose uptake. In common bean (*Phaseolus vulgaris*), auxin promoted sucrose transport in decapitated stems and whole shoots (Patrick, 1979). In sugar beet (*B. vulgaris*), indole acetic acid (IAA) strongly decreased active sucrose uptake in roots, whereas cytokinins enhanced the auxin-induced inhibition of sucrose uptake (Saftner and Wyse, 1984). The *SISUT2* gene contains two auxin responsive elements (AuxRE) in the promoter and in the tenth intron (He et al., 2008). Auxin-responsive elements (AuxRE) were also found in the promoter regions of *OsSUT3*, *OsSUT4*, *AtSUC1*, *AtSUC5*, *AtSUC6*, and *AtSUC9*. Auxin application to detached leaves affects *SUT1* gene expression in potato plants positively, both at transcriptional (Harms et al., 1994) and translational levels (He et al., 2008).

SISUT1 mRNA shows diurnal oscillation on RNA blots (Kühn et al., 1997), while real-time PCR analysis revealed circadian expression of all three known SUTs from potato (Chincinska et al., 2008). The tomato *SUT1* promoter contains an almost perfect “evening element,” which is a *cis*-acting motif common to circadian regulated genes (Harmer and Kay, 2005). Indeed the *SUT1* gene is preferentially expressed at the end of the light period similar to other circadian evening genes, and transcriptional control of *SUT1* expression follows a circadian rhythm. The *SUT2* gene from potato also follows a circadian oscillation pattern, with maximum transcript levels detectable in the early morning.

The promoter region of tomato *SUT2* revealed the presence of *cis*-regulatory elements necessary for regulation by GA, ethylene, light, ABA, and SA, wounding, salt and water stress, and pollen-specific expression (He et al., 2008). The *SISUT1* and *SISUT2* proteins have been immunolocalized to pollen tubes (Hackel et al., 2006), and *SISUT1* promoter::GUS studies in transgenic tobacco plants revealed *SUT1* promoter activity in phloem cells, guard cells, and trichomes (Weise et al., 2008). Therefore, *SUT1* promoter activity confirmed the *in silico* predictions of *cis*-regulatory elements.

Posttranscriptional Regulation of Sucrose Transporters

All three SUTs from solanacean species show a circadian rhythm pattern of expression under continuous light (Chincinska et al., 2008). The c-myc tagged *SoSUT1* gene fusion construct expressed under the cauliflower mosaic virus (CaMV) 35S promoter abolishes the normal circadian and diurnal patterns of *SUT1* expression (He et al., 2008). This infers that circadian regulation of the *SUT1* gene is based on the interaction of transcription factors binding to *cis*-regulatory upstream elements of the *SUT1* gene. Posttranscriptional control involving transcript stability is also plausible, since *SUT2* and *SUT4* transcripts from Solanaceae accumulate to high levels in 2 hours when *de novo* protein synthesis is inhibited by application of cycloheximide (He et al., 2008). Similarly, transcripts of *Arabidopsis AtSUT2/SUC3* accumulate significantly after a 3-hour cycloheximide treatment (Steinhauser et al., 2004).

The sucrose transporter *StSUT4* is involved in the phytochrome B-dependent shade avoidance response of potato plants (Chincinska et al., 2008). *StSUT4* mRNA stabilization appears to depend on the light quality, accumulating in response to shading or a reduced red/far red light ratio. Inhibitor studies using actinomycin A to determine the half-life of the *SUT4* mRNA under different light conditions reveal that under increased far red light or under shading conditions, the half-life of *SUT4* mRNA increases leading to accumulation of *StSUT4* mRNA (Liesche et al., 2011). *StSUT4* mRNA is potentially protected by far red-dependent RNA binding proteins from nucleolytic degradation under shading conditions. These data indicate that posttranscriptional processing of SUT mRNAs is tightly controlled.

SUT mRNAs are thought to be complexed with RNA-binding proteins since ribonucleoprotein complexes have been detected in the sieve-tube sap of several plant species (Ruiz-Medrano et al., 1999; Knop et al., 2001; Doering-Saad et al., 2002; Deeken et al., 2008). Phloem mobility was confirmed for several SUT mRNAs by various methods such as grafting, host plant-parasite interactions, and by laser microdissection combined with laser pressure catapulting. Phloem mobility was shown for *CmSUTP1* mRNA from melon (*Cucumis melo*; Roney et al., 2007), *SoSUT1* from spinach (*Spinacia oleracea*), *SISUT1* from tomato, *StSUT1* from potato, *NtSUT1* from tobacco (He et al., 2008), and *AtSUC2* and *AtSUT4* mRNA from *Arabidopsis* (Deeken et al., 2008). Phloem-mobile SUT transcripts are probably protected during long-distance transport in order to prevent ribonucleolytic degradation and likely move in form of ribonucleoprotein particles (RNP) through the phloem.

Posttranslational Control of Sucrose Transporters Expression

Regulation by Phosphorylation

The protein phosphatase inhibitor okadaic acid inhibited proton-motive force (pmf)-driven uptake of sucrose into plasma membrane vesicles from sugar beet leaves without affecting the amount of SUTs present in the vesicles. Okadaic acid appears to directly inhibit the sucrose cotransporter activity by maintaining the phosphorylated status of the cotransporter (Roblin et al., 1998). In addition to reducing *BvSUT1* transport activity, okadaic acid also decreased the *BvSUT1* protein and mRNA abundance and the relative transcription rate of the symporter gene. In contrast, kinase inhibitors increased sucrose transport, protein and mRNA abundance, and transcription (Ransom-Hodgkins et al., 2003). Thus, protein phosphorylation cascades appear to affect transcriptional activators of *BvSUT1* expression in a sucrose-dependent manner.

First evidence for the phosphorylation of the amino-terminal loop of SUTs arose from phosphoproteomic analyses of *Arabidopsis* plasma membranes (Nühse et al., 2004). Mass-spectrometric analysis of sucrose-induced phosphorylation changes revealed a phosphorylation site of the sucrose transporter *AtSUC1* at Ser-20 in the N-terminal region of the protein. Changes in relative phosphorylation of AtSUC1 in response to sucrose displayed as normalized relative ion intensities at different time points revealed a slight increase of this phosphopeptide after 30 minutes of sucrose supply (Niittyla et al., 2007). All SUTs from *Arabidopsis* with exception of *AtSUC2* contain this serine residue as a conserved putative phosphorylation site. Studies with chimeric transporters showed that the N-terminal region of SUTs is responsible for its substrate affinity (Schulze et al., 2000). Thus, amino-terminal phosphorylation of SUTs might represent a general feature of SUT regulation.

Regulation by Oligomerization

SUTs form homo- and hetero-oligomers when expressed in yeast (Reinders et al., 2002). Biochemical methods such as blue native polyacrylamide gel electrophoresis (PAGE), chemical cross-linking, immunoprecipitation, sodium dodecyl sulfate (SDS)-PAGE in the absence of the reducing agent DTT revealed the ability of the SUT1 protein from potato and tomato to dimerize *in planta*. SUT1 protein heterologously expressed in *Lactococcus lactis*, *X. laevis* oocytes, or yeast forms homodimers in a redox-dependent manner. The mechanistic relevance of SUT oligomerization is still not fully understood since monomers as well as dimers seem to function in sucrose transport (see Section 3.1).

Transgenic plants with reduced expression of *StSUT1* in the phloem show a dramatic reduction of the dimeric form of the protein in blue native PAGE, whereas the amount of the monomeric form of the SUT1 protein does not seem to change (Krügel et al., 2008), which suggests that the dimeric form of the protein predominates *in situ*. However, the sucrose uptake capacity of plasma membrane vesicles isolated from these plants is almost the same as in wild-type plants (Lemoine et al., 1996). In contrast, transgenic potato plants with constitutively reduced *StSUT1* expression show a reduction of the monomeric form of the protein, but not of the dimeric form in blue native PAGE (Krügel et al., 2008). The sucrose uptake capacity in plasma membrane vesicles isolated from these plants is dramatically reduced as compared to wild-type potato plants (Lemoine et al., 1996). This would support *in vivo* relevance of the SUT1 monomer for sucrose transport.

Evidence obtained with transgenic *SoSUT1*-plants argues in favor of functional significance of the dimeric form of the overexpressed protein. In blue native PAGE, only the dimeric form of the c-myc tagged overexpressed protein is detectable on two-dimensional gel electrophoresis following immunodetection with monoclonal c-myc antibodies (Liesche et al., 2008). The pmf dependent sucrose uptake in plasma membrane vesicles, isolated from these *SoSUT1-myc* overexpressing potato plants, is increased at least fourfold as compared to wild-type plasma membrane vesicles (Leggewie et al., 2003).

The redox environment also affects plasma-membrane targeting of a SUT1-GFP fusion protein expressed in yeast. The efficiency of plasma-membrane targeting in an oxidative environment could be increased due to higher ER-export rates when SUT1 protein is in the dimeric form (Krügel et al., 2008). Oligomerization as a prerequisite for efficient ER-export has been described for aquaporins (Zelazny et al., 2009) and the potassium channel KAT1 (Mikosch et al., 2006). Moreover, heterodimerization of PIP proteins in maize (*Zea mays*) and tetramerization of the potassium channel KAT1 are required for the efficient ER-export. The primary effects of redox reagents on sucrose transport activity are technically difficult to distinguish from changes in membrane potential, pmf, or plasma-membrane targeting of the transporter. Redox reagents appeared to have a slightly stimulating effect on the activity of *ZmSUT1* from maize (Sun et al., 2010), whereas decreased

sucrose transport activity of *StSUT1* was measured in the presence of redox reagents regardless of whether they were oxidizing or reducing (Wippel et al., 2010).

Using a combination of the yeast two hybrid split ubiquitin system, immunoprecipitation, and bimolecular fluorescence complementation interaction between the ER-anchored plant cytochrome b5 protein MdCYB5 from apple (*Malus domestica*) and the sucrose transporter MdSUT1 as well as the sorbitol transporter MdSOT6 was shown *in vivo* and *in vitro* (Fan et al., 2009). Interaction between the plant cytochrome b5 protein from apple or *Arabidopsis* and the two sugar transporters positively influences transporter affinity for the respective carbohydrates, sorbitol, or sucrose (Fan et al., 2009). In contrast, the yeast homologous cyt b5 protein did not increase carbohydrate affinity, which supports a plant-specific regulation mechanism. These data also show potential interactions between plasma membrane proteins and ER-anchored partners.

Sucrose Sensing and Signaling

Sucrose molecules have been postulated to constitute signals directing assimilate partitioning by regulation of their transporter activity. Sucrose accumulation and loss of sucrose symporter activity was directly correlated in plasma membrane vesicles isolated from sugar beet leaves (Chiou and Bush, 1998). Symporter activity dropped to 35–50% of water controls when leaves were fed with 100 mM sucrose and to 20–25% of controls with 250 mM sucrose. These sucrose-dependent changes in sucrose symporter activity were reversible (Chiou and Bush, 1998).

Later, transcript levels of *BvSUT1* turned out to be negatively affected specifically by sucrose (Vaughn et al., 2002). Nuclear run-on experiments demonstrated that decreased amounts of *BvSUT1* transcript are due to decreased transcriptional activity, and inhibitor studies revealed that both the *BvSUT1* mRNA and protein are rapidly degraded with half-lives of 108 minutes and 2.7 hours, respectively (Vaughn et al., 2002). In potato and tomato, a very short half-life was found for the SUT1 protein (Kühn et al., 1997), and the half-life of SUT transcripts varied between 69 and 131 minutes (He et al., 2008).

Sucrose Transport and Gibberellins

Not only the transcriptional regulation of SUT genes, but also the detailed analysis of the phloem sap content of various species revealed a potential link between sucrose transport and gibberellin signaling via the phloem translocation stream. Transcriptomic and proteomic studies on sieve tube content revealed the presence of many macromolecules engaged in gibberellin signaling and biosynthesis (see Chapter 10). Several cis-regulatory elements have been identified that could function in both sugar and gibberellin responses. The pyrimidine box motif is partially responsible for sucrose-mediated repression of genes and requires gibberellin for its induction (Washio, 2003). This motif was identified in the promoter region of three out of five SUTs from rice and of seven out of nine SUTs from *Arabidopsis* (Ibraheem et al., 2010). The GARE motif (gibberellin responsive element) is a *cis*-acting regulatory element involved in gibberellin responsiveness and partially involved in sucrose-mediated repression (Gubler and Jacobsen, 1992; Ogawa et al., 2003). Coexistence of regulatory elements involved in gibberellin and sugar responsiveness argues for the close interrelation between sucrose and GA signaling.

Sucrose Transport and Ethylene

Ethylene is a gaseous phytohormone that regulates plant growth and development. Ethylene-responsive genes contain ethylene-responsive elements within the promoter region that bind the ethylene responsive factor (ERF). Furthermore, an eight base pair sequence (ATTTCAAA) known as the ethylene-responsive enhancer element has been identified in the promoter region of ethylene responsive genes (Itzhaki et al., 1994). This ethylene responsive element (ERE) was identified in the 5' regulatory regions of *OsSUT2*, *OsSUT3*, *AtSUC5*, *AtSUC7*, *AtSUC8*, and *AtSUC9* (Ibraheem et al., 2010).

A link between ethylene regulation and SUT function is evidenced in rubber trees, sugar beet, and potato plants. Latex production in rubber trees (*Hevea brasiliensis*) takes place in phloem-localized laticifers (see Chapter 12). Sucrose plays an important role in latex production because it is the precursor of polyisoprene biosynthesis, and latex yield is also stimulated by ethylene (Dusotoit-Coucaud et al., 2009). SUTs have been localized in rubber tree laticifers, and three SUTs HbSUT1A, HbSUT2A, and HbSUT3 were shown to be specifically increased by ethylene or ethrel, an ethylene generator and latex stimulator (Dusotoit-Coucaud et al., 2009; Tang et al., 2010). The expression of HbSUT3 positively correlates with latex yield (Tang et al., 2010). Ethylene stimulation of latex production results in high sugar fluxes from the surrounding cells of the inner bark towards laticifers (Dusotoit-Coucaud et al., 2010).

Application of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) activates both ethylene production and sucrose transport in sugar beet root discs, and an inhibitor of ethylene production also inhibits sucrose uptake (Saftner, 1986). The relationship between ethylene and sucrose transport was analyzed in transgenic potato plants with reduced expression of the sucrose transporter *StSUT4* (K. Gier, U. Krügel, C. Kühn et al. unpublished). Accumulation of *StSUT4* transcripts is enhanced in potato wild-type plants after application of ethephon, an ethylene-generating compound. This implies that exogenously applied ethylene activates *StSUT4* gene expression. However, the transcript level of the endogenous ethylene biosynthetic enzyme ACC oxidase was significantly reduced in *SUT4*-inhibited plants, suggesting a reciprocal regulation of ethylene and SUTs (Chincinska et al., 2008). These results led to the speculation that ethylene production of *SUT4*-inhibited potato plants is reduced conferring the development of a characteristic phenotype, the complete loss of the shade-avoidance response.

Direct evidence for an interaction between SUTs and ethylene came from observations in *Arabidopsis*, where ethylene inhibits sucrose-induced anthocyanin accumulation (Jeong et al., 2010). Transcript levels of the sucrose transporter *AtSUC1* were enhanced in *Arabidopsis* plants treated with the ethylene-binding inhibitor silver nitrate or in ethylene insensitive *Arabidopsis* mutants. *AtSUC1* is thought to represent an integrator of signals in responses to sugar, light, and ethylene. Mutation of the *AtSUC1* gene lowered anthocyanin accumulation, soluble sugar content, and ethylene production in response to sucrose and light signals (Jeong et al., 2010).

The Role of Sucrose Transporters Outside the Phloem: Sucrose Transport and Pollen Development

The investigation of SUTs located outside the phloem, that is, in pollen and in growing pollen tubes could be helpful to understand the mechanisms of subcellular targeting and regulation of SUTs in phloem cells. Pollen cells are symplasmically isolated during maturation and germination, therefore

nutrient uptake necessarily occurs apoplasmically. Sugar loading in pollen occurs partially through the action of hexose transporters (Ylstra et al., 1998; Truernit et al., 1999; Ngampanya et al., 2003; Schneidereit et al., 2003; Scholz-Starke et al., 2003). However, *in vitro* germination of tobacco pollen was optimal when sucrose was used as a carbon source (Lemoine et al., 1999). ¹⁴C-Sucrose uptake and competition experiments revealed uptake of both sucrose and glucose (Lemoine et al., 1999). *In vitro* grown tomato pollen tubes also showed linear ¹⁴C-sucrose uptake even in the presence of an excess of unlabeled hexoses (Hackel et al., 2006), leading to the conclusion that the measured ¹⁴C-uptake is indeed due to uptake of intact sucrose and not due to the uptake of sucrose cleavage products (hexoses).

Pollen-specific SUTs have been identified. In tobacco, a pollen-specific sucrose transporter *NtSUT3* was isolated from a tobacco pollen cDNA library. The expression of the *NtSUT3* gene was restricted to pollen in mature flowers and pollen tubes after germination (Lemoine et al., 1999). Besides its localization in SEs, the tomato *SISUT2* gene was expressed in pollen and pollen tubes (Barker et al., 2000; Hackel et al., 2006). Inhibition of *SUT2* expression in transgenic tomato plants affected male fertility having severe negative effects on pollen morphology, development, germination, and pollen tube growth (Hackel et al., 2006).

Extracellular cleavage of sucrose by invertases plays an important part in pollen viability and male sterility (Goetz et al., 2001; Proels and Roitsch, 2009). Inhibition of the invertase *Lin5* or *Nin88* in transgenic tomato or tobacco plants, respectively, led to similarly negative changes in pollen morphology with reduced pollen germination as those observed in *SISUT2*-inhibited tomato plants (Goetz et al., 2001; Zanor et al., 2009). These studies suggest strong engagement of both extracellular cleavage and transport of sucrose in male sterility.

The *Arabidopsis* AtSUC1 protein could be involved in anther development and pollen tube growth (Stadler et al., 1999). The pollen of *Arabidopsis suc1* mutant plant is defective, resulting in aberrant segregation of reciprocal crosses and a decreased pollen germination rate. The expression of sucrose-regulated genes involved in anthocyanin biosynthesis is also affected in *suc1* mutant plants, leading to the assumption that AtSUC1 might be involved in sugar signaling and male gametophyte function (Sivitz et al., 2008). The *OsSUT1* gene of rice is also expressed in pollen, and the physiological role of *OsSUT1* was investigated in retrotransposon insertion lines. Only heterozygous *SUT1/sut1* plants could be obtained, and segregation of the progeny indicated that pollen grains containing a disrupted *SUT1* gene are dysfunctional (Hirose et al., 2010).

Future Directions

Further investigation of phloem-localized transporter proteins will contribute to a clearer view on carbohydrate processing in the highly specialized phloem cells. One major controversy in phloem sucrose transport pertains to the question if phloem loading of sucrose occurs at the plasma membrane of SEs, CCs, or both. Apart from immunolocalization studies, electrophysiological measurements using isolated protoplasts from both cell types (Ivashikina et al., 2003; Hafke et al., 2007) could help to elucidate this question.

Recent investigations on the interaction between SUTs and other proteins using yeast two hybrid split ubiquitin or coimmunoprecipitation provided novel information about regulation and targeting of SUTs (Fan et al., 2009; Krügel et al., 2011). Protein–protein interactions affect the affinity of SUTs (Fan et al., 2009). As many as 6% of the total SUT1-interacting proteins identified by mass spectrometry after coimmunoprecipitation are related to vesicle transport (Krügel et al., 2011), suggesting that the SUT1 protein undergoes vesicle trafficking. The SUT1 protein also shows

interaction with DRM-associated proteins that are components of raft-like microdomains (Krügel et al., 2011). Investigation of SUT-interacting proteins will shed more light on open questions, such as how the SUT1 protein that is expressed and perhaps translated in CCs can efficiently be transferred to the SE plasma membrane.

Innovative imaging methods have shown that lateral segregation of lipids and proteins exists in the plasma membrane. Perhaps, plant membrane microdomains represent signal transduction platforms similar to those documented for mammalian cells (Mongrand et al., 2010). Localization of typical raft proteins such as remorin or GPI-anchored proteins such as callose-binding protein at plasmodesmata indicates that raft-like microdomains might also accumulate in the plasma membrane lining plasmodesmata (Mongrand et al., 2010). Thus, characterization of SUTs that have been assigned to the DRM fraction will certainly help to investigate the communication between SEs and CCs and to renew insights on sieve-tube function.

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6 Cellular Basis of Electrical Potential Waves along the Phloem and Impact of Coincident Ca^{2+} Fluxes

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In 1973, Barbara Pickard published an excellent review that advanced almost prophetic speculations on electrical signaling in higher plants (Pickard, 1973). This review included many long-forgotten publications on electrical activities in plants. Burdon-Sanderson (1873), for instance, demonstrated that plants use action potentials (APs) as signals resulting in rapid leaf movements as is the case for the insectivorous Venus flytrap (*Dionaea muscipula*). His work disclosed a wealth of facts on APs in plants, including kinetic parameters such as rise times, duration, and rates of propagation (Pickard, 1973). A second mode of transmission, the variation potential (VP), was later discovered to propagate along petioles of the sensitive plant (*Mimosa pudica*) following a flame-stimulated AP (Houwink, 1935). A third mode of transmission, the system potential (SP), has been recently postulated (Zimmermann et al., 2009).

Far ahead of her time, Pickard (1973) speculated about function and impact of electrical signals in higher plants by raising numerous questions. Is phloem transport regulated by APs? Is there an electrically controlled calcium (Ca^{2+}) influx in phloem reminiscent of that in the sarcoplasmic reticulum of muscle fibers? Do APs release plant hormones or short-range control substances? These and other questions have only been incidentally addressed. This chapter focuses on the cellular ionic basis of electrical activities along the phloem and the impact of coincident Ca^{2+} fluxes on a variety of responses of sieve elements (SEs) and adjoining cells.

Electrical Long-Distance Signals in Higher Plants

In animals, fast transmission of electrical signals is mediated by APs that propagate in a wave-like fashion along neurons. In contrast, plants have evolved diverse electric activities or “signal

Abbreviations: AP, action potential; cADPR, cyclic ADP-ribose; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; CC, companion cell; CICR, calcium-induced calcium release; DACC, depolarization-activated Ca^{2+} -permeable channel; EPW, electrical potential wave; ER, endoplasmic reticulum; HACC, hyperpolarization-activated Ca^{2+} -permeable channel; IP₃, inositol 1,4,5-triphosphate; IP₆, myo-inositol hexakisphosphate; MSC, mechano-sensitive channel; NSCC, nonselective cation channel; PPU, pore-plasmodesma unit; SE, sieve element; SE-CC, sieve element-companion cell complex; SER, sieve element endoplasmic reticulum; SP, system potential; SWP, low-wave potential; VP, variation potential; VT, voltage transient

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types” along vascular pathways in response to stimuli (Pickard, 1973; Stahlberg and Cosgrove, 1997; Davies and Stankovic, 2006; Davies, 2006; Zimmermann et al., 2009). These signals transmit information over long distances to effect on essential physiological processes in remote target tissues or organs (Trebacz et al., 2006; Stahlberg et al., 2006). The signals exert remote control on gene expression (Wildon et al., 1992; Pena Cortes et al., 1995; Stankovic and Davies, 1997; Stankovic et al., 1998) and a multitude of physiological processes such as growth, respiration, water supply, photosynthesis, and synthesis of chemical signal messengers such as jasmonic acid, abscisic acid, and free radicals (reviewed in Fromm and Lautner, 2006; Trebacz et al., 2006; Pyatygin et al., 2008).

VPs or slow wave potentials (SWPs) and APs, together named electrical potential waves (EPWs), are long-distance systemic electrical signals that occur in response to various mechanical and physical stimuli (Stahlberg and Cosgrove, 1997; Davies, 2006). They are elicited by a wide variety of abiotic and biotic stimuli such as sudden cold or light treatments, squeezing, touching, cutting, heating, burning, and chewing by insects. Recently, a novel signal type known as wound-induced SPs was identified that is clearly distinct from the other EPWs (Zimmermann et al., 2009).

Propagation or generation of EPWs is tightly associated with the vascular system (Rhodes et al., 1996; Stahlberg et al., 2006; Davies, 2006; Furch et al., 2007; Hafke et al., 2009). Coincident electrical signals result in “mixed kinetics” (e.g., an overlap of AP and VP), which often hinders proper signal analysis (Stahlberg et al., 2006; Davies and Stankovic, 2006; Furch et al., 2007; Hafke et al., 2009). Distinction of the signal types is based on kinetic parameters such as amplitude, signal duration, and comparative shapes such as transient spikes or slow waves. Furthermore, stimulus specificity, defined as signal types that are related to specific stimuli and the ion fluxes mediating depolarization and repolarization of the electrical signals (Stahlberg et al., 2006) help to disclose EPW identity.

Action Potentials in Plants

APs are characterized by rapid transient spike-like changes of the resting membrane potential (Stahlberg and Cosgrove, 1997). The period between the onset of depolarization and the completion of repolarization lasting 1–50 seconds in most plants identifies an EPW as an AP (Pickard, 1973; Fromm, 1991). Like in nerve cells, APs in plants seem to obey an all-or-nothing law (Fromm and Spanswick, 1993; Pyatygin et al., 2008). If the plasma membrane depolarization exceeds a critical threshold, an AP or self-amplifying depolarization of required height or amplitude is generated that is independent of the stimulus strength. The AP propagates with a constant amplitude (Figure 6.1) and velocity (Zawadzki et al., 1991; Stankovic et al., 1998; Davies and Stankovic, 2006) of 0.5–10 cm s⁻¹ throughout the plant (Fromm, 1991; Wildon et al., 1992; Fromm and Spanswick, 1993; Fromm and Lautner, 2006). Also similar to nerve cells (Hille, 1992), APs of higher plants have a distinct absolute refractory period after the decay of excitation that can last from a few minutes up to several hours (Zawadzki et al., 1991; Fromm and Spanswick, 1993).

Sieve element–companion cell complexes (SE–CCs) are the conductive elements responsible for systemic transmission of APs (Samejima and Sibaoka, 1983; Fromm, 1991; Wildon et al., 1992; Fromm and Spanswick, 1993; Fromm and Bauer, 1994; Rhodes et al., 1996; Furch et al., 2007, 2009; Hafke et al., 2009) (Figure 6.1). Lateral transmission through adjacent cell layers occurs via plasmodesmata (Rhodes et al., 1996; Trebacz et al., 2006) (Figure 6.1), and transmission can be limited to organs or regions within the plant (Trebacz et al., 2006). For example, APs in narrow leaf lupine (*Lupinus angustifolius*) propagate both acropetally and basipetally along the stem, but do not reach roots or leaves (Zawadzki, 1980).

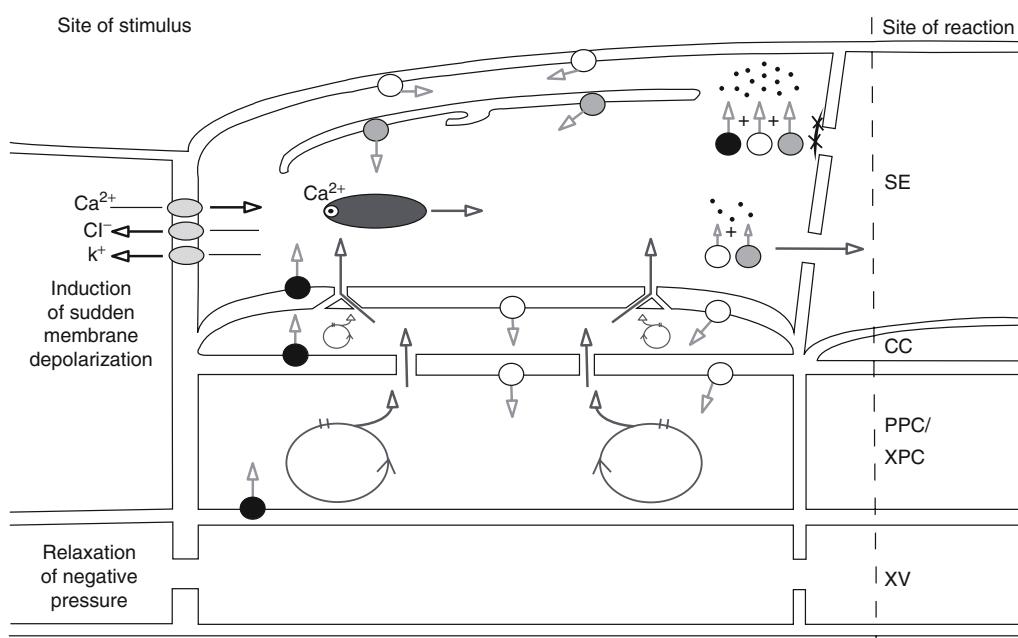


Figure 6.1 Ca^{2+} as the key link between electrical long-distance signaling and local chemical signaling. The events are presented for action and variation potentials in one diagram and actually occur simultaneously as in responses to burning [Dziubinska et al., 2001; Hlavackova et al., 2006; Furch et al., 2009]. Action potentials—brought about by Ca^{2+} influx, Cl^- efflux, K^+ efflux in succession—are triggered by several stimuli (upper left) and are transmitted by the phloem of which sieve elements (SEs) are the conducting modules. High amounts of Ca^{2+} influx (light-grey arrows), mediated by voltage-gated plasma membrane bound channels (white circles), enhance Ca^{2+} influx by stimulation of ER-bound Ca^{2+} -activated Ca^{2+} channels (grey circles). Propagation of action potentials via plasmodesmata in lateral directions induces Ca^{2+} influx by voltage-activated channels (white circles) in other vascular cells such as companion cells (CC) and phloem (PPC) and xylem parenchyma (XPC) cells with outputs varying with the structure/function organization of each cell type. Variation potentials are triggered (e.g., by wounding) by a sudden relaxation of the negative pressure that then propagates as a wave along xylem vessels (XVs). Disturbance of the water potential equilibrium results in water uptake and increased turgor of adjacent vascular cells located along the XVs. Their enhanced turgors activate mechanosensitive Ca^{2+} channels in xylem parenchyma and adjacent cells (black circles) leading to Ca^{2+} influx and subsequent potential waves. Variation potentials may also propagate symplasmically to SEs and induce Ca^{2+} influx including that from the ER cisternae in the sieve-element mictoplasm. It is unclear if Ca^{2+} influx in SEs is also elicited by substances produced in the vascular cells (dark-grey arrows) due to Ca^{2+} -triggered cascades (arrowed circles). These compounds may also play a role in long-distance signaling (dark-grey arrows in SEs). Furthermore, Ca^{2+} ions released into SEs may activate Ca^{2+} -binding proteins (dark-grey ovals) involved in long-distance signaling [Stahlberg and Cosgrove, 1997]. When the Ca^{2+} concentration in SEs (black dots) surpasses a threshold value, callose deposition is induced. Action potentials are usually unable to increase Ca^{2+} concentration to such an extent (grey + white circles) that the Ca^{2+} threshold is reached. However, in the case of burning, combinatory effects of action and variation potentials conferred by the aggregate contribution of the respective Ca^{2+} channels (white + grey + black circles) lead to blockage of long-distance and short-distance symplasmic transport. (Modified after van Bel et al., 2011). (For a color version of the figure, please see Plate 6.1.)

The selective propagation of APs indicates the existence of electrical barriers probably of anatomical origin that have not been investigated thus far. In general, the role of anatomical features in the propagation of APs has been virtually neglected. The spatial connectivity of vascular bundles, presence of isolated cell layers around conductive elements (Fleurat-Lessard and Roblin, 1982) as well as a reduced symplasmic coupling at the interface between SE-CCs and adjoining cells in transport phloem (van Bel and van Rijen, 1994; Rhodes et al., 1996; Hafke et al., 2005) should be examined more thoroughly in various species.

The orchestrated activity of voltage-dependent ion channels provides the basis for the fast AP kinetics (Lunevsky et al., 1983; Okihara et al., 1991; Zawadzki et al., 1991; Wayne, 1994; Fromm and Bauer, 1994; Davies, 2004). The ionic mechanisms underlying AP generation have been documented for algae (Lunevsky et al., 1983; Okihara et al., 1991; Homann and Thiel, 1994; Thiel et al., 1997), lower plants (Trebacz et al., 1994; Krol et al., 2003), and higher plants (Iijima and Sibaoka, 1981, 1982, 1985; Hodick and Sievers, 1988; Fromm and Spanswick, 1993; Fromm and Bauer, 1994; Opritov et al., 2002; Krol et al., 2004; Fisahn et al., 2004; Felle and Zimmermann, 2007; Furch et al., 2007, 2009).

AP mechanisms in algae bear strong resemblance to events that occur in higher plants (Trebacz et al., 2006). An initial membrane depolarization is triggered by a transient increase in cytoplasmic Ca^{2+} concentration brought about by gating of Ca^{2+} -permeable channels in the plasma membrane. Influx of Ca^{2+} leads to a chloride (Cl^-) efflux via Ca^{2+} -dependent anion channels located in the plasma membrane (Lunevsky et al., 1983; Tsutsui et al., 1986; Okihara et al., 1991; Homann and Thiel, 1994). There are several indications that additional Ca^{2+} is released from intracellular compartments during the transient Ca^{2+} increase. Signal molecules such as inositol 1,4,5-triphosphate (IP_3) may link the electrical events at the plasma membrane with the Ca^{2+} mobilization from the endoplasmic reticulum (ER) (Plieth et al., 1998; Wacke and Thiel, 2001; Wacke et al., 2003; Thiel et al., 2003).

Voltage-dependent potassium (K^+) channels open just before the steady-state potential for Cl^- ions is reached and mediate K^+ efflux that results in a repolarization to the resting value of the membrane potential (Homann and Thiel, 1994; Thiel et al., 1997). Apart from the ion fluxes, electrogenic proton (H^+)-pumps, at the plasma membrane are involved in the repolarization phase of APs. Since the repolarization is limited by the Nernst-potential for K^+ , the activity of H^+ -pumps must be increased to restore the membrane potential (Kishimoto et al., 1985).

In thallus tissue of scented liverwort (*Conocephalum conicum*) (Trebacz et al., 1994; Krol et al., 2003) and in higher plants, where Ca^{2+} and Cl^- channels are involved in depolarization and K^+ channels and H^+ -pumps involved in repolarization, APs are generated in a manner similar to that in algae (Trebacz et al., 1994, 2006 and Iijima and Sibaoka, 1985; Hodick and Sievers, 1988; Fromm and Spanswick, 1993; Fromm and Bauer, 1994; Lewis and Spalding, 1998; Opritov et al., 2002; Fisahn et al., 2004; Krol et al., 2004; Davies, 2006; Felle and Zimmermann, 2007; Furch et al., 2007, 2009; Pyatygina et al., 2008).

Like in algae, studies in liverworts and higher plants suggest that additional Ca^{2+} is mobilized from internal stores during APs (Krol et al., 2003, 2004; Furch et al., 2009). Cellular second messengers like the inositol phosphates IP_3 (Gilroy et al., 1990) and IP_6 (*myo*-inositol hexakisphosphate; Lemtiri-Chlieh et al., 2003) or cADPR (cyclic ADP-ribose; Leckie et al., 1998) may play a pivotal role as signals to release Ca^{2+} ions from endomembrane systems (Lemtiri-Chlieh et al., 2003; Kudla et al., 2010). Krol et al. (2003, 2004) reported inhibition of APs by the phospholipase C inhibitor neomycin in scented liverwort, *Arabidopsis*, sunflower (*Helianthus annuus*) and broadbean (*Vicia faba*). Phospholipase C is known to be involved in formation of IP_3 . Although IP_3 triggers Ca^{2+} release from endomembranes (Gilroy et al., 1990; Kudla et al., 2010), no candidate for an IP_3 receptor has been identified to date in the genomes of higher plants (Kudla et al., 2010). In SEs, Ca^{2+} -dependent forisome responses (Knoblauch et al., 2001) may depend on the liberation of Ca^{2+} from SE ER which points indirectly to involvement of internal Ca^{2+} stores in SE occlusion (Furch et al., 2009; Hafke et al., 2009; Thorpe et al., 2010).

In contrast to APs in animals, K^+ and Cl^- fluxes during APs in plants far exceed the theoretical ion charges required for loading the membrane capacitance (Gaffey and Mullins, 1958; Fromm and Spanswick, 1993; Trebacz et al., 1994). Therefore, net efflux of K^+ and Cl^- during APs might

play a part in regulating ion homeostasis (e.g., Mummert and Gradmann, 1991; Trebacz et al., 1994).

There is little information about location and nature of ion channels and pumps involved in AP transmission through the phloem. Dihydropyridin-sensitive voltage-dependent Ca^{2+} channels were localized to the phloem of tobacco (*Nicotiana tabacum*) and water lettuce (*Pistia stratiotes*) (Volk and Franceschi, 2000) and in more detail, to the plasma membrane and ER membranes of SEs of broad bean (Furch et al., 2009). Furthermore, H^+ -ATPases were localized via immunochemical methods to SEs and CCs in transport phloem of castor bean (*Ricinus communis*) (Langhans et al., 2001).

Phloem-localized K^+ channels of the AKT2/3 (*Arabidopsis* K^+ transporter) type were electrophysiologically characterized and related to AP repolarization (Marten et al., 1999; Bauer et al., 2000; Lacombe et al., 2000; Deeken et al., 2002). Weak inward rectifying currents matching the electrical characteristics of AKT2/3 channels were recorded in SE protoplasts of broad bean (Hafke et al., 2007). The increasing permeability of AKT2/3 channels at more alkaline pH values (Marten et al., 1999) as well as extracellular alkalinization observed during transmission of electrical signals (Zimmermann et al., 2009) point to participation of AKT2/3 channels in membrane repolarization. Moreover, passive K^+ fluxes contribute to a stabilization of the membrane potential during sucrose transport by charge compensation (Marten et al., 1999; Deeken et al., 2002; van Bel and Hafke, 2005). Therefore, it has been proposed that K^+ channels of the AKT2/3 type are involved in phloem loading and unloading of sucrose (Marten et al., 1999; Lacombe et al., 2000; Deeken et al., 2002; Gajdanowicz et al., 2011).

Variation Potentials in Higher Plants

VPs differ from plant APs in several ways. VPs do not obey the all-or-nothing law and are graded signals, of which amplitude and signal duration ranging from 10 seconds up to 30 minutes vary commensurate with the stimulus strength (Stahlberg and Cosgrove, 1997; Stahlberg et al., 2006). Propagation velocities of $0.1\text{--}1 \text{ cm s}^{-1}$ (Stahlberg and Cosgrove, 1997) and amplitude drop rapidly along the transmission path of VPs (Davies, 2004; Stahlberg et al., 2006). For example, a decrease in amplitude of $2.5\% \text{ cm}^{-1}$ has been observed in sunflower (*Helianthus annuus*) (Stahlberg et al., 2005). Thus, VPs attenuate with increasing distance from the site of stimulus and finally fade away (van Sambeek and Pickard, 1976).

Generation and propagation of VPs have only been detected in intact plants containing functional xylem, whereas APs can also be triggered in isolated plant organs (Stahlberg et al., 2006). Thus, VP generation seems to be tightly linked to physical processes in xylem vessels. A hydraulic pressure wave resulting from relaxation of negative pressure that propagates through the xylem vessels serves as a likely basis for VP generation (Stahlberg and Cosgrove, 1997). This relaxation leads to turgor disturbance of adjoining vascular parenchyma cells due to increased water uptake and in turn, to membrane depolarization (Malone and Stankovic, 1991; Stahlberg and Cosgrove, 1992, 1997; Mancuso, 1999; Davies, 2006) (Figure 6.1). Since the physical origin of VPs lies in the dead xylem vessels, VPs can pass dead or poisoned areas (Stahlberg et al., 2006). Spreading of the signal from xylem parenchyma to phloem is achieved by communicating turgor changes to adjacent cells and subsequent activation of mechanosensitive ion channels (van Bel et al., 2011) (Figure 6.1).

While fast depolarization during APs is mediated by ion channels, slow depolarization during VPs might originate from shutdown of electrogenic proton pumps at the plasma membrane. Experiments conducted in the presence of metabolic inhibitors as well as pH-dependent fluorescent dyes point to a mechanosensitive transient inhibition of electrogenic H^+ pumps during VPs (Stahlberg and

Cosgrove, 1992, 1996). Early studies (Reinhold and Kaplan, 1984) hinted at a pressure sensitivity of H⁺ pumps. Dense deposition of proton pumps was found on the plasma membrane of SEs (Langhans et al., 2001).

Mechanosensitive channels as found in the plasmamembrane of various cell types (for guard cells e.g., Cosgrove and Hedrich, 1991) could be involved in VPs (Davies, 2006; Davies and Stankovic, 2006). Mechanosensitive Ca²⁺-permeable channels reside on the plasma membrane of SEs in intact phloem tissue (Knoblauch et al., 2001; Furch et al., 2009) and isolated SE protoplasts (Hafke et al., 2007). A transient increase of Ca²⁺ in SEs through Ca²⁺-permeable channels during the VP-phase of an EPW has recently been reported (Furch et al., 2009) (Figure 6.1).

Hydraulic changes or voltage modulations could also give rise to production of signaling substances that are translocated in the xylem stream and trigger VPs in vascular cells along the pathway (e.g., Ricca's factor: Ricca, 1916; van Sambeek and Pickard, 1976; van Sambeek et al., 1976; Boari and Malone, 1993; Malone, 1996; Stahlberg and Cosgrove, 1997; Mancuso, 1999) (Figure 6.1). Oligosaccharides as well as the peptide systemin in solanaceous species are among potential second messengers triggering VP-like depolarizations (Thain et al., 1995; Moyen and Johannes, 1996). Given the hydraulic and chemical nature of VP generation, mechanosensitive and ligand-activated channels have been postulated to be involved in VP generation (Davies, 1993; Stankovic et al., 1997; Davies, 2006; Davies and Stankovic, 2006; Stahlberg et al., 2006) (Figure 6.1).

Stimulus-Specific Generation of APs and VPs?

Distinction between APs and VPs based solely upon their kinetic characteristics (Stankovic et al., 1997; Dziubinska et al., 2001) might be dubious since VPs often show AP-like kinetics (Stahlberg et al., 2006; Furch et al., 2008). Therefore, additional criteria such as stimulus specificity and strength needed to trigger the signals are invoked (Stahlberg et al., 2006). APs are triggered by nonwounding and natural stimuli (Trebacz et al., 2006; Stahlberg et al., 2006). This holds for the fast thigmonastic leaf movements in touch-sensitive plants (Sibaoka, 1969, 1991), the carnivorous Venus flytrap (Hodick and Sievers, 1988, 1989) and waterwheel plant (*Aldrovanda vesiculosa*) (Iijima and Sibaoka, 1981), as well as the complex thigmotropic and thigmonastic tentacle movements of sundews (several *Drosera* species) (Williams and Pickard, 1972; Williams and Spanswick, 1976). APs are also triggered by distant cooling using ice water (Fromm, 1991; Fromm and Bauer, 1994).

VPs are mostly triggered by damaging events such as burning (Houwink, 1935; van Sambeek and Pickard, 1976; Roblin, 1985; Wildon et al., 1992; Stankovic et al., 1998; Mancuso, 1999; Furch et al., 2007, 2010; Hafke et al., 2009), hot water (van Sambeek and Pickard, 1976), vigorous cutting (Mancuso, 1999; Furch et al., 2008; Hafke et al., 2009), strong crushing (van Sambeek and Pickard, 1976), electroshocks (Pickard and Minchin, 1990), and injury by chewing insects (Alarcon and Malone, 1994). A relationship between nonwounding/wounding stimuli and electrical response becomes apparent, when the stimulus strength varies under identical conditions. AP-like kinetics were observed when a plant is gently pushed using a forceps whereas, strong crushing with the same forceps resulted in VP-like kinetics (van Sambeek and Pickard, 1976).

However, a strict distinction of VPs and APs is hardly feasible in several instances. Strong injuries often trigger additional APs resulting in composite signals of APs and VPs (Davies and Stankovic, 2006; Furch et al., 2007, 2009; Hafke et al., 2009) (Figure 6.1). For example, a burning stimulus evokes composite signals (Houwink, 1935; van Sambeek and Pickard, 1976; Roblin, 1985; Roblin and Bonnemain, 1985; Stankovic et al., 1997, 1998) when recorded in close proximity of the

burning site (Davies and Stankovic, 2006; Furch et al., 2007, 2009, 2010; Hafke et al., 2009; Thorpe et al., 2010). With increasing distance from the site of stimulus, the two signals drift apart due to differences in propagation velocities and can be distinguished (Davies et al., 1991; Stankovic et al., 1998; Davies and Stankovic, 2006; Furch et al., 2010). Furthermore, dissimilar refractory periods enable unequivocal distinction of between AP and VP (Hafke et al., 2009).

EPWs Trigger Ca^{2+} Influx via Diverse Ca^{2+} Permeable Channels into Sieve Elements

Despite the different origin of APs and VPs and regardless of the engagement of voltage-dependent, mechanosensitive, or ligand-activated ion channels (Stahlberg and Cosgrove, 1997; Davies, 2006; Zimmermann et al., 2009), an essential feature of EPWs is the sharing of an initial Ca^{2+} influx (Trebacz et al., 2006; Davies and Stankovic, 2006; Demidchik and Maathuis, 2007; McAinsh and Pittman, 2009) into SEs (Furch et al., 2009; Hafke et al., 2009). Ca^{2+} activates numerous intracellular signaling cascades, and by implication, Ca^{2+} -permeable channels function as relay stations transforming long-distance electrical messages into local chemical intracellular signals (Knoblauch et al., 2001; Demidchik and Maathuis, 2007; Furch et al., 2009; Hafke et al., 2009; Kudla et al., 2010) by mediating Ca^{2+} influx from the extracellular space into the cell (Demidchik and Maathuis, 2007; Kudla et al., 2010) (Figure 6.1).

Specific Ca^{2+} -binding proteins (White and Broadley, 2003; Kudla et al., 2010) such as calcium-dependent protein kinases as found in SEs (Nakamura et al., 1993; Yoo et al., 2002) can be attached to the cytoskeleton (Malho et al., 1998). In this way, information provided by Ca^{2+} signatures is decoded and transformed into specific protein-protein interactions resulting in Ca^{2+} -dependent phosphorylation cascades and transcriptional responses that lead to downstream reactions (Luan et al., 2002; Sanders et al., 2002; Kudla et al., 2010).

Despite a wealth of data on Ca^{2+} -mediated processes in other cell types (White and Broadley, 2003; McAinsh and Pittman, 2009; Kudla et al., 2010) quantitative information is not available for Ca^{2+} -mediated signal cascades in SE-CCs. Development of new methods such as the isolation of SE protoplasts will facilitate a better understanding of Ca^{2+} effects in SEs (Hafke et al., 2007; Furch et al., 2009; Thorpe et al., 2010). Determining channel densities and distribution in SEs along with the quantification of Ca^{2+} resting values and the Ca^{2+} dynamics during the passage of EPWs has the potential to elucidate signal cascades such as those that appear to be involved in phloem-specific processes, for example SE occlusion in response to remote wounding (Furch et al., 2009).

Ca^{2+} Activities in Sieve Elements before, during, and after EPW Passage

In broad bean, steady-state levels of Ca^{2+} in sieve-tube sap collected via aphid stylectomy (Fisher and Frame, 1984) and in the cytoplasm of CCs and SE mictoplasm were measured to be in the range between 50 and 100 nM (Furch et al., 2009). These values are similar to those in other cell types having cytoplasmic Ca^{2+} -concentrations in the range of 10–200 nM (Malho et al., 1998; Trewavas, 1999), but contrast the results of previous studies where Ca^{2+} resting values in SEs were determined to be in the μM to mM range (Fromm and Bauer, 1994; Brauer et al., 1998). Values in the nM range indicate a classical role for Ca^{2+} as a signaling ion in SEs (White and Broadley, 2003; Furch et al., 2009; Hafke et al., 2009; Kudla et al., 2010). A strong distant burning stimulus triggers long-lasting EPWs (Furch et al., 2007, 2009; Hafke et al., 2009) that coincide with increased Ca^{2+} levels of 200 to 500 nM in CC cytoplasm, SE mictoplasm, and in the proximity of sieve plates (Furch et al., 2009).

The mictoplasm was originally defined as the mixture of cytoplasmic contents and the luminal fluid in SEs after disintegration of the vacuole (Engleman, 1965). For practical reasons, the definition was narrowed to the viscous SE cytoplasmic lining that is in open connection with the luminal sap (van Bel, 2003). Only in the mictoplasmic layer, Ca^{2+} concentrations are strongly enhanced during the passage of electropotentials in SEs.

The Ca^{2+} changes in SE mictoplasm are similar to those induced by various local stimuli such as rapid cooling in other cell types (Malho et al., 1998; Plieth, 2001). Elevated Ca^{2+} levels triggered by abrupt chilling are of special interest because most of what is known about stimulus-induced Ca^{2+} -elevations arose from imposing varying cooling regimes (Knight et al., 1996; Plieth, 1999; Plieth et al., 1999; Plieth, 2001; White, 2009). In root cells exposed to rapid chilling, both the magnitude of the electrical response (Minorsky and Spanswick, 1989; White, 2009) as well the Ca^{2+} increase (Plieth, 1999; Plieth et al., 1999) was related to the cooling rate, extent, and duration (White, 2009). Interestingly a cooling rate-dependent forisome response was observed in broad bean SEs (Thorpe et al., 2010), which implicate Ca^{2+} elevations in SEs as is found in response to different cooling regimes in root cells (Plieth, 1999; Plieth et al., 1999; White, 2009). Diverse relationships between character of the stimulus, electrical signals, and Ca^{2+} signatures in broad bean SEs are summarized in Table 6.1.

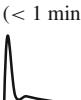
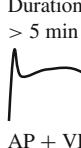
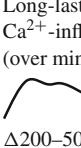
Character, intensity, and strength of the stimuli are transformed into specific Ca^{2+} signatures that could originate from the interaction of Ca^{2+} -permeable channels in the plasma membrane and endomembrane systems (Webb et al., 1996; Malho et al., 1998; Trewavas, 1999; Ng and McAinsh, 2003; White and Broadley, 2003; Demidchik and Maathuis, 2007; McAinsh and Pittman, 2009). Besides influx channels, Ca^{2+} efflux transporters such as Ca^{2+} ATPases at the plasma membrane and endomembranes or Ca^{2+} exchangers (McAinsh and Pittman, 2009) could modulate Ca^{2+} signatures during signaling (Pittman and Hirschi, 2003; McAinsh and Pittman, 2009). In contrast to other plant cells, Ca^{2+} influx and efflux in SEs (Furch et al., 2009) only depends on Ca^{2+} permeable channels or pumps operating at the plasma membrane or the sieve element reticulum (SER), but not at the tonoplast due to the absence of a vacuolar compartment (Sjölund, 1997; van Bel, 2003; van Bel et al., 2011).

Typical Ca^{2+} compartments in plant cells such as nuclei, mitochondria, Golgi apparatus, and plastids that often play a role in Ca^{2+} signaling (Pittman and Hirschi, 2003) are absent or highly reduced in SEs (Sjölund, 1997; van Bel., 2003). Furthermore, it has been speculated that soluble Ca^{2+} -buffering proteins fine tune and shape Ca^{2+} transients during signaling (McAinsh and Pittman, 2009). Together with Ca^{2+} -buffering components in the cytosol, Ca^{2+} -binding proteins associated with the cytoskeleton could be involved in Ca^{2+} -buffering during signaling (Malho et al., 1998).

Ca^{2+} -Permeable Channels of the Sieve Element Plasma Membrane

In animal cells, highly selective Ca^{2+} channels can mediate Ca^{2+} fluxes at the plasma membrane (Tsien et al., 1987; Tsien and Tsien, 1990), whereas in plant cells, nonselective cation channels (NSCCs) seem to be responsible for Ca^{2+} fluxes (Demidchik and Maathuis, 2007; McAinsh and Pittman, 2009). Three prominent groups of NSCCs are thought to be involved in the generation of stimulus-specific Ca^{2+} elevations: (1) hyperpolarization-activated Ca^{2+} -permeable channels (HACCs; Gelli and Blumwald, 1997; Hamilton et al., 2000; Kiegle et al., 2000); (2) depolarization-activated Ca^{2+} -permeable channels (DACCs) (Thuleau et al., 1994; Thion et al., 1998; White, 2009); and (3) mechanosensitive channels (MSCs) (Cosgrove and Hedrich, 1991; Ding and Pickard, 1993; Dutta and Robinson, 2004). Thus far, only MSCs were identified in SE plasma membranes (Knoblauch et al., 2001; Hafke et al., 2007).

Table 6.1 Several stimulus-specific electrical signals and corresponding Ca^{2+} signatures in sieve elements (SEs) (from top to bottom with increasing stimulus strength).

Stimulus	Protocol	Electrical signal	Ca^{2+} signature	Forisome response (F) Callose formation (C)	Comment/ literature for SEs
KCl	Distant 3–5 cm permanent	Duration: a few seconds  VT	Small Ca^{2+} transient (~ 60 s)  $\Delta 50 \text{ nM}$	—	[1]
Cutting	Distant 1 cm	Duration: up to 10 min  (AP?) VP (graded signal)	n.d.	F D, R n.d.	[2]
Cold shocks ΔT	Locally Cold shocks according to [6] and [7] or rapid cooling pulses according to [8] ΔT : 8–12°C	Duration: 10–30 min  “AP” according to [8] AP + H^+ -ATPase graded signal	“Fast” Ca^{2+} -spike (< 1 min)  $\Delta 400\text{--}2000 \text{ nM}$ [6], [7] Source: apoplast and internal storage [7]	F ΔT : 4–14°C D: 5–20 s R: 40–240 s threshold > 4°C C: n.d.	[3,4]
Burning stimulus	Distant 3–5 cm 3 s flame	Duration: > 5 min  AP + VP	Long-lasting Ca^{2+} -influx (over minutes)  $\Delta 200\text{--}500 \text{ nM}$	F D: 10–80 s R: 100–600 s K formation 15–25 min degradation: 60 min at the PPUs 2–3 h at the sieve plates	[1,2,5]
Cold cooling with defined cooling rates ($\Delta T/\Delta t$)	Locally cooling steps according to [6] and [8] $\Delta T/\Delta t$: –0.004°C/s to –0.44°C/s [6]	Duration: several minutes  “AP” + H^+ -ATPase graded signal	Long-lasting Ca^{2+} -Influx (over minutes) biphasic  $\Delta 200\text{--}800 \text{ nM}$ [6]	F $\Delta T/\Delta t$: –0.5 to –5°C/s [7] D: 50 – 10 s R: 5–30 min K n.d.	[3]

Notes: Stimuli were applied either at a distance or locally. Electrical signals were recorded from SEs intracellularly and Ca^{2+} signatures were obtained by use of fluorescent Ca^{2+} reporters. In case of locally applied cold stimulus, Ca^{2+} kinetics are supplemented with data taken from literature for other cell types. Besides the electrical and Ca^{2+} signatures, the forisome response (F) and callose formation (C) at the sieve pores and PPUs were used for identification of the stimulus strength based on different thresholds.

[1] Furch et al., 2009; broadbean; [2] Furch et al., 2008; broadbean; [3] Thorpe et al., 2010; broadbean; [4] Hafke et al., in preparation; [5] Furch et al., 2007; broadbean; [6] Plieth et al., 1999; *Arabidopsis*; [7] Knight et al., 1996; for tobacco and *Arabidopsis*; [8] Minorsky and Spanswick, 1989; pumpkin root cells. Note that Ca^{2+} signatures for cold shock were taken from other cell types and plants [6–8].

VT, voltage transient; VP, variation potential; AP, action potential; D, duration until complete forisome dispersion after stimulus; R, duration until complete forisome condensation after stimulus; n.d., not determined.

A long-lasting Ca^{2+} influx, as observed during the second phase of EPWs after remote burning in SEs (Furch et al., 2009) could be catalyzed by HACCs (Demidchik and Maathuis, 2007). In many cells, the resting value of the membrane potential is more positive than the activation voltage of HACCs (Demidchik et al., 2002; McAinsh and Pittman, 2009). However, it has been shown that the activation voltage of HACCs can be shifted to more positive membrane potentials in root cells (Demidchik et al., 2002; Demidchik and Maathuis, 2007; McAinsh and Pittman, 2009). This is brought about by Ca^{2+} influx in the physiological range (Demidchik et al., 2002) mediated by so-called voltage independent, nonspecific cation channels (White and Broadley, 2003; Demidchik and Maathuis, 2007). In analogy to such a mechanism, mechanosensitive Ca^{2+} channels in SEs that are briefly activated during the pressure wave of the VP as a response to a burning stimulus could shift the activation voltage of HACCs to a more positive membrane potential. Preliminary patch-clamp measurements on SE protoplasts point to the existence of hyperpolarization-activated Ca^{2+} -permeable channels in the plasma membrane of SEs (Hafke et al., unpublished results).

It has been proposed that Ca^{2+} -permeable channels act as primary temperature sensors (Minorsky and Spanswick, 1989; Monroy and Dhindsa, 1995; Plieth et al., 1999; White, 2009). An MSC with gradually increasing activity below 20°C was characterized in detail in plasma membranes of epidermal cells (Ding and Pickard, 1993). MSCs, activated by physical stretch or tensile forces (Demidchik and Maathuis, 2007) could mediate Ca^{2+} influx into SEs in reaction to abrupt temperature changes (Thorpe et al., 2010). MSCs in plants could share biophysical properties with transient receptor potential channels in mammals (Ramsey et al., 2006). These channels are Ca^{2+} permeable with different modes of activation integrating coincident stimuli such as temperature shocks and mechanical perturbation (Ramsey et al., 2006; Carpaneto et al., 2007).

Depolarization-activated Ca^{2+} -permeable channels (DACCs; Thuleau et al., 1994; Thion et al., 1998; Carpaneto et al., 2007; White, 2009) are possibly involved in cold-induced Ca^{2+} influx (Plieth et al., 1999; Plieth, 1999; White, 2009). In series of studies (White and Ridout, 1999; White, 2009), a DACC called the maxi cation channel (White, 2004, 2009) was identified as a candidate responsible for the generation of complex temperature-dependent Ca^{2+} signatures in root cells (Table 1; Plieth et al., 1999; Plieth, 1999; White, 2004, 2009). This channel is a potential player in temperature perception by SEs (Thorpe et al., 2010).

Passage of EPWs, Ca^{2+} Influx, and Sieve Element Occlusion Mechanisms

In light of the connections between electrical and chemical signals, the question arises about the impact of Ca^{2+} influxes accompanying EPWs on SE biology. Forisome dispersion and callose synthesis discussed in Chapter 7 are controlled by changes in Ca^{2+} concentration (King and Zeevaart, 1974; Colombani et al., 2004). Passage of EPWs in response to a remote burning stimulus leads to rapid dispersion of forisomes (Furch et al., 2007, 2009) and a stop in phloem transport (Thorpe et al., 2010). With a longer delay, callose production is initiated at the sieve pores and pore plasmodesmatal units (PPUs) in SEs at a distance from the site of burning (Furch et al., 2007). Application of potassium chloride, which causes a membrane depolarization of only a few mVs (Furch et al., 2009; Table 1), at a distance does not trigger forisome dispersion or callose synthesis. This suggests a relationship between Ca^{2+} influx and a tangible SE response. Despite the apparent coherence of these observations, Ca^{2+} increases of 200–500 nM in sieve-tube sap (Furch et al., 2009) triggered by burning does not meet the 50 μM threshold for forisome dispersion (Knoblauch et al., 2005; Furch et al., 2009). This discrepancy suggests that special conditions are required for forisome dispersion.

Ca²⁺ Hotspots as Result of a Limited Diffusion Range of Ca²⁺ Ions Released by Clustered Ca²⁺ Permeable Channels in SEs

Ca²⁺ concentrations of around 100 μM might only exist temporarily at the cytoplasmic channel mouth (Trewavas, 1999). Such high local Ca²⁺ concentrations have been detected in animal cells (Llinàs et al., 1992, 1995), but have not been documented yet for SEs. Fluorescent Ca²⁺-reporters, such as Oregon-Green-488-BAPTA-1 (Furch et al., 2009) acting as a mobile Ca²⁺ buffering dye, can dissipate such microgradients (Bolsover and Silver, 1991; Malho et al., 1998; Demuro and Parker, 2006) which impedes to detect their existence. The lack of forisome reaction with Ca²⁺ buffers like BAPTA (1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetra acetic acid) can, therefore, be regarded as indirect evidence for the existence of Ca²⁺ microdomains in SEs (Furch et al., 2009; Hafke et al., 2009).

Another prerequisite for establishing Ca²⁺ hotspots is an aggregation of Ca²⁺ channels (White and Broadley, 2003). The highest channel densities were found in the sieve-plate region and at PPUs probably to meet the high demand of Ca²⁺ necessary for the occlusion reactions (Furch et al., 2009; Hafke et al., 2009). Dihydropyridine-sensitive Ca²⁺ channels were localized at the plasma membrane and ER membranes in SEs (Furch et al., 2009). Hence, voltage changes in the SE plasma membrane (Furch et al., 2007, 2009) could directly recruit voltage-dependent Ca²⁺ channels at the ER as in several other cell types (Klüsener et al., 1995; Klüsener and Weiler, 1999; McAinsh and Pittman, 2009).

Similar to calcium-induced calcium release (CICR) at the tonoplast (Bewell et al., 1999; Sanders et al., 2002), Ca²⁺ influx through the SE plasma membrane could trigger Ca²⁺ release from putative Ca²⁺-dependent Ca²⁺ channels on the membranes of the ER cisternae (Furch et al., 2009; Hafke et al., 2009). Recruitment of internal Ca²⁺ stores is an established event during cold and heat shocks (Knight et al., 1996; Gong et al., 1998; White and Broadley, 2003). Circumstantial evidence also points to stimulus-induced Ca²⁺ recruitment from the ER as a prominent internal store in SEs (Furch et al., 2009; Thorpe et al., 2010; van Bel et al., 2011).

The responsiveness of forisomes depends on the forisome position inside SEs (Furch et al., 2009). In keeping with the channel density, it increases with the position in the SE and the degree of attachment to the membrane (Furch et al., 2009). Apart from deployment of the appropriate Ca²⁺ channels on the respective SE membranes, forisomes should be positioned in such a way to optimally exploit the Ca²⁺ hotspots. The forked forisome ends (Hafke et al., 2007) might be located at local microdomains where the maximum Ca²⁺ concentration is in the range of the threshold value (Furch et al., 2009; Hafke et al., 2009). In support of this idea, ultrastructural studies show that the forisome ends are inserted into the interstices of the SER stacks (Furch et al., 2009; Hafke et al., 2009) where an unstirred microenvironment would favor the establishment of Ca²⁺ hotspots or standing gradients. Thus, a narrow mechanical or electrical coupling of plasma membrane to ER (Hepler et al., 1990) in SEs possibly mediated by macromolecular anchors of unknown identity (Ehlers et al., 2000) is indispensable for forisome response.

Ca²⁺ Channels and the Presence of Cytoskeleton in Sieve Elements?

As in animal cells (Janmey, 1998; Lange and Gartzke, 2006), cytoskeletal elements in plant cells are connected to Ca²⁺-permeable channels and participate in Ca²⁺-dependent signal cascades (Trewavas and Malho, 1997; Mazars et al., 1997; Drøbak et al., 2004; Davies and Stankovic, 2006). Microtubules modulate the activity of DACCs (Mazars et al., 1997; Thion et al., 1998), and actin filaments are linked to MSCs (Wang et al., 2004; Zhang et al., 2007).

In spite of claims that a cytoskeleton is absent in SEs, there is some support for the contrary. Cytoskeletal disruptors such as latrunculin and oryzalin suppress electric and forisome responses to cold shocks (Hafke et al., unpublished results). These observations suggest interactions between a putative SE cytoskeleton, Ca^{2+} channels, and forisome reactions.

Electropotential Waves, Sieve-Element Cell Biology and Whole-Plant Responses

Differential Effects of APs and VPs in Intact Plants

The fast APs exert effects on gene expression in distant plant parts (Wildon et al., 1992; Herde et al., 1995; Stankovic and Davies, 1997; Rhodes et al., 2006). Moreover, APs can function as “precursors” and elicitors of the slower transmission of chemical signals (Zimmermann et al., 2009). Being single all-or-nothing phenomena, however, the information value of APs may be limited to acting as alarm signals for any kind of stress (Pyatygin et al., 2008; Zimmermann et al., 2009). As for VPs, stress and signal amplitude are correlated quantitatively, providing additional information on stimulus intensity. On the other hand, VPs seem to dampen more quickly than APs (Davies, 2004; Stahlberg et al., 2005, 2006), which has its logic, because information on wounding often is merely of local importance.

The superimposition of signals or “composite signals” could be of paramount physiological relevance to provide full-scale information on stresses, since composite signals would provide increased diversity of Ca^{2+} signatures enabling the plant to appropriately respond to the challenge. Temporal separation of APs and VPs could also assist in “formulating the right answer.” Dampening VPs along sieve tubes could establish a longitudinal Ca^{2+} gradient triggering local signal cascades according to diverse Ca^{2+} -dependent thresholds.

Impact of Ca^{2+} Channels on Reversible Sieve-Element Occlusion in Intact Plants

Sieve tubes form a pressurized symplasmic continuum that extends throughout the entire plant body; therefore, sieve-tube injuries present a lethal threat for the plant (van Bel, 2003). Injuries could cause the loss of large amounts of phloem sap and allow ingress of plant pathogens into sieve tubes, facilitating the systemic spread of infectious organisms (van Bel, 2003). In addition, severe injuries lead to collapse of pressure gradients in sieve tubes. To counteract the deleterious effects of injuries, plants have two time-shifted strategies to occlude sieve plates (Furch et al., 2007, 2010). Rapid occlusion is obtained by phloem-specific structural proteins such as the forisomes in Fabaceae (Furch et al., 2007, 2009) and the phloem proteins PP1 and PP2 in cucurbits (Furch et al., 2010). Slower occlusion is carried out by the synthesis of callose collars around the sieve pores. Both mechanisms are Ca^{2+} dependent (King and Zeevaart, 1974; Knoblauch et al., 2001) and reversible (Furch et al., 2007, 2009, 2010).

Collective Effects of Ca^{2+} Fluxes Generated by Sieve-Element Ca^{2+} Channels on Whole-Plant Function

The electrical conductivity of the SE plasma membrane combined with SE longevity and the low electrical resistance of large-perforated sieve-plates make sieve tubes ideal conduits for long-distance electrical signaling (van Bel and Ehlers, 2005). A high degree of electric sieve-tube insulation is

reached by sparse symplasmic connections (Kempers et al., 1998) and, hence, poor electrical coupling (van Bel and van Rijen, 1994) between SE-CCs and adjacent phloem parenchyma cells along most of the vascular pathway. However, electrical insulation of sieve tubes is likely to be less efficient as compared to the myelin sheath around axons in higher animals. Excess photoassimilates can be unloaded along the pathway into storage compartments through the few plasmodesmata (Patrick and Offler, 1996). Thus, quick filling of axial storage compartments implies an incomplete electrical insulation, which is the more plausible, because electrical currents are expected to pass through plasmodesmata with minuscule size exclusion limits. Loss of electrical current from SEs was inferred from depolarizations of adjacent parenchyma cells that coincide with EPWs in tomato petioles (Rhodes et al., 1996).

APs likely cover longer distances in specialized plants. In the touch-sensitive plant *Mimosa pudica*, for instance, petiolar sieve tubes are embedded in an insulating sclerenchyma sheath (Fleurat-Lessard and Roblin, 1982) that is interrupted in the pulvini. Increased numbers of plasmodesmata there provide ample electrical coupling to flexor parenchyma cells that readily react to electrical currents leaking from sieve tubes (Fleurat-Lessard and Bonnemain, 1978). This functional current loss might reveal the fundamental difference in the function of APs in plants. In contrast to the situation in animals, APs in plants do not seem to be primarily engaged in propagating electrochemical signals generated by minor ion displacements across the plasma membranes of the conducting cells. Instead, gating of ion channels in plants causes massive displacement of ions that elicit signaling cascades in cells along and at the end of the phloem pathway (Pyatygina et al., 2008). Thus, leakage of current is inherent to the principal function of electrical propagation in plants.

Effects of APs have been observed a considerable distance away from the site of stimulus in species that are less specialized for electrical transmission. Drought-mimicking treatments in 60-cm-tall maize (*Zea mays*) plants induced APs that resulted in modified gas exchange rates in leaves (Fromm and Fei, 1998). APs also propagated over several decimeters in lupin (Zawadzki, 1980) and sunflower (Dziubinska et al., 2001), where APs remained restricted to the axis (Zawadzki, 1980; Dziubinska et al., 2001). It is unclear whether there is a discontinuity of an unknown nature or if vascular branching in the nodes is responsible for electrical shunting and corresponding current loss that interferes with the propagation.

Future Directions

In contrast to the wealth of information available for other cell types, knowledge is limited as for ion channels, carriers, and pumps engaged in electrical signaling and triggering signal cascades along the phloem. Ca^{2+} -permeable channels at the SE plasma membrane seem to function as relay stations between long-distance physical stimuli and local cellular responses of SEs and, probably CCs. The transport systems at the plasma membrane of SEs could be similar to those in other cell types; however, the propagation of electrical long-distance signals along highly pressurized sieve tubes might require unique properties.

Fundamental questions remain to be answered. Which channels are responsible for long-lasting Ca^{2+} fluxes into SEs during VP phase? What is the nature and three-dimensional structure of the Ca^{2+} -permeable channels in SE-CCs? What is the distribution of Ca^{2+} -permeable mechanosensitive and voltage-dependent channels over the SE-CC surfaces? Yet other questions pertain to the involvement of Ca^{2+} -permeable channels of the ER in signaling cascades. It is well established for other cell types that cytosolic Ca^{2+} is elevated due to Ca^{2+} mobilization from both external and internal Ca^{2+} stores. Since the ER is the only Ca^{2+} store in SEs, additional Ca^{2+} release from ER

is needed to provide sufficient Ca^{2+} mobilization to exceed the threshold for specific downstream reactions.

Patch-clamp studies on isolated SE protoplasts are expected to become highly useful for identification and characterization of membrane transport events, in particular Ca^{2+} -mediated processes at the SE plasma membrane. The limited intracellular machinery of SE protoplasts would enable a less disturbed and, hence, sharper distinction of interactions between plasma membrane and ER membranes than in “conventional” cells. Therefore, SE protoplasts might become excellent tools to investigate intracellular cascades in SEs provided that the signaling cascades remain intact during the laborious preparation of these protoplasts.

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7 Sieve Element Occlusion

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Transport and communication within unicellular organisms are restricted to short distances and can be driven by diffusion or by means of the cytoskeleton when directional transport is required. However, the evolution of multicellular organisms required specific tissues to transport photoassimilates and signaling molecules to distant locations within a reasonable timeframe. In most large organisms, microfluidic tube systems have evolved to serve long-distance transport. The utility of these systems is illustrated by convergent development of similar transport systems among living organisms. A good example is the phloem of higher plants and large red and brown algae. The construction of both phloem types bears a striking similarity even though their common ancestor lacked vascular tissue.

The phloem microfluidic system in higher plants is composed of specialized elongated cells called sieve elements (SEs). Individual SEs are interconnected via sieve plates. Sieve plates are perforated cell walls containing sieve-plate pores of up to 5- μm diameter. Ontogenetically sieve-plate pores represent modified plasmodesmata, which increase in diameter during sieve-tube development to provide a connection of low resistance in the mature tube. To further decrease resistance in the tube system, major parts of the cellular equipment of SEs, such as the nucleus and the vacuole are degraded at maturity. Remaining organelles are usually located at the margins of the cells. Multiple interconnected SEs form a network of tubes spanning throughout the plant, permitting rapid translocation of assimilates and signals.

Despite the obvious success of microfluidics networks, concentrating high amounts of essential nutrients, such as sugars and amino acids into a tube system comes with inherent risks. Mechanically severing the tubes could result in draining the system and nutritionally depriving the organism. Furthermore, a virtually unlimited supply of highly concentrated assimilates attracts an armada of pests. Phloem-feeding insects such as aphids, whiteflies, and leafhoppers have specialized to feed from sieve tubes, while other herbivores such as chewing insects or mammals constantly injure the system. Disregarding biotic or abiotic factors, it is obvious that evolution must have led to counter measures to preserve the integrity of the system.

Abbreviations: CalS, callose synthase; ER, endoplasmic reticulum; GSL, glucan synthase-like; MRI, magnetic resonance imaging; PP1, phloem protein 1; PP2, phloem protein 2; P-protein, phloem-specific protein; SEO, sieve-element occlusion; SE-plastid, sieve-element plastid; STEP, sieve-tube exudate protein

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Despite the importance of preserving the sieve tube system, the current knowledge of cellular events that occur in response to sieve tube damage is limited. The unique anatomical features of the sieve tubes and resulting difficulties with *in vivo* observations have created roadblocks to understanding these responses. The phloem is embedded in a thick layer of ground tissue and is often covered by a sclerenchyma cap, which prevents direct observation of its cellular features. Furthermore, ground tissue is often composed of cells that contain chloroplasts or other pigmented organelles, such as anthocyanins in vacuoles, and periderm, all of which decrease the optical properties to the point of invisibility. Other noninvasive visualization methods such as infrared microscopy or magnetic resonance imaging (MRI) that allow deeper penetration of the tissue lack the necessary resolution that is required to visualize subcellular structures.

Structural observations of SEs within the phloem require more careful tissue preparation as compared to other cell types. When a sieve tube is severed, the low resistance of sieve tubes in combination with an exceptionally high turgor (Turgeon, 2010) causes an immediate effect at a distance from the wound. Since proper fixation of the tissue requires small sample sizes, it is unclear whether ultrastructural observations of the phloem ever truly reflected the *in vivo* state of sieve tubes. The use of modern light microscopic instrumentation, such as confocal microscopy, has provided useful insights through multidimensional reconstructions and real-time observations as to the location and dynamics of sieve-tube components. Unfortunately, the limited resolution of confocal microscopy restricts its use in providing detailed ultrastructural data. The numerous structural and preparative challenges in observing the phloem have strongly influenced interpretations of the events leading to sieve tube occlusion.

This chapter will summarize the current knowledge of mechanisms that exist to prevent nutrient loss from the sieve tube system after injury, with a focus on biotic and abiotic factors that lead to a mechanical disruption. The mechanisms preventing sieve tube occlusion during feeding by piercing/sucking insects is discussed in Chapter 15.

Callose

Callose Biosynthesis

Callose is a β -1,3 glucan with widespread distribution in higher plants and is most often associated with cellular events such as cell division, pollination, and hypersensitive responses as well as structural components such as plasmodesmata, cell walls, and sieve plates of the phloem (Hong et al., 2001a; Enns et al., 2005; Dong et al., 2008; Xie et al., 2010; Barratt et al., 2011; Xie et al., 2011). Callose has also been identified in sieve-plate homologs of brown algae, mosses, and seedless vascular plants (Behnke and Sjolund, 1990).

Partially purified cellulose synthases were found to synthesize both cellulose and callose (Jacob and Northcote, 1985). Further purification studies separated the activities of cellulose and callose synthases (CalS; Kudlicka and Brown, 1997; Verma and Hong, 2001). Similar to cellulose synthase, CalS are transmembrane proteins ($\ddot{\text{O}}$ stergaard et al., 2002) composed of two hydrophobic domains and a cytoplasmic loop (Hong et al., 2001b). Twelve members of the CalS gene family have been identified in *Arabidopsis*. They have been called *callose synthase* 1–12 (*CalS1–12*) or *glucan synthase-like* 1–12 (*GSL1–12*). Although the gene family names are synonymous, the numerical tags for the 12 different *CalS* and *GSL* genes do not correspond with each other (Table 7.1; Enns et al., 2005). At present, a consensus has not been reached on which nomenclature should be used.

Table 7.1 Comparison of the synonymous gene families callose synthase (*CalS*) and glucan synthase-like (*GSL*) in *Arabidopsis*

Callose synthase (<i>CalS</i>)	Glucan like synthase (<i>GSL</i>)	Cellular involvement
<i>CalS1</i>	<i>GSL6</i>	Cytokinesis
<i>CalS2</i>	<i>GSL3</i>	Unknown
<i>CalS3</i>	<i>GSL12</i>	Unknown
<i>CalS4</i>	<i>GSL9</i>	Unknown
<i>CalS5</i>	<i>GSL2</i>	Pollination, microsporogenesis
<i>CalS6</i>	<i>GSL11</i>	Unknown
<i>CalS7</i>	<i>GSL7</i>	Phloem-specific wound callose synthesis
<i>CalS8</i>	<i>GSL4</i>	Unknown
<i>CalS9</i>	<i>GSL10</i>	Pollination, microsporogenesis
<i>CalS10</i>	<i>GSL8</i>	Cytokinesis, pollination, microsporogenesis
<i>CalS11</i>	<i>GSL1</i>	Pollination, microsporogenesis
<i>CalS12</i>	<i>GSL5</i>	Pollination, microsporogenesis

Because callose genes encode for callose synthesizing proteins, *CalS* will serve as the primary designation in this chapter.

In *Arabidopsis*, specific *CalS* function in different cellular processes. During cytokinesis callose synthesized by *CalS1* and *CalS10* is deposited at newly forming cell plates (Hong et al., 2001a). *CalS* that are active during pollination and microsporogenesis are encoded by *CalS5*, *CalS9*, *CalS10*, *CalS11*, and *CalS12* (Xie et al., 2010). Pathogen-induced wounding activates transcription of *CalS12* with subsequent callose deposition in mesophyll cell walls (Dong et al., 2008; Xie et al., 2011). *CalS7* encodes the *CalS* responsible for phloem-specific callose synthesis in response to SE wounding (Barratt et al., 2011; Xie et al., 2011). It has been proposed that *CalS7* is part of a *CalS* complex in combination with other enzymes such as UDP-glucose synthase and sucrose synthase (Verma and Hong, 2001).

Callose Deposition in Sieve Tubes

The temporal occurrence of callose depositions on sieve plates has been separated into three distinct categories: (1) dormancy callose, (2) definitive callose, and (3) wound callose. Dormancy callose is deposited on sieve plates at the end of a growing season and is removed in the spring to reactivate sieve tubes (Evert and Derr, 1964; Aloni and Peterson, 1997). Definitive callose usually occurs on plates of senescing sieve tubes (Evert and Derr, 1964). Wound callose is deposited in response to mechanical injury and deposition can be temporary (Esau and Cheadle, 1961; Evert and Derr, 1964; Eschrich, 1975).

Dormancy Callose

Dormancy callose seals the dormant SEs by targeted deposition in sieve pores and lateral sieve areas (Behnke and Sjolund, 1990) or by almost completely filling the lumen of the SE (Evert and Derr, 1964). However, the pores still remain slightly open throughout the dormancy period.

The removal of dormancy callose can occur prior to the breaking of the leaf buds or after bud break and reactivation of the vascular cambium. The timing of this removal appears to be species specific, and auxin has been shown to facilitate the removal of dormancy callose in certain species

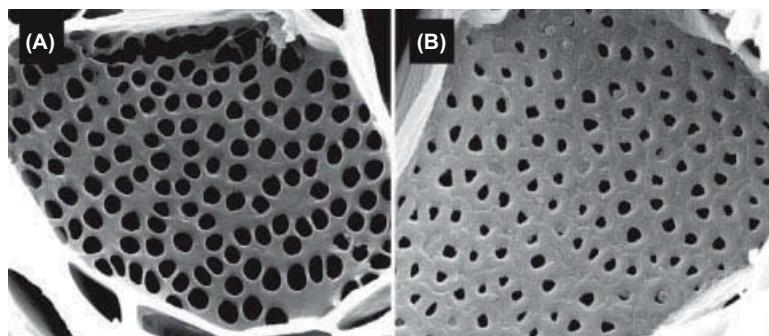


Figure 7.1 Scanning electron micrographs of green bean sieve plates before and after sieve element (SE) injury. (A) Sieve-plate pores are free of callose in uninjured conducting SEs. (B) When the SE is severely injured callose can fill the pores, constricting phloem flow and preventing assimilate loss. (From Mullendore et al. (2010); Copyright American Society of Plant Biologists.)

(Aloni and Peterson, 1997). In other species, however, endogenously applied auxin can promote synthesis and accumulation of callose on sieve plates (Aloni and Peterson, 1997).

Wound Callose

Since sieve-plate pores represent constrictions in the sieve-tube channel, occlusion mechanisms that target sieve-plate conductivity will be most efficient (Mullendore et al., 2010). In 1885, Fischer reported that sieve plates in excised tissue that had been quickly killed and fixed by boiling showed less callose deposition than sieve plates that had not been boiled before examination. This was a first indication that callose on sieve plates is formed in response to injury. Subsequent investigations supported this finding (Esau and Cheadle, 1961; Evert and Derr, 1964; Esau, 1969; Eschrich, 1975). It is evident in electron micrographs that callose is specifically generated around sieve-plate pores (Figure 7.1). As a SE membrane integral enzyme complex, CalS utilizes sugars from the symplast and produces callose to the apoplasmic space (Verma and Hong, 2001). Deposition at sieve-plate cell walls leads to decreasing pore diameter attended by increasing resistance of the tube system and reduced flow velocity (Mullendore et al., 2010).

The speed of callose deposition determines the efficiency of occlusion and has been the subject of several investigations. Callose deposition in response to phloem-feeding insects has been reported to occur within several hours (Kusnierszyk et al., 2008) or a day (Hao et al., 2008; Saheed et al., 2009). Mechanical wounding and burning leads to reactions within minutes on sieve plates and plasmodesmata (Furch et al., 2007; Nakashima et al., 2003). Callose formation may be reversible if wounding does not lead to cell death (Furch et al., 2007, 2010).

All of these findings, however, are based on the observation of aniline blue fluorescence on the sieve plates or plasmodesmata. Aniline blue is a callose-specific dye and such fluorescence signals indicate that callose has been deposited, but not necessarily that the sieve pores or plasmodesmata have been blocked in regards to transport. Based on the same methods, Eschrich (1956; 1965) and Currier (1957) concluded that callose deposition on sieve plates can occur within seconds.

Mullendore et al. (2010) used a scanning electron microscope-based technique to investigate sieve-plate pores and the progress of callose formation at high resolution. Initially callose deposition rates were in the range of 25–60 nm/s, but decrease over time in some species. Total occlusion was achieved in bamboo (*Phyllostachys nuda*) and green bean (*Phaseolus vulgaris*) after 10–20 minutes, but sieve pores in pumpkin (*Cucurbita maxima*) were never fully occluded.

P-Proteins

The nomenclature for proteins that accumulate in SEs is notoriously confusing and generally unsatisfactory. Historically, phloem proteins (P-proteins) were defined as “proteinaceous material in the phloem that is sufficiently characteristic when observed with the electron microscope to warrant a special term” (Cronshaw, 1975). While the term P-protein primarily refers to structural proteins, a large number of soluble proteins have been identified in sieve-tube exudates from numerous species (see Chapter 8). Since most of the soluble proteins are invisible in electron micrographs, these proteins do not fall under the strict definition of P-proteins. Phloem-specific proteins became the accepted terminology for nonstructural proteins that are characteristic of the phloem (Cronshaw and Sabnis, 1990), and more recently, the term sieve-tube-exudate-proteins (STEPs) became fashionable (Schobert et al., 1995, 1998). This term does not discriminate between soluble and structural proteins that are found in sieve-tube exudates. The focus on structural proteins in this chapter will use the term P-protein according to the definition by Cronshaw (1975).

During early SE ontogeny, proteins assemble into filaments or tubules that aggregate into one or more P-protein bodies (Cronshaw and Esau, 1967; Cronshaw and Esau, 1968; Esau, 1969; Behnke, 1974; Cronshaw, 1975). In about 10% of the dicots, these bodies remain as nondispersive P-protein bodies in the sieve tube lumen at maturity (Behnke, 1991). In the remaining 90% of the dicot families the protein bodies disperse once autolysis of the nucleus is initiated.

Dispersive P-Protein Bodies

The development of SEs before the opening of the sieve-plate pores is well understood because standard procedures for electron microscopy preparation, fixation, and embedding preserve the integrity of the young SE with minimal artifacts. In this state, SEs are still connected by plasmodesmata only. The challenge begins when sieve pores open and the mechanical conductance between SEs increases (Knoblauch and Peters, 2010). The surge occurring in the sieve tubes during tissue sectioning causes displacement and structural alterations of sieve-tube components. In mature SEs, P-proteins have been characterized as being amorphous, crystalline, filamentous, tubular, and fibrillar (Cronshaw and Sabnis, 1990; Evert, 1990).

The best-described dispersive P-proteins are the phloem protein 1 and 2 (PP1 and PP2) from pumpkin, because ample amounts of PP1 and PP2 can easily be obtained from exudates of cut stems or petioles. PP1 is a 96 KDa protein that forms filaments, and PP2 is a 25 KDa dimeric lectin that binds covalently to PP1 (Bostwick et al., 1992; Golecki et al., 1999). The subunits are synthesized in companion cells and transported via the pore-plasmodesma units (see Chapter 2) into the SEs. PP1 and PP2 subunits have been shown to move with the assimilate stream (Golecki et al., 1999; Dinant et al., 2003). *PP1* belongs to a family that is found exclusively in Cucurbitaceae (Clark et al., 1997), while *PP2* belongs to a large gene family found in many angiosperm taxa (Bostwick et al., 1992; Dinant et al., 2003; Beneteau et al., 2010). Immunolabeling showed PP1 to be part of a large protein plug covering the sieve plates of pumpkin in sectioned tissue (Clark et al., 1997).

Fibrillar and filamentous P-proteins have been observed in various angiosperms as a fine mesh-work within the lumen (Cronshaw, 1975; Ehlers et al., 2000), agglomerations in parietal layers (Evert and Deshpande, 1969; Evert et al., 1969) or as densely packed structures filling the sieve-plate pores (Yapa and Spanner, 1972; Robidoux et al., 1973; Johnson et al., 1976). Location and appearance of P-proteins within the sieve-tube lumen is one of the most controversial topics in phloem biology. The variety of structures observed is related to different degrees of injury-induced artifacts, but there are

controversial opinions regarding which structures truly represent the uninjured translocating state of SEs.

In order to solve some of the problems, a method for *in vivo* observation of broad bean (*Vicia faba*) sieve tubes by confocal microscopy was developed (Knoblauch and van Bel, 1998). Imaging of translocating SEs confirmed that P-protein aggregates in parietal positions (parietal P-proteins). Injury by intense laser light or impalement of microcapillaries revealed that aggregates detach from the sidewalls and move with the translocation stream. Often the aggregated protein precipitates and forms a layer of P-protein at the sieve plate. Strong mechanical injury can lead to disruption of SE-plastids whose contents contribute to plug formation.

Nondispersible P-Proteins

Nondispersible P-protein bodies mainly occur in woody angiosperms and are classified according to their morphology (Behnke, 1991). They can exist alongside dispersive P-protein bodies in the same species. They occur in the SEs of about 10% of the dicots, their morphology is family specific and of taxonomic significance. The refractive index of nondispersible P-protein bodies often differs from the cytoplasm, making them visible in the light microscope without staining.

As early as 1891, Strasburger described spindle or barrel shaped bodies in the SEs of faboid legumes. Due to their distinctive shape, these structures were later called “crystalline P-protein bodies” (Wergin and Newcomb, 1970). On the basis of cytological studies, some investigators suggested that crystalline P-proteins undergo a transition from a crystalloid to a dispersed state during SE differentiation (Wergin and Newcomb, 1970; Palevitz and Newcomb, 1971) and therefore, belong to the group of dispersive P-proteins. Other investigators argued that the apparent dispersal is an artifact attributable to turgor loss during tissue preparation for electron microscopy (Fisher, 1975; Lawton, 1978). Analogous to other nondispersible P-proteins, crystalline P-proteins were regarded as static inclusions with unknown functions (Sabnis and Sabnis, 1995).

Forisomes

The discovery of a dynamic behavior of crystalline P-proteins was the first demonstration of a function of P-proteins. The transition of crystalline P-protein bodies from a crystalloid to a dispersed state has been demonstrated to be a reversible and rapid (<1 second) contractile reaction in which an elongated low-volume state switches into a longitudinally contracted, spherical high-volume conformation (Figure 7.2; Knoblauch et al., 2001). Since the bodies are not crystalline they were renamed forisomes (“gate body”) to more accurately reflect their structural composition (Knoblauch et al., 2003). The conformational change of forisomes is associated with a dramatic volume increase of several hundred percent (Knoblauch et al., 2001; Peters et al., 2006). The presence of divalent cations appears to be the decisive factor in forosome contractility. Cycling between a high-volume and low-volume state can be induced in isolated forisomes by repetitive exchange of bathing media containing either calcium (Ca^{2+}) or chelators (Knoblauch et al., 2003). Other divalent cations such as strontium and barium, but not magnesium, are equally effective in inducing forosome reaction.

Forosome reaction coincides *in vivo* with plasma membrane leakage induced through mechanical injury or permeabilizing substances such as Triton X-100 (Knoblauch et al., 2001). Similarly, abrupt turgor changes imposed by osmotic shock, supposedly also leading to membrane leakage, cause the conformational switch. The transformation from low-volume to high-volume states seems to be

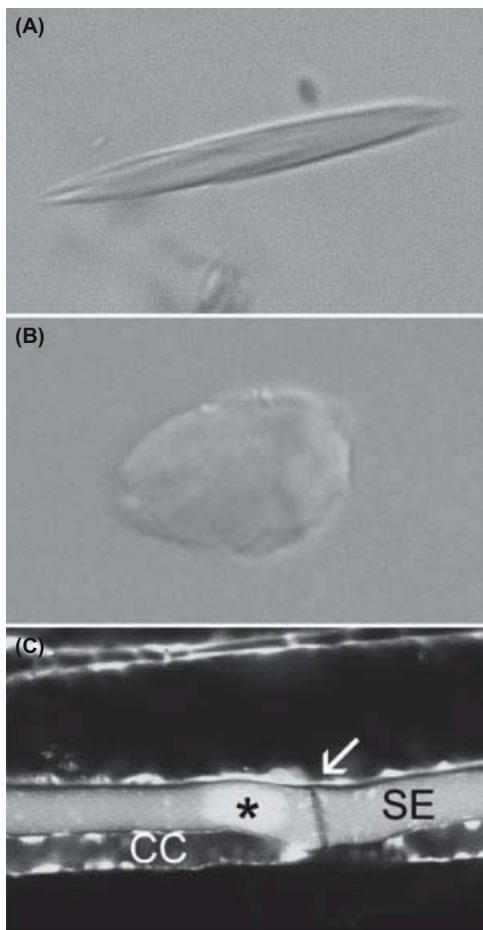


Figure 7.2 Forisome reactivity in response to free calcium and sieve element (SE) injury. (A) In the absence of free calcium ions, isolated broad bean forisomes rest in a low-volume state. (B) The addition of free calcium ions causes the forisome to switch into a high-volume state. (C) Broad bean SE with their companion cells (CC). After injury, the forisome (asterisk) located in front of the sieve plate (arrow) blocks flow by turning into the high-volume state. (Reprint of Figure C with permission by the American Society of Plant Biologists. Figure 7.2C from Knoblauch et al. (2001); Copyright American Society of Plant Biologists.)

associated with a blockage of the translocation stream since a large plug at the sieve plate is formed (Figure 7.2C); Knoblauch et al., 2001).

Three genes have been isolated that encode proteins involved in forisome formation. The corresponding gene family has been designated *Sieve Element Occlusion* (*SEO*; Pélassier et al., 2008). There are several highly conserved motifs between the three genes, and the percentage of identical amino acids in paired alignments ranges from 41.8% to 50% (Pélassier et al., 2008). *SEO* related genes of unknown function have been identified in several other nonlegume taxa including *Arabidopsis* (Pélassier et al., 2008; Rüping et al., 2010). Interestingly, no known calcium-binding site was identified in any of the *SEO* or *SEO*-related genes and they lack similarity to cytoskeletal elements (Pélassier et al., 2008). A potential thioredoxin fold was predicted as a structural component of *SEO* and *SEO*-related genes (Rüping et al., 2010), but the significance of this motif is unclear.

In vitro experiments revealed that forisomes possess some remarkable properties that attracted attention from life and material scientists alike (Mavroidis and Dubey, 2003; Huck, 2008). Besides their ability to undergo conformational changes without ATP as an energy source, forisomes differ from other protein actuators by generating significant and equal forces during contraction as well as expansion (Knoblauch et al., 2003; Warmann et al., 2007). Since forisome reaction can be induced by pH changes, amperometric ion titration allows electrical control of forisome volume change and force generation in artificial systems (Knoblauch et al., 2003).

Ultrastructurally, forisomes consist of longitudinal aligned filaments in the resting, low-volume state. This alignment leads to a prominent cross-striation of 12-nm periodicity (Wergin and Newcomb, 1970) and the high degree of organization is reflected in a birefringence of the protein bodies (Peters et al., 2007). During the transition into the high-volume state, the filaments separate, the striation disappears, and no ultrastructural order remains evident in transmission electron micrographs (Palevitz and Newcomb, 1971; Lawton, 1978) or the polarization microscope (Peters et al., 2007). The observed structural disorder in the high-volume state might be due to fixation- and dehydration-induced artifacts during preparation for transmission electron microscopy. Interestingly, nondispersible P-protein bodies found in other plant families do not react to divalent cations, although their ultrastructure appears very similar to forisome ultrastructure (Behnke, 1991; Knoblauch et al., 2001). This difference may help to identify the reactive domains in forisomes. Revealing sequence information of nondispersible P-protein bodies would provide the opportunity to narrow down potential reactive domains in forisomes, ultimately leading to the identification of the mutations that turned a static protein body into a reactive structure.

Sieve Plate Pore Occlusion: A Concerted Effort?

Increasing size and complexity of plants has led to adaptations in the overall geometry and structure of sieve tubes. Fast growing plants, especially vines, usually contain exceptionally large sieve-plate pores in large diameter sieve tubes. For example, the sieve tubes in the phloem of pumpkin stems exceed 50- μm diameter and sieve-plate pore diameters reach 5 μm (Mullendore et al., 2010). Another strategy to lower resistance is to increase the length of SEs to reduce the number of sieve plates in the sieve tube (Thompson and Holbrook, 2003). This is typical for many grasses (Poaceae) that contain SEs with relatively small diameters, often below 10 μm , but with lengths exceeding 1 mm.

The difference in sieve-tube morphology certainly has an impact on occlusion strategies and it appears that most plant species do not rely on a single mechanism. This is supported by the observation that green bean, which contains forisomes, also produces callose to occlude pores. While the forisome reaction occurs within less than a second, complete occlusion of pores by callose requires 20 minutes in green bean (Mullendore et al., 2010). Therefore, it has been proposed that P-proteins serve as an initial line of defense and that callose represents a more rigid and permanent protection against nutrient loss after mechanical injury (van Bel, 2003; Furch et al., 2007, 2010). In other dicot families that do not contain forisomes, the role in occlusion of callose and P-proteins appears more as a complementary action. In severed sieve tubes of most dicots, a large plug at the sieve plate can be visualized within less than a minute after wounding (Figure 7.3). Electron micrographs show that this plug is primarily composed of P-proteins, which often traverse the pores. Depending on the amount of injury, remnants of sieve element plastids (SE-plastids) and endoplasmic reticulum (ER) membranes can participate in plug formation. Transmission electron micrographs suggest that the following onset of callose formation compresses the material located in the pores until a tight seal is secured at the sieve plate.

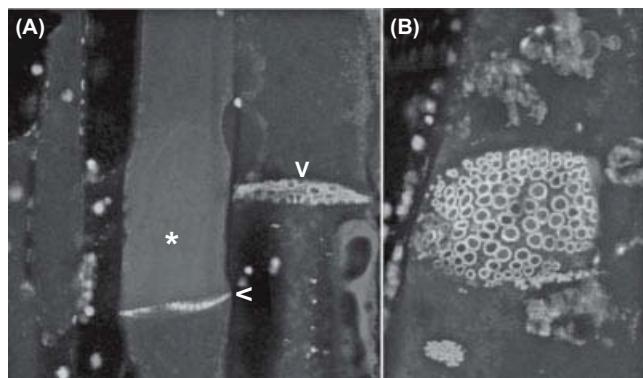


Figure 7.3 Confocal images of squash sieve elements (SEs) and sieve plates after SE injury. (A) Two sieve tubes are present in longitudinal view. The one on the left shows protein precipitation (asterisk) in front of a sieve plate (arrow heads). Cross-sectional view of a sieve plate in an injured SE shows that P-proteins fill the pores (red) and callose is deposited around these proteins (blue rings). (For a color version of the figure, please see Plate 7.1.)

Interestingly, P-proteins have never been observed in the Poaceae, which are regarded as one of the most evolutionary advanced plant families. Sieve tubes in grasses within this family typically have relatively small SE diameters and small sieve-plate pores. This led investigators to hypothesize that the P-proteins have been lost secondarily (Eleftheriou, 1990) and sieve-tube occlusion relies solely on callose formation. A constant rate of callose formation was observed in bamboo and full occlusion of the sieve pores was achieved in about 10 minutes following wounding (Mullendore et al., 2010).

Speculation about the primary function of P-proteins is widespread throughout the phloem literature (Sabnis and Sabnis, 1995; van Bel, 2003; Kehr, 2006; Furch et al., 2010). There is little doubt that P-proteins participate in sieve-tube occlusion and that forisomes have specifically evolved for this purpose. However, the conclusion that the primary function of P-proteins, especially filamentous P-proteins, is entirely for sieve-tube occlusion might be drawn too quickly. The phloem is a high-pressure system and in the event of wounding that severs the sieve tube, the sudden pressure release results in a strong surge that sweeps sieve-tube components towards the sieve plate. Extreme pressure drops also disrupt membranes that can become components of the occlusion plug. Thus, occlusion appears to be a random event where all released material accumulates at the sieve plate. While disrupted intercellular structures participate in plug formation, sieve-tube occlusion is not necessarily the primary function of such structures. The Fabaceae contain both forisomes that are dedicated for occlusion and filamentous P-proteins. It would be surprising, if these structural proteins accumulate in sieve tubes solely for occlusion purposes.

Since various SE occlusion mechanisms seem to be employed by different plant families, the question of efficiency arises. Insights into effectiveness of occlusion mechanisms can be drawn from the results of aphid stylectomy experiments, which is a commonly used technique to collect sieve-tube sap. Once an aphid stylet has penetrated an SE, the stylet is severed with a blade or laser near the insect's head and the sieve-tube exudate is collected from the severed stylet for analysis. Interestingly, this method does not work well in all plants. In a comparative study on 45 plant species and cultivars, Fisher and Frame (1984) found that 28 of the 35 nonleguminous species or cultivars examined exuded sieve-tube sap for periods ranging from 30 minutes to several days after severing the stylet. Of the 12 taxa without appreciable exudation, 9 were legumes. Only

one of ten legumes kept exuding for more than 15 minutes, but always ceased to do so before 30 minutes. Clearly, legumes were capable of terminating sieve-tube exudation rapidly, while most nonleguminous plants lacked this ability. These results support the hypothesis that legumes are equipped with a unique mechanism that allows rapid and very effective blockage of individual sieve tubes. Furthermore, phloem-feeding insects must actively overcome this occlusion mechanism as is revealed in Chapter 15.

All members of the Poaceae examined, including important crop plants such as maize (*Zea mays*), barley (*Hordeum vulgare*), and wheat (*Triticum aestivum*) proved to be excellent phloem-sap bleeders in stylectomy experiments and with the exception of millet (*Panicum miliaceum*) that exuded for 1 hour, Poaceae kept exuding for more than 1 day (Fisher and Frame, 1984). The observations that these species exude for prolonged periods indicates that callose formation does not function as a sieve tube occlusion mechanism in stylectomy experiments and suggests that callose occlusion mechanisms might rely on different signals or signal strength.

Electropotential waves are known to propagate along sieve tubes in response to mechanical or physical stimuli that are accompanied by increases in intracellular calcium (see Chapter 6; Fromm, 1991; Fromm and Spanswick, 1993). Recently electropotential waves have been linked to sieve-tube occlusion. Burning, but not cutting of a leaf, has been reported to result in distant blockage of phloem flow by both forisomes and callose (Furch et al., 2007). Electropotential waves also have been detected after cutting, but the resulting Ca^{2+} influx into sieve tubes was insufficient to trigger forisome reaction at distant locations 3–4 cm from the cutting site (Furch et al., 2009). Local mechanical injury does trigger the forisome reaction (Knoblauch et al., 2001); thus, the signal strength and the resulting occlusion appear to be distance dependent.

The long-standing observation that phloem flow stops after localized chilling (Pickard and Minchin, 1990; Pickard and Minchin, 1992), has also been linked to the forisome reaction by induction of increased intracellular calcium levels (Thorpe et al., 2010). The calcium threshold for forisome reaction *in vitro* is about 50 μM (Knoblauch et al., 2003). Callose synthesis seems also to be calcium dependent (Hong et al., 2001a), but a threshold for the phloem-specific CalS in *Arabidopsis* CalS7 has not been determined.

Future Directions

Critical for a better understanding of sieve-tube occlusion mechanisms and phloem structure/function relations in general will be the development of sophisticated *in vivo* observation techniques and protocols. Of specific interest will be P-proteins and SE-plastids, which are unique to the phloem and very prominent in sieve tubes. The description of *SEO* genes which encode forisome proteins (Pélissier et al., 2008) might provide a starting point to identify the building blocks of P-proteins in other plant families, since *SEO*-related genes have been identified in various angiosperms (Pélissier et al., 2008; Huang et al., 2009; Rüping et al., 2010). Fusion of *SEO*-related proteins with fluorescent marker proteins will reveal if *SEO* related genes also encode for P-proteins. The investigation of SE-plastids on the other hand remains difficult. So far isolation attempts for detailed functional analysis have failed (Knoblauch and Peters, 2010).

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Section C

Long-Distance Signaling

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8 Sieve Elements: Puzzling Activities Deciphered through Proteomics Studies

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Phloem tissue fulfils a multitude of functions, including transport of photoassimilates, remobilization of nitrogen compounds, long-distance signaling for developmental transitions, transmission of silencing signals, establishment of systemic resistance against pests and pathogens, and propagation of electrical signals in response to wounding. It regulates sink demands with respect to resource availability from various source regions of the plant. As a result of its intimate contact with distantly located organs, the phloem represents an integrative long-range communication pathway. This vital role of interorgan coordination is based on the phloem capability of controlled loading, transport, and unloading of metabolites, mineral nutrients, proteins, peptides, RNA molecules, and hormones, as well as to transduce and amplify a range of signals.

Our knowledge on the phloem has tremendously improved with seminal case studies that established: (1) a working model for the mechanism of symplasmic trafficking of macromolecules from companion cells (CCs) into sieve elements (SEs) through plasmodesmata (PD) that involves macromolecular chaperones (Lough and Lucas, 2006) for targeted and nontargeted transport of proteins through PD (Xoconostle-Cázares et al., 1999; Crawford and Zambryski, 2000, 2001); (2) the involvement of different subcellular routes to engage the PD pathway *via* the cytoskeleton or the endoplasmic reticulum (ER) (Martens et al., 2006; Lucas et al., 2009); (3) the contribution of different cell types in cell-to-cell transport within the phloem, including CCs, phloem parenchyma, and SEs, the interaction between cells based on thermodynamic differences (Sjölund, 1997; van Bel, 2003a, 2003b; Hafke et al., 2005); (4) the respective contribution of apoplastic and symplasmic routes for loading of metabolites and specialization of collection, transport, and delivery phloem compartments (Oparka and Turgeon, 1999; Oparka and Santa Cruz, 2000; van Bel, 2003b); and (5) specific mechanisms that can reversibly and transiently occlude sieve tubes in response to injury

Abbreviations: CC, companion cell; EFP, extrafascicular phloem; eIF-5A, eukaryotic initiation factor 5A; ER, endoplasmic reticulum; FP, fascicular phloem; GlcNAc, *N*-acetyl glucosamine; NCAP, noncell-autonomous protein; PD, plasmodesmata; PI, protease inhibitor; PM, plasma membrane; PP1, phloem protein 1; PP2, phloem protein 2; PTB, polypyrimidine tract-binding protein; RBP, RNA-binding protein; RNP, ribonucleoprotein; ROS, reactive oxygen species; SE, sieve element; SEO, sieve element occlusion protein; SER, sieve element reticulum

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to maintain the thermodynamic driving force in sieve tubes over long distances (Knoblauch and Peters, 2010).

However, signals and activities taking place in sieve tubes are largely unknown. Considerable efforts have been invested in describing the protein content of sieve-tube exudates, which provides a picture of this highly organized network at specific spatial and temporal points. Sieve-tube exudate represents mobile components transported under high hydrostatic pressure, that is, by mass flow, through the sieve-tube system. Hundreds of proteins are present in this exudate (Fisher et al., 1992; Sakuth et al., 1993; Balachandran et al., 1997). The identification of these proteins has been urged by the need to unravel the basic cellular processes acting specifically in the SEs in long-distance transport of macromolecules, long-range signaling in defense response and maintenance of enzymatic activities in those enucleate cells. Thus proteome studies of the sieve-tube exudates has shed new light on previous reports on mRNA and small RNA populations present in the vascular bundles, phloem tissues, CCs and sieve-tube sap (see Chapter 9; Birnbaum et al., 2003; Ivashikina et al., 2003; Vilaine et al., 2003; Schrader et al., 2004; Yoo et al., 2004; Divol et al., 2005; Zhao et al., 2005; Doering-Saad et al., 2006; Pommerrenig et al., 2006; Omid et al., 2007; Buhtz et al., 2008; Deeken et al., 2008; Kehr and Buhtz, 2008; Le Hir et al., 2008; Buhtz et al., 2010).

Methods Used to Collect Sieve-Tube Sap

Different approaches have been developed to sample the phloem translocation stream. In large part, the method used depends on the plant species and characteristics of their vasculature. The main traits determining the method of choice are: (1) the volume of sieve-tube sap that spontaneously exudes when an incision is made into the vascular system and the duration of this exudation; (2) the availability of species-compatible phloem-feeding insects, especially aphids or planthoppers, to collect sieve-tube exudate by stylectomy; and (3) the quantity and purity of sieve-tube sap components essential for the planned experiments.

Phloem “bleeders,” such as the cucurbits, castor bean (*Ricinus communis*) and yucca (*Yucca filamentosa*), exude large volumes of sieve-tube sap from excised tissues. Other species, such as oilseed rape (*Brassica napus*), can also be used for bleeding from its cut stem yields only small volumes (Giavalisco et al., 2006). Stylectomy has been mainly used to sample the phloem of monocots (Kennedy and Mittler, 1953; Downing and Unwin, 1977; Kawabe et al., 1980). This method yields minute volumes of pure sieve-tube sap; experiments have been conducted on oat (*Avena sativa*), barley (*Hordeum vulgare*), and maize (*Zea mays*) using the bird cherry-oat aphid (*Rhopalosiphum padi*) and on rice using the brown planthopper (*Nilaparvata lugens*). Stylectomy appears to be more difficult to adapt to eudicots (van Bel et al., 2011a), although it has been used on *Arabidopsis* using the green peach aphid (*Myzus persicae*) (Doering-Saad et al., 2002; Deeken et al., 2002; Hunt et al., 2009).

A simple method, termed EDTA-facilitated exudation, was developed by King and Zeevaart (1974). Excised petioles are immersed in exudation buffer containing the chelator EDTA in order to reduce occlusion of the sieve plate pores. It was proposed that EDTA acted by preventing either rapid aggregation of P-proteins or slower deposition of callose. This method has been broadly used for a range of herbaceous and woody plant species. Although cutting the petiole will certainly cause some level of wounding, this technique is generally considered reliable for analysis of sugars, amino acids, and inorganic ions. Recently, this approach was also applied to analyze RNA and protein contained in the sieve-tube sap (Deeken et al., 2008; Gaupels et al., 2008; Dafoe et al., 2009). For an evaluation of the advantages and disadvantages associated with each method used to sample

sieve-tube sap, the reader is referred to recent reviews by Gaupels et al. (2008), Turgeon and Wolf (2009) and Dinant et al. (2010). Since the use of EDTA might cause alterations in the mobility of some phloem sap proteins, or leakage from other tissues, or extraction of contents from the apoplasm, a more thorough evaluation of this technique for proteome analysis is still needed (van Bel et al., 2011a).

Overview of Sieve-Tube Sap Composition

The most abundant components in the sieve-tube sap are sugars, such as sucrose, raffinose-family oligosaccharides, or polyols, depending on the plant species; sugar levels can range from 300 to 1000 mM. A range of amino acids have been identified in the sieve-tube sap; concentrations in the 200–400 mM range have been reported (Dinant et al., 2010). Inorganic ions such as potassium can accumulate up to 125 mM in sieve-tube sap (Deeken et al., 2002). A comparison of protein concentrations in sieve-tube exudates collected from various species established a range of 0.1–30 mg mL⁻¹ (Fisher et al., 1992; Sakuth et al., 1993; Schobert et al., 1998; Hayashi et al., 2000; Suzui et al., 2006), although higher values, even up to 100 mg mL⁻¹, were reported for pumpkin (*Cucurbita maxima*; Richardson et al., 1982). Values for abundant proteins can be in the range of 10 µg mL⁻¹, such as thioredoxin h (TRX h) or acyl-CoA-binding protein of rice (Fukuda et al., 2005; Suzui et al., 2006), although higher concentrations of up to 10 mg mL⁻¹ have been recorded for the phloem proteins 1 and 2 (PP1 and PP2) in the sieve-tube sap of pumpkin (Read and Northcote, 1983).

Qualitative variations in sieve-tube sap composition due to the organ sampled (petiole or stems), plant age (petioles of young or mature leaves), location on the plant, and experimental conditions have been reported for pumpkin, melon (*Cucumis melo*), wheat (*Triticum aestivum*), and oilseed rape (Richardson et al., 1982; Fisher et al., 1992; Kehr et al., 1999; Giavalisco et al., 2006; Malter and Wolf, 2010). In pumpkin, variations in protein composition between sieve-tube sap collected from the fascicular (bundle) phloem (FP) and the extrafascicular phloem (EFP) suggest that different functions may be associated with these two phloem systems (Zhang et al., 2010a). In barley, marked differences in protein concentrations were dependent on the method used to collect the sieve-tube exudate (Gaupels et al., 2008), reflecting the need for caution when interpreting data based on these various sampling techniques.

The presence of many hundreds of soluble proteins in the sieve-tube sap is now well established (Fisher et al., 1992; Balachandran et al., 1997; Gaupels et al., 2008; Lin et al., 2009). Mass spectrometry studies and immunodetection with specific antibodies have revealed a wide range of phloem proteins in monocot and eudicot species, including both woody and herbaceous plants (Table 8.1). Most studies have focused on identifying phloem proteins in plants without applying any stress, besides collect of sieve-tube exudates; however, variations in sieve-tube sap protein content in response to drought (Walz et al., 2002), wounding (Dafoe et al., 2009) and virus infection (Malter and Wolf, 2010) have been reported.

The most extensive analyses of the proteome of sieve-tube exudates have been performed in cucurbits (Schobert et al., 1998; Haebel and Kehr, 2001; Walz et al., 2002; Walz et al., 2004; Lin et al., 2009; Malter and Wolf, 2010; Zhang et al., 2010a; Cho et al., 2010). Collectively, experiments performed on sieve-tube exudates of cucurbits have established a large proteomics database for phloem translocation stream comprised of over a thousand proteins. Analysis of this phloem proteome provides important clues on how the phloem acts as a component of the plant communication system (Lin et al., 2009). In addition, these studies established that the sieve-tube system represents a highly active metabolic compartment, particularly with respect to response to

Table 8.1 A survey of proteins detected in sieve-tube exudates collected from various angiosperm species

Plant species ^a	Proteins	Identification ^b	Collect ^c	Comments	Reference
<i>Cucurbitaceae</i>					
<i>C.max.</i>	>100	nd	Bleed (st)		Balachandran et al., 1997
<i>C.max.</i>	9	Ab	Bleed		Schobert et al., 1998
<i>C.max.</i>	4	MS	Bleed (pt, st)	Comparison between organs	Kehr et al., 1999
<i>C.max.</i>	17	MS	Bleed (pt, st)		Haebel and Kehr, 2001
<i>C.max.</i>	13	MS	Bleed (st)	Response to drought	Walz et al., 2002
<i>C.max.</i>	29	MS	Bleed (st)		Walz et al., 2004
<i>C.max.</i>	1209	MS	Bleed (st)		Lin et al., 2009
<i>C.max.</i>	47	MS	Bleed (st)	Field conditions	Cho et al., 2010
<i>C.max.</i>		MS	Bleed (st), diss	FP and EFP	Zhang et al., 2010b
<i>C.me.</i>	14	MS	Bleed (pt, st)	FP and EFP from CMV-infected and transgenic plants	Malter and Wolf, 2010
<i>C.mos.</i>	n.d.	MS	Bleed (st)		Lin et al., 2009
<i>C.s.</i>	11	MS	Bleed (st)	Response to drought	Walz et al., 2002
<i>C.s.</i>	16	MS	Bleed (st)		Walz et al., 2004
<i>Euphorbiaceae</i>					
<i>R.c.</i>	>100	nd	Bleed		Sakuth et al., 1993
<i>R.c.</i>	5	Ab	Bleed (hyp)		Schobert et al., 1995
<i>R.c.</i>	9	Ab	Bleed (hyp)		Schobert et al., 1998
<i>R.c.</i>	18	MS	Bleed (hyp); styl		Barnes et al., 2004
<i>Fabaceae</i>					
<i>R.p.</i>	11	Ab	Bleed		Schobert et al., 1998
<i>L.a.</i>	>100	nd	EDTA		Hoffmann-Benning et al., 2002
<i>Malvaceae</i>					
<i>T.p.</i>	6	Ab	Bleed (hyp)		Schobert et al., 1998
<i>Brassicaceae</i>					
<i>A.t.</i>	24	MS	EDTA (pt)	Flowering plants	Beneteau et al., 2010
<i>A.t.</i>	287	MS	EDTA (pt)	Flowering plants	Batailler et al., 2012
<i>B.n.</i>	103	MS	Bleed (st)	Flowering plants	Giavalisco et al., 2006
<i>Lamiaceae</i>					
<i>P.o.</i>	16	MS	EDTA	Flowering and nonflowering plants	Hoffmann-Benning et al., 2002
<i>Salicaceae</i>					
<i>P.t. × P.d.</i>	48	MS	EDTA (st)	Response to wounding	Dafoe et al., 2009
<i>Poaceae</i>					
<i>H.v.</i>	n.d.	nd	EDTA, Styl	Comparison of two collect methods	Gaupels et al., 2008
<i>O.s.</i>	>150	nd	Styl (leaf)	Phosphorylation status	Nakamura et al., 1993
<i>O.s.</i>	6	Ab	Styl (leaf)		Schobert et al., 1998
<i>O.s.</i>	107	MS	Styl (leaf)		Aki et al., 2008
<i>T.a.</i>	>200	nd	Styl (leaf)	Comparison between organs	Fisher et al., 1992
<i>T.a.</i>	5	Ab	Styl (leaf)		Schobert et al., 1998
<i>Asparagaceae</i>					
<i>Y.f.</i>	6	Ab	Bleed		Schobert et al., 1998

n.d., not determined; CMV, cucumber mosaic virus.

^aPlant species: *C.max.*, pumpkin (*Cucurbita maxima*); *C.s.*, cucumber (*Cucumis sativus*); *C.me.*, melon (*Cucumis melo*); *C.mos.*, squash (*Cucurbita moschata*); *R.c.*, castor bean (*Ricinus communis*); *L.a.*, lupine (*Lupinus albus*); *P.o.*, perilla (*Perilla ocmoides*); *P.t. × P.d.*, poplar hybrid (*Populus trichocarpa × Populus deltoides*); *H.v.*, barley (*Hordeum vulgare*); *O.s.*, rice (*Oriza sativa*); *A.t.*, *Arabidopsis* (*Arabidopsis thaliana*); *B.n.*, oilseed rape (*Brassica napus*); *T.a.*, wheat (*Triticum aestivum*); *Y.f.*, yucca (*Yucca filamentosa*); *R.p.*, black locust (*Robinia pseudoacacia*); *T.p.*, linden (*Tilia platyphyllos*).

^bIdentification method: Ab, proteins identified using specific antibodies or antisera; MS, mass spectrometry; nd, not determined.

^cCollect method: EDTA, EDTA-facilitated exudation from petioles (pt) or stems (st); Bleed, bleeding from stems (st), petioles (pt) or hypocotyl (Hyp); Styl, stylectomy from leaves (leaf); Diss, dissection from stem (st).

oxidative stress (Walz et al., 2002; Cho et al., 2010; Lin et al., 2009). Lastly, analysis of the proteins contained within the phloem proteome indicates a key role for the phloem in systemic defense against insects and pathogens (Walz et al., 2004; Malter and Wolf, 2010; see Chapters 11–16).

Sieve-Tube Sap Proteome: An Integral Component of the Plant Communication System

Noncell-Autonomous Proteins and Large Protein Complexes in Sieve Elements

A variety of proteins involved in protein-protein interactions, including the chaperone GroEL (Schobert et al., 1995), cyclophilins (Schobert et al., 1998; Barnes et al., 2004; Gottschalk et al., 2008; Batailler et al., 2012), and heat shock proteins (Schobert et al., 1995; Aki et al., 2008) have been identified in the sieve-tube sap. The discovery that several of these proteins are able to traffic cell to cell, therefore representing noncell-autonomous proteins (NCAPs), and also have the capacity to facilitate cell-to-cell transport of other proteins and RNA (Balachandran et al., 1997; Xoconostle-Cázares et al., 1999), represented a major breakthrough in plant cell biology (Table 8.2).

Although no RNase activity has been detected in sieve-tube exudates (Sasaki et al., 1998; Doering-Saad et al., 2002), it is assumed that mRNA molecules and small RNAs are translocated in the SEs as ribonucleoprotein (RNP) complexes. Subsets of sieve-tube sap proteins appear to be involved in RNA trafficking in the phloem and, consistent with this function of the phloem, RNA-binding proteins (RBP) have been identified in sieve-tube exudates. For example, in pumpkin sieve-tube sap, at least 82 proteins annotated as RNA-binding were described (Lin et al., 2009). They include phloem protein CmPP2, CmPP16, phloem small RBP CmPSRP1 (Xoconostle-Cázares et al., 1999; Yoo et al., 2004; Lin et al., 2009), glycine-rich RBPs (such as GRP7) (Barnes et al., 2004; Aki et al., 2008; Lin et al., 2009; Batailler et al., 2012), the translationally controlled tumor-associated protein (TCTP) (Giavalisco et al., 2006; Aki et al., 2008; Hinojosa-Moya et al., 2008; Lin et al., 2009; Malter and Wolf, 2010; Batailler et al., 2012), and in pumpkin the RBP CmRBP50 (Ham et al., 2009). Several putative components of the protein synthesis machinery presenting RNA-binding activities, such as eukaryotic initiation factor eIF-5A, eIF-4A, and RNA helicases were also identified (Aki et al., 2008; Lin et al., 2009; Ma et al., 2010). Consistent with long-distance trafficking of RNA in sieve tubes, a number of phloem proteins have been proposed to function in the unfolding and refolding of RNA molecules during their trafficking through the PD that connect CCs and SEs (Xoconostle-Cázares et al., 1999; Yoo et al., 2004; Lough and Lucas, 2006).

Insight into the nature of the protein complexes that mediate in long-distance translocation of RNA species has recently been provided by studies on the pumpkin RBP50. A combination of co-immunoprecipitation (co-IP) and cross-linking experiments identified the phloem proteins that participate in forming a CmRBP50-based RNP complex (Ham et al., 2009). CmRBP50 is a member of the polypyrimidine-track-binding protein (PTB) family and gel mobility-shift studies confirmed that RNA binding and RNP complex formation was dependent on the presence of pyrimidine track motifs in the mRNA molecules bound within the phloem RNP complexes.

The specificity of mRNA bound by the CmRBP50 RNP complex was demonstrated by the observation that, of the several thousand mRNA species detected in the pumpkin phloem translocation stream, only six transcripts were detected in the protein complex purified by co-IP. Importantly, all six mRNA species contained PTB motifs, and, interestingly, four of the six mRNA encoded transcription factors (Ham et al., 2009). The two most abundant transcripts were *CmPP16* and the pumpkin *GIBBERELLIC ACID-INSENSITIVE* ortholog *CmGAIP*, both of which have previously been shown to traffic from source leaves into the plant apex and developing leaves (Ruiz-Medrano

Table 8.2 NCAP and phloem proteins involved in trafficking or other activities in SE

Function	Proteins	Species ^a	Characteristics	Putative roles	Reference
<i>Structural proteins</i>					
PP1	<i>C. max.</i>	Phloem filament	Component of P-proteins	Clark et al., 1997	
PP2	<i>C. max.</i>	Phloem lectin	Component of P-proteins	Bostwick et al., 1992	
For1/SEO1-3	<i>V.f., G.m.</i>	Forosome components	Component of P-proteins	Noll et al., 2007; Pelissier et al., 2008	
<i>RNA binding—ribonucleoprotein complexes</i>					
PP2	<i>C.s.</i>	<i>In vitro</i> binding to viroid RNAs	Translocation of RNAs	Gomez and Pallás, 2001;	
	<i>C.me.</i>	Binding to viroid, viral, and cellular RNAs		Owens et al., 2001; Gomez and Pallás, 2004	
	<i>C.max.</i>	Binding to 7S cellular mRNA		Gomez et al., 2005	
CmPP16	<i>C.max.</i>	Binding to mRNA, Entry of mRNA in SE	Translocation of RNAs	Ham et al., 2009	
CmPSRP1	<i>C.max., C.s., L.a.</i>	Binding to small RNAs		Xoconostle-Cázares et al., 1999	
RBP50	<i>C.max.</i>	Part of a larger complex, binding to mRNAs	Ribonucleoprotein complex, translocation of RNAs	Yoo et al., 2004	
				Ham et al., 2009	
<i>Protein binding—protein large complexes</i>					
CmPP16	<i>C.max.</i>	Binding to eIF-5A, binding to TCTP and NCAPP1	Shoot-ward and root-ward movement of phloem proteins	Aoki et al., 2005; Taoka et al., 2007	
NCAPP1	<i>N.t.</i>	Binding to phloem sap proteins	Role in development	Lee et al., 2003; Taoka et al., 2007	
PP2	<i>A.t.</i>	Binding to phloem sap proteins	Recognition of glycosylated proteins	Beneteau et al., 2010	
RBP50	<i>C.max.</i>	Binding to \approx 200 kDa complex	Large ribonucleoprotein complex	Ham et al., 2009	
Hsc70-1 eIF-4A	<i>C.max.</i> <i>C.max.</i>	Binding to RBP50 Binding to eEF2 and eIF4A	Translational complex	Ham et al., 2009 Ma et al., 2010	

<i>RTM1</i>	<i>A.t.</i>	jacalin	Protein complex restricting virus movement	Chisholm et al., 2001; Cossion et al., 2010
<i>RTM2</i>	<i>A.t.</i>	small heat shock protein	Protein complex restricting virus movement	Chisholm et al., 2001; Cossion et al., 2010
<i>RTM3</i>	<i>A.t.</i>	meprin and TRAF-homology domain	Protein complex restricting virus movement	Cossion et al., 2010
<i>Binding to virus particles</i>				
	PP1 PP2		Interaction with CMV Interaction with CABYYV	Requena et al., 2006 Bencharki et al., 2010
<i>Translation and protein turnover</i>				
	Eif-5A CmPS-1	<i>C.max.</i> <i>C.max.</i>	Translation factor Serpin (serine proteinase inhibitor)	Ma et al., 2010 Yoo et al., 2000; la Cour Petersen et al., 2005
<i>Signaling</i>				
FT	<i>C.max</i>	Florigen	signaling	Lin et al., 2007
FT	<i>A.t.</i>	Florigen	signaling	Corbesier et al., 2007
FT	<i>O.S.</i>	Florigen	signaling	Aki et al., 2008
CmCPK1	<i>C.max.</i>	Calmodulin-like protein kinase	signal transduction	Yoo et al., 2002
Dir1	<i>S.e.</i>	LTP	signal transduction	Mitton et al., 2009
<i>Others</i>				
Cyclophilin	<i>R.c.</i>	Protein folding	Refolding of NCAPP	Gottschalk et al., 2008
CmPP36	<i>C.max.</i>	Cytochrome b(5) reductase	cofactor for iron reduction	Xoconostle-Cázares et al., 2000
RcPRO1	<i>R.c.</i>	Profilin	cytoskeleton formation	Schober et al., 2000
RPP10	<i>O.S.</i>	Acyl-CoA-binding proteins	lipid metabolism	Suzui et al., 2006
RPP13	<i>O.S.</i>	Thioredoxin h	Antioxidant	Ishiwatari et al., 1995;
Glutaredoxin	<i>R.c.</i>	Glutaredoxin		Ishiwatari et al., 1998
CCH	<i>A.t.</i>	Copper homeostasis factor	Redox regulation	Szederkényi et al., 1997
			Metal homeostasis	Mira et al., 2001a

CABYYV, Cucurbit aphid-borne yellows virus.

^a*C.max.*, pumpkin (*Cucurbita maxima*); *C.s.*, cucumber (*Cucumis sativus*); *C.m.*, melon (*Cucumis melo*); *R.c.*, castor bean (*Ricinus communis*); *L.a.*, lupine (*Lupinus albus*); *N.t.*, tobacco (*Nicotiana tabacum*); *P.o.*, perilla (*Perilla ocytioides*); *O.s.*, rice (*Oriza sativa*); *A.t.*, Arabidopsis (*Arabidopsis thaliana*); *B.n.*, oilseed rape (*Brassica napus*); *T.a.*, wheat (*Triticum aestivum*); *Y.f.*, yucca (*Yucca filamentosa*); *R.p.*, black locust (*Robinia pseudoacacia*); *T.p.*, linden (*Tilia platyphyllos*); *V.f.*, fava bean (*Vicia faba*); *G.m.*, soybean (*Glycine max*); *S.e.*, tomato (*Solanum esculentum*).

et al., 1999; Haywood et al., 2005). The stability of this CmRBP50-based RNP complex was demonstrated by the fact that co-IP experiments performed on sieve-tube sap collected from cucumber (*Cucumis sativus*) scions grafted onto pumpkin stock plants identified all of the pumpkin proteins and four of the mRNA species that were shown to constitute this specific RNP complex (Ham et al., 2009). Figure 8.1 presents a model of this pumpkin RBP50 RNP complex.

As the phloem translocation stream contains numerous RBP, it will be important for future studies to identify the RNA substrates and molecular composition of additional RNP complexes. Of equal importance, it will be interesting to learn where these RNP complexes are assembled; that is, in the CCs or within the sieve tubes. Furthermore, it will be important to discover the nature of the molecular signals carried by these long-distance RNP complexes that allow them to be recognized by their target cells or tissues. The mechanisms involved in the symplasmic exchange of macromolecules between SEs and CCs, their subsequent transport to other surrounding cells and their selective exit along the pathway (Foster et al., 2002; Aoki et al., 2005) still remains to be deciphered, as well as those acting in CCs as check-point control cells for macromolecular “hopping” (van Bel et al., 2011b). Finally, although several phloem-mobile mRNA species have been shown to mediate developmental events within sink tissues (Kim et al., 2001; Haywood et al., 2005; Banerjee et al., 2006), a great deal remains to be learned in terms of the evolution and function of this class of transcripts acting over long distance. A combination of proteomics and functional genomics studies should help to accelerate our understanding of this important frontier of plant biology.

The Ubiquitin-Proteasome System: A Role in Protein Degradation or Signaling?

Components of the proteasome-mediated degradation pathway, including ubiquitin-activating, ubiquitin-conjugating, and ubiquitin-ligase enzymes, as well as subunits of the 26S proteasome have been reported within sieve-tube exudates for various plant species (Schobert et al., 1995; Hoffmann-Benning et al., 2002; Barnes et al., 2004; Walz et al., 2004; Giavalisco et al., 2006; Aki et al., 2008; Lin et al., 2009; Batailler et al., 2012). In pumpkin, 166 proteins involved in proteasome-mediated protein degradation were detected in the soluble fraction of sieve-tube exudates, including all subunits of the 26S proteasome (Lin et al., 2009). This study, in particular, provides strong support for the hypothesis that the functional, enucleate sieve-tube system has retained the capacity for proteolysis. This is consistent with the observation in *Nicotiana benthamiana* that plants affected in the expression of *RPN9*, the ortholog of one subunit of the 26S proteasome found in the pumpkin sieve-tube sap, exhibited alterations in phloem development and in hormone signaling (Jin et al., 2006).

In plants, the ubiquitin-26S proteasome system also targets numerous signaling pathways linked to phytohormones (Vierstra, 2009). The phloem translocation stream carries various phytohormones, including auxin, cytokinins, abscisic acid, gibberellins (GA), jasmonates (JA), and methylsalicylates (see Chapter 11; Zeevaart, 1977; Allen and Baker, 1980; Hoad, 1995; Kamboj et al., 1998; Rocher et al., 2006; Chhun et al., 2007; Jager et al., 2007; Truman et al., 2007). It still remains to be established whether the ubiquitin–proteasome system in sieve-tubes functions in protein degradation, hormone signaling, or other processes involved in cell survival.

Phytohormones and Signaling

In addition to various hormones, numerous enzymes associated with hormone production and signaling have been detected in sieve-tube sap. For example, aminocyclopropane-carboxylate (ACC)

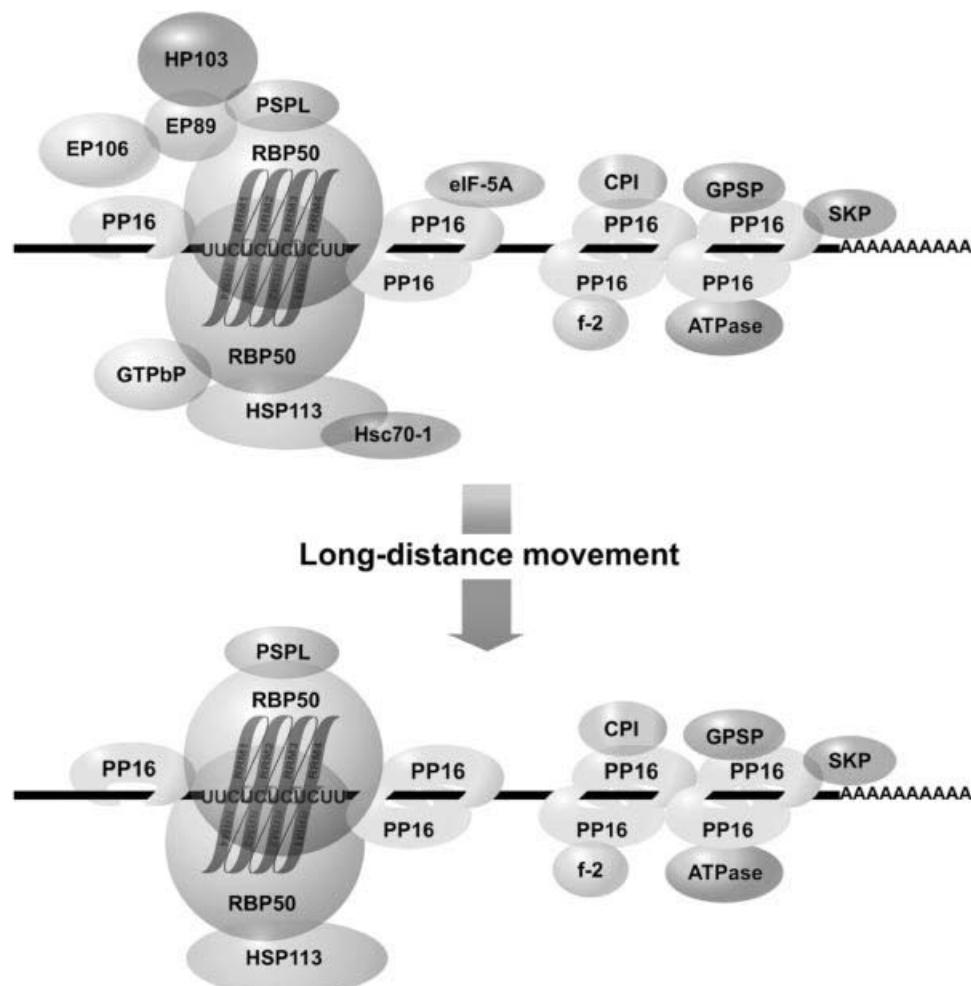


Figure 8.1 Model of a phloem *Cucurbita maxima* RNA-binding protein 50 (CmRBP50)-based ribonucleoprotein (RNP) complex that delivers mRNA species transcribed within source leaves into sink tissues. The pumpkin RBP50 binds to polypyrimidine-tract-binding (PTB) motifs located within a set of six phloem-mobile, polyadenylated transcripts. CmPP16-1/2 binds both to RBP50 and the target mRNA, forming the core of the RNP complex. In addition, GTP-binding protein GTPbP, heat shock-related protein HSP113 and cognate heat shock protein Hsc70-1 bind to RBP50; these proteins might function to chaperone the RNP complex to and through the companion cell (CC)-sieve element (SE) plasmodesmata (PD). Another set of four proteins, composed of the 89-kD expressed protein (EP89), the 103-kD hypothetical protein (HP103), the 106-kD expressed protein, and the phosphoinositide-specific phospholipase-like protein (PSPL) are shown interacting with RBP50. Eukaryotic initiation factor 5A (eIF-5A) is an abundant protein in the pumpkin phloem sap (Ma et al., 2010) that binds transiently to PP16-1/2, but the function of eIF-5A in RBP50-RNP complex formation remains to be elucidated. Regions outside the PTB motifs are bound by PP16-1/2 along with five additional proteins: (1) Cys proteinase inhibitor (CPI), (2) the Csf-2-related protein (Cmf-2), (3) the 44-kD putative ATP-binding protein (ATPase), (4) the glutathione-regulated potassium-efflux system protein (GPSP), and (5) the shikimate kinase precursor (SKP). The lower image shows the composition of the phloem-mobile RBP50-based RNP complex based on co-immunoprecipitation results obtained using phloem sap collected from cucumber scions grafted onto pumpkin stock plants. (Reproduced from Ham et al. (2009) with permission from the American Society of Plant Biologists.) (For a color version of the figure, please see Plate 8.1.)

synthase and ACC oxidase, both enzymes involved in ethylene synthesis, were identified in sieve-tube exudates from pumpkin and cucumber (Walz et al., 2004). Several enzymes potentially involved in the synthesis of auxin from tryptophan were identified in sieve-tube exudates from *Arabidopsis*, including methyl indole-3-acetate methylesterases (MES3 and MES3), indole-3-acetonitrile nitrilase (NIT2), and an indoleacetamide hydrolase (AMI1) (Batailler et al., 2012). Other examples are allene oxide synthase (AOS), allene oxide cyclase (AOC), and lipoxygenase (LOX) required for the synthesis of JA (Avdiushko et al., 1994; Hause et al., 2003; Walz et al., 2004), and the GA-3 oxidase and GA-7 oxidase involved in GA production (Cho et al., 2010).

There is also evidence for the long-range transport of auxin in the phloem from source to sink tissues (Cambridge and Morris, 1996; Bhalerao et al., 2002; Marchant et al., 2002; Ljung et al., 2005). Cytokinin is also transported both in the phloem and in the xylem (Hirose et al., 2008). However, the mechanisms involved are so far poorly characterized. The identification of enzymes acting on their biosynthesis within the sieve tubes suggests that modification of phytohormone accumulation or degradation may take place along the translocation pathway. This could be the basis for some regulatory steps, including relayed amplification of the signal, or molecular “hopping” within the sieve-tube conduit or with surrounding cells (van Bel et al., 2011b).

It will be important to establish whether these sieve-tube-located enzymes participate in the amplification of hormonal signals or the production of secondary messengers, as demonstrated in the case of JA signaling in response to wounding in tomato (Hause et al., 2003; Stenzel et al., 2003; Wasternack et al., 2006). In this case, the occurrence of AOC in SEs, together with the preceding enzymes in JA biosynthesis, LOX and AOS, and the preferential generation and transport in the phloem of JA, which is a main signal molecule in wound response, make it a remarkable system for signal amplification (Wasternack et al., 2006).

Calcium, Kinases, and Signal Transduction Pathways

Components of various signal transduction pathways, including calcium-signaling networks, have been identified in sieve-tube sap. A role for calcium in SE signaling is now well established. For example, electropotential waves triggered by burning stimuli are accompanied by changes in SE calcium ion (Ca^{2+}) levels through both plasma membrane (PM) and ER Ca^{2+} channels (Furch et al., 2009). Calcium-binding proteins, such as calmodulin, annexins, calreticulin, and calcium-dependent protein kinases, are present in the sieve-tube sap, with the annexins often at high levels (Nakamura et al., 1993, 1995; Yoo et al., 2002; Barnes et al., 2004; Giavalisco et al., 2006; Nakamura et al., 2006; Aki et al., 2008; Dafoe et al., 2009; Lin et al., 2009). Calreticulin, a calcium-buffering protein associated with the ER (Jia et al., 2009), has been consistently reported in most surveys of the sieve-tube sap proteomes. Annexins are a family of cytosolic proteins that bind to phospholipids in a calcium-dependent manner and could mediate in the process of free Ca^{2+} release (Laohavosit et al., 2009). Annexins could function in Ca^{2+} -signaling pathways and signaling cascades involving reactive oxygen species (ROS; Mortimer et al., 2008).

Biochemical studies performed on sieve-tube sap proteins have revealed that many of these proteins appear to undergo posttranslational modifications, such as phosphorylation and glycosylation (Avdiushko et al., 1997; Yoo et al., 2002; Taoka et al., 2007). Phosphorylation of phloem proteins was first established in rice (*Oryza sativa*) and cucumber (Nakamura et al., 1993; Avdiushko et al., 1997) and later shown to recruit either calcium-dependent or calcium-independent protein kinases (Yoo et al., 2002; Kumar and Jayabaskaran, 2004) that are abundant in some sieve-tube sap samples, especially in pumpkin (Lin et al., 2009). A significant number of glycosylated proteins

have also been found in sieve-tube sap (Taoka et al., 2007). A combination of phosphorylation and *O*-linked *N*-acetyl glucosamine (GlcNAc) glycosylation has been shown to regulate recognition and trafficking of CmPP16 through the NCAPP1 pathway (Taoka et al., 2007). Posttranslational modifications might thus regulate the entry into the sieve-tube system of NCAPs involved in long-distance signaling.

Metabolic Activities Reveal Oxygen Status of Phloem Tissues

Low Oxygen Tensions and Adaptation to Primary Metabolism

The phloem represents a specialized transport tissue with high metabolic activity to fuel processes such as the active loading of soluble carbohydrates into the phloem translocation stream and to maintain the high sugar concentration gradient. Glycolytic activity has been reported in sieve-tube sap of both black locust (*Robinia pseudoacacia*) and squash (*Cucurbita pepo*) (Kenneke et al., 1971; Lehmann, 1973a, 1973b), in contrast to the weak enzymatic activities reported in castor bean sieve-tube sap (Geigenberger, 2003). A full complement of glycolytic intermediates was identified in the sieve-tube sap of castor bean (van Dongen et al., 2003), consistent with reports of organic acids and precursors, such as glucose-6P and fructose-6P, in the cucurbit sieve-tube sap (Richardson et al., 1982; Fiehn, 2003).

These activities would likely translate into high local rates of oxygen (O_2) consumption. However, O_2 access to phloem cells can be restricted with O_2 tensions falling from 21% (v/v) in air and at the leaf surface to 7% (v/v), giving rise to a hypoxic state in the sieve tubes. The decrease in O_2 would lead to a progressive reduction in the phloem energy balanced with adaptive consequences on metabolism (van Dongen et al., 2003). Metabolic changes could include: coordinated inhibition of glycolysis and respiration in response to low O_2 tension; inhibition of nucleotide biosynthesis within phloem tissues; a need to conserve energy and O_2 ; inhibition of ATP formation via oxidative phosphorylation; and biosynthesis of alanine, GABA, oxoglutarate, and succinate, whose syntheses have been proposed to play a role in counteracting cytoplasmic acidification in low O_2 conditions (van Dongen et al., 2003). Hence, falling internal O_2 leads to a switch to pathways that consume less ATP and utilize O_2 more efficiently, to conserve energy. One example in phloem cells is the preferential breakdown of sucrose by sucrose synthase (SuSy) and UDP-glucose pyrophosphorylase (UGPase), which requires only one molecule of inorganic pyrophosphate (PPi) instead of invertase and hexokinase, which use two molecules of ATP (Geigenberger, 2003; van Dongen et al., 2003).

Analysis of phloem proteomics databases identified a pool of enzymes that potentially function during hypoxic metabolism. In the pumpkin sieve tubes, enzymes annotated in the functional categories related to glycolysis, fermentation, oxidative/nonreductive pentose phosphate cycle, Krebs cycle, and gluconeogenesis were highly represented (Figure 8.2; Lin et al., 2009). Enzymes such as triose phosphate isomerase, enolase, glyoxalase, NAD-dependent glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate mutase, phosphoglycerate kinase, UGPase, fructose biphosphate aldolase, phosphogluconate dehydrogenase, and malate dehydrogenase have frequently been reported in the sieve-tube sap (Espartero et al., 1995; Barnes et al., 2004; Giavalisco et al., 2006; Aki et al., 2008; Lin et al., 2009; Batailler et al., 2012). Enzymes involved in amino acid metabolism were also highly represented. For example, the presence of alanine and aspartate amino transferases suggests a function in nitrogen recycling in relation to carbon availability within the sieve-tube sap (Batailler et al., 2012).

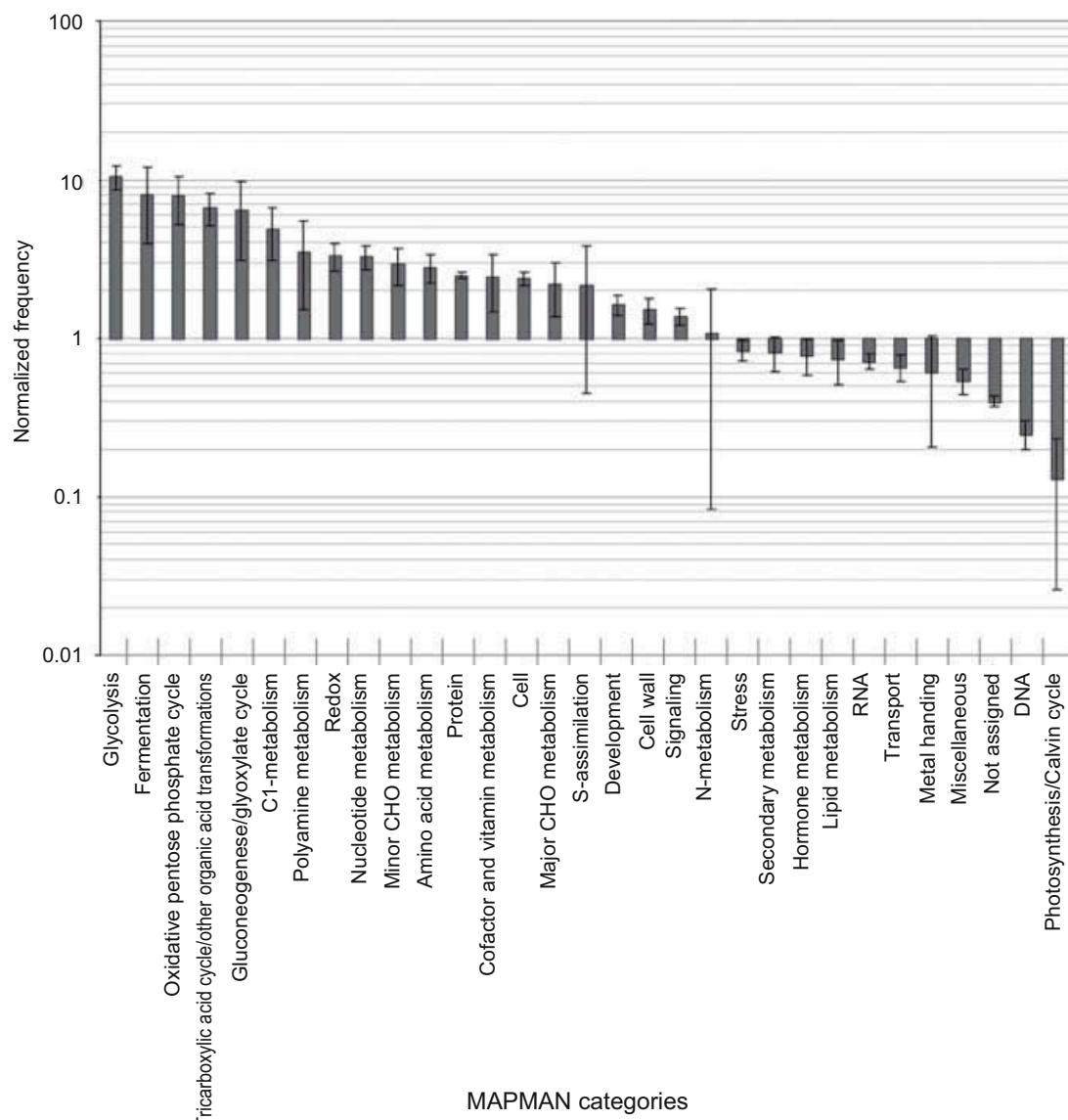


Figure 8.2 Functional categories for proteins identified in phloem exudates analyzed from pumpkin stems. Normalized frequencies for the phloem proteome of pumpkin (\pm bootstrap standard deviation) were calculated using the BAR Classification SuperViewer program (http://bar.utoronto.ca/ntools/cgi-bin/ntools_classification_superviewer.cgi). *Arabidopsis* accession numbers for the closest orthologs were employed for these analyses. In this histogram, values above 1 represent proteins that are highly represented in the pumpkin proteome, whereas values below 1 are poorly represented.

These findings support the hypothesis that phloem tissues use primary metabolic pathways adapted to their hypoxic environment. Further, this specialized metabolism might reflect an important role the SE-CC plays in remobilization and recycling of metabolites that have been retrieved from the apoplasm and the surrounding phloem parenchyma cells (van Bel, 2003b; Hafke et al., 2005).

Production of Metabolites Acting on Defense and Detoxification

Enzymes in pathways that generate secondary metabolites involved in plant defense have been detected in sieve-tube sap. For instance, enzymes acting on the glucosinolate biosynthetic pathway were reported in the Brassicaceae (Giavalisco et al., 2006; Batailler et al., 2012). Enzymes involved in the synthesis of phenylpropanoid-derived plant defense compounds, such as phenylcoumaran benzylic ether reductase, were reported in poplar (Dafoe et al., 2009).

Interestingly, the release of defense compounds in cucurbits has been proposed to occur in the EFP, based on the identification of many secondary metabolites in EFP exudates (Fiehn, 2003; Tolstikov et al., 2007). The vascular system of cucurbits is indeed spatially distinguishable into FP and EFP (Crafts, 1932). FP, which is responsible for sugar transport (Zhang et al., 2010a), is contained within vascular bundles, and EFP, thought to function in signaling, defense, and transport of amino acids and secondary metabolites (Tolstikov et al., 2007), is distributed as scattered elements throughout the cortex, peripheral to the vascular bundles, and around rings of sclerenchyma. Proteome studies of cucurbits have been carried out on sieve-tube exudates collected from both the FP and EFP. Interestingly, a recent study of proteins and metabolites of sieve-tube exudates collected separately from FP and EFP of pumpkin suggested important differences in their content (Zhang et al., 2010a). In addition, the FP proteome was proposed to represent a newly identified functional system within cucurbits that is distinct from the extensively studied proteome of phloem exudates. For example, PP1 and PP2, the two most abundant proteins of the EFP, were not detected in the FP proteome (Zhang et al., 2010a). Thus these two phloem tissues may have evolved divergent functions, indicating additional specializations within a single plant (Zhang et al., 2010a). Since FP is the only pathway for long-distance trafficking present in most plant species, to what extent the proteome of the EFP in cucurbits reflects that of the FP from other plant species still needs to be examined carefully (Turgeon and Oparka, 2010; van Bel et al., 2011b).

Oxidative Metabolism, Antioxidant Defense, and Control of Redox State

A wide range of ROS and redox-related proteins have been reported in the sieve-tube sap of a number of plant species. Importantly, the sieve-tube sap of all plant species examined contains antioxidant proteins (Ishiwatari et al., 1995; Szederkényi et al., 1997; Ishiwatari et al., 1998; Hoffmann-Benning et al., 2002; Walz et al., 2002, 2004; Giavalisco et al., 2006; Aki et al., 2008; Dafoe et al., 2009; Lin et al., 2009; Beneteau et al., 2010; Cho et al., 2010; Batailler et al., 2012). Several ROS-scavenging enzymes have been identified, including Cu/Zn superoxide dismutase (SOD), peroxidase (PX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (Walz et al., 2002). Activity stains and photometric assays showed that SOD, DHAR, and PX activity was detectable in sieve-tube exudates in cucumber and pumpkin. This activity increased in response to drought treatment, after 1 week without watering plants (Walz et al., 2002). Other proteins such as TRX h and glutathione S-transferase (GST) have also been found. GST can detoxify radicals and TRX h, an abundant protein found in the sieve-tube exudate of rice, is involved in disulfide reduction and proposed to act on the repair of oxidatively damaged proteins in SEs (Ishiwatari et al., 1995). The sieve-tube sap typically also contains high concentrations of nonenzymatic radical scavengers, such as glutathione, ascorbate, or citrulline.

These enzymes and radical scavengers have been proposed to function in the protection of SE components against ROS produced either by mitochondria or by endomembrane systems as normal

by-products of primary metabolism (Walz et al., 2002). A number of these proteins, such as PX, GST, SOD, MDHAR, and TRX, could be associated with oxidative metabolism induced by the lack of O₂, their expression being upregulated in response to hypoxia (Blokhina and Fagerstedt, 2010). They may be important components of the primary adaptive metabolism associated with a low O₂ tension in SEs.

Since the SEs can be the sites of pathogen infections, their function in the detoxification of ROS produced by the PM NAD(P)H oxidase system, as a first reaction to contact with potential pathogens, has also been proposed (Walz et al., 2002). These antioxidant systems could also facilitate systemic redox signaling acting on coordinated responses required in growth, development or plant defense (Diaz Vivancos et al., 2010).

Another class of proteins frequently identified in sieve-tube exudates is the metal-ion-binding metallothioneins (Barnes et al., 2004; Aki et al., 2008; Batailler et al., 2012). Such proteins play a central role in essential trace element homeostasis and in metal detoxification. Due to a high content of thiol groups, these proteins also function as potent antioxidants by scavenging ROS and have been proposed to diminish ROS damage and increase the tolerance to abiotic stresses (Xue et al., 2009; Zhu et al., 2009; Wang et al., 2010; Hassinen et al., 2011). Ferredoxin and copper chaperone homeostasis (CCH) factor are other metal-binding proteins found in sieve-tube sap (Mira et al., 2001a; Giavalisco et al., 2006; Aki et al., 2008). In *Arabidopsis*, CCH, which exhibits antioxidant activity, accumulates predominantly in SEs and was proposed to play a role in copper reallocation during senescence, in addition to the possibility that CCH may participate in the protection of the vascular system from oxidant toxic molecules (Mira et al., 2001a, 2001b).

The Phloem Plays a Key Role in Plant Defense

Defense Proteins and Response to Biotic and Abiotic Stresses

Several insect groups, including aphids, whiteflies, and planthoppers, are phloem-feeding insects that have developed sophisticated systems to prevent SE occlusion by callose or P-proteins (see Chapters 7 and Chapter 15; Will et al., 2007, 2009). Aphid species are also known to transmit plant viruses and they are the most common vectors of viral diseases. Aphid-vectored viruses therefore could induce responses influencing plant–aphid interactions and *vice-versa* (Goggin, 2007). In addition, phloem tissue is the pathway for systemic infection of plant viruses and some phloem-limited bacteria (see Chapter 13 and 14).

Numerous sieve-tube sap proteins are predicted to be involved in stress and defense (Kehr, 2006). These include proteins induced by wounding, such as members of the major latex protein/ripening-related protein (MLP/RRP) subfamily, such as CSF-2 and SN-1, and the peptidase SLW-1 and the β-glucosidase SLW-3 (Walz et al., 2004), related to proteins induced in tomato by silverleaf whitefly (*Bemisia tabaci*) feeding. Myrosinases and myrosinase-binding proteins, involved in the activation of glucosinolates, compounds deterrent for insect feeding in the Brassicaceae (Mewis et al., 2006), were identified in sieve-tube exudates collected from oilseed rape and *Arabidopsis* (see Chapter 12; Giavalisco et al., 2006; Batailler et al., 2012).

Defense proteins, such as putative leucine rich repeat (LRR) proteins and pathogen resistance (PR) proteins have also been identified in sieve-tube sap (Dafoe et al., 2009; Beneteau et al., 2010; Batailler et al., 2012). Additional proteins, frequently described as being upregulated in response to biotic or abiotic stresses, such as major pollen allergen Bet v I, putative thiamin biosynthetic enzyme DJ-1/ThiJ, or universal stress protein (USP) have also been reported (Giavalisco et al., 2006; Aki

et al., 2008; Malter and Wolf, 2010; Batailler et al., 2012), although the functions of these phloem proteins remain to be established.

Currently, our ability to directly relate changes in sieve-tube sap proteomes to biotic and abiotic stress responses is limited. Infection of melon by *cucumber mosaic virus* revealed induced accumulation of a limited number of phloem proteins, including enolase, TCTP, cognate heat shock protein Hsc70, and MLP (Malter and Wolf, 2010). Two sieve-tube sap proteins, pop3/SP1 protein, and a thaumatin-like protein, were reported to accumulate in poplar (*Populus deltoides*) in response to wounding (Dafoe et al., 2009). These findings suggest that viral infection and wounding might not induce major changes in sieve-tube sap protein composition. Drought treatment in pumpkin induced an increase in sieve-tube exudate activity of SOD, DHAR, and a PX (Walz et al., 2002), demonstrating that phloem responses to stress may rely on the increase in activity of enzymes already present at a steady state level in sieve tubes.

Lectins and Storage Proteins

A range of lectins, a class of proteins that reversibly and nonenzymatically bind specific carbohydrates, have been identified in the sieve-tube sap, including chitin-binding PP2, mannose-binding proteins, curculins, jacalins, and proteins related to the legume lectins and ricins (Smith et al., 1987; Walz et al., 2004; Giavalisco et al., 2006; Aki et al., 2008; Lin et al., 2009; Zhang et al., 2010a; Beneteau et al., 2010). Plant lectins that recognize sugars that are typically not present in the host species are thought to function in plant defense against insects, bacteria, or fungi (Vasconcelos and Oliveira, 2004; De Hoff et al., 2009). Indeed, several lectins have antibiotic effects on aphids (Rahbé et al., 1995) and it has been proposed that phloem lectins may participate in the defense against phloem-feeding insects. Alternatively, lectins binding to portions of complex sugar moieties of endogenous glycosylated proteins could regulate intra- or intercellular signaling, or protein trafficking between CCs and SEs (Taoka et al., 2007; Beneteau et al., 2010). Plant viruses that frequently hijack the cellular machinery for their own benefit may recruit such proteins for long-distance trafficking.

An example of the complex functions of phloem lectins is illustrated by studies on the PP2. PP2 is abundant in the sieve-tube exudates of pumpkin, melon, cucumber, and oilseed rape (Gomez and Pallás, 2001; Walz et al., 2004; Gomez et al., 2005; Giavalisco et al., 2006). This dimeric lectin, binding to GlcNAc oligomers was initially characterized in pumpkin, cucumber, and melon (Sabnis and Hart, 1978; Read and Northcote, 1983; Dinant et al., 2003). In *Arabidopsis*, PP2-A1 binds to GlcNAc oligomers, high-mannose N-glycans, and 9-acetyl-N-acetylnuraminic sialic acid (Beneteau et al., 2010). In *Arabidopsis*, PP2-A1 has a negative effect, *when taken up from diets*, on the growth of two aphid species, pea aphid (*Acyrtosiphon pisum*) and green peach aphid (Beneteau et al., 2010), which has been confirmed *in vivo* (Zhang et al., 2011). In addition, PP2-A1 plays a positive role in virus transmission by aphids (Bencharki et al., 2010).

In cucurbits, CmPP2 binds to the RNA of various plant viruses or viroids (Gomez and Pallás, 2001; Owens et al., 2001; Gomez et al., 2005), which may assist them for systemic transport within the plant. CmPP2 also binds a large range of mRNAs (Ham et al., 2009), indicating a possible role in the translocation of endogenous RNAs in the phloem translocation stream. Thus, sieve-tube sap proteins could be recruited by plant pathogens, such as viruses, to assist in their long-distance movement or insect acquisition and vectoring. There is, however, only circumstantial evidence for the activity of endogenous phloem proteins in deterring insect feeding or systemic movement of plant pathogens.

Various proteins annotated as storage proteins are present within sieve-tube exudates, including in *Arabidopsis* germins, cupins, and vegetative storage proteins (Beneteau et al., 2010; Batailler et al., 2012), and in pumpkin, several late embryogenesis abundant proteins (Lin et al., 2009).

Protease Inhibitors and Protein Turnover

Protease inhibitors (PIs), including serine, cysteine, and aspartic PIs, have been identified as abundant constituents of sieve-tube sap in a range of plant species (Murray and Christeller, 1995; Christeller et al., 1998; Schobert et al., 1998; Kehr et al., 1999; Yoo et al., 2000; Dannenhoffer et al., 2001; Haebel and Kehr, 2001; Barnes et al., 2004; Walz et al., 2004; la Cour Petersen et al., 2005; Aki et al., 2008; Dafoe et al., 2009; Lin et al., 2009). Phloem PIs could serve as defense molecules in response to insect feeding. Plant PIs can block herbivore insect protease activity and several PIs, such as oryzacystatin or pea Bowman-Birk PI, were reported to be active against aphids (Rahbé et al., 2003a, 2003b; Ribeiro et al., 2006). Evidence that phloem-feeding insects have digestive proteases in their gut make it tempting to speculate that phloem PIs play an important defensive role against phloem-feeding insects and other herbivores (Yoo et al., 2000; Kehr, 2006).

Regulating protein turnover in the fully differentiated, enucleate SEs is a potentially important feature of the sieve-tube system. Phloem PIs are also attractive candidates to prevent unregulated protein degradation in mature SEs and to regulate control of selective cell death during SE differentiation (Dannenhoffer et al., 2001; la Cour Petersen et al., 2005; Hao et al., 2008). For instance, the cucumber phloem serpin (*serine proteae inhibitor*) CmPS-1, a sieve-tube PI highly resistant to turnover, was proposed to protect sieve-tube proteins against proteolytic activity (la Cour Petersen et al., 2005).

Although early reports indicated that cucurbit sieve-tube exudates lack proteolytic activity (Chino et al., 1991), recent proteome studies detected aminopeptidases and proteases in sieve-tube sap of pumpkin and *Arabidopsis* (Lin et al., 2009; Batailler et al., 2012). In addition, identification of the entire machinery for a functional 26S proteasome within the pumpkin sieve-tube sap (Lin et al., 2009) suggests that selective degradation of ubiquitinated phloem proteins occurs within the sieve tubes.

Structural Components of Sieve Elements

SE Structural Proteins

Analysis of phloem proteome databases indicates that certain structural constituents, including cytoskeletal elements are not abundant. However, several cytoskeleton-related proteins, such as profilin, actin, and actin depolymerizing factor, are routinely detected in sieve-tube exudates (Schobert et al., 1998, 2000; Walz et al., 2004; Giavalisco et al., 2006; Aki et al., 2008; Dafoe et al., 2009; Lin et al., 2009). In castor bean, profilin is an abundant phloem protein that could function to prevent actin filament formation within mature SEs (Schobert et al., 2000). This hypothesis is supported by earlier observations that microfilaments and microtubules were not detected in the lumen of mature SEs (Evert, 1990); however, alternate scenarios are possible, supported by the observation of cytoskeleton elements in the vicinity of the sieve plates (Evert, 1990). For example, the cytoskeleton may be present but not visible due to the shortcomings in preservation techniques used in the preparation of the samples for transmission electron microscopy, or it may be organized with a different structure than is typical in most cells. Their presence might be critical for the trafficking of macromolecules through the PD and though sieve plates, which remains to be explored.

Table 8.3 Examples of membrane proteins identified in sieve elements or companion cells

Class	Example	Species ^a	Reference
<i>Membrane components</i>			
GPI-anchored protein	SE-ENOD	<i>A.t.</i>	Khan et al., 2007
Membrane protein	RCI2A	<i>A.t.</i>	Thompson and Wolniak, 2008
<i>Metabolite transport</i>			
Sucrose transporter	SUC/SUT	<i>A.g., A.t., S.t., L.e., O.s., P.m., Z.m., A.m.</i>	Stadler et al., 1995; Stadler and Sauer, 1996; Kühn et al., 1997; Weise et al., 2000; Noiraud et al., 2000; For more references, see Kühn and Grof, 2010
Sucrose sensor	SUT2	<i>S.e.</i>	Barker et al., 2000
Hexose transporter	VvHT1	<i>V.v.</i>	Vignault et al., 2005
Polyol transporter	AgMAT PmPLT	<i>A.g., P.m.</i>	Ramsperger-Gleixner et al., 2004; Klepek et al., 2005; Schneider et al., 2006; Juchaux-Cachau et al., 2007; Pommerenig et al., 2007
Amino acid transporter	AAP2	<i>A.t.</i>	Zhang et al., 2010b
Proton pump-ATPase	AHA3	<i>A.t., R.c.</i>	DeWitt and Sussman, 1995; Langhans et al., 2001
Metal-nicotianamine transporter	OsYSL2	<i>O.s.</i>	Koike et al., 2004; Ishimaru et al., 2010
Cyclic nucleotide-gated channel	CNGC19	<i>A.t.</i>	Kugler et al., 2009
<i>Ion and water transport</i>			
Potassium channels	VFK1 AKT2/3	<i>A.t., V.f.</i>	Lacombe et al., 2000; Ache et al., 2001; Deeken et al., 2002
ABC transporter	ALS3	<i>A.t.</i>	Larsen et al., 2005
Sulfate transporter	Sultr1.3 SULTRs	<i>A.t., Pta</i>	Yoshimoto et al., 2003; Dürr et al., 2010
Nitrate transporter	NRT1.7	<i>A.t.</i>	Fan et al., 2009
Calcium channels		<i>N.t., P.s., V.f.</i>	Volk and Franceschi, 2000; Furch et al., 2009
Aquaporins		<i>Z.m., S.o.</i>	Barrieu et al., 1998; Fraysse et al., 2005
<i>Phytohormone transport</i>			
Purine/cytokinin permease	PUP2	<i>A.t.</i>	Bürkle et al., 2003
Auxin influx carrier	AUX1	<i>A.t.</i>	Kleine-Vehn et al., 2006

^aA.g., celery (*Apium graveolens*); A.m., mask flower (*Alonsoa meridionalis*); A.t., *Arabidopsis (Arabidopsis thaliana)*; S.e., tomato (*Solanum lycopersicum*); N.t., tobacco (*Nicotiana tabacum*); L.e., *Lycopersicon esculentum*; O.s., rice (*Oriza sativa*); P.m., common plantain (*Plantago major*); Pta, poplar hybrid (*Populus tremula × Populus alba*); P.s., water lettuce (*Pistia stratiotes*); R.c., castor bean (*Ricinus communis*); S.o., spinach (*Spinacia oleracea*); S.t., potato (*Solanum tuberosum*); V.f., fava bean (*Vicia faba*); V.v., grape vine (*Vitis vinifera*); Z.m., maize (*Zea mays*).

As expected, membrane proteins were not represented in the soluble proteome obtained from sieve-tube exudates, although PM proteins from the SEs are essential for the basic loading functions of the SEs (see Chapters 4 and 5; Table 8.3). Proteins associated to other membranes, such as those from the sieve element reticulum (SER) as well as peripheral sieve-tube proteins, remain largely uncharacterized (Ehlers et al., 2000). In pumpkin sieve-tube exudates, proteins associated with coated membranes and clathrin-coated vesicles were identified, indicating a capacity to recycle PM

components. In addition, proteins potentially acting in vesicle trafficking and membrane dynamics were also found, suggesting active exchanges between endomembrane system components (Lin et al., 2009). In any event, the recent development of a protocol to isolate functional SE protoplasts should allow for a comprehensive characterization of the SE membrane proteins (Hafke et al., 2007) and should allow a more comprehensive analysis of SE transporters and membrane-anchored proteins.

Sites of Sieve-Tube Sap Protein Synthesis

Differentiation of SEs is linked to a partial autolysis with the dismantlement of the nucleus, vacuole, and a progressive reorganization of the rough ER into a stacked SER. Typically, when differentiation is complete, the level of polyribosomes diminishes; indeed, ultrastructural studies performed on differentiated SEs have failed to visualize ribosomes. Such ultrastructural observations have given rise to the dogma that the translational machinery is absent from mature SEs. Few proteins that are produced early in SE development have been identified. One example is the sieve element occlusion protein (for1/SEO1), a component of the forisomes, which in the Fabaceae accumulates in immature SEs and remains as large bodies in mature SEs (Noll et al., 2007; Pelissier et al., 2008). Another protein that accumulates early in development and persists in enucleate SEs is a GPI-anchored early-nodulin-like protein, SE-ENOD, a protein identified using a monoclonal antibody, produced against an enriched SE preparation (Khan et al., 2007). This protein is extensively processed through the membrane system and anchored in the SE PM. SE-ENOD is a member of the plastocyanin-like copper-binding domain protein family. Plastocyanins and ENODs have been implicated in a number of developmental processes, including the formation of a specialized cell surface, although the exact function of SE-ENOD in phloem SEs remains to be established (Khan et al., 2007).

A breakthrough in understanding the intricate relationship between CCs and SEs came with the discovery that several proteins present in mature SEs, such as the pumpkin phloem lectin, PP2 (Bostwick et al., 1992), and TRX h in rice (Ishiwatari et al., 1998) were produced in CCs prior to trafficking into the neighboring SEs through the interconnecting PD. These findings provided the foundation for the idea that the phloem operates as a unique symplasmic domain in which NCAPs can traffic cell-to-cell and long distance, which is now supported by numerous studies conducted on a range of SE proteins (Xoconostle-Cázares et al., 1999, 2000; Lee et al., 2003; Aoki et al., 2005; Haywood et al., 2005; Lough and Lucas, 2006; Taoka et al., 2007) (Figure 8.3).

Even though targeted and nontargeted trafficking of NCAPs can occur across the CC–SE interface (Crawford and Zambryski, 2001), what remains to be established is the species of proteins synthesized in CCs that enter into mature SEs (Figure 8.4). Evidence for differential distribution of proteins in CCs and SEs has been obtained for rice (Fukuda et al., 2005). A recent study that described the translatomes of various cell types revealed the presence of 798 and 480 specific transcripts in CCs of the shoot and root, respectively, with only limited overlap (Mustroph et al., 2009). Comparing proteins detected in sieve-tube sap with those deduced from the translatome of CCs also showed only partial overlap (Batailler et al., 2012). The differential distribution of proteins between SEs and CCs provides additional evidence for a selective mechanism controlling the intercellular trafficking of proteins into the phloem.

Presence of the Translational Machinery in Sieve Tubes: An Enigmatic Puzzle

Surprisingly, many of the proteins required for assembly of the translational machinery were identified in sieve-tube exudates collected from pumpkin stems (Lin et al., 2009). The types of proteins

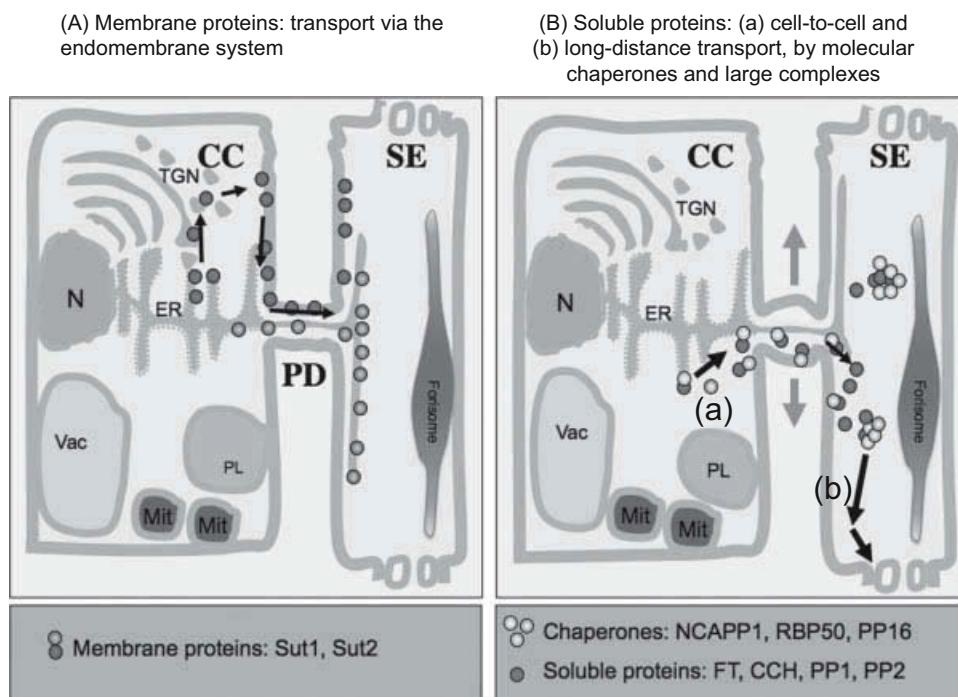


Figure 8.3 Models of noncell-autonomous protein (NCAP) entry into the enucleate sieve-tube system *via* the companion cell (CC)-sieve element (SE) plasmodesmata (PD). (A) Membrane proteins play an essential role in mediating the trafficking of proteins and RNA species to various intracellular and intercellular destinations. In the context of the CC–SE complex (CC–SE), it has been proposed that the plasma membrane (PM) and endoplasmic reticulum (ER) function as major routes for the delivery of proteins synthesized in CCs and translocated through the PD to SE endomembranes (Martens et al., 2006). Examples of such proteins are the PM-located sucrose transporters (e.g., SUT2) that are present both on the PM of the CC and SE (Barker et al., 2000). (B) Soluble proteins present in sieve-tube sap gain entry into the SE from the CC *via* targeted or nontargeted trafficking through PD. Selective trafficking involves the action of molecular chaperones and PD receptors that function to guide proteins along the cytoskeleton or the ER to and through the PD. Examples of such proteins are NCAPP1 (receptor) and CmPP16/PP2 (chaperones). NCAPs trafficked along this pathway are released into the lumen of the SE and move long distance by bulk flow in the translocation stream. Most soluble proteins and protein complexes present in phloem exudates are thought to belong to this class and participate in both (a) cell-to-cell transport and (b) long-distance transport. In addition to (A) and (B) scenarios, sieve-tube proteins can also be synthesized and accumulate within immature SE, then persist through SE maturation, and function in fully differentiated SE, such as the SE-ENOD proteins (Khan et al., 2007) (not shown in the figure). CC, companion cell; SE, sieve element; PD, plasmodesmata; PL, plastids; Mit, mitochondria; Vac, vacuole; N, nucleus; ER, endoplasmic reticulum; TGN, trans Golgi network. (For a color version of the figure, please see Plate 8.2.)

detected included aminoacyl-tRNA synthetases, ribosomal proteins, as well as translation, elongation, and termination factors. This unexpected finding questions the dogma that protein synthesis does not occur in the mature enucleate SEs. An important clue to decipher the role of translation machinery in SEs might be the discovery of the presence of the eukaryotic initiation factor, eIF-5A, that has been detected in sieve-tube exudates of pumpkin (Lin et al., 2009) as well as other species, such as rice and oilseed rape (Giavalisco et al., 2006; Aki et al., 2008). In a recent study of the pumpkin eIF-5A gene family, protein overlay and gel filtration chromatography experiments performed on sieve-tube exudates revealed that these eIF-5A proteins specifically interacted with a number of other translation factors, including elongation factor 2 (EF2) (Ma et al., 2010). These

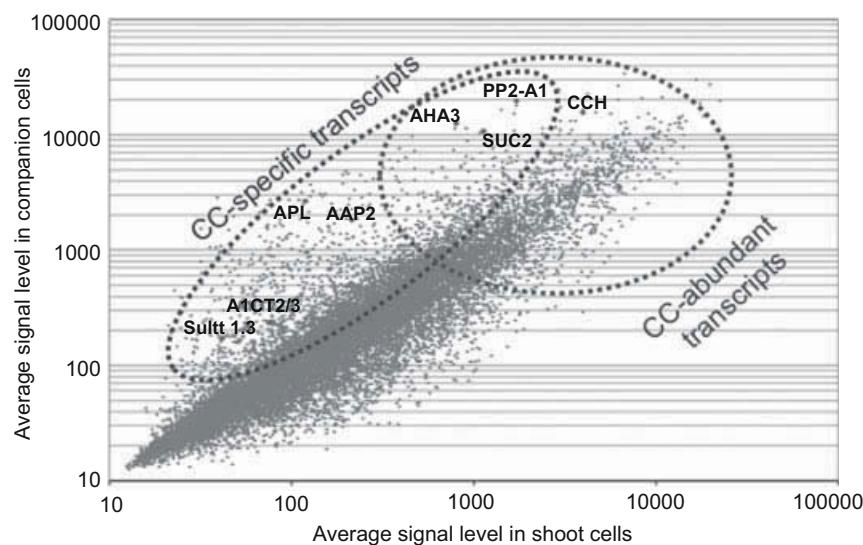


Figure 8.4 Translatome of companion cells (CCs) and other cell types from shoot tissues of *Arabidopsis*. Data are presented on a scatter plot comparison of CC and shoot expression profiles. Expression values were obtained from Mustroph et al. (2009, Supplementary materials). Normalized expression values for each gene probe set in CCs (i.e., SSUC2C set of values, obtained from cells sorted using pSUC2-GFP fusion in the shoot) are plotted on the Y-axis and the equivalent values for expression in shoot cell types are plotted on the X-axis (average signals for STotC, S35SC, SRBC, SS2-2C, SGL2C, SCERC, SKATC datasets). Both axes are logarithmic. Representative genes specifically expressed in the phloem are highlighted in bold.

findings are consistent with the hypothesis that mature SEs could have retained the capacity for protein synthesis.

Additional roles for eIF-5A became apparent from studies on phloem RNP complexes. A pumpkin 50-kD PTB-RBP (CmRBP50) mediates the formation of a very stable RNP that moves long distance through the pumpkin sieve-tube system. Although CmIF-5A is not a *bona fide* component of the CmRBP50-RNP complex, chemical cross-linking studies suggested that it was involved in the formation of this RNP complex (Aoki et al., 2005; Ham et al., 2009). This raises the question as to whether additional components of the protein translational machinery might also have been co-opted to perform other functions perhaps associated with long-distance delivery of RNAs to target sink tissues.

The phloem translocation stream is known to carry a population of 20–25 nucleotide small RNAs (see Chapter 9; Yoo et al., 2004; Buhtz et al., 2008, 2010). The presence of a slightly larger class of small RNAs was recently reported; an important constituent of this size class appeared to be truncated tRNAs. *In vitro* translation experiments demonstrated that addition of these truncated tRNAs inhibited protein synthesis *in vitro* (Zhang et al., 2009). Although the meaning of these results is unclear, this may indicate that a tight regulation of the translation machinery is operating in mature SEs.

In *Arabidopsis*, sieve-tube exudates collected using EDTA-facilitated exudation lacked many components of the translation machinery (Batailler et al., 2012). This could well reflect the difficulties associated with collecting sieve-tube sap from this species or artifacts due to the use of EDTA for exudation. Alternatively, it is possible that mature SEs of cucurbits may have maintained the capacity for protein synthesis.

Future Directions

It is well established that the enucleate sieve-tube modules contain a complex population of proteins and RNA species. Availability of genomes for graft-compatible species from which analytical quantities of sieve-tube sap can be collected now opens the way for experiments that can separate these proteins and RNAs into pools that act locally *versus* those that move long distance in the translocation stream. Functional genomics studies will then be needed to explore the roles played by these phloem proteins. Identification of the proteins involved in the local maintenance of the enucleate sieve-tube system will provide important insights into the evolution of the phloem of the angiosperms. Furthermore, identifying the proteins involved in this maintenance process will open the door to exploring regulatory networks that underlie the operation of the phloem translocation stream.

Turnover of integral membrane proteins located in the SE PM and SER, as well as in plastid and mitochondrial membranes, is an important area for future study. A subset of these proteins could be delivered across the PM continuum through the connecting PD between the CCs and the SEs (Figure 8.3). However, if mature SEs have indeed retained functional translational machinery, such membrane proteins could be generated within these cells. This would require the retention of a Golgi-secretory pathway within mature SEs; if such components were to be detected, this would establish the sieve-tube system as a remarkable feat of evolution.

Detection of all subunits required for the formation of the 26S proteasome in sieve-tube sap suggests a high level of coordination in the trafficking of these proteins into the sieve-tube system. Understanding the mechanism by which the phloem 26S proteasome is assembled will yield important information regarding protein import and macromolecular assembly in the enucleate SEs. This information will also open the door to studies on the potential roles played by the ubiquitin-26S proteasome system in both protein turnover and the evolution of signaling pathways that are probably unique to the phloem.

The concept of protein and RNA delivery through the phloem translocation stream to target tissues and organs is now well established. Much remains to be learned, however, regarding the molecular mechanisms responsible for controlling the exchange of these phloem-mobile macromolecules with the surrounding tissues. With respect to trafficking of RNA, studies are needed to further establish the nature of the RNP complexes involved in long-distance delivery and transfer of the bound message to the target cells. Functional genomics studies are also urgently needed to explore the role of these long-distance macromolecular-signaling agents in the orchestration of plant growth and development at the whole-plant level.

Lastly, interrogation of existing phloem proteomes has clearly indicated that the sieve-tube system contains a complex array of enzymes. Dissecting the functions of these enzymes and developing the metabolic networks that operate within the sieve-tube system will be essential if we are to understand the events involved in phloem maintenance and function. Such studies should focus on establishing a comprehensive picture of the metabolic networks that operate along the entire translocation pathway from the source to the various sinks located in the shoot and root regions of the plant.

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9 Endogenous RNA Constituents of the Phloem and Their Possible Roles in Long-Distance Signaling

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Short- and long-distance communication networks are essential to coordinate diverse biological activities within all living organisms, including higher plants. Intra- and intercellular messenger RNA (mRNA) transport is an important component of cellular communication to coordinate cell differentiation and plant growth (Haywood et al., 2002; Ding et al., 2003; Kim and Pai, 2009). Several plant transcription factors, such as the floral transcription factor *LEAFY* and *APETALA1* or the *KNOTTED1*-like homeobox transcription factor, have the ability to move over short distances from cell-to-cell through plasmodesmata and are involved in meristem initiation and maintenance (Lucas et al., 1995; Sessions et al., 2000).

More recently it was suggested that mobile silencing signals from endogenous *trans*-acting (ta) short-interfering (si) RNA (ta-siRNA) loci might produce local gradients of target gene expression to enable correct leaf polarization in *Arabidopsis* (Chitwood et al., 2009; Dunoyer et al., 2010a). An unexpected observation was that specific mRNAs such as *CmNACP* can be delivered even to distant plant organs through the phloem (Ruiz-Medrano et al., 1999). Meanwhile many RNAs, proteins, and ribonucleoprotein (RNP) complexes found in the phloem translocation stream have become attractive candidates to serve as information transmitters (Ruiz-Medrano et al., 1999). For some of them a role in long-distance signaling has been proposed (Oparka and Cruz, 2000; Lough and Lucas, 2006; Kehr, 2009; Dinant and Lemoine, 2010).

Comprehensive studies concentrating on phloem RNA composition revealed different types of RNAs. Initially mRNAs were found in several plant species (Kehr and Buhtz, 2008; Harada, 2010). Subsequently nonprotein coding RNAs such as ribosomal RNAs (rRNA) and low molecular weight RNAs smaller than 300 nucleotides (nts) including transfer RNAs (tRNAs), micro RNA (miRNA), and siRNAs that are usually summarized as small RNAs (smRNAs) were identified in sieve-tube exudates of various plants (Kragler, 2010). It is important to note that while many RNAs have been

Abbreviations: EDTA, ethylenediaminetetraacetic acid; GFP, green fluorescent protein; ISH, *in situ* hybridization; miRNA, micro RNA; MPSS, massively parallel signature sequencing; mRNA, messenger RNA; nt, nucleotide; RNA, ribonucleic acid; RNP, ribonucleoprotein; rRNA, ribosomal RNA; RT-PCR, reverse transcription-polymerase chain reaction; SAGE, serial analysis of gene expression; siRNA, short-interfering RNA; smRNA, small RNA; snRNA, small nucleolar RNA; SOLiD, supported oligo ligation detection; SUT1, sucrose transporter 1; ta-siRNA, *trans*-acting short interfering RNA; tRNA, transfer RNA

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identified in the sieve-tube sap, neither the ability to move *in vivo* nor an impact on remote organs or tissues has been demonstrated for most of the RNAs. Moreover, detailed mechanisms of the translocation of endogenous macromolecules through the phloem are still not well understood, and proposed models that assume translocation as RNP complexes need to be experimentally validated.

This chapter provides an overview of the experimental strategies that have been successfully employed to identify RNAs in sieve-tube sap and a summary of the different types of RNAs that have been identified. In addition, the methods to examine RNA mobility and potential physiological roles RNAs might play in the complex long-distance communication network of higher plants will be discussed.

Methods for Identifying Phloem RNAs

The first convincing indications that RNAs occur in sieve elements were obtained by *in situ* hybridization (ISH) studies (Kühn et al., 1997). Later, comprehensive studies of the RNA composition of sieve-tube exudates were primarily based on the isolation of total RNA, conventional cloning, and RNA gel blot analysis. For experimental validation, reverse transcription-PCR (RT-PCR), RNA gel blots, or *in situ* approaches were performed (Ruiz-Medrano et al., 1999; Ishiwatari et al., 2000; Doering-Saad et al., 2006; Omid et al., 2007). Classical cloning approaches have been replaced by high-throughput sequencing that efficiently generates comprehensive data sets. The following paragraphs will focus on the description of methods that were successfully used (Figure 9.1).

Methods to Localize Specific RNAs in Tissue Sections

ISH has been an important and powerful tool to detect a limited number of specific transcripts and smRNAs in tissue sections. The technique, however, is laborious and can be prone to artifacts due to extensive pretreatment steps. Nevertheless, ISH has been frequently used to determine temporal and spatial localization of RNAs in vascular tissue sections at tissue- and cell-specific levels. It has been employed to localize mRNAs in sieve elements in a wide range of species, including *Solanum* species (Kühn et al., 1997; Banerjee et al., 2006), rice (*Oryza sativa*; Ishiwatari et al., 1998; Ishiwatari et al., 2000), pumpkin (*Cucurbita maxima*; Ruiz-Medrano et al., 1999), cucumber (*Cucumis sativus*; Xoconostle-Cazares et al., 1999), castor bean (*Ricinus communis*; Doering-Saad et al., 2006), and apple (*Malus domestica*; Kanehira et al., 2010). ISH studies helped to establish the important role of *BEL5* in potato (*Solanum tuberosum*) tuber formation (Banerjee et al., 2006). Moreover, ISH confirmed the presence of transcripts identified from sieve-tube sap-enriched cDNA libraries in phloem tissue (Doering-Saad et al., 2006) and convincingly demonstrated that the phloem lectin PP2 is transcribed in companion cells and not in sieve elements (Bostwick et al., 1992; Dinant et al., 2003). Recently, ISH was used to visualize the accumulation of *MpSLR/IAA14* mRNA in phloem tissue of apple shoots (Kanehira et al., 2010).

In situ RT-PCR of RNA to detect transcripts in tissue sections is a more direct localization technique than ISH (Ruiz-Medrano et al., 1999; Kim et al., 2001; Haywood et al., 2005) (Table 9.1). The presence of *CmNACP* mRNA within the companion cell–sieve element complex of pumpkin (Ruiz-Medrano et al., 1999) and phloem mobility of the *PFP-LeT6* fusion transcript in heterografting studies was verified with this technique (Kim et al., 2001).

In situ techniques have been optimized for smRNA detection in different plant tissues using short, radioactively or nonradioactively labeled DNA, RNA, or, the very sensitive, locked nucleic acid oligonucleotide probes. The spatial and, to some extent, the temporal patterns of miRNA

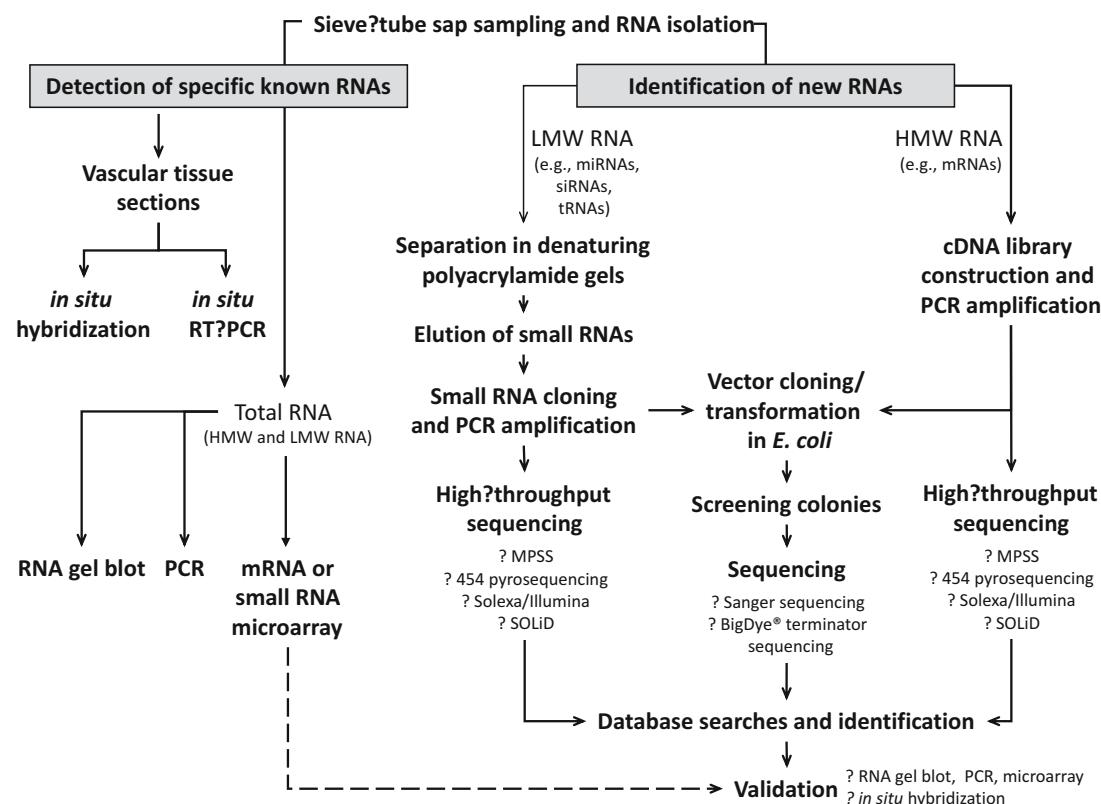


Figure 9.1 Overview of the experimental strategies that have been applied to analyze known or to identify new RNAs in the phloem. *Left-hand side:* Specific known RNAs can be detected by *in situ* methods within tissue sections. Alternatively, sieve-tube sap can be sampled and can be used for the detection of known RNAs by RNA blots, PCR strategies, or microarray hybridization. *Right-hand side:* RNAs of different size classes isolated from sieve-tube sap can also be subjected to different cloning and sequencing procedures in order to identify new RNAs. Generally, all experiments need to be repeated in independent biological replications using a different detection method to validate the presence of a specific RNA in the phloem. HMW, high molecular weight; LMW, low molecular weight; mRNA, messenger RNA; miRNA, micro RNA; MPSS, massively parallel signature sequencing; RT-PCR, reverse transcription-PCR; siRNA, short-interfering RNA; SOLiD, supported oligo ligation detection; tRNA, transfer RNA.

accumulation in the phloem of *Nicotiana benthamiana*, *Arabidopsis* (Valoczi et al., 2006), and apple (Varkonyi-Gasic et al., 2010) have been confirmed.

Methods to Analyze RNAs in Sieve-Tube Sap Samples

As reviewed in Chapter 8, the general terms “phloem sample,” “phloem exudates,” or “sieve-tube sap” commonly used in the literature actually encompass a wide variety of soluble extracts obtained by sampling methods that differ in labor-intensiveness, the injury induced, invasiveness, precision, and levels of dilution or contamination of samples. Sieve-tube sap sampling is generally difficult to perform and depending on the plant species and the biological questions to be answered, some methods are better suited than others. Independent of the sampling method, potential contamination

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Table 9.1 RNAs that have been localized to sieve elements or identified in sieve-tube sap samples

RNA class	Plant species	Sampling/detection method	Number of RNAs	Reference(s)
mRNA	<i>O. sativa</i>	Stylectomy/RT-PCR	3	Sasaki et al., 1998
	<i>C. maxima</i>	Spontaneous exudation/ <i>in situ</i> RT-PCR	1	Xoconostle-Cazares et al., 1999
	<i>C. maxima</i>	Spontaneous exudation/cDNA library	10	Ruiz-Medrano et al., 1999
	<i>S. lycopersicum</i>	No sampling/ <i>in situ</i> RT-PCR	1	Kim et al., 2001
	<i>H. vulgare</i>	Stylectomy/RT-PCR	3	Doering-Saad et al., 2002
	<i>C. maxima</i>	Spontaneous exudation/RT-PCR	1	Haywood et al., 2005
	<i>R. communis</i>	Spontaneous exudation/cDNA library	158	Doering-Saad et al., 2006
	<i>B. napus</i>	Spontaneous exudation/RT-PCR	1	Giavalisco et al., 2006
	<i>C. melo</i>	Spontaneous exudation/cDNA library, RT-PCR	986	Omid et al., 2007
	<i>A. thaliana</i>	EDTA exudation/transcript microarray, RT-PCR	14	Deeken et al., 2008
siRNA	<i>H. vulgare</i>	Stylectomy/cDNA AFLP	8	Gaupels et al., 2008
	<i>C. maxima</i>	Spontaneous exudation/RT-PCR	6	Ham et al., 2009
	<i>B. oleracea</i>	Spontaneous exudation/microarray, RT-PCR	7	Yang and Yu, 2010
	<i>C. maxima</i> , <i>A. thaliana</i>	Spontaneous exudation/RNA gel blot, PCR	11	Yoo et al., 2004; Dunoyer et al., 2010a
miRNA		No sampling/labeling, bombardment		
	<i>C. maxima</i>	Spontaneous exudation/cloning and sequencing	3	Yoo et al., 2004
	<i>L. althus</i>	Spontaneous exudation/cloning and sequencing/RNA blot	1, 8	Atkins and Smith, 2007; Rodriguez-Medina et al., 2011
	<i>B. napus</i>	Spontaneous exudation/cloning and 454 sequencing	32 (18 families)	Buhtz et al., 2008
tRNA	<i>B. napus</i> , <i>C. maxima</i>	Spontaneous exudation/RT-PCR	2	Pant et al., 2008
	<i>B. napus</i>	Spontaneous exudation/Solexa/Illumina sequencing	18 (7 families)	Pant et al., 2009
	<i>B. napus</i>	Spontaneous exudation/miRNA microarrays	161 (37 families)	Buhtz et al., 2010
	<i>M. domestica</i>	Stylectomy/RT-PCR	11	Varkonyi-Gasic et al., 2010
Other RNAs	<i>C. maxima</i>	Spontaneous exudation/SAGE	151	Zhang et al., 2009
	<i>R. communis</i>	Spontaneous exudation/cDNA library	25	Doering-Saad et al., 2006
Other RNAs	<i>B. napus</i>	Spontaneous exudation/cloning and 454 sequencing	144	Buhtz et al., 2008
	<i>C. maxima</i>	Spontaneous exudation/SAGE	413	Zhang et al., 2009

must be assessed to guarantee high-quality samples. Moreover, data interpretation should take possible low levels of contamination into account. This is an important concern in the case of RNA analyses, especially when highly sensitive detection and amplification protocols are used. Quality control to assess contamination by the contents of nonphloem cells has been achieved by sugar measurements, in which high amounts of sucrose and the absence of the monosaccharides glucose and fructose is regarded as indicative for a high content of sieve-tube fluid. Also the lack of photosynthesis-related transcripts or proteins can serve as a purity indicator for phloem samples (Doering-Saad et al., 2006; Giavalisco et al., 2006; Buhtz et al., 2008). In the case of insect stylectomy, clear indicators for contaminating effects of insect feeding have not been established.

Detection of Specific RNAs by RNA Gel Blotting

RNA gel blotting (also called “northern blotting”) enables the detection of specific RNAs in sieve-tube samples and the comparison with different other plant organs or tissues. While this classical technique is quite easy to use, its sensitivity is low compared to other detection methods like ISH, PCR techniques, or microarray hybridization. RNA gel blotting was useful to assess the quality of sieve-tube exudates from oilseed rape (*Brassica napus*; Buhtz et al., 2008) and to characterize different phloem transcripts in pumpkin (Xoconostle-Cazares et al., 1999, 2000; Ruiz-Medrano et al., 1999). smRNAs can easily be analyzed by RNA gel blots without sensitivity problems (Yoo et al., 2004; Buhtz et al., 2008; Zhang et al., 2009), and the technique has been used to compare the abundance of miRNAs in sieve-tube sap with other tissues or their reaction to nutrient starvation (Buhtz et al., 2008; Pant et al., 2008). This technique was also applied to detect mobile miRNAs in *Arabidopsis* grafts (Buhtz et al., 2010). In a recent study, RNA gel blot assays confirmed the presence of truncated rRNAs, tRNAs, and small nucleolar RNAs (snRNAs) in sieve-tube sap exudates of pumpkin (Zhang et al., 2009).

Detection of Phloem RNAs by PCR-Based Methods

Sieve-tube sap mRNAs and miRNAs from several plant species have been analyzed by PCR-based protocols. Transcripts were detected in sieve-tube sap of rice collected by insect stylectomy (Sasaki et al., 1998) and to confirm phloem location of transcripts from sieve-tube exudates of cucurbits (Ruiz-Medrano et al., 1999; Haywood et al., 2005) and brassicas (Giavalisco et al., 2006; Yang and Yu, 2010). Because of their high sensitivity, PCR-based approaches require relatively low amounts of sieve-tube sap samples. This enables to studies of an increased number of transcripts as compared to RNA gel blotting (Doering-Saad et al., 2002; Omid et al., 2007).

Phloem miRNAs have been analyzed by PCR-related methods such as stem-loop RT-PCR (Chen et al., 2005) and polyadenylation-based RT-PCR (Shi and Chiang, 2005). The highly sensitive stem-loop RT-PCR assays identified different miRNA families in cucumber, pumpkin, rape, and apple from as little as 0.1 µl of sieve-tube sap (Varkonyi-Gasic et al., 2007, 2010; Pant et al., 2008). Polyadenylation-based RT-PCR was used to monitor the expression of five nutrient-responsive phloem miRNA families in oilseed rape (Buhtz et al., 2010).

Comprehensive Analysis of Phloem RNAs by Microarrays

Microarray platforms developed for mRNA and smRNA profiling have proven useful to compare the relative accumulation of many specific RNAs in different samples in parallel as well as to verify high-throughput sequencing data. A comparative transcript profiling study was conducted of sieve-tube sap samples collected by ethylenediaminetetraacetic acid (EDTA)-facilitated exudation and vascular tissue obtained by laser microdissection (Deeken et al., 2008). This study, performed in *Arabidopsis*, showed clear differences between phloem tissue and sieve-tube EDTA exudate,

and yielded a number of interesting transcripts that were suggested to be potential mobile signals (Deeken et al., 2008). Recently, transcript microarray and RT-PCR analysis with sieve-tube exudates from *Brassica oleracea* also defined potential systemically acting mRNAs (Yang and Yu, 2010). The results suggest that transcripts of at least two floral regulators are present in sieve-tube exudate (Yang and Yu, 2010). Additionally, a recent study with oilseed rape has demonstrated the applicability of customized smRNA microarrays containing probes of all known plant miRNAs for profiling smRNA accumulation under full nutrition and different nutrient stress conditions in sieve-tube sap and organs such as leaf and root (Buhtz et al., 2010). These miRNA arrays revealed the presence of members from 37 miRNA families in sieve-tube sap of plants and enabled the characterization of nutrient-specific miRNAs.

Comprehensive Identification of Sieve-Tube RNAs by Cloning and Sequencing Strategies

Cloning and sequencing strategies generate comprehensive cDNA sequence libraries that can be used to identify known RNAs and discover additional RNAs that are present in sieve-tube sap. Different cloning strategies have been applied to RNAs of diverse classes in all size ranges. cDNA libraries derived from polyadenylated transcripts in sieve-tube sap samples have been generated by standard reverse transcription protocols. Since sieve-tube exudates can contain only traces of mRNA, amplification of cDNA by PCR prior to cloning and sequencing was required in castor bean (Doering-Saad et al., 2006), melon (*Cucumis melo*; Omid et al., 2007), and barley (*Hordeum vulgare*; Gaupels et al., 2008).

Successful cloning and sequencing of smRNAs is dependent on protocols that have been adapted from those established for nonphloem tissues (Chappell et al., 2006; Lu et al., 2007). Optimized for sieve-tube sap samples, such approaches identified smRNAs such as si- and miRNAs with sizes between 18 and 25 nts in healthy and virus-infected pumpkin plants (Yoo et al., 2004) and oilseed rape (Buhtz et al., 2008). Longer RNAs with lengths between 30 and 90 nts were cloned and sequenced from the phloem of pumpkin by a modified serial analysis of gene expression (SAGE) approach (Zhang et al., 2009). Although these classical cloning approaches generated valuable information, they did not reveal the full complexity of the smRNA population in sieve-tube sap.

Recently developed “deep sequencing” technologies such as “massively parallel signature sequencing” (MPSS), 454 pyrosequencing, sequencing-by-synthesis (Solexa/Illumina), or sequencing by “supported oligo ligation detection” (SOLiD) dramatically facilitated the analysis of smRNAs (Lu and Souret, 2010). High-throughput sequencing techniques have become a basic tool for comprehensive analysis of differentially regulated smRNAs in all types of organisms and tissues, including the phloem. Two studies applied 454 pyrosequencing (Buhtz et al., 2008) or Solexa/Illumina sequencing technology (Pant et al., 2009) to identify thousands of nonredundant smRNA sequences in phloem samples from oilseed rape grown under normal conditions or under phosphorous and nitrogen deficiency. A third study analyzed sieve-tube sap from *Hop stunt viroid*-infected cucumber plants to characterize viroid-derived smRNAs by 454 pyrosequencing (Martinez et al., 2010), as reviewed in more detail in Chapter 13. These few studies have already produced comprehensive datasets of phloem smRNAs that far outmatch the results obtained by all earlier conventional cloning approaches.

RNAs Identified in Sieve-Tube Sap

More than 40 years ago, nucleic acids were unexpectedly found by biochemical analyses of TCA-precipitated phloem exudates of cucurbits and *Robinia pseudoacacia* (Ziegler and Kluge, 1962;

Table 9.2 RNAs for which long-distance translocation has been demonstrated

RNA class	Functional category	RNA name(s)
mRNAs	Development	BEL1 and BEL5 (tuber) ^a , CmGAIP (leaf) ^b , CmNACP (meristem) ^c , DELLA-GAI (leaf) ^b , FT (flowering) ^d , PFP-LeT6 (leaf) ^e , Aux/IAA ^{f,g} , SAUR ^f , FVE (flowering) ^h , AGL24 (flowering) ^h
	RNA transport	CmPP16 ⁱ
	Others/unknown	3 unknown RNAs ^f
siRNAs	PTGS	CP SqMV ^j , IR71 ^k , PAI ^l
	Development	172 ^m
	Nutrient allocation	395 (S) ⁿ , 399 (P) ^{n,o,p}

^aBanerjee et al., 2006. ^bHaywood et al., 2005. ^cRuiz-Medrano et al., 1999. ^dLi et al., 2009. ^eKim et al., 2001. ^fOmid et al., 2007. ^gKanehira et al., 2010. ^hYang and Yu, 2010. ⁱXoconostle-Cazares et al., 1999. ^jYoo et al., 2004. ^kDunoyer et al., 2010a. ^lMolnar et al., 2010. ^mKasai et al., 2010. ⁿBuhtz et al., 2008. ^oPant et al., 2009. ^pLin et al., 2008.

Kollmann et al., 1970). At that time, the observed DNA/RNA fractions were thought to be contaminants from the cells adjacent to the enucleate sieve elements that were introduced during phloem sampling. This view persisted until 1997 when sucrose transporter 1 (SUT1) transcripts were localized by ISH in sieve elements (Kühn et al., 1997). Since then, other mRNAs, rRNAs, and low molecular weight RNA such as tRNA and smRNA species (miRNAs, siRNAs) have been identified in sieve-tube exudates (Yoo et al., 2004; Buhtz et al., 2008; Kehr and Buhtz, 2008; Zhang et al., 2009). RNAs are probably stable in this compartment, because sieve elements lack RNase activity (Sasaki et al., 1998; Doering-Saad et al., 2002; Gaupels et al., 2008; Zhang et al., 2009).

It is important to note that during the last decade hundreds of RNAs have been identified in phloem-derived samples (Table 9.1); however, experimental evidence for phloem mobility has only been obtained for few and these RNAs are currently the best candidates to have a role in long-distance signaling (Table 9.2).

mRNAs in Sieve-Tube Sap

Numerous cellular mRNAs have been discovered in phloem samples obtained by different sampling methods (summarized in Figure 9.1). *ACTIN*, *ORYZACYSTATIN*, and *THIOREDOXIN* transcripts in rice (Sasaki et al., 1998; Ishiwatari et al., 2000) and a *PROTON ATPase* mRNA in barley (Doering-Saad et al., 2002) were detected in sieve-tube samples obtained by insect stylectomy. *THIOREDOXIN* mRNA was found in phloem exudate of oilseed rape (Giavalisco et al., 2006), and the mRNA of the virus movement protein homolog *CmPP16* was found in pumpkin sieve-tube exudate (Xoconostle-Cazares et al., 1999). Eight mRNAs from barley including mRNAs encoding *PROTEIN KINASE*, *CARBONIC ANHYDRASE*, *WIRIA*, and a *PLANTHOPPER-INDUCIBLE* protein were identified in aphid stylectomy-derived samples (Gaupels et al., 2008).

Several mRNAs known to be involved in developmental processes such as *BEL1* mRNA in potato; *CmGAIP*, *DELLA-GAI*, *CmNACP* transcripts in pumpkin; and *PFP-LeT6* mRNA in tomato have been characterized (Ruiz-Medrano et al., 1999; Kim et al., 2001; Haywood et al., 2005; Banerjee et al., 2006). Comprehensive datasets of mRNAs in sieve-tube exudates are available for several plant species (Table 9.1) including melon (Omid et al., 2007), castor bean (Doering-Saad et al.,

2006), and *B. oleracea* (Yang and Yu, 2010). The contamination-prone EDTA-facilitated exudation technique allowed the identification of potentially phloem-localized transcripts in the model plant *Arabidopsis* (Deeken et al., 2008). It is estimated that more than 1000 different mobile mRNAs occur in the phloem translocation stream (Lucas and Lee, 2004). The identity of most of these mRNAs, their mobility within the phloem, and their functions remain to be elucidated in future studies.

siRNAs in Sieve-Tube Sap

siRNAs play important roles in plant development, gene silencing, pathogen defense mechanisms, and other stress responses. siRNAs of 21–24 nts in length are produced from double-stranded RNAs that originate from endogenous transposons or exogenous nucleic acids from viruses or transgenes. siRNAs can be involved in RNA-mediated DNA or histone methylation (chromatin modification) and can cleave mRNA targets or act as translation inhibitors to regulate eukaryotic gene expression (Katiyar-Agarwal et al., 2006).

Yoo et al. (2004) sequenced hundreds of sieve-tube smRNAs from healthy pumpkin plants and primarily observed sequences with lengths of 21, 23, and 24 nts. Database homology searches identified a number of 21-nt short RNAs as being complementary to *CUCURBIT TRANSPOSON-LIKE 1* and 2 genes, suggesting that they are indeed siRNAs. Additionally, several sequences had similarities to ESTs, but whether they are siRNAs or belong to other classes of smRNAs is unknown. In the same study, a transgene-derived coat protein siRNA accumulating in the phloem of spontaneously silenced pumpkin plant lines expressing the coat protein of *Squash Mosaic Virus* identified both sense and antisense coat protein siRNA in similar amounts in sieve-tube sap (Yoo et al., 2004). Sequencing smRNAs in the sieve-tube sap of *Cucumber Yellow Cloverovirus*-infected pumpkin plants revealed high levels of viral-derived siRNAs (Yoo et al., 2004). Deep-sequencing analysis of *Hop stunt viroid*-derived smRNAs from cucumber phloem exudate showed preferential accumulation of 22-nt smRNA species (Martinez et al., 2010). Other results suggest that the phloem translocation stream contained 24-nt siRNAs derived from inverted repeat loci like *IR71* or inverted repeat phosphoribosylanthranilate isomerase (Dunoyer et al., 2010a; Molnar et al., 2010). siRNAs for which phloem transport was demonstrated are listed in Table 9.2.

miRNAs in Sieve-Tube Sap

Plant miRNAs are involved in diverse developmental processes, signal transduction, and protein degradation by regulating target genes (Jones-Rhoades and Bartel, 2004; Jones-Rhoades et al., 2006). Besides their important role in controlling plant development, miRNAs are essential components of regulatory networks involved in hormone-signaling (Mallory and Vaucheret, 2006). miRNAs accumulate in response to both biotic stresses such as bacterial or viral infections (Navarro et al., 2006; Bazzini et al., 2009), or abiotic stresses such as drought, salt stress, or nutrient starvation (Yang et al., 2007; Covarrubias and Reyes, 2010). Mature miRNAs originate from long polyadenylated single-stranded primary transcripts that are processed in two steps by Dicer-like 1 RNase III enzyme to form a 21-nt miRNA/miRNA* duplex that is subsequently methylated by the methyltransferase HEN1. The mature miRNA strand becomes incorporated into the RNA-induced silencing complex to regulate target gene expression by mRNA transcript cleavage or inhibition of translation, while

Table 9.3 List of miRNAs that have been found in sieve-tube sap, with focus on known nutrient deficiency-responsive miRNAs

	miRNA number
miRNAs found in more than one species	mi156 ^{a,b,c,d} mi159 ^{a,b,c,d} mi160 ^{a,b,c} mi162 ^{a,b,c} mi164 ^{a,b,d} mi166 ^{b,d} mi167 ^{a,b,c} mi168 ^{a,b,d} mi169 ^{a,b,c,d} mi172 ^{a,b,c} mi390 ^{a,b,c} mi393 ^{a,b,c} mi398 ^{a,b,c} mi399 ^{a,b}
Known nutrient-responsive miRNAs	mi395 ^{b,d} mi397 ^b mi398 ^{a,b,c} mi399 ^{a,b,d} mi408 ^b mi2111 ^b

^a*C. maxima*: Yoo et al., 2004; Pant et al., 2008; Varkonyi-Gasic et al., 2010.

^b*B. napus*: Buhtz et al., 2008, 2010; Pant et al., 2009.

^c*M. domestica*: Varkonyi-Gasic et al., 2010.

^d*L. albus*: Atkins and Smith, 2007; Rodriguez-Medina et al., 2011.

the strands complementary to the mature miRNAs, called miRNA*^s, are rapidly degraded (Chen, 2005).

In early studies, miRNAs were localized to vascular tissues by ISH (Juarez et al., 2004; Valoczi et al., 2006). While these early studies failed to clearly localize miRNAs to sieve elements, subsequent techniques including sequencing, PCR, and miRNA microarray studies enabled unequivocal identification of miRNAs in sieve-tube exudates. miRNAs, including those localized by ISH, have been identified in phloem exudates from pumpkin (Yoo et al., 2004) and white lupin (*Lupinus albus*; Atkins and Smith, 2007; Rodriguez-Medina et al., 2011), but most sequences were obtained thus far from oilseed rape (Buhtz et al., 2008, 2010; Pant et al., 2009) and apple (Varkonyi-Gasic et al., 2010). Many of the same miRNAs turned out to be present in sieve-tube sap samples collected from oilseed rape, apple, and pumpkin (Yoo et al., 2004; Buhtz et al., 2008, 2010; Varkonyi-Gasic et al., 2010), indicating that miRNA composition of sieve-tube sap might be conserved among unrelated plant species (Table 9.3).

Some of the sieve-tube sap miRNAs (miR159, miR160, miR164, miR166, miR167, miR319) are known to function in the regulation of developmental processes and could be involved in the systemic regulation of vegetative and floral development (Mallory et al., 2004). Other phloem miRNAs (miR395, miR397, miR398, miR399, miR408, miR2111) are known to be induced by nutrient starvation in nonphloem tissues; four of them were also shown to respond to nutrient availability in sieve-tube sap. These include the sulfate deficiency-responsive miR395, the low copper-dependent miR398, and phosphate deficiency-responsive miR399 and miR2111 (Buhtz et al., 2008, 2010; Pant et al., 2009). Additional miRNAs (miR169, miR164, miR168, miR393) responsive to other abiotic and biotic stress conditions were found in sieve-tube sap.

Detailed analyses of sieve-tube sap miRNAs showed that phloem miRNA patterns clearly differed from those of surrounding tissues and whole organs such as leaf or root. miR168 and miR169 were highly enriched in phloem exudates as compared to other tissues in oilseed rape (Buhtz et al., 2008, 2010) and pumpkin (Varkonyi-Gasic et al., 2010), whereas other miRNAs (miR172 or miR400) were underrepresented in sieve-tube sap samples (Buhtz et al., 2008). Interestingly, miR171 was never convincingly detected in sieve-tube sap of pumpkin, oilseed rape, or apple, and this observation together with other results (Parizotto et al., 2004) suggests that this miRNA acts in a strictly cell-autonomous manner. In addition to the mature miRNAs, complementary miRNA* strands were sequenced and confirmed in sieve-tube sap samples of oilseed rape (Buhtz et al., 2008; Pant et al., 2009), indicating that miRNAs might travel long-distance as double-stranded molecules. Whether miRNA*^s themselves have a biological function is so far unclear, but a regulatory activity of miRNA* species and their localization in argonaute complexes have been shown previously (Mi et al., 2008; Okamura et al., 2008).

tRNAs and Other RNAs in Sieve-Tube Sap

A recent study characterized the content of small noncoding RNAs ranging between 30 and 90 nts in pumpkin sieve-tube sap by a modified SAGE cloning approach (Zhang et al., 2009). Many of the sequences matched to rRNAs and specific tRNAs with minor proportions of spliceosomal small nucleosomal RNA, processing-related smRNAs, and protein transport-associated signal recognition particle RNAs. As compared to leaf tissue, higher amounts of rRNAs and small nucleosomal RNA fragments were detected in sieve-tube sap (Zhang et al., 2009). Earlier studies of oilseed rape and castor bean (Doering-Saad et al., 2006; Buhtz et al., 2008) had identified rRNA in phloem-derived samples providing strong evidence that rRNAs are authentic constituents of sieve-tube sap. However, it cannot be completely excluded that some of them originate from the cut surface during sampling. In the case of sieve-tube sap tRNAs additional evidence suggests they are functional: both full-length tRNAs and truncated phloem-specific fragments were shown to inhibit translation in *in vitro* assays (Zhang et al., 2009).

Sequence studies revealed a high number of smRNA sequences in the range of 21–24 nts with no strong homologies to database entries (Buhtz et al., 2008). Some of these unknown smRNAs were significantly more abundant in sieve-tube sap as compared to leaf or root tissue (Buhtz et al., 2010). Whether these smRNAs have a phloem-specific function, are translocated, or develop physiological functions in distant tissues is unclear.

Methods for Studying RNA Transport in Sieve Tubes

To assign a signaling function to a specific sieve-tube RNA molecule, it is essential to demonstrate both its presence in sieve-tube samples and the capacity for long-distance transport in a living plant system. Systemic RNA transport has been demonstrated through grafting experiments, transient gene expression movement assays, and parasitic plant interactions, as discussed in more detail in the following sections.

Demonstrating RNA Transport by Grafting Experiments

The joining of vegetative tissues from two or more plants by grafting has been a widely used technique to optimize the properties of horticultural species (Harada, 2010). Reestablishing the continuity of the vascular system between the scion (shoot) and rootstock is essential to obtain a successful graft union. Therefore, grafting is restricted to closely related species. Typically, grafting is accomplished between plant parts taken from different genotypes of a single species (homografts), but can also be accomplished by joining tissues from different species (heterografts). Homografting is normally more successful than heterografting. For example, while homografts of *Arabidopsis* or tomato had a success rate of up to 100%, heterografts between *Arabidopsis* and different *Brassica* species or *Arabidopsis* and tomato showed different levels of incompatibility (Flaishman et al., 2008). Because the vascular systems of the scion and rootstock are joined at the graft union, grafting has become a well-accepted technique to provide evidence for the phloem mobility of macromolecules and their potential role as signaling compounds in living plants.

Related plant species, mutant and wild-type plants, or tissues in different physiological states, for example induced and not induced to flower, can be combined as grafting partners. Grafting has provided conclusive evidence of long-distance messengers involved in photoperiodic regulation

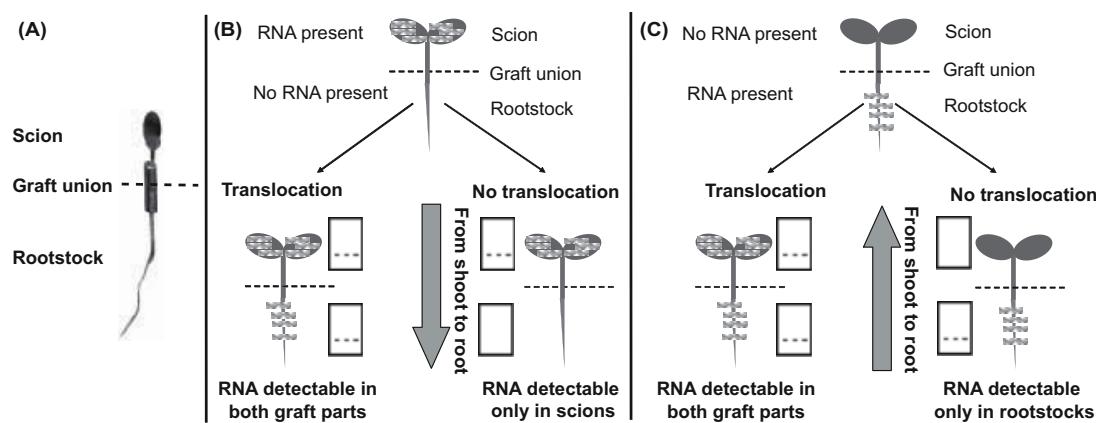


Figure 9.2 General scheme of reciprocal grafting experiments to study RNA long-distance translocation in plants. (A) Shows a grafted *Arabidopsis* seedling, the graft unit is stabilized by a plastic tube. (B) Setup for studying RNA transport from shoot to root. A scion that contains a specific RNA is grafted onto a rootstock that does not contain this particular RNA. After formation of successful graft units, it is analyzed whether this RNA is detectable in the rootstock or not, for example by RNA blots or PCR-based methods. If the RNA is found in both parts of the graft it is a strong indication that this RNA is phloem-mobile. (C) Reciprocal grafting to examine RNA transport from root to shoot. A scion that is devoid of a specific RNA is grafted onto a rootstock that does contain this particular RNA. After formation of successful graft units, it is analyzed whether this RNA is detectable in the scion or not, for example by RNA blots or PCR-based methods. If the RNA is found in both parts of the graft it is a strong indication that this RNA is phloem-mobile. Performing both reciprocal grafts yields information about the general mobility of a particular RNA and also about the direction of RNA transport. (For a color version of the figure, please see Plate 9.1.)

of flowering (Chailakhyan, 1936; Zeevaart, 1958), demonstrated the systemic spread of viruses (Wisniewski et al., 1990; Bertaccini and Bellardi, 1992), and provided important insights into phytohormone transport and action (Proebsting et al., 1992), apical dominance (Mapelli and Kinet, 1992), and nodule formation (Lohar and VandenBosch, 2005). Transport between graft partners is inferred from the appearance of a compound in either rootstock or scion that normally would not contain this compound (Figure 9.2). Reciprocal grafting in which scion and rootstock are exchanged allows translocation to be studied in alternate directions.

Most studies addressing phloem mobility of mRNAs have been performed in cucurbits by heterografting of the closely related species cucumber and pumpkin (Xoconostle-Cazares et al., 1999) or melon and pumpkin (Omid et al., 2007). Translocation of mRNA can be detected due to small sequence differences between homologous transcripts of the two grafted species. Grafting techniques have been refined to allow experiments to be conducted with *Arabidopsis* (Rhee and Sommerville, 1995; Turnbull et al., 2002; Ayre and Turgeon, 2004; Flajshman et al., 2008; Notaguchi et al., 2009). Micrografting of *Arabidopsis* validated the mobility of two miRNAs (miR395 and miR399) by grafting either an overexpressor (Pant et al., 2008) or a miRNA biosynthesis mutant (Buhtz et al., 2010) with a wild-type plant.

Demonstrating RNA Phloem Transport by Particle Bombardment, Microinjection, or Transient Expression of Molecules

An alternative technique to study cell-to-cell as well as long-distance movement of RNAs is the direct introduction of specific RNA molecules by microinjection, particle bombardment, or transient

gene expression experiments. These methods allow observation of the movement of silencing from the initial targeted cells into the surrounding tissue as well as systemically over long-distance *via* the phloem.

Injecting RNA together with different proteins directly into cells demonstrated that specific sieve-tube sap proteins can alter the size exclusion limit of mesophyll cell plasmodesmata to mediate cell-to-cell movement of specific RNAs (Balachandran et al., 1997). Also binding preferences for certain RNA classes have been revealed with this approach (Xoconostle-Cazares et al., 1999; Yoo et al., 2004).

The spread of gene silencing has been studied by particle bombardment of leaves of intact *Arabidopsis* plants (Dunoyer et al., 2010b). It could be shown that *in vitro* transcribed green fluorescent protein (GFP)-derived siRNA duplexes were mobile and triggered GFP silencing when introduced into plants constitutively expressing GFP (Dunoyer et al., 2010b). This approach was also suitable to demonstrate long-distance mobility when bombardment was located near veins. These experiments moreover suggested that siRNAs themselves and not other forms of RNAs can move into neighboring cells (Dunoyer et al., 2010b). A similar system has been created by the local and transient expression of siRNAs by *Agrobacterium* infiltration. Here, gene silencing was initiated in *N. benthamiana* constitutively expressing GFP by infiltration of a single leaf, with *Agrobacterium tumefaciens* containing GFP sequences in a plant transformation vector, and the spread of silencing could be followed by the disappearance of GFP fluorescence (Hamilton and Baulcombe, 1999).

Demonstrating RNA Transport in Natural “Grafts” Formed by Parasitic Plants

A natural alternative to grafting is provided by the interactions of certain parasitic plant species and their hosts. The obligate parasitic plant dodder (*Cuscuta* sp.) establishes continuous connections with the host plant’s phloem system (Lee, 2009) through which nutrients, viruses (Hosford, 1967), proteins (Haupt et al., 2001), and mRNAs (Roney et al., 2007; David-Schwartz et al., 2008; Westwood et al., 2009) are translocated from host to parasite. Macromolecules most likely move through symplastic connections between phloem and parasite (Dörr and Kollmann, 1995; David-Schwartz et al., 2008). Studies of root parasitic plants from the *Orobanchaceae* demonstrated that gene silencing signals are translocated from host to parasite, corroborating a general mode of RNA transfer in these plant–plant systems (Tomilov et al., 2008; Westwood et al., 2009).

An unexpected observation that “nonphloem” transcripts such as *RUBISCO* mRNA also appeared to move between host and parasite (David-Schwartz et al., 2008), indicates that host–parasite systems might not accurately reflect the normal translocation events that typify nonparasitic plants. Because of this result, the mRNAs identified in parasitic plant interactions have not been categorized as reliable phloem-mobile RNAs (Table 9.2).

Evidence for RNA Movement and Possible Functions of Phloem-Mobile RNAs

A large number of RNAs from many different classes have been identified in the phloem of different plant species (Table 9.1). This number will certainly increase in the future. Phloem mobility of an RNA species, however, is a prerequisite for implying a role in long-distance signaling, but has only been demonstrated for a small subset of these RNAs (Table 9.3). For example, mobility could only be confirmed for 6 out of 43 melon sieve-tube sap mRNAs (Omid et al., 2007). RNAs that are

found in sieve-tube sap but are not phloem-mobile are unlikely to function in signaling processes. The following sections will focus on possible functions of RNAs for which phloem mobility was demonstrated unequivocally.

Phloem-Mobile mRNAs

Mobile mRNAs detected in sieve-tube sap of different species belong to a broad range of functional classes. The functions of the so-far-known phloem-mobile mRNAs are related to different aspects of developmental regulation, signal transduction, and stress or defense responses (Omid et al., 2007; Kehr and Buhtz, 2008).

The developmental regulators *KNOTTED1*-like, *BEL1*, and *GAI* mRNAs each induced significant phenotypic changes in the recipient tissue in grafting experiments (Kim et al., 2001; Haywood et al., 2005; Banerjee et al., 2006). *FT* mRNA (Li et al., 2009) in addition to or together with FT protein (Jaeger and Wigge, 2007; Tamaki et al., 2007) move through the phloem to influence flowering time. Grafting *Arabidopsis* wild-type scions onto transgenic overexpressor rootstocks provides data that supports the identification of two other potential floral regulators *AGL24* and *FVE* (Yang and Yu, 2010).

Some of the phloem-mobile transcripts, *Aux/IAA* and *SAUR*, seem to be related to auxin signaling and accumulate upon auxin treatment in auxin-response assays (Omid et al., 2007). *Aux/IAA* repressor proteins are known to be negative regulators of auxin signaling, while functions of proteins from the large and highly conserved *SAUR* family are not exactly clear (Paponov et al., 2009). Detailed studies to dissect the relevance of long-distance transport of auxin-related mRNAs have yet to be conducted. Meanwhile, transcript delivery into target cells was proposed to control the expression of auxin-regulating genes and thus auxin concentration (Omid et al., 2007). The roles of long-distance signaling in the regulation of developmental processes will be covered in more detail in Chapter 10.

CmPP16 from pumpkin is the only mobile transcript that seems to be unrelated to development (Xoconostle-Cazares et al., 1999). Its cell-to-cell and long-distance mobility has been confirmed by both microinjection experiments and heterografting studies between pumpkin and cucumber (Xoconostle-Cazares et al., 1999). The transcript moved together with the encoded protein (*CmPP16* protein) that is an analogue to viral movement proteins. It has RNA-binding properties that enable the transport of sense and antisense RNAs, including its own transcript (Xoconostle-Cazares et al., 1999). For all other mRNAs found in sieve-tube sap, mobility studies have either not been conducted, movement could not be demonstrated (Omid et al., 2007), or their function is unknown. Future studies are necessary to identify additional transcripts with potential long-distance signaling functions.

Phloem-Mobile siRNAs

It is well established that gene silencing induced by transgene expression or virus infection systemically spreads among plant organs. Systemic gene silencing was initially observed in tobacco grafts, where a sequence-specific silencing signal could cross graft unions from silenced tobacco rootstocks and lead to gene silencing in nonsilenced scions. The appearance of silencing correlated with the appearance of siRNAs directed against the transgene in the systemically silenced scion. Similarly,

transient induction of local gene silencing entailed a systemic gene silencing response (Palauqui et al., 1997; Voinnet and Baulcombe, 1997; Ruiz et al., 1998; Hamilton et al., 2002; Baulcombe, 2004). The silencing signal was translocated from silenced host plants to parasitic plants forming phloem connections with their hosts (Tomilov et al., 2008). It was further demonstrated that siRNAs are present in phloem exudate of spontaneously silenced pumpkin plants, but not in nonsilenced plants. In addition, siRNAs were detected in wild-type cucumber scions heterografted onto silenced pumpkin rootstocks, nourishing the idea that siRNAs themselves act as systemic silencing signals (Yoo et al., 2004).

Other studies using different experimental strategies provided indications that siRNAs were the silencing-transmitting compounds. However, these studies never fully ruled out that longer transcripts or aberrant RNAs induce systemic gene silencing (Voinnet, 2005). Further evidence for long-range mobility of siRNAs was recently provided by grafting experiments between wild-type *Arabidopsis* plants and a T-DNA insertion mutant at the siRNA generating inverted repeat *IR71* (Dunoyer et al., 2010a). These mutants are unable to produce certain siRNAs. When wild-type scions were grafted onto mutant *ir71* rootstocks, siRNAs from the entire inverted repeat could be detected in roots, restricting the mobile molecules to endogenous siRNAs or siRNA precursor transcripts. It appears that *Arabidopsis* siRNAs of all size classes move cell to cell and long distance (Dunoyer et al., 2010a). Deep-sequencing analysis of *Arabidopsis* grafts using wild-type scions and *dcl2 dcl3 dcl4* triple mutant rootstocks, impaired in the silencing pathway, demonstrated that siRNAs from thousands of genetic loci could move through graft unions (Molnar et al., 2010). Moreover, mobile siRNAs are probably translocated as the double-stranded form and their action in promoting DNA methylation in target cells are functionally indistinguishable from locally synthesized siRNAs (Dunoyer et al., 2010a, 2010b; Molnar et al., 2010).

Transfer of siRNAs between cells and organs observed after spontaneous transgene or virus silencing most likely functions as a systemic defense mechanism against foreign DNA. Since endogenous mobile siRNAs induce epigenetic effects in target tissues (Molnar et al., 2010), they might affect meristems, where siRNAs could reinforce silencing of transposons or initiate epigenetic changes in response to environmental adaptation or stress. In developing reproductive tissues, these epigenetic modifications could even be passed on to the next generation to influence its stress adaptation potential (Molnar et al., 2010).

Phloem-Mobile miRNAs

In contrast to siRNAs, miRNAs were long believed to act solely in a locally restricted manner (Dunoyer et al., 2007). Recent studies demonstrated that specific miRNAs can indeed move over long distances through the phloem. Similar to floral induction, grafting experiments suggested that potato plants grown under inductive short-day photoperiods synthesize a tuber-inducing stimulus that is produced by leaves and translocated to stolons to induce tuberization. In potato, miR172 mainly accumulated in the phloem of stems, and grafting of miR172 overexpressor scions onto wild-type rootstocks increased the number of tubers, indicating that a miR172-regulated signal or, more likely, miR172 itself is phloem-mobile (Martin et al., 2009). The latter assumption was corroborated by recent grafting experiments in *N. benthamiana* showing that miR172 was translocated from rootstocks to scions (Kasai et al., 2010). As mentioned before, *BEL5* mRNA is also phloem-mobile and appears to play a role as a long-distance signal influencing tuberization (Lee and Cui, 2009). The function of miR172 and *BEL5* mRNA in tuber induction is described in more detail in Chapter 10.

Another development-related miRNA that has been implicated with long-distance signaling is miR166. This miRNA is expressed during maize (*Zea mays*) leaf development, where it accumulates within phloem tissue and is thought to regulate development of lateral organs by targeting *HD-ZIP III* mRNAs (Juarez et al., 2004). While its long-distance transport is supported by the presence of miR166 in sieve-tube sap of oilseed rape and white lupin (Atkins and Smith, 2007; Buhtz et al., 2008, 2010), translocation of this miRNA has as yet not been demonstrated.

In contrast, there is clear evidence that two of the nutrient deficiency-responsive miRNAs, miRNA 395 and 399, are translocated across graft unions. Together with miR398 they constitute the three miRNAs that accumulated in sieve-tube sap of oilseed rape under different nutrient deficiencies (Buhtz et al., 2008). miR395 controls sulfate assimilation and allocation by regulating the expression of ATP sulfurylases and the low-affinity sulfate transporter SULTR2.1 (Jones-Rhoades and Bartel, 2004). SULTR2;1 is a low-affinity sulfate transporter located in the root vasculature (Kataoka et al., 2004) that is thought to be involved in sulfur retrieval from the apoplast and sulfur root-to-shoot translocation (Kataoka et al., 2004; Kopriva, 2006). Under sulfate limitation, miR395 accumulates to suppress the expression of its target genes, which leads to a change of sulfate allocation between roots and shoots and as a consequence to an increase in the level of sulfate in shoots (Liang et al., 2010). Surprisingly, the expression of *SULTR2;1* was found to increase in roots of *Arabidopsis* under sulfur starvation (Liang et al., 2010). It was recently suggested that this observed discrepancy could be explained by the cell type-specific, spatially separated expression of miR395 (expressed in phloem companion cells) and *SULTR2;1* (found in xylem parenchyma cells). This spatial expression pattern could switch off *SULTR2;1* expression in phloem cells in order to restrict SULTR2;1 to the xylem (Kawashima et al., 2009). Recently, the role of miR395 in the regulation of sulfate assimilation has been analyzed in more detail (Kawashima et al., 2011). miR399 participates in the maintenance of phosphate homeostasis by regulating the expression of *PHO2*, an mRNA for an ubiquitin-conjugating E2 enzyme (Aung et al., 2006; Bari et al., 2006). Accumulation of miR399 under phosphate limitation reduces *PHO2* transcript accumulation. miR399 overexpression resulted in increased levels of phosphate in shoots (Fujii et al., 2005; Bari et al., 2006).

Grafting studies demonstrated that miR395 and miR399 are translocated from shoots to roots. Reciprocal grafting experiments between miR399 overexpressing and wild-type *Arabidopsis* plants grown on full nutrition media showed that mature miR399 and not its precursor is translocated from shoots to roots (Pant et al., 2008). Moreover, shoot-to-root translocation was shown to reduce the amount of its target *PHO2* mRNA in roots. Similar results were obtained in *N. benthamiana* (Lin et al., 2008; Pant et al., 2008). An alternative approach utilized the miRNA processing mutant *hen1-1* that is impaired in miRNA methylation and therefore in the formation of mature miRNAs. Grafts between wild-type *Arabidopsis* and *hen1-1* mutant plants confirmed the previous observation that miR399 can be translocated from shoot to root and downregulate *PHO2* expression in roots when the grafted plants were grown under phosphate starvation. Using the same grafting system, translocation of miR395 under sulfur deprivation could be demonstrated (Buhtz et al., 2010). Also here, the transport of miR395 was observed only in the direction from wild-type shoots to *hen1-1* roots. This translocation had a significant negative effect on the accumulation of its target mRNAs, especially obvious in the case of *APS4* (Buhtz et al., 2010). miR171, which was not detected in sieve-tube sap samples in earlier studies (Buhtz et al., 2008; Pant et al., 2009), was used as a negative control and was found to be immobile, indicating that only specific miRNAs are phloem-translocatable. This feature could be restricted to miRNAs expressed in companion cells, although analyses of the cell type-specific expression of miRNAs in support of this assumption are still missing. Alternatively, sieve element-targeted miRNAs could require specific target sequences or

structural characteristics to enter sieve elements, which seems difficult to envision given the short length of miRNAs.

It is important to note that reciprocal grafting demonstrated unidirectional movement of miR395 and miR399 from shoot to root. This is likely influenced by source-sink relationships in the small plants employed, where the root represents the major sink. It is not excluded, however, that miRNAs move in other directions when different source-sink relations prevail. For example, movement of miR172 from rootstock to scion was observed using a different grafting strategy that included removal of all source leaves in *N. benthamiana* (Kasai et al., 2010).

Although the evidence for phloem mobility of miR395 and miR399 is compelling, the functional significance of their translocation is still a matter of debate. The necessity to deliver these miRNAs to roots seems obscure given the fact that both can be produced in roots with highly similar expression patterns, mainly accumulating in root vascular tissues (Aung et al., 2006; Kawashima et al., 2009; Buhtz et al., 2010). It was proposed that miRNA translocation serves as a proactive means of communication between organs that experience stress and organs that still have sufficient nutrient supply. This would allow plants to coordinate nutrient deficiency responses and to precisely redirect nutrient flows between organs (Buhtz et al., 2010).

Other miRNAs abundant in sieve-tube sap might be interesting candidates as long-distance signaling molecules during biotic and abiotic stresses, because they were found to be stress-responsive in nonphloem tissues. Members of the phloem-enriched miR169 family respond to abscisic acid, heat, and drought (Li et al., 2008, 2010; Zhao et al., 2009), and both miR164 and miR168 are influenced by virus infection (Csorba et al., 2007; Bazzini et al., 2009). miR393 targets a helix-loop-helix transcription factor and auxin receptors, and this miRNA was recently shown to be induced by nitrate (Vidal et al., 2010) and bacterial elicitors (Navarro et al., 2006). Hence, it is tempting to assume that signaling processes involved in stress responses depend on systemic translocation of miRNAs. However, except for miR395 and miR399 proof of phloem mobility and systemic effects on target genes is still missing for the other candidates.

Phloem-Mobile tRNAs and Other RNAs

To date, only one study described the presence of mature tRNAs, rRNAs, and other RNAs in the size range of 30–90 base pairs in sieve-tube sap (Zhang et al., 2009). The lack of corroborating data is likely the result of other experiments concentrating on RNAs of larger (mRNAs) or smaller (siRNAs and miRNAs) size. Larger rRNAs and ribosomal proteins have been identified in other studies on sieve-tube contents (Doering-Saad et al., 2006; Giavalisco et al., 2006; Buhtz et al., 2008; Lin et al., 2009; Ma et al., 2010). Phloem mobility of tRNAs and rRNAs has not been demonstrated. Occurrence of components of the translational machinery, however, was unexpected because of the widely accepted dogma that differentiated sieve elements of angiosperms are incapable of transcription and translation.

The identification of rRNAs, tRNAs, and several sieve-tube sap proteins related to translation (Ma et al., 2010) raises the interesting possibility that protein synthesis might be possible in enucleate sieve tubes. However, certain caveats cannot be ignored. Some essential components required for translation such as certain tRNAs have not been detected in sieve-tube sap (Zhang et al., 2009), and a wealth of microscopic studies confirms the absence of rough endoplasmic reticulum and functional ribosomes in fully differentiated sieve tubes (Sjolund, 1997). In addition, biochemical studies using radioactive labeling to measure protein turnover did not hint at any translational activity in sieve tubes of wheat (*Triticum aestivum*; Fisher et al., 1992). Another argument against active

translation in mature sieve elements is the observation that small amounts of sieve-tube sap RNA and artificial tRNA fragments resembling the tRNA halves found in phloem samples act as efficient and nonspecific inhibitors of translation in *in vitro* assays (Zhang et al., 2009).

These observations led to the suggestion that sieve-tube sap tRNAs are probably not involved in translation, but on the contrary interfere with the assembly of functional ribosomes and therefore inhibit translational activity (Zhang et al., 2009). Furthermore, it has been proposed that specific tRNAs could serve as a systemic source for cytokinin production, as several sieve-tube sap detectable tRNAs are cytokinin precursors or might be signal molecules informing sink tissues about the metabolic status of source tissues (Zhang et al., 2009; Kragler, 2010). Moreover, tRNAs (and rRNAs) could be transported to distant tissues and somehow influence the assembly or function of the translational machinery there.

All in all, the exact *in vivo* functions of intact tRNAs and tRNA fragments in sieve tubes remain to be elucidated. Likewise, the origin and the potential functions of the other translation-related RNAs such as rRNAs and as yet unknown RNAs wait to be discovered. As mentioned earlier, it cannot be excluded that some of the RNAs found in sieve-tube samples might not be of functional importance.

Import and Transport Mechanism of Phloem-Mobile RNAs

RNAs in sieve elements are thought to be imported from the adjacent companion cells through the connecting pore-plasmodesmata units (Lough and Lucas, 2006). Import of RNAs from companion cells into sieve elements is probably mediated by specific RNA-binding proteins that have chaperone function. RNA-specific chaperones could alter the macromolecular structure of RNAs to facilitate their passage through plasmodesmata and stabilize them during translocation (Yoo et al., 2004; Lough and Lucas, 2006; Ham et al., 2009). RNA stability, however, does not seem to be a primary concern within the translocation stream since RNase activity was not detected in sieve-tube exudate (Sasaki et al., 1998; Doering-Saad et al., 2002; Gaupels et al., 2008; Zhang et al., 2009).

RNAs appear to translocate within sieve tubes according to the metabolic flux from source to sink. The concept of uni-directional RNA transport is supported by the observation that the systemic spread of silencing could be altered by manipulating source-sink relationships (Tournier et al., 2006). Recently, a model of a phloem-mobile RNP complex, consisting of phloem protein CmRBP50 homooligomers complexed with six mRNAs and several interacting proteins, was proposed as a mechanism to mediate the long-distance transport of RNA in the phloem (Ham et al., 2009). The protein components of long-distance RNA transporting complexes, including RNA-binding proteins that facilitate trafficking from companion cells into sieve elements and large multi-RNA containing RNP complexes, is discussed in more detail in Chapter 8.

smRNAs also seem to be translocated with the aid of RNA-binding proteins. Microinjection experiments demonstrated that 25-nt siRNA molecules were unable to spread between mesophyll cells through plasmodesmata by simple diffusion. However, mobility could be achieved by co-injection with the smRNA-binding sieve-tube sap protein CmPSRP1 (Yoo et al., 2004). CmPSRP1 had a higher binding affinity for single-stranded than for double-stranded siRNAs and only mediated trafficking of single-stranded siRNAs. In addition, the protein had a binding preference for smRNA molecules in the size range of 20–40 nt (Yoo et al., 2004). Furthermore, single-stranded and double-stranded specific RNase digestion assays (Yoo et al., 2004; Buhtz et al., 2008) suggest that single-stranded smRNA is the phloem-dominant form. On the other hand, the detection of complementary strands of mature si and miRNAs (Yoo et al., 2004; Buhtz et al., 2008) argue in

favor of the functional importance of smRNA duplexes and the phloem transport of siRNAs as double-stranded molecules (Dunoyer et al., 2010b). A similar mechanism could exist for miRNAs. The capability for cell-to-cell and long-distance movement seems not to be restricted to a limited size class of siRNAs in *Arabidopsis*, since endogenous siRNAs of all sizes were shown to be mobile in grafting experiments (Dunoyer et al., 2010a). Whether siRNAs are transported as RNP complexes or as unbound molecules has yet to be determined.

RNA-binding proteins have been primarily identified in sieve-tube sap of cucurbits, especially in pumpkin. Many proteins with theoretical RNA-binding capacity have been identified in sieve-tube sap of many different species including melon, castor bean, and oilseed rape (Barnes et al., 2004; Gomez et al., 2005; Giavalisco et al., 2006). The prevalence of these proteins indicates that RNAs are generally translocated through the sieve tubes as RNP complexes. However, some of the proteins, in particular phloem lectins, bind RNA in a nonspecific manner (Gomez et al., 2005). These proteins could, for example, act in viral defense by binding and disarming viral nucleic acids.

Future Directions

During the last decade, a growing number of RNAs has been localized in sieve elements *in situ* or identified in sieve-tubes from different plant species. Hence, RNAs could constitute another component in addition to metabolites, phytohormones, peptides, and proteins of the complex system in place for systemic exchange of information between plant organs. Although translocation studies have as yet only been performed for a small subset of RNAs, solid evidence emerges that siRNAs in general and several, but not all, mRNAs and miRNAs are indeed phloem-mobile. Phloem-translocated mRNAs seem to be mainly involved in the regulation of developmental decisions such as meristem transformation into flowers, leaves, or tubers. Phloem-mobile siRNAs spread gene silencing and act as an immunization mechanism against viruses and other foreign or aberrant genetic information. These siRNAs may also pass epigenetic information to meristems or even to the plants offspring. Mobile miRNAs appear to function in integrating nutrient deficiency responses and possibly a range of other reactions to abiotic and biotic challenges and in fine-tuning of developmental processes like flowering or tuberization. It is essential, however, to demonstrate *in vivo* mobility of each single candidate molecule to convincingly assign potential long-distance signaling functions to the wealth of RNAs in sieve-tube samples. Since not all mRNAs found in phloem samples are phloem-mobile a function, if any, distinct from signaling must be assumed where mobility cannot be verified.

Recent studies also identified unexpected RNA and polypeptide components in sieve-tube samples usually associated with the translational apparatus, challenging the common notion that enucleate sieve elements are incapable of transcription and translation. However, the majority of results from biochemical and functional studies and the failure to identify the complete set of components necessary for translation undermine the validity of this idea. Although most studies provide evidence that contamination by nonphloem cells is low to negligible, a low level of leakage from cut surfaces during sampling cannot be fully excluded.

To identify additional long-distance signaling molecules, detailed studies have to be performed to elucidate which of the sieve-tube sap RNAs is phloem-mobile. Furthermore, it will be essential to establish the molecular components and mechanism of RNA long-distance transport in noncucurbit species to confirm that RNP complexes represent a general phloem shuttle for RNAs. Effects of each translocatable RNA molecule in target tissues will need to be carefully dissected. Finally, assignment of functions for individual phloem-mobile RNAs will be essential to substantiate the hypothesis

that specific RNAs indeed play essential roles in controlling and integrating diverse important physiological, developmental, and pathogenesis-related processes at the whole plant level.

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10 The Effect of Long-Distance Signaling on Development

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Plants have developed a unique long-distance signaling pathway that takes advantage of connections in the vascular system, predominantly the phloem. This information superhighway transports sugars, metabolites, proteins, and RNAs via a combination of plasmodesmata and the sieve-tube system. The delivery of these information molecules has been implicated in regulating developmental processes, responses to biotic stresses, delivery of nutrients, and as a vehicle used by viruses for systemic infection (Lough and Lucas, 2006).

The role of long-distance transport mediated by the phloem in impacting development is probably best represented by the transport of photosynthate from leaves in the form of sugar to various sink organs throughout the plant. The movement of these sugars is regulated in the collection phloem of source organs, during transport, and in the release phloem of sink organs (Dinant and Lemoine, 2010). In potato, for example, the onset of tuberization is accompanied by a switch from apoplastic to symplastic unloading (Viola et al., 2001). In elongating stolons prior to tuber formation, apoplastic phloem unloading predominates. During the earliest stages of tuberization, symplastic transport is predominant in the subapical region of the stolon tip. The disruption of dormancy and the activation of bud growth in potato were also associated with the induction of a symplastic connection (Viola et al., 2007). Symplastic gating utilizing a regulated surveillance system (Lucas et al., 2001; Ding et al., 2003; Hancock et al., 2008) very likely controls the allocation of carbon resources and developmental signals to both meristematic and vegetative tissues.

As part of an elaborate signaling system, the phloem translocation stream contains a large population of RNA molecules and proteins, suggesting that plants use mobile RNAs and proteins to integrate developmental processes at the whole-plant level. Because of the phloem's involvement in the production and collection of signal molecules in source organs and in transporting and releasing them to sink organs, the phloem exhibits great potential to facilitate interorgan coordination and

Abbreviations: AP1, APETALA1; CaMV, cauliflower mosaic virus; cds, coding sequence; CO, CONSTANS; CmGAI, *Cucurbita maxima* gibberellic acid insensitive; CmPP16, *Cucurbita maxima* phloem protein 16; CmRBP50, *Cucurbita maxima* RNA-binding protein 50; FD, FLOWERING LOCUS D; FL, full length; FT, FLOWERING LOCUS T; GA, gibberellic acid; GAS, galactinol synthase; GID1, GIBBERELLIC ACID INSENSITIVE DWARF1; GFP, green fluorescent protein; LD, long day; POTH1, potato homeobox 1; RNP, ribonucleoprotein; SAM, shoot apical meristem; SCL14, SCARECROW-LIKE14; SD, short day; SP6A, Self-pruning 6A; SLY1, SLEEPY1; StBEL5, *Solanum tuberosum* BEL5; SUC2, sucrose-proton symporter 2; TALE, three-amino acid loop extension; UTR, untranslated region; Vg1, Vera RNA-binding protein; YFP, yellow fluorescent protein

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to mediate processes of development. This chapter focuses on three model systems as primary examples of noncell autonomous movement of protein or full-length mRNAs that impact plant development. The first example is the long-distance transport of FLOWERING LOCUS T (FT) protein to the apical shoot to induce flowering. The second example involves a RNA–protein complex of pumpkin (*Cucurbita maxima*) that moves *CmGAI* and other full-length mRNAs. In this system, a polypyrimidine tract-binding protein, designated CmRBP50, serves as the core RNA-binding protein in a phloem–mobile complex. The final example is the transport of *Solanum tuberosum* *BEL5* (*StBEL5*) RNA from leaves to stolon tips to activate tuber formation in potato (*Solanum tuberosum*). *StBEL5* is a transcription factor that functions in tandem with Knotted1-like proteins. These examples represent complex and dynamic phloem transport systems involving RNA–protein and protein–protein interactions that facilitate directed movement and maintain the integrity of the signal molecules.

The Protein of FLOWERING LOCUS T Functions as the Mobile Floral Signal

Flowering in plants has long been confirmed to be regulated by a graft-transmissible compound that moves from leaves, the site of photoperiod perception, to the shoot apical meristem (SAM), the site of activation for the floral pathway. For many years, however, the identity of this floral activator, designated florigen, has remained a mystery. Recent genetic and molecular studies have identified the protein of FT as the main component of the mobile floral elicitor (reviewed by Turck et al., 2008). FT is expressed in the phloem tissues of cotyledons and leaves (Takada and Goto, 2003; Yamaguchi et al., 2005) and encodes a 20-kD protein with homology to phosphatidylethanolamine-binding protein or Raf kinase inhibitor protein (Kobayashi et al., 1999; Kardailsky et al., 1999). Ectopic overexpression of FT by the 35S RNA promoter of cauliflower mosaic virus (CaMV) (35S::FT) causes a precocious-flowering phenotype (Kobayashi et al., 1999; Kardailsky et al., 1999), whereas knockout mutants of FT caused delayed flowering (Koornneef et al., 1998).

CONSTANS (CO) is a transcription factor that is regulated by photoperiod and induces the transcription of FT in the vascular tissue of leaves. CO contains two conserved protein domains: a zinc finger domain and the CCT (CONSTANS CO-LIKE, TIMING OF CAB 1) domain (Putterill et al., 1995; Robson et al., 2001; Griffiths et al., 2003). The target element of CO was recently identified in upstream sequences of the FT gene of *Arabidopsis*. CO-mediated induction of FT required sequences between 4.0 and 5.7 kb upstream of the FT transcription start site (Adrian et al., 2010). Consistent with FT being the primary target of CO in leaves, CO expression induces activation of the FT promoter in the phloem companion cells of leaves (Takada and Goto, 2003; An et al., 2004). FT protein is uploaded into the sieve elements either by diffusion through plasmodesmata or by an unidentified active transport mechanism (Turck et al., 2008). FT unloading from the phloem and transport within the apex probably involves cell-to-cell transport through plasmodesmata, but this process remains relatively unknown.

FT mRNA is translated in leaves and moves through the phloem system to the SAM where it interacts with the transcription factor, FLOWERING LOCUS D (FD) (Figure 10.1). FD is a bZIP transcription factor preferentially expressed in the shoot apex. FD cannot work alone but depends on its FT partner to promote flowering. This tandem complex then binds to the promoter of the floral identity gene, APETALA1 (AP1), to activate its transcription. In a widely accepted model, FT directly regulates the activity of FD at the shoot apex by formation of a transcriptional complex, even though induction of FT RNA expression in response to photoperiod occurs predominantly in leaves. This discrepancy between the spatial pattern of FT transcription and translation and the

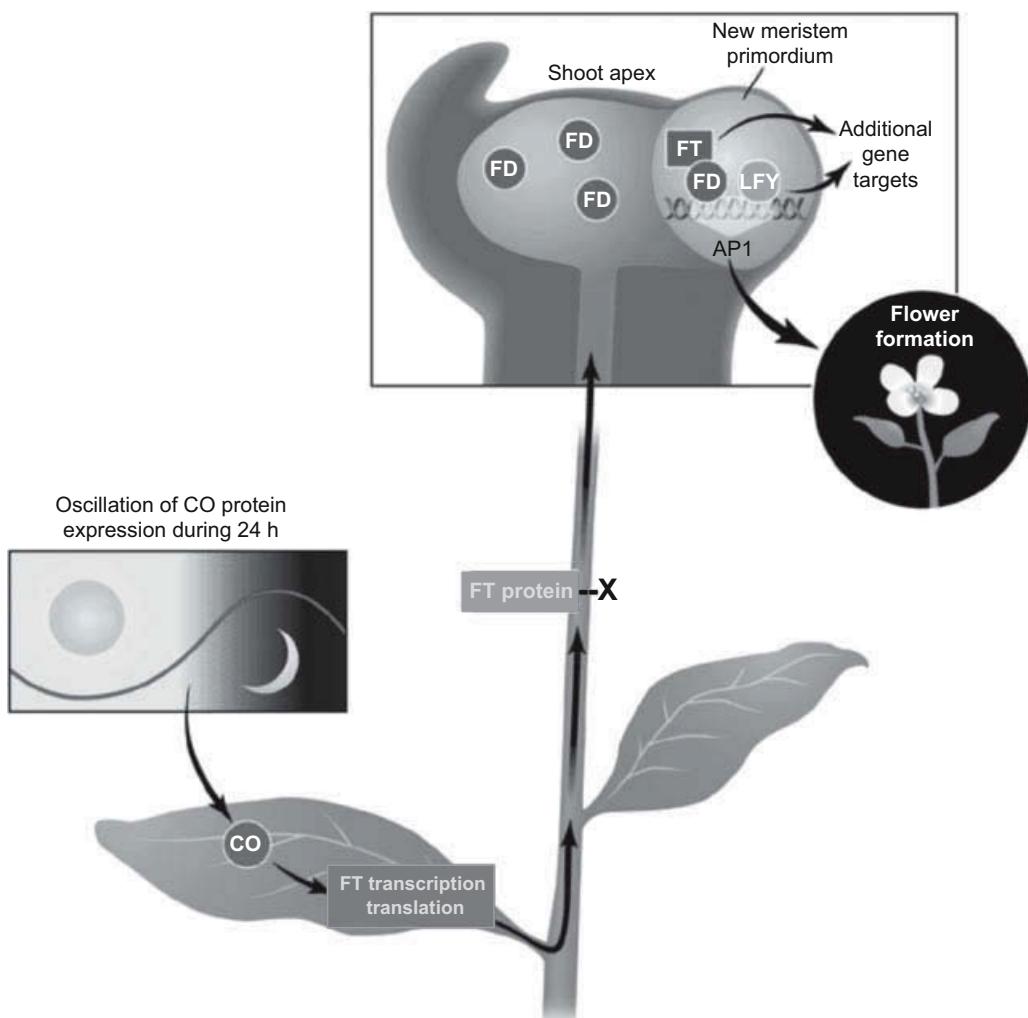


Figure 10.1 The FT protein signals activation of the floral pathway. In response to the appropriate day length (inset), accumulation of the transcription factor CONSTANS (CO) activates transcription of FT in the leaf. In the leaf, FT mRNA is translated, and FT protein moves through the phloem upward with putative unknown chaperone proteins (X) to the shoot apex. In the SAM, the FT protein interacts with the transcription factor FD. This tandem complex then activates floral pathway genes such as *API* to initiate floral development. The FT-FD complex acts redundantly with the transcription factor LEAFY (LFY) to activate *API*. FD, FLOWERING LOCUS D; FT, FLOWERING LOCUS T. (Modified from Blázquez, 2005.) (For a color version of the figure, please see Plate 10.1.)

location of its functional activity suggests that the FT protein may serve as the mobile floral signal. Several lines of evidence support the premise that FT protein moves from leaves to the SAM to induce flowering.

FT protein was identified by mass spectrometry in the sieve-tube sap of the dicot squash (*Cucurbita moschata*; Lin et al., 2007) and the monocot rice (*Oryza sativa*; Aki et al., 2008). The definitive experiment employed a MYC epitope:FT fusion protein expressed under the control of the

phloem-specific sucrose-proton symporter 2 (*SUC2*) promoter in transgenic *Arabidopsis*. The FT fusion protein moved to the SAM and rescued a loss of function *ft* mutant restoring the flowering phenotype (Jaeger and Wigge, 2007). Although it has been clearly established that long-distance FT protein movement is required for flowering, what mechanism controls FT movement to the shoot apex? One possibility is that FT protein interacts with other mobile proteins to facilitate delivery of the florigen complex to the SAM. Studies on the direction of movement of phloem proteins indicate a diverse range of possibilities for bidirectional movement that could influence the ultimate destination of the FT protein (Aoki et al., 2005). It is likely that protein chaperones or other escort partners also exist to facilitate transport across the surveillance system that protects the SAM. In a remarkable security system, numerous plant viruses can move freely throughout the plant but cannot transverse the gateway that leads into the SAM. By utilizing viral RNA transport and disrupting posttranscriptional gene silencing, Foster et al. (2002) demonstrated the existence of a zone of surveillance that regulates the entry of viral RNA and signaling molecules into the shoot apex. This putative surveillance system regulates cell-to-cell trafficking and acts a filter to protect the shoot apex from detrimental infectious agents such as viral RNA or protein.

How does the FT signal so easily cross this gateway? Both direction and surveillance imply the existence of chaperone proteins that are essential for directing FT to its final destination. Support for the concept of specific locations for transport gateways for FT is demonstrated by the use of various nonnative promoters and fusion tags. An FT-green fluorescent protein (GFP) fusion complements an *ft* mutation when expressed in the SAM or the major veins of the leaf by the *SUC2* promoter but not when expressed in the minor veins of leaves by the galactinol synthase (*GAS1*) promoter (Corbesier et al., 2007). When FT was fused to yellow fluorescent protein (YFP), the fusion protein did not induce early flowering when expressed under the *SUC2* promoter but did induce flowering when gene expression was directed by the *CaMV35S* promoter (Mathieu et al., 2007). By activating a viral peptidase, free FT protein was released from the YFP-fusion driven by *SUC2* and then was able to induce early flowering. These results suggest that depending on the location of expression, fusions to FT might interfere with normal transport of the mobile protein signal. Such interference could be mediated by transport through gateways located at strategic junctions distributed throughout the plant (Lucas et al., 2001). To date, however, the only confirmed protein partner of FT is FD and the interaction between the protein partners occurs exclusively in the SAM and functions in the transcriptional regulation of *API* (Abe et al., 2005; Wigge et al., 2005). As discussed previously in this section, FT is dependent on FD for its function in floral activation.

FT is also known to play a number of roles in regulating vegetative development (Böhlenius et al., 2006; Shalit et al., 2009; Rinne et al., 2011). Recent studies have clearly shown the function of a mobile FT signal in controlling tuber formation. The expression of the FT rice homolog in photoperiod-sensitive potato genotypes induces these plants to tuberize independent of day length conditions (Abelenda et al., 2011). This rice protein is graft transmissible, is detected in stolon tips, and acts as a strong signal for inducing tuber formation. It has been proposed that the potato homolog of FT, Self-pruning 6A (SP6A), is translated in leaves and moves through potato plants growing under short days (SDs) to induce tuber formation (Navarro et al., 2011). Overexpression lines of SP6A were able to tuberize under noninductive long-day conditions, whereas silencing of SP6A strongly delayed tuberization under inductive, short-day conditions (Navarro et al., 2011). Together these results provide strong evidence for a role of SP6A as the long distance mobile signal for tuber induction. The self-pruning/FT family in potato contains two other FT homologs. One is expressed in mature tubers and may function in the control of tuber sprouting. A third homolog, designated Self-pruning 3D, is expressed in correlation with a photoperiod-neutral transition to flowering (Xu et al., 2011).

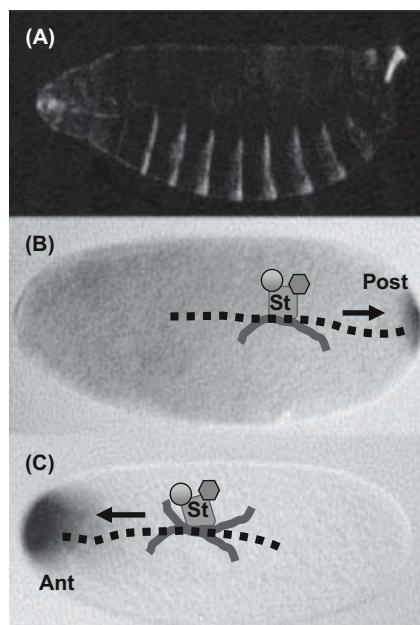


Figure 10.2 Staufen model for intracellular RNA transport in *Drosophila*. (A) Dark-field image of the developing embryo of *Drosophila*. (B) Movement of Staufen–*oskar* RNA complex to the posterior (Post) end of the developing oocyte. (C) Movement of Staufen–*bicoid* RNA complex to the anterior (Ant) end of the developing oocyte. Two *bicoid* transcripts (grey lines) bind to Staufen. St, Staufen. The grey circle and hexagon represent other proteins in the complex. The dotted lines represent the oocyte microtubules essential for localization. (Reprinted from Irion et al. (2006) and reproduced with the permission of Daniel St Johnston.)

Full-Length mRNA as a Long-Distance Signal

In animals, mRNAs are transported within the cell in a tightly regulated process to facilitate their function. With the extensively studied Staufen protein of *Drosophila* (St Johnston et al., 1991; Ferrandon et al., 1994), binding to the *bicoid* and *oskar* mRNAs occurs to determine their anterior or posterior localization in the egg cell (Figure 10.2). This directed localization involves RNA–protein, RNA–RNA (Ferrandon et al., 1997) and protein–protein interactions (Elvira et al., 2006) during transport and repression of translation of the mRNAs. Repression of translation insures that the mobile mRNAs are functional only at the target site (King et al., 2005). Commonly, it is the untranslated regions (UTRs) that function in binding to proteins that facilitate movement of a transcript (Jansen, 2001), in mediating RNA stability (Derrigo et al., 2000; Lee and Jeong, 2006), or in regulating the efficiency of translation (Gualerzi et al., 2003; Barreau et al., 2006). Without the protection of these RNA-binding proteins, naked, cellular RNA molecules quickly fall prey to degradative processes (Shyu et al., 2008).

The transport and localization of full-length mRNA serves as a dynamic cellular tool for optimizing and targeting protein expression. Du et al. (2007) have succinctly outlined the advantages of localizing RNA. First, transporting RNA requires less energy than transporting a protein. Second, localizing RNA provides a degree of spatial control over protein synthesis, ensuring that the protein is not translated at any other location than the functional site (Boggs, 2006). Finally, mobilizing RNA and regulating translation can facilitate the formation of protein gradients to establish cellular

polarity (Figure 10.2) or asymmetric patterns of cell division (Cosma, 2004). The polarity of the developing *Drosophila* embryo is determined by the transport and localization of the select RNAs, *oskar*, *bicoid*, *gurken*, and *nanos*. This chaperone-mediated localization leads to morphogen gradients establishing the pattern of embryogenesis (Ephrussi et al., 1991; Mahowald, 2001). Localization of the RNA of *nanos* is not as tightly controlled as in the examples with *oskar* and *bicoid*. Its RNA is distributed more diffusely in the posterior end of the embryo. Instead, fine-tuning of the *nanos* protein gradient is enhanced by repressing translation and restricting accumulation of the protein to the posterior end (Crucks et al., 2000).

Unlike cell-autonomous animal systems, plants move RNA and proteins from the cell of origin through the phloem to target sites where environmentally regulated and developmental processes are activated. Techniques involving grafting showed that specific mRNAs can move long distances through the phloem and numerous full-length mRNAs have been identified in the sieve-tube system (see Chapter 9) of several plant species (Asano et al., 2002; Vilaine et al., 2003; Omid et al., 2007; Deeken et al., 2008; Gaupels et al., 2008). Only a few, however, have been confirmed to be transported to distantly located sink organs (Kehr and Buhtz, 2008). These include *CmGAI*, *CmNACP*, and *CmPP16* from pumpkin (Ruiz-Medrano et al., 1999; Xoconostle-Cazares et al., 1999; Haywood et al., 2005); *DELLA-GAI* from *Arabidopsis* (Haywood et al., 2005); *PFP-LeT6* from tomato (Kim et al., 2001); and *StBEL5*, a BEL1-like transcription factor, is expressed in potato (Banerjee et al., 2006). The long-distance transport of the transcripts of *GAI* and *StBEL5* and their respective roles in development has been discussed in the following section.

Mobile *GAI* RNA

Gibberellin insensitive (GAI) encodes a protein that belongs to the GRAS family of transcriptional regulators, and functions as a negative regulator of gibberellic acid (GA) responses (Peng et al., 1997; Pysh et al., 1999; Richards et al., 2001). GAI restricts plant growth presumably by modifying transcriptional programming. This family of repressors is characterized by the conserved N-terminal DELLA domain that serves as a receiver domain for activated GA INSENSITIVE DWARF1 (GID1) GA receptors (Willige et al., 2007). The GA signal is perceived by GID1, which is a soluble protein that is localized to both cytoplasm and nucleus. Binding of GA to GID1 enhances the interaction between GID1 and DELLA, resulting in rapid degradation of DELLA via the ubiquitin-proteasome pathway. A specific ubiquitin E3 ligase complex (SCFSLY1/GID2) is required to recruit DELLA for ubiquitination and subsequent degradation by the 26S proteasome. In *Arabidopsis*, the GA dependent degradation of GAI is facilitated by an interaction with SLEEPY1 (SLY1) an F-box protein that functions as the degradation substrate receptor subunit of the E3 ubiquitin ligase SCFSLY1. The F-box domain is a conserved motif that facilitates protein–protein interaction in the proteasome degradation pathway. The SLY1 and GAI interaction also occurs when the DELLA domain is deleted. These results indicate that the DELLA domain functions as a binding domain for GA-activated GID1 and is not involved in the interaction with SLY1. In the GA response pathway, GAI functions as a repressor of elongation growth (Sun, 2000; Willige et al., 2007).

The *Arabidopsis* gibberellic acid-insensitive (*gai*) mutant displays a dark green dwarf phenotype (Koornneef et al., 1985). Sequence analysis of the *Arabidopsis* *gai-1* mutant revealed that this allele has a 17-amino acid deletion from the conserved DELLA domain, rendering mutant *gai* and *rga* (repressor of GA) proteins insensitive to gibberellin-induced proteolysis. Plants expressing mutant DELLA repressors are GA insensitive, dark-green, late-flowering dwarfs (Peng and Harberd, 1997; Dill and Sun, 2001; Silverstone et al., 2001; Fleck and Harberd, 2002; Itoh et al., 2002;

Dill et al., 2004). The truncated form of GAI acts as a gain-of-function mutant that can inhibit some components of the GA signaling pathway (Peng et al., 1997). All mutants carrying deletions in the DELLA domain exhibited a semidominant dwarf phenotype (Silverstone et al., 1998; Peng et al., 1999; Ikeda et al., 2001; Chandler et al., 2002; Wen and Chang, 2002). Expression of *Arabidopsis gai* in rice yielded a dwarf phenotype, suggesting that GAI is sufficiently conserved between plant families to allow it to function in a heterologous genome (Peng et al., 1999; Fu et al., 2001).

Direct evidence that GAI mRNA circulates throughout the plant via the phloem was obtained by the characterization of mRNA in sieve-tube sap of mature pumpkin. Heterografting experiments with cucumber (*Cucumis sativus*) as the scion and pumpkin as the rootstock demonstrated that pumpkin *CmNACP*, *CmGAIP*, and *CmPP16* RNAs moved selectively upward into developing apical tissues of the cucumber scion (Ruiz-Medrano et al., 1999). *In situ* RT-PCR experiments confirmed that all three mRNA molecules were present within the sieve tubes. In contrast to the many plant RNA viruses that are excluded from the apical meristem, these early results established the existence of a select subset of full-length mRNAs that move through the phloem vascular system into meristematic tissues. In this early experimental system, however, an altered phenotype was not correlated with movement of these RNAs. To confirm that mobile *Arabidopsis GAI* RNA transported through the phloem to the shoot apex affects plant development, an engineered dominant gain-of-function construct for both pumpkin (*CmGAI*) and *Arabidopsis DDELLA-GAI* genes was employed (Haywood et al., 2005). Delivery of *CmGAIP* and *DDELLA-GAI* RNA to shoot apices was confirmed in heterografts of pumpkin and cucumber (Figures 10.3A, B, and C). The dominant gain-of-function constructs also mediated highly reproducible changes in leaf phenotype when expressed in transgenic tomato lines (Figure 10.3D). In addition, this study demonstrated that sink strength does not regulate *GAI* RNA delivery.

Results from several studies indicate that *GAI* RNA entry into functional sieve elements occurs via a selective process. Control over *GAI* RNA delivery and movement, via the phloem, appears to be regulated by sequence motifs conserved between plant families. By using grafting and RT-PCR analyses, motifs in the coding sequence (cds) and the 3' UTR of the *GAI* RNA of *Arabidopsis* displayed functional roles in regulating RNA movement (Huang and Yu, 2009). Sequences of *GAI* RNA were identified that were necessary and sufficient to target the RNA of GFP for long-distance movement, suggesting that specific sequences of *GAI* mediate long-distance trafficking.

Recently, an RNA–protein complex that transports *GAI* RNA of pumpkin has been identified that contains 6 mRNAs and up to 16 proteins (Ham et al., 2009). In addition to *GAI*, other phloem transcripts identified in this complex were *PP16-1*, *SACREDCROW-LIKE14 (SCL14)*, *SHOOT MERISTEMLESS*, *ETHYLENE RESPONSE FACTOR*, and *Myb*. *GAI* and *SCL14* are members of the GRAS family of transcription regulators involved in GA signaling. This ribonucleoprotein complex is phloem mobile moving across pumpkin and cucumber heterograft unions, but the precise role of the proteins in this complex in transport and RNA metabolism is still not entirely clear.

The Long-Distance Transport of a *BEL1*-like mRNA Regulates Tuber Development

Tuber formation in potatoes is a complex developmental process involving a number of important regulatory systems (Fernie and Willmitzer, 2001; Martínez-García et al., 2001; Hannapel et al., 2004). It has long been known that under short-day conditions receptors in the source leaves perceive the light cue and activate a transmissible signal that moves from the leaf through the phloem down to the stolon to induce growth in the subapical region of the stolon (inset, Figure 10.4). Under SDs, tubers form, whereas under LDs, they do not. This graft-transmissible signal initiates

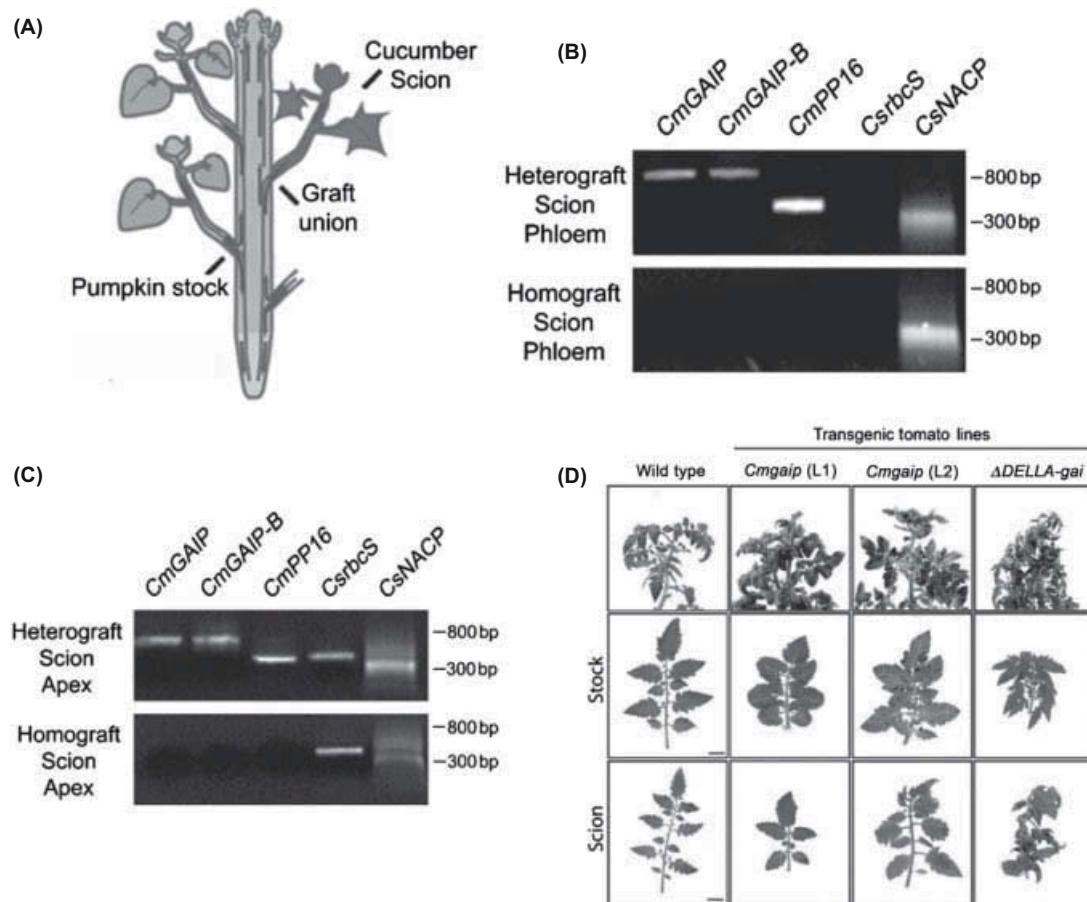


Figure 10.3 Long-distance translocation of CmGAIP and CmGAIP-B transcripts into the vegetative apex of cucumber scions grafted onto pumpkin stocks. (A) Diagram illustrating the arrangement of the grafting system used to assay for phloem long-distance transport of macromolecules. A graft-compatible but genetically distinguishable receiver shoot apex (termed the scion) is attached (graft union) onto the main axis of the donor plant (termed the stock); this system is referred to as a heterograft. (B) RT-PCR analysis, using gene-specific primers directed against RNA collected from phloem sap of cucumber scions (upper panel), amplified both CmGAIP and CmGAIP-B transcripts. Controls: CmPP16 RNA formed a positive control for phloem-mobile transcripts derived from the pumpkin stock. Absence of a CsrbcS product confirmed the lack of wound-induced contaminating RNA from surrounding tissues. Lower panel: RT-PCR failed to amplify the pumpkin transcripts using RNA collected from the phloem sap of cucumber scions grafted onto cucumber stocks (termed a homograft). A phloem-mobile cucumber RNA, CsNACP, was included as a positive control in both hetero- and homograft assays. (C) RT-PCR analysis, using gene-specific primers directed against RNA collected from vegetative apices of cucumber scions grafted onto pumpkin stocks; note that both CmGAIP and CmGAIP-B transcripts were detected in these assays (upper panel). Parallel RT-PCR analysis performed on homografted cucumber scions (control) failed to amplify the pumpkin transcripts (lower panel). Cucumber CsrbcS and CsNACP primer pairs were used to amplify these transcripts as positive controls. (D) Phloem-mediated delivery of Cmgaip and DDELLA-gai RNA induces developmental phenotypes in a tomato graft system. Phenotypic analysis of tomato leaves that develop after grafting of wild-type scions onto the indicated stocks. Upper panel: general morphology of wild-type tomato and transgenic lines expressing P35S:Cmgaip (L1 and L2; two representative transgenic lines) and P35S:DDELLA-gai. Middle panel: Leaf morphology of stock leaves. Lower panel: Scion leaf phenotype represented by the third leaf that developed postgrafting. Scale bars 1/4 2.5 cm; common to stock and scion leaves. (From Haywood et al., 2005.) (For a color version of the figure, please see Plate 10.2.)

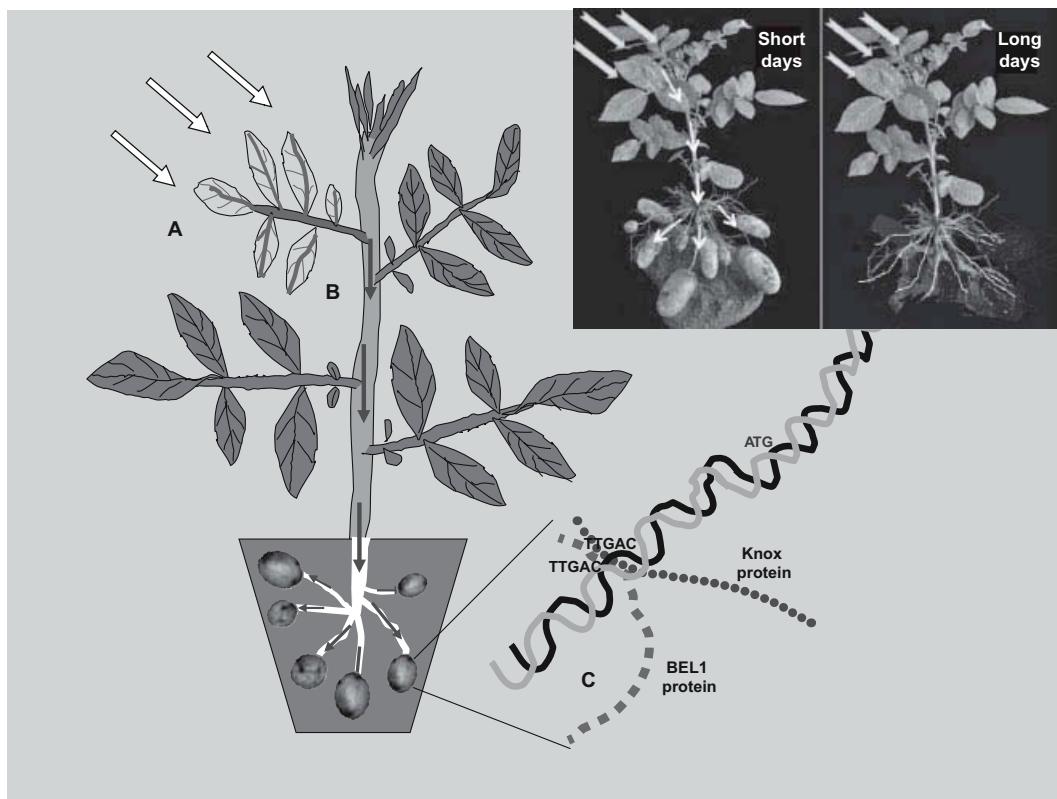


Figure 10.4 The *StBEL5* RNA signaling pathway for tuber formation. Short-day photoperiod conditions are inductive for tuber formation, whereas long days are noninductive (inset). This signaling pathway is based on the initial transcriptional activation by light (A, white arrows with black outline) of the *StBEL5* gene in the veins of leaves and petioles (dash-grey). A short-day photoperiod facilitates movement of the *StBEL5* RNA through the petiole junction into the stem via the phloem (B, dark-grey arrows) by mediating the activation or expression of appropriate RNA-binding proteins. Under these conditions, RNA may then be escorted to site-specific targets, like stolon tips, via protein chaperones. Enhanced translation of BEL5 (dash-grey line) then occurs in the stolon tip leading to binding to a Knox protein partner (dot grey line) and subsequent activation of transcription and regulation of select target genes by interaction with the tandem TTGAC motif (Chen et al., 2004) of the promoter (C). (For a color version of the figure, please see Plate 10.3.)

processes that stimulate cell division and expansion as well as a change in the orientation of cell growth in the subapical region of the stolon tip (Xu et al., 1998). These developmental activities ultimately lead to starch accumulation and formation of the tuber. Moreover, the onset of tuberization is accompanied by a switch from apoplastic to symplastic unloading (Viola et al., 2001), which might impact movement of the signal molecule that induces tuber formation.

As one approach to identifying this mobile signal, research efforts have focused on the role of transcription factors in regulating tuber formation. One important group of transcription factors includes the TALE superfamily. In plants, these include members from the BEL1-like and the KNOTTED1-like families: both families of transcription factors are involved in pattern formation and maintenance of the SAM (Reiser et al., 2000; Kanrar et al., 2008). The BEL1-like family of transcription factors is ubiquitous among plant species and these proteins interact with KNOTTED1-like transcription factors to regulate a range of developmental processes (Müller et al.,

2001; Chen et al., 2003; Smith and Hake, 2003; Kumar et al., 2007; Pagnussat et al., 2007; Kanrar et al., 2008; Ragni et al., 2008).

Recent studies focusing on phloem mRNAs of potato suggest that the tuberization signal may be a full-length mobile RNA (Banerjee et al., 2006). *StBEL5*, a member of the BEL1-like family of transcription factors, is one of several mobile mRNAs of potato that is transported through the phloem (Müller et al., 2001; Chen et al., 2003; Smith and Hake, 2003). The results of studies in transgenic plants provide a positive correlation between the accumulation of *StBEL5* RNA and tuber formation. Overexpression of *StBEL5* has consistently produced plants that exhibit overall increased vigor and enhanced tuber production (Chen et al., 2003; Banerjee et al., 2006, 2009). RNA detection methods and grafting experiments have been used to demonstrate that *StBEL5* transcripts are present in phloem and move across a potato graft union to localize in stolon tips, the site of tuber induction (Banerjee et al., 2006; Yu et al., 2007). RNA movement originates in the veins of source leaves and petioles (Figure 10.4A) and is induced by a short-day photoperiod, regulated by the UTRs, and correlates with enhanced tuber production (Figure 10.4B). Promoter activity of *StBEL5* has been observed in companion cells and phloem parenchyma of petioles (Banerjee et al., 2006). Whereas photoperiod mediates the movement of *StBEL5* RNA, transcription of the *StBEL5* gene in leaves is activated by either red or blue light, regardless of photoperiod or light intensity (Chatterjee et al., 2007).

Evidence for Movement of BEL5 RNA through the Phloem

The promoter of the *StBEL5* gene is activated by light in the veins of leaves but is not active in stems (Chatterjee et al., 2007). Despite this promoter activity profile, an abundant amount of *StBEL5* RNA could be detected in stems supporting the idea that the RNA was being transported from leaves to stems through the phloem (Banerjee et al., 2006). If this were the case, then *BEL5* RNA would be present in cells within the stem phloem tissue. This hypothesis was confirmed by *in situ* hybridization of stem and stolon sections with probes specific for *StBEL5* and laser capture microdissection (Banerjee et al., 2006). Both techniques verified that *BEL5* RNA plus *POTH1* (potato homeobox 1), the *KNOTTED1*-type protein partner of *BEL5*, are present in phloem cells. Laser capture microdissection and profiling of RNA from sieve-tube sap confirmed that six other *StBEL* RNAs were also present in the phloem stream (Yu et al., 2007; Campbell et al., 2008). These observations suggest the intriguing possibility that several of the *BEL1* RNAs of potato participate in phloem-mediated transport to regulate development and to respond to environmental cues. In potato, genes for thirteen *BEL1*-like transcription factors have been identified (Lin and Hannapel, unpublished data).

Movement of *StBEL5* transcripts was first confirmed by grafting an overexpression line for a full-length *StBEL5* construct to a wild-type plant (Banerjee et al., 2006). In three separate plants, the *StBEL5* RNA moved from the transgenic scion into the wild-type stock accumulating specifically in the stolon tip. As with the previous positive correlations with tuber enhancement, transport of transgenic *StBEL5* RNA was correlated to an increase in tuber production on the grafted plants (Banerjee et al., 2006).

The Role of Untranslated Regions in RNA Transport

The 3' UTR of the *StBEL5* transcript is critical in mediating RNA transport, stability, and translation (Banerjee et al., 2006, 2009). Numerous other studies on mobile RNAs have confirmed the functional

role of the 3' UTR in recognizing RNA-binding proteins that regulate metabolism and movement (Ferrandon et al., 1994; Corral-Debrinski et al., 2000; Padmanabhan and Richter, 2006; Irion and St Johnston, 2007). In the Staufen–*bicoid* complex, a well-studied protein–RNA interaction, the localization of *bicoid* RNA requires specific binding to proteins in an endosomal sorting complex to an element in the 3' UTR (Irion and St Johnston, 2007). In *Saccharomyces cerevisiae*, *ASH1* mRNA (a transcription repressor) is localized to the bud tip of daughter cells, where it plays key roles in development (Olivier et al., 2005). The localization of these transcripts depends on interactions between localization elements in *ASH1*, some of which are located within the 3' UTR and are recognized by the RNA-binding protein, She2p. Recall that motifs in the cds and the 3' UTR of the mRNA of *AtGAI* display functional roles in regulating movement (Huang and Yu, 2009). In the oocyte of *Xenopus laevis*, *Vg1* RNA is transported to the vegetal cytoplasm, where localized expression of the encoded protein is critical for embryonic polarity (King et al., 2005). Interaction between specific repeated motifs within the 3' UTR and RNA-binding proteins mediates the targeted localization of *Vg1* RNA to the vegetal pole of the developing embryo (Lewis et al., 2004).

Our understanding of the long-distance movement of full-length RNAs in plants and the sequences that mediate this transport process continues to expand (Kehr and Buhtz, 2008; Banerjee et al., 2009; Ham et al., 2009; Huang and Yu, 2009). On the basis of animal models, these sequences likely form recognition stem/loop structures designated *zip code elements* that function in recognizing RNA-binding proteins (reviewed in Jansen, 2001) and are expected to be most predominant in the 3' UTR (Macdonald and Kerr, 1997; Chartrand et al., 1999; Corral-Debrinski et al., 2000; Thio et al., 2000). Viroid RNA motifs have also been identified that facilitated selective RNA movement through plant cells (Qi et al., 2004; Zhong et al., 2007, 2008). These short sequences most likely mimic endogenous plant RNA motifs that are recognized by cellular factors for transport. In the following section, the role of UTRs in mediating the long-distance transport of *BEL1-like* mRNAs of potato has been examined in more detail.

Untranslated Regions of StBEL5 Mediate Transcript Mobility

StBEL5 RNA has a large 3' UTR of 503 nt with several conserved motifs. These regions include clusters of CUUC (polypyrimidine tract motif) and UAGUA motifs (Banerjee et al., 2009) and are conserved in putative *BEL5* orthologs from tomato and tobacco as well as in *StBEL11* and *StBEL29*. Does the 3' UTR of *StBEL5* play a role in regulating *StBEL5* mRNA movement? To address this question, the relative accumulation of *StBEL5* RNAs in whole plants of transgenic lines that expressed only the cds (*StBEL5-cds*) or the cds plus both UTRs (*StBEL5-FL*) was compared. In these experiments, transcription of both constructs was regulated by the *CaMV35S* promoter. Transgenic plants with each of the two constructs were grown under SD conditions and RNA extracted from new leaves (Figure 10.5A) and 5.0-mm stolon tips (Figure 10.5B) and RNA for the *BEL5* construct was quantified. In samples from SD plants, more PCR product for the full-length transcript was detected in stolons compared with leaves (Figure 10.5C), whereas the yield of product for the *BEL5* cds RNA from leaf and stolon exhibited a ratio of 1:1 (Figure 10.5C). When transgenic lines expressing the full-length *BEL5* transcript were grown under long-day (LD) conditions, preferential accumulation of the transgenic RNA in stolons did not occur and the ratio of leaf:stolon RNA was 1:1 (Figure 10.5C).

To confirm that the preferential accumulation of full-length *BEL5* RNA, regulated by the *CaMV 35S* promoter, was due to photoperiod-mediated movement, the leaf-abundant GAS promoter from

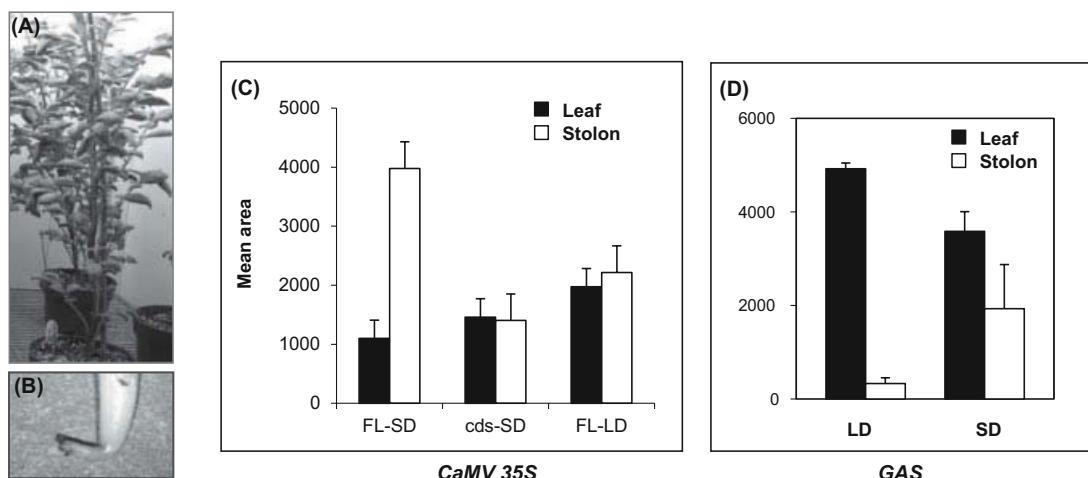


Figure 10.5 Effect of *StBEL5* UTRs and photoperiod on long-distance transcript movement into stolon tips. (A) Movement of transcripts with and without the UTRs (FL and cds, respectively) was assayed in three transgenic plants (for each construct) grown under short-day (SD) conditions for 14 days or long days (LD). RNA was extracted from recently matured source leaves. (B) Stolon tips (0.5 cm in length) on plants described in A were used to extract RNA. (C) RNA levels measured by one-step RT-PCR in leaf and stolon tissues obtained from transgenic potato lines described in A; constructs driven by the cauliflower mosaic virus (*CaMV*) 35S promoter. (D) As in C, except that full-length *StBEL5* transcripts were driven by the leaf-abundant galactinol synthase (GAS) promoter that in potato is active only in the minor veins. Values represent Mean \pm SEM for three biological replicates. (Reprinted from Banerjee et al. (2006); Copyright American Society of Plant Biologists, www.plantcell.org.) (For a color version of the figure, please see Plate 10.4.)

melon (*Cucumis melo*; Ayre et al., 2003) was used to regulate *StBEL5* expression. With this promoter, in the samples from plants grown under SD conditions, the ratio of quantified *StBEL5* RNA that moved to stolons in relation to the source leaf was sevenfold greater in SD compared with LD plants (Figure 10.5D), indicating enhanced mobility of the *StBEL5* RNA under SD conditions. This increased RNA mobility was correlated with earliness (more tubers after 10 d of SD inductive conditions) and enhanced tuber yields (Banerjee et al., 2006). Collectively, these studies demonstrated that addition of the UTRs facilitated the transport of *BEL5* RNA into stolon tips and that a short-day photoperiod enhances this process.

A recent report has demonstrated the role of potato microRNA, *miR172*, in regulating *BEL5* RNA levels and affecting tuber development (Martin et al., 2009). Levels of *miR172* were higher under tuber-inducing SDs than under noninductive LDs and were upregulated in stolons at the onset of tuberization. This microRNA was detected in vascular bundles and its overexpression in potato enhanced *BEL5* transcript levels and activated tuberization under LDs. These results suggest that phloem-mobile microRNAs may mediate the *BEL5* long-distance signaling pathway for tuber formation.

If the *BEL5* UTRs mediate long-distance movement, then it should be possible to fuse both UTRs to a less mobile RNA and enhance its overall mobility. This hypothesis was tested by developing a chimeric construct containing both *StBEL5* UTRs fused to the cds of a less mobile *BEL1*-like RNA, *StBEL14* (Figure 10.6A, BEL14 + BEL5 UTRs). A construct consisting of the full-length *StBEL14* sequence without the *StBEL5* UTRs was used as a base-line control (Figure 10.6A, FL-BEL14). As a positive control, movement was assayed for a transgenic line previously characterized (Banerjee et al., 2006) expressing the full-length *StBEL5* transcript (Figure 10.6A, FL-BEL5). To insure that

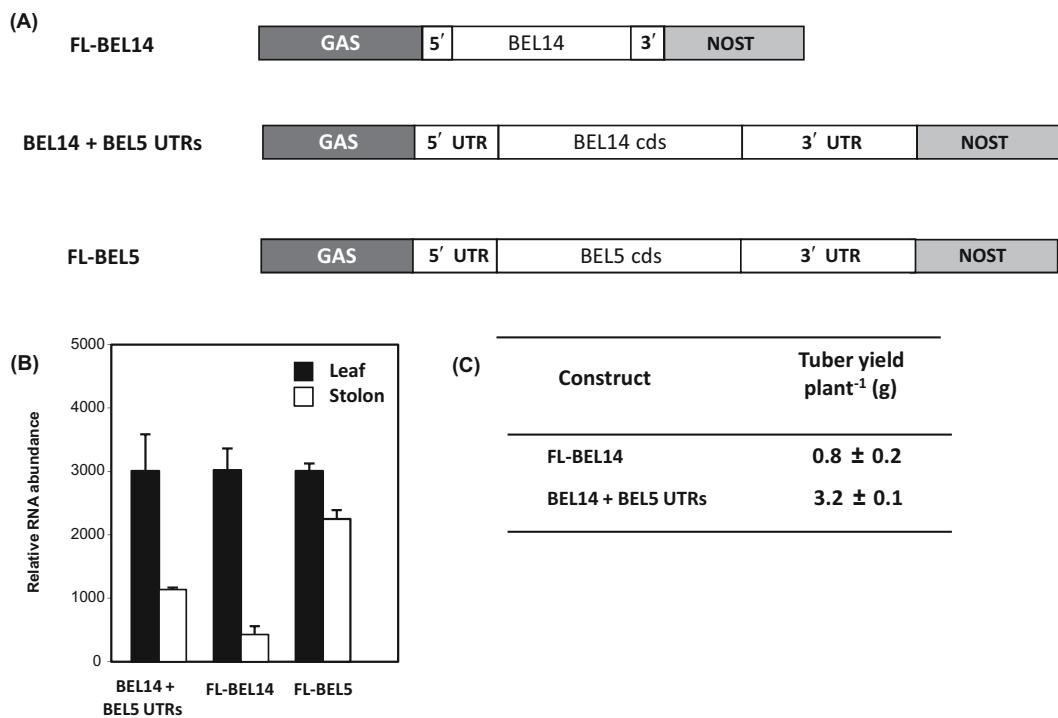


Figure 10.6 Three RNA constructs driven by the leaf-abundant *GAS* promoter were tested for their capacity to move from leaf veins into stolon tips. (A) FL-BEL14 contains the *BEL14* cds plus both native *BEL14* UTRs; *BEL14* + both *StBEL5* UTRs replacing the *StBEL14* UTRs; FL-BEL5, full-length *StBEL5*. *StBEL14* was chosen as a test RNA because it is not abundant in stems or stolons (B). Relative RNA accumulation in new leaves and 0.5 cm samples from stolon tips quantified for transgenic potato lines expressing the constructs shown in (A). Three transgenic plants, per construct, were grown under short day conditions for 12 days prior to RNA extraction. (C) Transgenic plants assayed in B were used to measure tuber yield. All values represent Mean \pm SEM for three clones of one independent transgenic line per construct. NOST, nopaline synthase terminator; cds, coding sequence. (Reprinted from Banerjee et al. (2009); Copyright American Society of Plant Biologists, www.plantphysiol.org.)

the source of most of these transgenic RNAs was the source leaves (similar to the *StBEL5* promoter), the leaf abundant *GAS* promoter was used for all three constructs.

The most efficient movement of transgenic RNA was observed with the full-length *StBEL5* RNA (Figure 10.6B). The difference in RNA accumulation in leaves relative to stolon tips between transgenic lines with *BEL14* plus the *StBEL5* UTRs or *BEL14* without these UTRs was 2.7-fold. Hence, almost threefold more *BEL14* transcripts were translocated into stolon tips in the presence of the *StBEL5* UTRs. Such enhanced movement was correlated with a fourfold increase in tuber yield for the *BEL14* + *BEL5* UTR lines compared with the *FL-BEL14* lines (Figure 10.6C). Clearly, addition of the UTRs of *BEL5* to another, less mobile, *BEL* RNA can enhance its phloem mobility and targets the chimeric RNA to a specific organ.

Future Directions

The long-distance transport of FT protein to the apical shoot to induce flowering, *StBEL5* RNA from leaves to stolon tips to activate tuber formation in potato, and the CmRBP50 ribonucleoprotein

(RNP) complex of pumpkin that moves *CmGAI* and other full-length mRNAs are all remarkable examples of long-distance trafficking of signal molecules.

In these model systems for noncell autonomous movement of protein or full-length mRNAs, it is readily apparent that escort factors that facilitate transport are likely involved in controlling both the direction of movement and the final destination of the mobile complex. The existence of such a complex is a potentially new paradigm in plant biology. Couple this discovery with the fact that posttranscriptional regulation mediated by protein–mRNA complexes occurs widely among eukaryotes (Sanchez-Diaz and Penalva, 2006; Keene, 2007; Halbeisen et al., 2008) and it is clear that RNA stability, transport, and localization are important mechanisms in plants for regulating development in response to environmental cues.

Still there are many questions as yet unanswered. Are phloem-mobile RNAs translated in sieve elements? What is the function of these numerous mobile RNAs and proteins? Do stress responses mobilize such molecules? What mechanisms determine the direction of transport and final destination? What is the nature of the mobile protein/protein and RNP complexes, and what posttranslational processes regulate the stability and location of these complexes? For example, both glycosylation and phosphorylation are important aspects of recognition for phloem proteins and control their capacity for cell-to-cell movement through the plasmodesmata (Taoka et al., 2007). Phosphorylation of CmRBP50 was required to maintain stability of the phloem–mobile complex (Ham et al., 2009). The determination of factors regulating symplastic gating at the molecular level and the extrapolation of these findings to developmental systems are key issues to be addressed.

The major challenges that lie ahead for plant biologists are to (1) confirm the identity of phloem-mobile RNAs from among the thousands of transcripts expressed in phloem cells, (2) identify zip code elements present in mobile RNAs that mediate binding to specific escort proteins, (3) characterize the numerous protein/protein and RNA–protein complexes that are integral to this remarkable long-distance transport network, and (4) understand how these complexes function to regulate mobility and localization and how they impact development and source/sink transitions.

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11 Plant Defense and Long-Distance Signaling in the Phloem

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In plants, responses to local stresses are translocated systemically to impact stress tolerance in distant tissues and organs. Such messaging invariably leads to enhanced stress resistance in the systemic organs in subsequent encounters with the same or similar stressors. Systemic signals by definition are produced at the site of stress exposure and transported or propagated over long distances. They are perceived in distant plant tissues and initiate systemic stress resistance through priming or induction of defense responses.

This chapter provides an overview of how distant tissues and organs are alerted to possible attacks by signals that move through vascular bundles. The initial sections will discuss the systemic wound response (SWR), systemic acquired resistance (SAR), and systemic acquired acclimation (SAA). Subsequent sections will review the systemic signals associated with SWR, SAR, and SAA that are thought to move, at least partially, through the phloem.

Abbreviations: ABA, abscisic acid; AOC, allene oxide cyclase; AOS, allene oxide synthase; APX, ASCORBATE PER-OXIDASE; AtGSNOR, *Arabidopsis thaliana* S-nitrosoglutathione reductase; AtPep, *Arabidopsis thaliana* signal peptide; AzA, azelaic acid; AZI, AZELAIC ACID INDUCED; BA, benzoic acid; CA, cinnamic acid; CaMV, cauliflower mosaic virus; CC, companion cell; CDR, CONSTITUTIVE DISEASE RESISTANCE; DAB, diaminobenzidine; DAF-2 DA, 4,5-diamino-fluorescein diacetate; *dir1*, DIR1 mutant; DIR, DEFECTIVE IN INDUCED RESISTANCE; *fad7*, FAD7 mutant; FAD, FATTY ACID DESATURASE; G3P, glycerol-3-phosphate; *gly1*, allelic to *sfd1*; GSNO, S-nitrosoglutathione; GSNOR, S-nitrosoglutathione reductase; H₂O₂, hydrogen peroxide; HBA, hydroxybenzoic acid; HR, hypersensitive response; HypSys, hydroxyproline-rich systemins; ICS, isochorismate synthase; JA, jasmonic acid; *jai-1*, JASMONIC ACID INSENSITIVE-1 mutant; JA-Ile, JA-isoleucine; LOX, lipoxygenase; MeJA, methyljasmonate; MeSA, methylsalicylate; NahG, naphthalene hydroxylase G; NO, nitric oxide; NOS, nitric oxide synthase; NR, nitrate reductase; (ns)LTP, (nonspecific)lipid transfer protein; O₂⁻, superoxide; OPDA, oxophytodienoic acid; PAL, phenylalanine ammonia lyase; pCMBS, p-chloromercuribenzene sulfonate; PEPR, PEPTIDE RECEPTOR; PIN, PROTEINASE INHIBITOR; PR, PATHOGENESIS-RELATED; PROPEP, PROPEPTIDE; R, resistance gene; RBOH, RESPIRATORY BURST OXIDASE HOMOLOG; ROS, reactive oxygen species; SA, salicylic acid; SAA, systemic acquired acclimation; SABP, SALICYLIC ACID BINDING PROTEIN; SAMT, SALICYLIC ACID METHYLTRANSFERASE; SAR, systemic acquired resistance; SE, sieve element; SFD, SUPPRESSOR OF FATTY ACID DESATURASE DEFICIENCY; *spr-2*, mutant SUPPRESSED IN 35S::PROSYSTEMIN-MEDIATED RESPONSES; SWR, systemic wound response; TMV, tobacco mosaic virus; TNV, tobacco necrosis virus; ZAT, ZINC FINGER PROTEIN OF *ARABIDOPSIS THALIANA*.

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Responses to Long-Distance (Systemic) Signaling

Systemic Wound Response

The SWR is triggered after localized attack of the plant by herbivorous insects and protects distal plant parts from subsequent infestation. Plants respond to chewing insects by activating a battery of defense genes. The most prominent wound-induced markers to date are the proteinase inhibitor (*PIN*) genes (Koiba et al., 1997; Schilmiller and Howe, 2005). In tomato (*Solanum lycopersicum*), the model plant for investigation of wound responses, *PIN1* and *PIN2* are induced upon plant colonization with herbivorous larvae of tomato hornworm (*Manduca sexta*) or mechanical wounding using a forceps (Green and Ryan, 1972; Koiba et al., 1997). The expressed proteins are inhibitors of the digestive proteinases of insects, and overexpression of *PIN2* reduced feeding and growth of the larvae (Koiba et al., 1997).

Exogenous application of jasmonic acid (JA) or methyl jasmonate (MeJA) induces *PIN* expression. This effect was suppressed in wounded JA-deficient mutants, suggesting that JA derivatives are involved in the regulation of *PIN* genes (Farmer et al., 1992; Li et al., 2002). Moreover, extensive microarray analyses in *Arabidopsis* wild-type plants and mutants in the JA perception or biosynthetic pathway demonstrated that the expression of defense genes after attack by herbivores was largely dependent on JA signaling (Reymond et al., 2004).

Almost 40 years ago, Green and Ryan (1972) found that wounding and insect attack induced *PIN* expression in both local and distant leaves. Since this breakthrough paper, many studies were undertaken to identify the systemic signal(s) involved in establishing the SWR. Among the candidate signals were the peptide hormone systemin that has been found exclusively in Solanaceae, JA derivatives, ethylene, abscisic acid (ABA), electrical signals, and reactive oxygen species (ROS) (for reviews see Schilmiller and Howe, 2005; Wasternack, 2007).

Systemic Acquired Resistance

SAR is a form of inducible disease resistance that is triggered in systemic tissues of plants undergoing a localized infection of shoot organs (Vlot et al., 2008a; Shah, 2009). This mode of signaling originates from bacterial, fungal, or viral infections that are battled by a specific defense pathway, namely the salicylic acid (SA) pathway (Vlot et al., 2009). Accumulation of SA in the systemic, healthy tissue of infected plants is required for SAR to occur and the resulting enhanced systemic resistance is active against all pathogens that are normally fended off via the SA pathway.

During an incompatible plant-pathogen interaction, recognition of a so-called pathogen-derived effector by a plant *RESISTANCE* (*R*) gene product quickly leads to accumulation of defense-related molecules, including SA and ROS, to relatively high concentrations (reviewed in Vlot et al., 2009). This, in turn, leads to the death of infected cells and cells surrounding the infected site in a hypersensitive response (HR) (Mur et al., 2008a) and SAR. The nature of long-distance signals mediating SAR is largely unknown, although there are several good candidates (Table 11.1), including derivatives of the plant hormones SA, JA, and auxin (Park et al., 2007; Truman et al., 2007, 2010), peptides (Xia et al., 2004), and lipids or lipid-derived molecules, including azelaic acid (AzA) (Jung et al., 2009) and glycerol-3-phosphate (Chanda et al., 2011). Additional signals include RNAs (Deeken et al., 2008), nitric oxide (NO), and/or S-nitrosylated peptides/proteins (Rustérucci et al., 2007; Gaupels et al., 2008).

Table 11.1 Relationship of signaling compounds with systemic responses and published research related to systemic transport or to the transport routes of the signals

Compound	Systemic response	Detected in SEs or STEs	Selected references (phloem related)
JA	SWR/SAR	Yes	Li et al., 2002; Hause et al., 2003; Truman et al., 2007; Chaturvedi et al., 2008; Glauser et al., 2009; Koo et al., 2009
SA	SAR	Yes	Yalpani et al., 1991; Shulaev et al., 1995; Mölders et al., 1996; Smith-Becker et al., 1998; Rocher et al., 2006, 2009
MeSA	SAR	Yes	Park et al., 2007; Rocher et al., 2009
AzA/AZI1	SAR	Yes	Jung et al., 2009
DIR1/glycerolipid-derived compound/G3P	SAR	Yes	Maldonado et al., 2002; Nandi et al., 2004; Chaturvedi et al., 2008; Mitton et al., 2009; Chanda et al., 2011
Systemin	SWR	Yes	McGurl et al., 1992, 1994; Narváez-Vásquez et al., 1995; Li et al., 2002; Lee and Howe, 2003
CDR1-derived peptide	SAR	No	Xia et al., 2004
H ₂ O ₂ /other ROS	SWR/SAR/SAA	No	Alvarez et al., 1998; Karpinski et al., 1999; Choi et al., 2007; Rossel et al., 2007; Miller et al., 2009
NO/GSNO/nitrated or S-nitrosylated proteins	SAR	Yes	Song and Goodman, 2001; Rustérucci et al., 2007; Gaupels et al., 2008
Electrical signaling	SWR	Yes	Wildon et al., 1992; Rhodes et al., 1996

AzA, azelaic acid; AZI1, azelaic acid insensitive 1; CDR1, constitutive disease resistance 1; DIR1, defective in induced resistance 1; GSNO, S-nitrosoglutathione; G3P, glycerol-3-phosphate; JA, jasmonic acid; MeSA, methyl salicylate; NO, nitric oxide; ROS, reactive oxygen species; SA, salicylic acid; SAA, systemic acquired acclimation; SAR, systemic acquired resistance; SEs, sieve elements; STEs, sieve-tube exudates; SWR, systemic wound response.

It is still under debate if and, if so, which SAR signals move by phloem transport. Early girdling experiments in cucumber that blocked phloem but not xylem transport showed that functional phloem is essential for SAR (Guedes et al., 1980). On the other hand, SAR in *Arabidopsis* does not appear to be restricted to leaves of the same orthostichy that are directly connected to the inoculated leaf (Kiefer and Slusarenko, 2003). Therefore, it is speculated that SAR signals are transported via the phloem and can in part be distributed by lateral transfer between anastomosing sieve tubes belonging to different orthostichies (Kiefer and Slusarenko, 2003) or by phloem-to-xylem transfer and subsequent xylem transport (van Bel and Gaupels, 2004; Rocher et al., 2006). Moreover, long-distance signaling within and between plants via air-borne volatile compounds has increasingly become of interest in recent years in relation to SWR and SAR (van Bel and Gaupels, 2004; Heil and Ton, 2008). Additional systemic signals, such as electrical potential and ROS, might move independently of the phloem by rapid cell-to-cell transport, which may affect amplification and propagation of the systemic signal (Wildon et al., 1992; Miller et al., 2009).

Systemic Acquired Acclimation

In addition to systemic responses to pests and pathogens, plants must systemically adapt to local abiotic stressors such as excess light. This signaling event is called systemic acquired acclimation

(SAA). If during the course of a day single leaves of a plant are exposed to intense sunlight, excess excitation energy can cause an electron overload of the photosynthetic electron transport chain, photoinhibition, and consequently accumulation of harmful ROS from the Mehler reaction (Rossel et al., 2007; Foyer and Noctor, 2009). Strikingly, the light-exposed leaf is able to transmit messages about the stress imposed to systemic leaves triggering them to better adapt to the excess light conditions by expression of genes involved in antioxidant defense and regulation of photosynthesis (Karpinski et al., 1997, 1999). Recently, the stress-inducible and ROS-sensitive transcription factors zinc finger protein of *Arabidopsis thaliana* (ZAT10) and ZAT12 of *Arabidopsis* have been shown to play a role in SAA (Rossel et al., 2007; Miller et al., 2009). Although H₂O₂ and ABA were suspected to be involved in SAA signaling via the phloem, the nature of the systemic signal(s) is still unknown (Karpinski et al., 1999; Rossel et al., 2007).

Phloem-Mobile Defense Signals

The identification of phloem-mobile systemic signals should meet the following criteria: candidate compounds should (i) accumulate in sieve-tube exudates after local stress, (ii) demonstrate phloem mobility, and (iii) induce stress resistance systemically if exogenously applied or overexpressed. To date, only SA/methyl salicylate (MeSA), JA derivatives, the lipid-derived molecule AzA and the peptide systemin were shown to be involved in phloem-based systemic signaling. However, even for these signals, many questions regarding the exact mode of transport, action in distant organs, and/or interaction with other signals remain unanswered. Other compounds such as ROS and NO will be discussed because of initial links to systemic signaling and phloem transport.

Jasmonic Acid

Lipids and fatty acids are primary metabolites of all living cells and mainly engaged in energy metabolism and membrane formation. They also comprise a versatile class of signaling compounds often referred to as lipid-derived signals. The oxylipin JA is a particularly well studied signaling molecule with multiple roles in plant development and flowering as well as in responses to herbivory and pathogen infection (Wasternack, 2007; Browse, 2009; Koo and Howe, 2009).

Upon insect chewing or mechanical injury, JA is rapidly synthesized from linolenic acid. After release of linolenic acid from chloroplast membranes by phospholipase activity and oxidation by lipoxygenase (LOX), the oxo-fatty acids are further processed by allene oxide synthase (AOS) and allene oxide cyclase (AOC) to give oxophytodienoic acid (OPDA). Subsequently, OPDA is reduced by OPDA reductase, after which shortening of one side chain in three β-oxidation cycles results in the formation of JA (reviewed by Wasternack, 2007; Browse, 2009).

Recent studies established that not JA itself but the JA-isoleucine (JA-Ile) conjugate that is synthesized by the enzyme JAR1, and other structurally related JA-amino acid conjugates represent major bioactive jasmonates (Staswick and Tirayaki, 2004; Katsir et al., 2008; Melotto et al., 2008). Furthermore, the stereochemistry of JA-Ile conjugates seems to be an important factor in determining activity of this compound. It is proposed that conversion of the (–) and (+) isomers of JA-Ile can impact bioactivities, and epimerization might play a role in regulating overall JA-mediated responses (Fonseca et al., 2009). JA and its derivatives were shown to be involved in local and systemic induction of the wound-marker gene *PIN2* (Li et al., 2002), while several lines of evidence suggest an essential role of JA in systemic signal transduction during the SWR (Koo and Howe, 2009).

As an initial indication for a role of JA in phloem-based signaling, enzymes of the JA biosynthesis pathway namely LOX, AOS, and AOC were localized to the sieve element (SE) plastids of tomato plants (Hause et al., 2000, 2003). Moreover, JA was detected in midvein tissue of wounded leaves (Stenzel et al., 2003; Glauser et al., 2008) and in sieve-tube exudates from pathogen-infected plants (Truman et al., 2007; Chaturvedi et al., 2008). The transport route of MeJA *in planta* was investigated using radioactive isotope tracing. In tobacco (*Nicotiana tabacum*), ^{11}C -labeled MeJA applied to a mature leaf moved both in phloem and xylem pathways with a vigorous exchange between the two vascular tissues (Thorpe et al., 2007). In contrast, ^{14}C -JA seemed to be mobile only in the phloem of *Nicotiana sylvestris* (Zhang and Baldwin, 1997). This discrepancy could be explained by the different chemical properties of MeJA and JA (Thorpe et al., 2007).

The collective studies discussed in the previous paragraph have established that JA is produced and transported in the phloem (Figure 11.1). The intriguing question whether JA itself acts as the essential systemic signal in SWR was addressed by employing JA mutants in grafting experiments

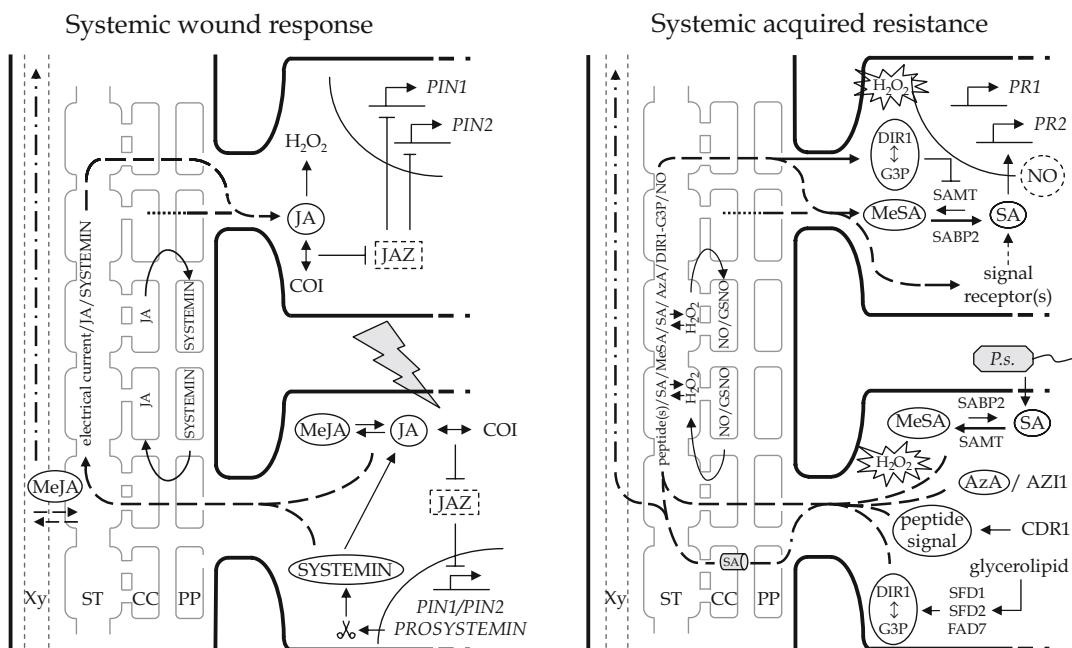


Figure 11.1 Hypothetical models integrating mobile signals related to systemic wound response and systemic acquired resistance and their systemic transport. Transport is indicated from a source to a sink leaf, including exchange of signals between phloem and xylem. Signal propagation along the route is indicated by curved arrows. Signaling upstream (in source leaf) and downstream (in sink leaf) from the transported signals are rudimentarily summarized. Upon wounding (flash arrow) JA is induced and interacts with COI (CORONATINE INSENSITIVE), which in turn induces degradation of the transcriptional repressor JAZ (JASMONATE ZIM-DOMAIN; for details on additional signaling partners and mechanisms, see Koo and Howe, 2009). *PR1* and *PR2* are induced downstream from SA via a redox-dependent molecular mechanism that is in part regulated by NO/S-nitrosylation (for details on additional signaling partners and mechanisms, see Vlot et al., 2009). *P.s.*, *Pseudomonas syringae*; PP, phloem parenchyma; CC, companion cell; ST, sieve tube; Xy, xylem tissue; broken lines indicate transport with arrowheads indicating direction; solid arrows indicate direct or indirect induction; dotted arrow from signal receptors to SA indicates presumed induction; solid circles indicate systemic signals; dotted boxes or circles indicate regulation of signaling; SA in tube indicates active transport mechanism; star shape indicates an oxidative burst.

(Li et al., 2002). Whereas the tomato suppressed in 35S::prosystemin-mediated responses (*spr-2*) mutant is impaired in JA biosynthesis, the *jai-1* mutant is insensitive to JA without JA biosynthesis being affected. Grafted mutant plants were wounded at the lower stock leaf and *PIN2* expression was analyzed in systemic scion leaves. Significantly, systemic induction of *PIN2* could be observed only when a *spr-2* scion was grafted onto a *jai-1* rootstock, but not in the reciprocal graft. This indicates that production of JA in the wounded leaf and perception of JA in systemic leaves is pivotal for induction of SWR (Li et al., 2002).

The most straightforward explanation for these results would be phloem transport of JA from the site of injury to distant leaves where it elicits defense gene expression. However, current findings do not support this hypothesis. In wounded *Arabidopsis* plants, rapid JA and JA-Ile accumulation resulted, at least in part, from *de novo* synthesis of these compounds in systemic leaves rather than transport from the damaged leaf (Koo et al., 2009). Moreover, two independent studies revealed that the speed of the signal triggering systemic jasmonate synthesis after a remote stimulus was in the range of 2 or 3.4–4.5 cm/min (Glauser et al., 2009; Koo et al., 2009). This would argue against signal translocation in the assimilate stream that usually flows at much lower rates of 0.5–1 cm/min (Geiger et al., 1969; Christy and Fisher, 1978; Fisher, 1990), although velocities of up to 25 cm/min have been reported (Baker and Milburn, 1989).

Wound-triggered systemic *PIN2* expression in tomato was independent of phloem mass flow, but correlated with systemic electrical signaling (Wildon et al., 1992). Rapid transduction of the systemic signal was, at least to some extent, dependent on the phloem connections (Rhodes et al., 1996; Glauser et al., 2009). After wounding a single *Arabidopsis* leaf, JA increased to ninefold higher levels in orthostichous systemic leaves—that is, leaves with direct vascular connections—than in nonorthostichous leaves (Glauser et al., 2009). Given that the low impedance in the sieve tubes would facilitate electrical signal propagation, it is tempting to speculate that upon wounding a rapid electrical signal propagates through the phloem and elicits JA synthesis and *PIN2* expression along the phloem pathway (Figure 11.1; Stankovic and Davies, 1996; Fromm and Lautner, 2007). Also wound-induced hydraulic signals in the xylem are linked to electrical signaling by mechanosensory ion channels in vascular parenchyma cells (Fromm and Lautner, 2007). In summary, a role of JA in SWR is likely, although it remains unclear if the hormone serves as the primary systemic signal.

Much less is known about a putative function of JA in SAR. Interestingly, at 5-hour after infection with an avirulent strain of *Pseudomonas syringae* JA was detected in sieve-tube exudates, suggesting phloem transport of this hormone during early SAR signaling (Truman et al., 2007). Moreover, exogenous application of JA induced SAR and SAR was attenuated in *Arabidopsis* JA biosynthesis and response mutants (Truman et al., 2007). However, in independent experiments by other research groups, JA mutants did not display reduced SAR as compared to wild-type plants (Attaran et al., 2009). Infiltration of JA and MeJA did not induce SAR and JA did not copurify with a SAR-inducing HPLC fraction of sieve-tube exudates from *P. syringae* infected plants although these exudates indeed displayed elevated levels of JA (Chaturvedi et al., 2008; Xia et al., 2010).

Current knowledge infers that JA might be an early phloem-mobile alarm signal systemically regulating JA-responsive genes upon pathogen infection (Truman et al., 2007), but it does not appear to be a long-distance signal in SAR.

Salicylic Acid

SA mediates resistance in aerial tissues against viruses and (hemi-) biotrophic bacteria and fungi. The bulk of SA that is induced by pathogens is synthesized in the chloroplast from isochorismate

following synthesis of isochorismate from chorismate by the enzyme ISOCHORISMATE SYNTHASE 1 (ICS1). Chorismate, which is derived from the shikimate pathway, also is converted to phenylalanine via multiple enzymatic steps. A minor source of pathogen-induced SA likely is derived from the phenylpropanoid pathway upon conversion of phenylalanine by phenylalanine lyase (PAL) into cinnamic acid (CA). SA biosynthesis and metabolism have been reviewed (Garcion and Métraux, 2006; Vlot et al., 2009).

Biological Significance in Long-Distance Signaling

SA is the principal regulatory hormone involved in SAR; SA-related signaling in the primary inoculated leaf contributes to SAR signal generation (Vlot et al., 2009) and accumulation of SA in systemic leaves is essential to trigger SAR (Vernooij et al., 1994). SA moves systemically in plants (Shulaev et al., 1995; Mölders et al., 1996), nevertheless does not appear to be the mobile signal triggering the onset of SAR in systemic plant parts. The latter was most strikingly shown by using transgenic plants expressing the bacterial SA hydroxylase NahG that degrades SA to catechol. *NahG* plants cannot accumulate SA or mount an SAR response. SAR experiments in grafts of wild-type and *NahG* tobacco plants revealed that an *NahG* rootstock is capable of generating a SAR signal that triggers SAR in a wild-type scion (Vernooij et al., 1994). *Vice versa*, an *NahG* scion is not capable of mounting SAR in response to a wild-type SAR signal. Therefore, SA is not required for SAR signal generation, but is essential for SAR signal perception and/or propagation in the systemic tissue.

It is somewhat difficult to reconcile SA mobility in plants (see Section 2.2.2.) with the fact that it is not the systemic SAR signal. In addition to disease resistance, SA is involved in thermotolerance and flowering (reviewed in Vlot et al., 2009). Its long-distance transport may be essential for these processes in addition to disease resistance. In light of its function as a signaling molecule, it also is of interest that SA is propagated by a positive feedback loop. In this loop, SA cooperates with various signaling partners, including H₂O₂, to enhance SA signaling (Vlot et al., 2009). This, in turn, leads to the hypothesis that mobile SA may propagate SA signaling along its route, for example, in phloem companion cells (CCs), thereby strengthening the SA-dependent SAR signal (Figure 11.1). Such a scenario is supported by findings of *de novo* synthesis of SA and 4-hydroxybenzoic acid (4-HBA), in petioles and stems of cucumber plants upon infection of leaves with *P. syringae* pv *syringae* (Smith-Becker et al., 1998). Accumulation of SA and 4-HBA in sieve-tube exudates from infected plants continued to rise as compared to their levels in similar exudates from uninfected plants long after leaf excision.

Since the discovery that SAR triggered by infection of *Arabidopsis* leaves with *P. syringae* also depends on SA (Summermatter et al., 1995), *Arabidopsis* genetics has been used to unravel the signal(s) that cause SAR. One of the most promising candidate SAR signals was originally identified in tobacco and is a derivative of SA: MeSA (Park et al., 2007). Sieve-tube exudates from tobacco mosaic virus (TMV)-infected tobacco leaves contain MeSA, also when these leaves express *NahG*. Moreover, conversion of SA to MeSA by salicylic acid-methyltransferase 1 (SAMT1) is required in inoculated tissues for SAR to occur. At the same time, the enzymatic active site of the MeSA esterase salicylic acid binding protein2 (SABP2) (SA-BINDING PROTEIN2), which converts MeSA to SA, is necessary only in the systemic plant parts, presumably for SAR signal perception. Earlier studies have shown that MeSA can induce SAR-like disease resistance (Shulaev et al., 1997), but is not biologically active itself (Seskar et al., 1998). Together, this would make SABP2 the receptor of the SAR signal MeSA converting it to the active compound SA (Figure 11.1) (Vlot et al., 2008a). Although under discussion (Vlot et al., 2008b, 2009; Attaran et al., 2009; Liu et al., 2010, 2011), the SAR signal MeSA appears to be conserved between plant species and plays a significant role in SAR in tobacco (Park et al., 2007), *Arabidopsis* (Vlot et al., 2008b; Park et al., 2009; Liu et al.,

2010, 2011), and potato (*Solanum tuberosum*) (Manosalva et al., 2010). In *Arabidopsis* new findings suggest the existence of at least two signaling mechanisms leading to SAR, one dependent on MeSA and one dependent on light (Liu, Von Dahl, and Klessig, personal communication). The requirement of MeSA for a successful SAR response in *Arabidopsis* depends on the time of day of the primary infection: an inverse correlation was observed between the requirement of MeSA for SAR and the length of light exposure following the initial SAR trigger.

Long-Distance Mobility

Definitive proof of SA long-distance mobility came from two studies using radio-labeled compounds. In tobacco, $^{18}\text{O}_2$ -labeling of one leaf undergoing TMV infection resulted in the accumulation in systemic leaves of SA, of which up to 70% was $^{18}\text{O}_2$ -labeled. Since the systemic tissue was kept in ambient ($^{16}\text{O}_2$ -containing) air, systemic $^{18}\text{O}_2$ -labeled SA had originated from the infected leaf (Shulaev et al., 1995). In cucumber (*Cucumis sativus*), ^{14}C -labeled benzoic acid (BA) fed to cotyledons, was converted to ^{14}C -SA upon infection of the cotyledons with tobacco necrosis virus (TNV). Up to 14% of the SA accumulating in distant organs was radio labeled and had therefore originated from the infected tissue (Mölders et al., 1996). Transport of SA was further supported by the presence of ^{14}C -SA and the absence of ^{14}C -BA in sieve-tube exudates. These data show that during SAR at least a portion of systemically accumulating SA is transported from the infected tissue to systemic organs. It is difficult to determine exactly the amount of transported SA, because the labeled compounds are diluted by unlabeled *de novo* synthesized SA, particularly in the ^{14}C -BA feeding experiment (Mölders et al., 1996). Moreover, labeling studies, in which ^{14}C -labeled SA precursors were fed to systemic leaves of TNV-infected cucumber, showed that part of SA having accumulated originates from *de novo* synthesis in the systemic leaves (Meuwly et al., 1995).

Depending on the plant species, sieve-tube loading of compounds occurs apoplasmically or symplasmically. In the latter case, SA could freely diffuse through plasmodesmata from the mesophyll to the sieve tubes. Most plant species studied in relation to SAR, including tobacco and *Arabidopsis*, but not cucurbits, are apoplastic phloem loaders (Bürkle et al., 1998; Gottwald et al., 2000). Yalpani et al. (1991) first described accumulation of SA in sieve-tube exudates taken from TMV-infected tobacco leaves and advanced that the physicochemical properties of SA are optimal for phloem transport. In support of these early studies, Rocher et al. (2006) detected high levels of SA in sieve-tube exudates of the symplasmic–apoplastic phloem loader *Ricinus communis* (castor bean) in a pH-dependent manner upon feeding of SA to cotyledons. Loading of SA onto castor bean phloem most likely occurs at least in part from the apoplast via (a) pH-dependent carrier system(s) (Rocher et al., 2006, 2009) (Figure 11.1). Rocher et al. (2006, 2009) reached this conclusion based on three findings. (1) Prediction of the physicochemical properties of BA, 3-HBA, and 4-HBA leads to the conclusion that these compounds are better able to diffuse through membranes than SA. However, in *in planta* experiments SA was taken up by castor bean phloem more efficiently than either of the BA derivatives. (2) By contrast, poorly diffusible SA derivatives were relatively efficiently phloem mobile. (3) A chemical inhibitor (pCMBS) of carrier activities essential for phloem loading of sucrose inhibited accumulation of radio-labeled SA in veins of castor bean cotyledons.

Rocher et al. (2006) detected levels of SA in xylem sap that were more than 100-fold lower than in sieve-tube sap collected from the same castor bean seedlings. The authors suggest that this might reconcile apparent phloem mobility of the SA/SAR signal with the observation that SAR is not limited to orthostichous leaves (Kiefer and Slusarenko, 2003) if SA is transported from infected mature leaves via phloem and is redistributed to other mature leaves via xylem transport. In order to move from the phloem to the xylem vessels, SA/MeSA must cross at least one plasma membrane. In this light, it is of interest to note that the potential SAR signal MeSA is

considerably more membrane permeant than SA, but less likely to move via the phloem stream and even modeled as “xylem mobile only” (Rocher et al., 2009), although it was detected in sieve-tube exudates from TMV-infected tobacco leaves (Park et al., 2007). Due to its ability to diffuse through membranes, MeSA can freely move between phloem and xylem to induce SAR in orthostichous and nonorthostichous tissues (Figure 11.1).

Lipid-Derived Molecules Other Than JA

Genetic studies in *Arabidopsis* hinted at involvement of lipid-derived molecules other than JA in SAR signaling cascades. For example, a role in SAR was recently implied for the C9 metabolite AzA (Jung et al., 2009). AzA was found in sieve-tube exudates from SAR signal-emitting *Arabidopsis* leaves and was able to induce defense in wild-type plants both in AzA-treated and systemic leaves. Expression of a gene encoding, a predicted secreted protease inhibitor/seed storage/lipid transfer protein, azelaic acid induced1 (AZI1), was induced by AzA. Reciprocal experiments analyzing defense gene induction by sieve-tube exudates from infected wild-type and *azil1* mutant plants showed that AZI1 is required for generation or transmission of SAR signals, but not for their systemic perception (Figure 11.1; Jung et al., 2009).

Another predicted lipid transfer protein (LTP), Defective in induced resistance 1 (DIR1), does not appear to be chemically related to AZI1, but is essential for SAR (Maldonado et al., 2002). Sieve-tube exudates from infected wild-type plants induced defense gene expression in wild-type plants, whereas similar exudates from *dir1* plants did not. Conversely, *dir1* mutant plants responded normally to sieve-tube exudates from infected wild-type plants. This shows that DIR1 also is required for SAR signal generation or transmission, but not for its perception in systemic plant parts. Similar to *dir1*, several mutants were affected at the level of chloroplastic glycerolipid metabolism displayed normal local resistance responses, but no SAR induction (Nandi et al., 2004; Chaturvedi et al., 2008). Sieve-tube exudates from infected leaves of the *sfd1*, *sfd2*, or *fad7* mutants also did not induce defense gene expression in wild-type plants, whereas all mutants displayed a normal response to similar exudates from wild-type plants. The *FAD7*-dependent SAR signal is capable of inducing pathogen resistance in plant species as diverse as tomato and wheat demonstrating strong conservation of this defense signal in the plant kingdom (Chaturvedi et al., 2008). Potential cross-species conservation of the *DIR1*-dependent SAR signal was recently hypothesized after finding a *DIR1* homolog in sieve-tube exudates from tomato plants (Mitton et al., 2009).

Sieve-tube exudates from infected *dir1* plants reconstituted defense-inducing activity of similar exudates from *sfd1* or *fad7* plants indicating that DIR1 may act in concert with a glycerolipid-derived signal to trigger SAR (Figure 11.1; Chaturvedi et al., 2008). Although an independent study could not demonstrate a correlation between *FAD7* and SAR (Xia et al., 2010), a role of *suppressor of fatty acid desaturase deficiency* (SFD1) in SAR remains undisputed. A recent study showed that the DIR1-dependent (and possibly SFD1-dependent) signal likely fortifies the MeSA-dependent SAR signal by shifting the equilibrium between MeSA and SA in the systemic tissue toward the active defense-inducing compound SA (Figure 11.1; Liu et al., 2011). The DIR1-dependent SAR signal does so by repressing expression of the gene encoding the enzyme that supports methylation of SA. Liu et al. (2011) hypothesized that this is essential for MeSA-mediated SAR in tobacco and *Arabidopsis*.

Intriguing new findings suggest that accumulation of glycerol-3-phosphate (G3P) downstream from SFD1 is essential for SAR (Chanda et al., 2011). Although G3P alone did not induce SAR, its addition to sieve-tube exudates from infected *gly1* (allelic to *sfd1*), mutant plants restored the SAR inducing capacity of the otherwise inactive exudates. The interdependence of DIR1 and SFD1

or *FAD7*-dependent SAR signals was challenged by findings that the AzA signal requires *DIR1* and SA, but not *SFD1* or *FAD7* for priming of defense (Jung et al., 2009). However, the plant-derived factor essential for SAR downstream from G3P appears to be DIR1 (Figure 11.1; Chanda et al., 2011). Chanda et al. (2011) reached this conclusion based on two findings: (1) G3P did not reconstitute SAR inducing activity in sieve-tube exudates from infected *dir1* mutant plants, and (2) G3P significantly enhanced the SAR inducing capacity of recombinant DIR1 protein.

Other nonspecific (ns)LTPs similar to AZA1 and DIR1 play a role in resistance as so-called pathogenesis-related proteins (PR14 family; Van Loon and Van Strien, 1999). Roles in SAR have been documented for pathogen-derived LTPs, termed elicitors, being complexed with sterol lipids (Osman et al., 2001). Also, expression of either of two pepper LTPs in rootstocks of grafted tobacco plants induced resistance in wild-type scions against at least two different pathogens (Sarowar et al., 2009). It is largely unknown which lipid substrates activate or are carried by these 7–9 kDa nsLTPs. Tobacco LTP1 binds JA with high affinity and treatment of decapitated tobacco plants with an LTP1-JA complex significantly enhanced resistance of the plants against *Phytophthora parasitica* (as measured by evaluating internal necrotic symptoms inside of infected stems; Buhot et al., 2004). It is compulsory to study LTP-lipid complexes to further unravel the role of lipids in inducible stress resistance in plants.

Peptides

The first peptide hormone identified in plants was named systemin due to its functioning in systemic induction of *PIN* genes upon local wounding (Ryan et al., 2002). Tomato systemin is an 18-amino-acid peptide derived by proteolytic cleavage from prosystemin (Pearce et al., 1991). Antisense silencing of the prosystemin gene directed by the cauliflower mosaic virus (CaMV) 35S promoter largely suppressed systemic *PIN* expression whereas overexpression under control of the same promoter caused constitutive expression of defense genes suggesting an essential role of systemin in SWR (McGurl et al., 1992, 1994).

Notably, defense genes were also induced in wild-type scions grafted onto an unwounded *CaMV35S::PROSYSTEMIN* rootstock. These findings inferred that either systemin itself or a systemin-induced signal traveled from rootstock to scion (Ryan et al., 2002). Systemic mobility of systemin was observed after application of ¹⁴C-labeled systemin to a local wound site (Narváez-Vásquez et al., 1995).

Recent studies argue against systemin acting as a long-distance message in the SWR. When JA mutant scions were grafted onto *CaMV35S::PROSYSTEMIN* plants, *PIN2* was expressed in scions unable to synthesize JA, but not in scions defective in JA perception. Thus, JA perception but not synthesis is essential for systemin-induced systemic defense gene expression (Li et al., 2002). In contrast, *PIN2* expression was not affected in mutant scions defective in systemin perception when grafted onto wounded WT rootstocks inferring that the peptide signal is not perceived in distant leaves (Lee and Howe, 2003).

PROSYSTEMIN is expressed in phloem parenchyma cells and the unidentified systemin receptor is likely to be phloem-localized (Narváez-Vásquez and Ryan, 2004; Hind et al., 2010). Since JA is produced within the CC-SE complex, Stenzel et al. (2003) proposed a tight interaction of JA and systemin in wound signal transduction through the phloem. According to a current model, systemin and JA interact through a positive feedback loop to propagate long-distance signaling in the SWR (Figure 11.1) (Ryan and Moura, 2002; Schilmiller and Howe, 2005). However, the exact mode of systemin/JA interaction is still ambiguous.

Systemin is present in tomato, potato, black nightshade (*Solanum nigrum*), and bell pepper (*Capsicum annuum*), but not in tobacco. Other 15–20 amino-acid peptides were identified in tobacco and tomato. These hydroxyproline-rich systemins (HypSys) have the interesting feature that two peptides in tobacco and three peptides in tomato were released from a single precursor (Pearce et al., 2001; Pearce and Ryan, 2003). Although HypSys and systemin do not share sequence homology, they cooperatively regulated SWR in tomato (Narváez-Vásquez et al., 2007). Additional research is required to establish a role for wound-responsive peptide signals in plants other than the *Solanaceae*.

A family of *Arabidopsis* peptides similar to systemin called *AtPep1-6* is thought to help propagate stress defense signaling (reviewed by Ryan et al., 2007). Expression of the respective precursor genes propeptide (*PROPEP1-6*) is regulated by SA and JA. In addition, the PROPEP-derived signaling peptides *AtPep1-6* induce expression of their precursor genes, indicating a positive feedback mechanism. Since the plasma membrane-bound receptor-like kinase peptide receptor1 (PEPR1) and its close homolog PEPR2 are responsible for perception of *AtPep1* and several of its family members (Yamaguchi et al., 2006; Krol et al., 2010), the *AtPeps* are thought to propagate stress defense signaling by cell-to-cell communication (Ryan et al., 2007). It will be interesting to determine whether the *AtPeps* are phloem-mobile and can transfer “danger” signals to the systemic tissue.

The first hint regarding the activity of peptide signals in systemic disease resistance arose from the characterization of a gain-of-function *Arabidopsis* mutant affected in the constitutive disease resistance1 (*CDR1*) locus (Figure 11.1; Xia et al., 2004). *CDR1* encodes an aspartic protease that resides in the apoplast. Overexpression of *CDR1* from a transgene driven by a chemically inducible promoter activates SA-mediated disease resistance both in *CDR1*-expressing leaves and systemically in wild-type, non-*CDR1*-expressing scions micrografted onto *CDR1*-expressing rootstocks. A bioassay using size exclusion chromatography fractions of intercellular washing fluids from *CDR1*-expressing tissue revealed that a 3–10 kDa peptide induces expression of the defense-related *PR2* (pathogenesis-related gene2) gene both locally and systemically. The identity of this *CDR1*-dependent peptide SAR signal as well as its transport mode has yet to be established, but it is of significance that the undefined peptide can move across a graft junction (Xia et al., 2004). *CDR1* and its function in defense are conserved between *Arabidopsis* and rice (*Oryza sativa*) (Deo Prasad et al., 2009, 2010). This recent discovery will stimulate additional studies to find the *CDR1* substrate and product as well as its mode of transport and action during SAR.

Reactive Oxygen Species

ROS are regarded as general stress signals in plant responses to adverse environmental conditions, wounding, and pathogen infection (Jaspers and Kangasjärvi, 2010; Torres, 2010). ROS arise from the photosynthetic and respiratory electron transport chains as well as from enzymatic reactions catalyzed by oxidases and peroxidases (Foyer and Noctor, 2009). Rapid accumulation of ROS during the stress-induced oxidative burst is often mediated by isoenzymes of the NADPH oxidase respiratory burst oxidase homolog (RBOH, Torres, 2010). This enzyme produces superoxide radicals (O_2^-) that are rapidly dismutated to hydrogen peroxide (H_2O_2) by superoxide dismutase.

The water-soluble H_2O_2 has been implicated in systemic signaling events in SAR (Figure 11.1). Infection of an *Arabidopsis* leaf with avirulent *P. syringae* induced a local oxidative burst and HR. Systemic periveinal microbursts, micro-HRs detectable only at microscopic level, and consequently SAR occurred within 1–2 hours after infection. Strikingly, SAR could be induced by injection of the H_2O_2 -generating glucose/glucose oxidase system that was suppressed by the NADPH oxidase

inhibitor diphenyliodonium and by catalase prompting the authors to suggest H₂O₂ as a candidate phloem-mobile signal (Alvarez et al., 1998). An extracellular peroxidase has been identified as an alternative source of H₂O₂ in local and systemic ROS signaling during SAR in pepper plants (Choi et al., 2007).

By using transgenic *Arabidopsis* expressing the reporter gene *LUCIFERASE* under control of the H₂O₂-inducible *ascorbate peroxidase2* (*APX2*) promoter Karpinski et al. (1999) uncovered a crucial function of vascular H₂O₂ in eliciting SAA in response to excess light stress. In most studies, diaminobenzidine (DAB) staining was employed for detection of H₂O₂ in systemic leaves of SWR-, SAR-, and SAA-induced plants. However, unambiguous proof for systemic (phloem-based) transport of H₂O₂ from stressed to unstressed leaves is missing. In fact, Rossel et al. (2007) did not observe DAB staining in systemic leaves after induction of SAA. Since mutants defective in ABA, JA, and SA signaling were capable of establishing SAA, the nature of the systemic signal remains unknown.

Similar to other signals' detection of H₂O₂ in the vascular tissue does not necessarily mean that this ROS is a systemic signal. For example, during SWR in tomato, H₂O₂ accumulates in the vascular bundles of systemic leaves probably acting as a second messenger of JA in defense gene induction (Orozco-Cárdenas et al., 2001). Due to their high reactivity, ROS generally are not likely to be systemically translocated without signal amplification by continuous induction of ROS production along the transport route. Signal transduction by ROS is controlled both on the level of production as well as degradation. Therefore, ascorbate peroxidase in the vascular bundle sheath cells of *Arabidopsis* (Karpinski et al., 1999) and the complete antioxidant system present in SEs of cucurbits (Walz et al., 2002) might regulate local, vascular-internal, and systemic ROS signaling.

In the context of signal amplification, it is noteworthy that Miller et al. (2009) reported on rapid signal transduction in *Arabidopsis* by ROS amplification/propagation processes. ROS signaling was investigated by use of transgenic plants expressing the *LUCIFERASE* reporter gene under control of the ROS-inducible *ZAT12* promoter. Systemic signaling was elicited by wounding, excess light, and by temperature or salt stress, and moved rapidly at a rate of 8.4 cm/min both in acropetal and basipetal direction. Using signaling mutants it was shown that the ROS signal was dependent on the RBOH isoform D, but independent of ethylene, JA, and SA. Interaction of ROS, calcium, and electrical signals in systemic stress signaling has not been investigated further and the exact route of signal transport is unknown.

Nitric Oxide

During the past decade NO has emerged as an important signal molecule in plant stress responses (Del Río et al., 2004; Besson-Bard et al., 2008; Neill et al., 2008; Leitner et al., 2009). For instance, NO accumulation was detected upon wounding of *Arabidopsis* and sweet potato (*Ipomea batatas*) leaves (Jih et al., 2003; Huang et al., 2004). In the defense against pathogens, NO plays well-documented parts in establishing HR as well as in the formation of cell-wall appositions during powdery mildew interactions with barley (*Hordeum vulgare*) (Mur et al., 2006). Moreover, NO is a potent regulator of defense-related gene expression (Grün et al., 2006) and enzyme activity (Clark et al., 2000; Navarre et al., 2000; Igamberdiev et al., 2006).

Use of mammalian nitric oxide synthase (NOS) inhibitors revealed that a NOS-like enzyme was activated during plant stress reactions and induced defense gene expression in *Arabidopsis* (Foissner et al., 2000; Zeidler et al., 2004). Although arginine-consuming NOS-like NO production has been demonstrated in various plant species, no plant NOS could be identified so far (Besson-Bard et al.,

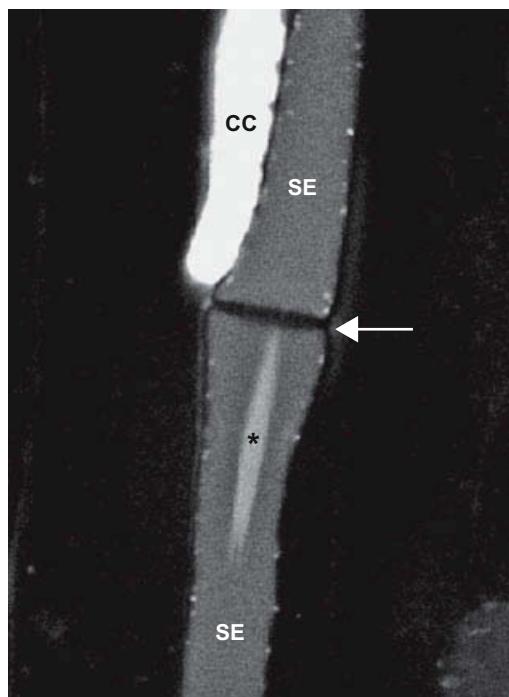


Figure 11.2 Nitric oxide (NO) production in the phloem of *Vicia faba* as detected by 4,5-diaminofluorescein diacetate (DAF-2DA). The exposed intact phloem tissue was treated with 1 mM H₂O₂ and NO-specific DAF-2DA fluorescence (black to white gradient indicates low to bright fluorescence) was observed with a confocal laser scanning microscope 10 minutes after treatment. A representative image of H₂O₂-induced fluorescence in a companion cell (CC) and two sieve elements (SE) of *V. faba* is shown. The spindle-shaped forisome is a specific feature of Fabaceae. After treatment with 1 mM H₂O₂ the forisome dispersed within 1 minute in a calcium-dependent manner occluding the sieve element and recondensed within 10 minutes. Asterisk, forisome. Arrow head indicates the position of the sieve plate between the two SEs.

2008; Leitner et al., 2009). Other workers claimed that nitrite is a source of NO in plants, derived either nonenzymatically at high pH or via nitrate reductase (NR) activity (Del Río et al., 2004; Neill et al., 2008).

In view of its high mobility, NO has been proposed to be a systemic stress signal (Durner and Klessig, 1999; Foissner et al., 2000; van Bel and Gaupels, 2004; Gaupels et al., 2008; Neill et al., 2008). In support of this suggestion, using the specific NO-sensing fluorochrome 4,5-diaminofluorescein diacetate (DAF-2 DA), NO was detected in vascular bundles where it was shown to function in senescence, cell wall lignifications, salt stress response, and pathogen defense (Corpas et al., 2004; Gabaldón et al., 2005; Requena et al., 2005; Valderrama et al., 2007). In a detailed microscopic study of intact phloem, NO synthesis was shown to occur in CCs of broad bean (*Vicia faba*) (Figure 11.2; Gaupels et al., 2008). SA and H₂O₂ induced a strong NO-specific DAF fluorescence in CCs that was dependent on calcium and could be blocked by inhibitors of NOS, but not NR. Moreover, NO was microscopically detected in SEs suggesting systemic transport of NO or NO-binding compounds.

To date, two publications have reported the involvement of NO signaling in SAR. Injection of NO donors into tobacco leaves reduced the size of lesions caused by TMV on treated and systemic

nontreated leaves (Song and Goodman, 2001). Also, local treatment with NOS inhibitors or an NO scavenger attenuated SAR in distant leaves (Song and Goodman, 2001).

S-nitrosoglutathione (GSNO) is known to be a natural reservoir and carrier of NO and might act as a phloem-mobile signal. Accordingly, Rustérucci et al. (2007) found that *Arabidopsis* antisense lines of the GSNO catabolizing GSNO reductase (GSNOR) displayed elevated resistance and constitutive SAR. They also observed that GSNOR was primarily located in CCs and proposed that inhibition of GSNOR, facilitating the accumulation and transport of GSNO in sieve tubes, might be an important factor in the generation of SAR (Figure 11.1). In systemic leaves GSNO could induce resistance gene expression according to its local effect on tobacco leaves (Durner et al., 1998). However, this hypothesis is challenged by the fact that T-DNA insertion mutants of *AtGSNOR1* displayed decreased SA levels and compromised *R* gene-dependent resistance, basal resistance, as well as nonhost resistance (Feechan et al., 2005).

The most commonly investigated NO modifications of proteins are nitration of tyrosine residues and S-nitrosylation of cysteine residues (Leitner et al., 2009). Immuno-blot analyses with nitrotyrosine antibodies identified nitrated proteins in sieve-tube exudates of pumpkin plants that were wounded (Gaupels, 2006) or watered with 10 mM H₂O₂ (Gaupels et al., 2008). Similarly, Valderama et al. (2007) visualized *in situ* nitrated and S-nitrosylated proteins in the vascular tissue of salt-stressed olive (*Olea europaea*) plants. NO-binding phloem proteins were not identified in either of these studies.

NO and ROS interact with each other and multiple other signaling molecules increasing the complexity of these systemic signal transduction pathways. For instance, H₂O₂ and NO cooperate in pathogen defense (Mur et al., 2006) and abiotic stress responses (Neill et al., 2008). NO might facilitate H₂O₂ signaling by inhibiting the antioxidant enzymes ascorbate peroxidase and catalase (Clark et al., 2000; Zeier et al., 2004). In addition, 100 and even 10 μM H₂O₂ induced NO synthesis in the phloem of broad bean (Gaupels et al., 2008). Finally, ROS and NO are known to cooperate with ethylene, JA, and SA in stress signaling (Mur et al., 2008b; Neill et al., 2008; Foyer and Noctor, 2009; Jaspers and Kangasjärvi, 2010; Torres, 2010).

Future Directions

Various signaling compounds that are involved in inducible stress resistance responses are transported systemically through the vasculature (Figure 11.1). JA and systemin, but also electrical signals and possibly H₂O₂, play major parts in SWR, whereas SA, LTPs, and associated lipid-derived signals, peptides, NO, and H₂O₂ are mainly associated with SAR.

A single essential phloem-mobile signal has not been unequivocally identified for any of the systemic signaling events. This is due to several reasons that complicate phloem research. The phloem tissue is not readily accessible creating difficulties in isolating homogeneous samples. Phloem samples are most commonly analyzed in plant species such as castor bean or cucurbits that readily exude sieve-tube contents from cut leaves or stems. Samples from other plants such as *Arabidopsis* and members of the *Solanaceae* are typically obtained as EDTA-facilitated sieve-tube exudates from petioles. In both cases, exudates can be contaminated by wound-related cellular signals from nonvascular cells (van Bel and Gaupels, 2004; Gaupels et al., 2008b; Turgeon and Wolf, 2009). Therefore, only a relatively few studies have screened for systemic signals by analyzing changes in the composition of sieve-tube exudates after onset of long-distance signaling.

A further difficulty in the quest for systemic signals is attributed to distinguishing between local, phloem-internal, and long-distance signals. This issue could be addressed by using mutants defective

in the synthesis and perception of the signal in grafting experiments. Alternatively, phloem-targeted expression of genes involved in the production or degradation of a candidate signal in transgenic plants could be a useful approach to analyze systemic signaling.

An increasing number of reports indicate that phloem signaling is complex and probably involves a network of messenger molecules and self-amplification loops along the pathway rather than a single translocated signal. If different signals are present in plants at the same time, these signals must interact before, during, or after long-distance transport, and the outcome of such interactions will determine the final systemic response. For instance, the JA and systemin signals together presumably strengthen SWR by positive feedback propagation of the combined signal. ABA was also suspected to function in SWR, although the exact mode of action and possible interaction with JA is still ambiguous (Peña-Cortés et al., 1995). Similarly, SA, H₂O₂, and NO could form a positive feedback loop that would propagate the SAR signal as it moves along the vasculature.

In addition to positive feedback regulation or synergism between signals, signaling events are often regulated by negative feedback loops or antagonism between different signaling pathways. JA and SA form a classic example of two antagonistic signaling pathways. By inference, plants that have adopted an SWR state can no longer adequately defend themselves against pathogen attack and *vice versa*. Another antagonistic interaction takes place between SA and auxin signaling (Wang et al., 2007), whereas auxin signaling appears to be a prerequisite for SAR (Truman et al., 2010). To reconcile these antagonistic interactions with a possible positive regulatory role of jasmonates and auxin in SAR, Truman et al. (2007, 2010) suggest a spatial or temporal separation of different long-distance signals.

Thus, a sequence or even parallel signaling events might occur rather than a single signal cascade or network (Figure 11.3). In this regard, it should be noted that wounding induced rapid signaling by electrical potential waves and H₂O₂ within 1–5 minutes (Wildon et al., 1992; Miller et al., 2009).

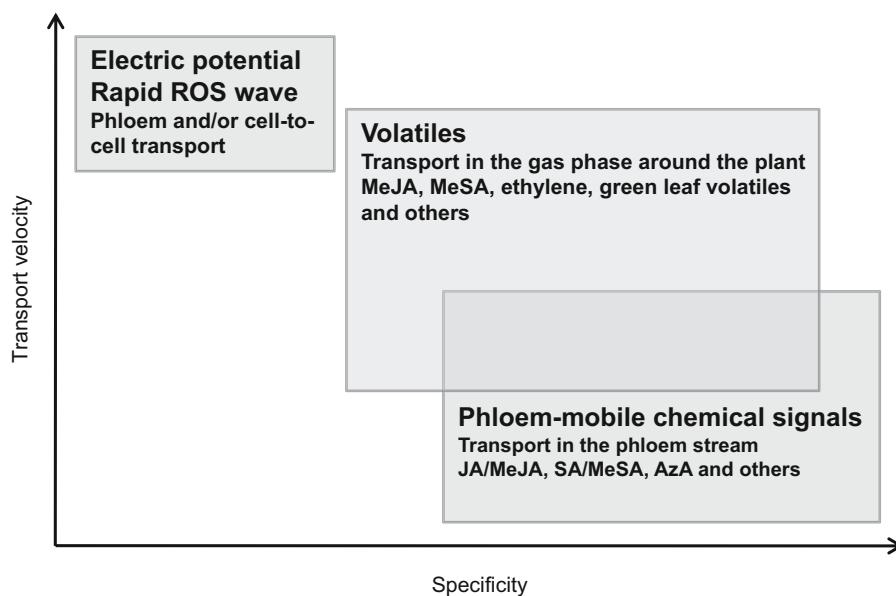


Figure 11.3 Hypothetical model ranking systemic signals according to their transport velocity and signal specificity.

H_2O_2 induced a specific set of genes independently of JA, SA, and ethylene. This rapid H_2O_2 signaling was also elicited by high light intensity, temperature stress, and salt treatment suggesting that it is a nonspecific stress response. A subsequent, wound-specific signaling event is then mediated by phloem transport and systemic perception of JA (Li et al., 2002) and possibly other chemical signals.

In view of the available data, it is tempting to hypothesize that rapid signaling by electrical potential waves and H_2O_2 is part of a general stress response that allows the plant to adapt to impending stress by shifting from primary to secondary metabolism (Figure 11.3). This response is rapid, nonspecific, and probably transient. The second response is slower, but more specific since a combination of chemical signals likely communicates more details about the original stress sensed by the local leaf to distant leaves conferring specific and long-lasting stress resistance. The signals are transported through the phloem that allows safe and targeted translocation as well as signal amplification along the route. Concomitant emission of a mixture of volatiles such as ethylene and so-called green leaf volatiles (Heil and Ton, 2008) would facilitate spreading of the message beyond the orthostichy and would add a higher degree of specificity as well as functional overlap as in the case of air-released MeJA and MeSA with phloem-mobile signals (Figure 11.3; Heil and Ton, 2008). Taken together, a picture is emerging in which vascular tissues, particularly the phloem, integrate complex signal networks including ROS, reactive nitrogen species, electrical signals, and defense hormones for amplification and propagation of systemic alarm signals.

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Section D

Biotic Interactions with the Phloem

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12 Role of Phloem Metabolites in Plant Defense

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While the primary function of phloem is the transport of energy-rich molecules from source to sink, many plants have evolved phloem-born defense strategies against herbivores and pathogens. These strategies involve both physical and chemical defenses that can require the involvement of multiple cell types. Sieve elements and associated cells contain a multitude of defensive compounds and proteins as the first line of defense against phloem-feeding insects and other predatory insects. Specialized structures of primary or secondary phloem such as laticifers and resin ducts provide an additional line of defense. These cell types are warehouses of secondary metabolites that provide both physical and chemical barriers against a plethora of invading organisms.

Several defensive tactics are employed by plants in response to challenge, including anatomical changes within phloem cell architecture, the constitutive or inducible accumulation of toxic metabolites and proteins, and the creation of physical barriers in the form of exuding latex or resin. Whether defense strategies are physical or chemical in nature, the sequence of events leading to structural changes or metabolite production are highly complex and in some cases require the coordinated participation of different cell types within the phloem and associated tissues.

Sieve-Tube Sap Provides Both Food and Fight

While the primary function of phloem is nutrient translocation, cell types within this tissue have been implicated as important contributors to the synthesis, distribution, and release of myriad defense compounds in many plants. For example, the sieve-tube sap of some plants is known to contain proteins and small metabolites that deter feeding herbivores and exhibit toxicity toward pathogens. Examples of defense secondary metabolites found in sieve-tube sap and exudates of specialized phloem tissues are described in Table 12.1. Studying the contents of *Arabidopsis* phloem has revealed a plethora of defense compounds that are covered in more detail in Chapters 8 and 11. In turn,

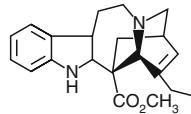
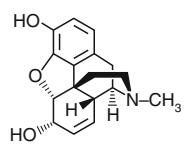
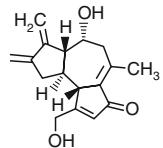
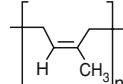
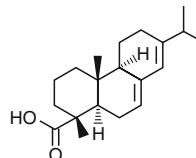
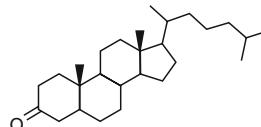
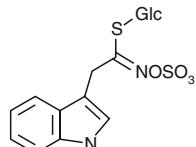
Abbreviations: D-AB1, 1,4-dideoxy-1,4-imino-D-arabinitol; ABC, ATP-binding cassette; BIA, benzylisoquinoline alkaloid; DMAPP, dimethylallyl diphosphate; HCN, hydrogen cyanide; HSS, homospermidine synthase; IPP, isopentenyl diphosphate; M-cell, myrosinase-containing cell; MDR, multidrug resistance; MIA, monoterpane indole alkaloid; MLX56, mulatexin; Mir1-CP, maize insect resistance 1-cysteine protease; PP, polyphenolic phloem parenchyma; S-cell, sulfur-rich cell

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Table 12.1 Selected phloem metabolites with putative or established roles in plant-insect interactions. Included are taxa in which these compounds are commonly found. The ketone steroid cholestan-3-one accumulates in engineered tobacco (Behmer et al., 2010) but is otherwise present only in trace amounts. The location within the phloem may represent the site of metabolite biosynthesis or accumulation.

Compound class	Taxa	Tissue/cellular location	Example	Structure
Monoterpene indole alkaloid	<i>Catharanthus roseus</i> (Madagascar periwinkle) Apocynaceae	Biosynthesis in internal phloem parenchyma, epidermal cells, mesophyll idioblasts and laticifers	Catharanthine	
Benzylisoquinoline alkaloid	<i>Papaver somniferum</i> (opium poppy) Papaveraceae	Biosynthesis in sieve elements, accumulation in laticifers	Morphine	
Sesquiterpene lactone	<i>Lactuca sativa</i> (lettuce) Asteraceae	Accumulation in laticifers	Lactucin	
Isoprenoid	<i>Hevea brasiliensis</i> (Para rubber tree) Euphorbiaceae	Accumulation in laticifers	Natural rubber	
Monoterpene	Coniferales and Taxales (conifers)	Accumulation in resin ducts and blisters of secondary phloem	(-)- α -Pinene	
Diterpenoid	Coniferales and Taxales (conifers)	Accumulation in resin ducts and blisters of secondary phloem	Abietic acid	
Ketone steroid	Transgenic <i>Nicotiana tabacum</i> (tobacco) Solanaceae	Presence in phloem exudate (sap)	Cholestan-3-one	
Glucosinolate	Brassicaceae	Accumulation in phloem-associated S-cells; presence in phloem exudate (sap)	Indol-3-yl-methyl glucosinolate	

phloem-feeding insects such as aphids have developed counter-strategies allowing them to overcome some phloem-born defenses such as sieve element occlusion that is discussed in Chapter 15.

Toxic quinolizidine alkaloids are associated with the phloem of wild lupins and prevent the feeding of most generalist insects (Lee et al., 2007). However, certain specialist feeders and even some generalist pests such as the green peach aphid (*Myzus persicae*) have developed tolerance to lupin alkaloids (Cardoza et al., 2006). Other defensive metabolites found in sieve-tube sap include glucosinolates (Halkier and Gershenson, 2006; Hopkins et al., 2009) and probably cyanogenic glucosides (Jørgensen et al., 2005, 2011), although laticifer transport of cyanogenic products is also possible in plants such as cassava (Santana et al., 2002). Phloem transport of defensive compounds including sulfur-containing metabolites often follows their biosynthesis, which occurs in specialized cells proximal to phloem sieve elements.

Glucosinolates are sulfur- and nitrogen-rich specialized metabolites that accumulate in many members of the Brassicaceae. The best studied model for glucosinolate metabolism is *Arabidopsis*, which accumulates these compounds in the vacuoles of sulfur-rich cell (S-cells). S-cells are located on the distal periphery of the phloem area of the vascular bundles in leaves and flower stalks (Koroleva et al., 2000, 2010), which spatially separates them from myrosinase enzymes (Figure 12.1). Myrosinases are thioglucosidases occurring in idioblast cells (i.e., M-cells) of the phloem parenchyma and guard cells (Halkier and Gershenson, 2006). Tissue damage causing cellular disruption mixes the contents of S-cells and neighboring M-cells, exposing glucosinolates to myrosinases and causing a so-called “mustard oil bomb.” Glucosinolate hydrolysis results in the release of thiocyanates, isothiocyanates, and nitriles that exhibit insect-deterring properties (Bones and Rossiter, 1996; Halkier and Gershenson, 2006; Hopkins et al., 2009). Glucosinolate breakdown products are known to deter generalist herbivores and pathogens especially when tissue damage occurs as the result of feeding or infection. The green peach aphid partially circumvents this defensive mechanism while feeding on *Arabidopsis* and other crucifers by inserting its stylet cell by cell to reach phloem sieve elements (Tjallingii and Hogen Esch, 1993) (Figure 12.2). Such a feeding habit allows the aphid to avoid bringing glucosinolate-containing sap into contact with myrosinase from nearby M-cells (Andréasson et al., 2001; Husebye et al., 2002; Thangstad et al., 2004). Nevertheless, some glucosinolate hydrolysis does occur during passage through the gut of the insect where myrosinase-independent postdigestive breakdown of indole glucosinolates has been shown to elicit strong antifeeding effects on green peach aphid (Kim et al., 2008).

Complex plant–herbivore relationships involving phloem sterols have also been examined (Behmer and Nes, 2003; Janson et al., 2009). Arthropods, including phloem-feeding insects, are unique among animals because they do not synthesize essential sterols. Many insects generate sterols such as cholesterol by metabolizing plant sterols obtained during feeding. A recent study examining the sterol profiles of different sieve-tube saps and insect responses to various sterols found that aphids reared on tobacco containing high levels of “atypical” sterols (e.g., 3-ketone steroids) also exhibited severely reduced reproduction and high mortality (Behmer et al., 2010). Atypical steroids are found at very low levels in wild-type tobacco (e.g., < 1% of total sterols in sieve-tube sap). Transformation of tobacco with a gene encoding 3-hydroxysteroid oxidase elevated the level of 3-ketone steroids to 58% and 88% of total sterols in leaf extracts and sieve-tube sap, respectively. The decreased fitness of aphids reared on this transgenic tobacco suggests that although certain plant sterols are essential to insect health, other sterols might function as defensive metabolites.

Beyond small molecules, defensive proteins have also been localized to sieve-tube sap and are discussed in Chapter 8. The direct effects of sieve-tube sap proteins on feeding insects have not been extensively investigated, although certain proteins appear to play defensive roles in plant–insect interactions (Kehr, 2006). Recent evidence suggests that a cysteine protease in maize (*Zea mays*)

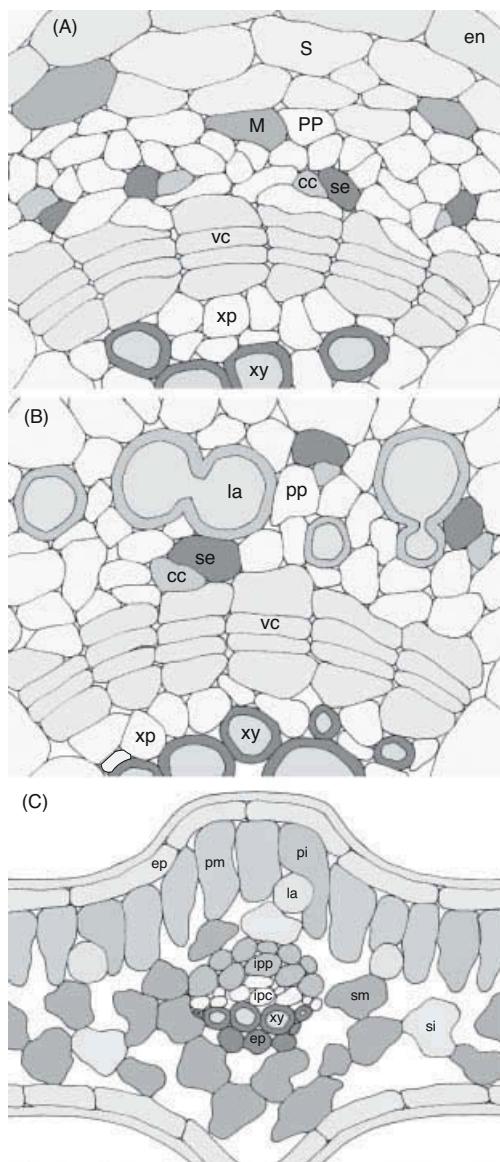


Figure 12.1 Schematic representations of multicell models for the biosynthesis and accumulation of defense compounds. (A) In *Arabidopsis thaliana* flower stalks, glucosinolate-rich S-cells (S) are located at the distal periphery of the phloem of vascular bundles. Expression of thioglucoside glucohydrolase 1 (myrosinase) was shown to occur in specialized phloem myrosinase cells (M) (Husebye et al., 2002). Although glucosinolates and myrosinase enzymes are normally stored in different compartments, tissue disruption by herbivores mixes these two components releasing defensive, bioactive hydrolysis products. Other abbreviations: companion cells (cc), endodermis (en), phloem parenchyma (pp), sieve elements (se), vascular cambium (vc), xylem parenchyma (xp), xylem tracheids (xy). (B) In opium poppy (*Papaver somniferum*) the production of benzylisoquinoline alkaloids such as morphine requires the coordinated participation of companion cells (cc), sieve elements (se) and laticifers (la). Biosynthetic enzymes are synthesized in companion cells and transferred to sieve elements, where alkaloid biosynthesis occurs. These alkaloids are then stored in neighboring laticifer networks. Other abbreviations: phloem parenchyma (pp), vascular cambium (vc), xylem parenchyma (xp), xylem vessels (xy). (C) The production of monoterpenoid indole alkaloids in Madagascar periwinkle (*Catharanthus roseus*) leaves involves numerous cell types, including adaxial internal phloem parenchyma (ipp), laticifers (la) within the palisade mesophyll (pm), idioblasts of palisade and spongy mesophyll (pi and si, respectively) and upper epidermis (ep). Isoprenoid precursors are synthesized in internal phloem parenchyma, and translocation of intermediates to epidermal cells allows the complete biosynthesis of antifungal catharanthine, which is then secreted into the waxy cuticle. In contrast, vindoline biosynthesis requires the additional participation of laticifer and idioblast cells. Other abbreviations: adaxial internal phloem conducting elements (ipc), xylem vessels (xy), abaxial external phloem (ep), spongy mesophyll (sm). (For a color version of the figure, please see Plate 12.1.)

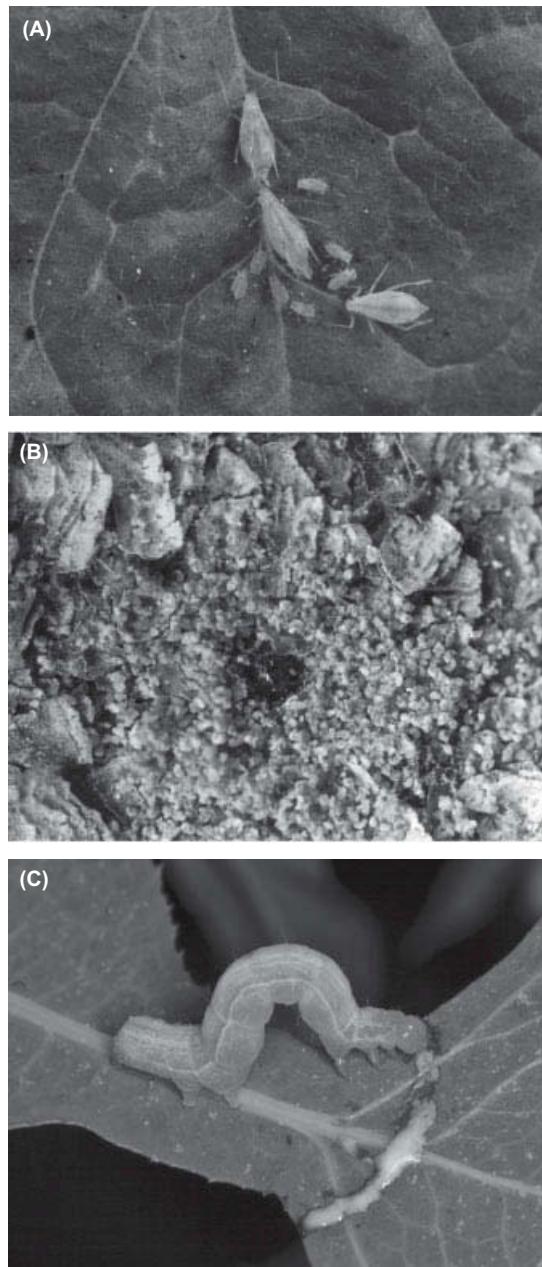


Figure 12.2 Examples of plant-insect interactions involving plant defensive metabolites. (A) Green peach aphids align along leaf vascular tissue to feed on sieve-tube sap. Sap of cruciferous plants often contain glucosinolates, the hydrolysis products of which are toxic or possess antifeedent qualities. However, the careful feeding habit of aphids maintains glucosinolate hydrolysis at a minimum, since hydrolyzing enzymes are compartmentalized separately in the phloem and are only released upon tissue damage (see the text). (B) A pine beetle burrows into the bark of a conifer stem. The secondary phloem of conifers harbors a minefield of anatomical structures containing chemical defenses such as polyphenolics and oleoresin. (C) A cabbage looper engages in trenching behavior while feeding on the leaf of prickly lettuce. The larva chews furrows across leaf vascular tissue, releasing latex containing toxic metabolites such as sesquiterpene lactones. Later, the larva will feed beyond the cut sites where latex outflow is minimal. (Photo courtesy of Dr. David Dussourd (copyright).) (For a color version of the figure, please see Plate 12.2.)

contributes to the defense response against insect herbivory. Mir1-CP is a unique, 33-kD protease that accumulates in maize lines genetically resistant to several lepidopteran pests (Lopez et al., 2007; Zhu-Salzman et al., 2008). Upon ingestion, Mir1-CP either disrupts (Pechan et al., 2002) or permeabilizes (Mohan et al., 2006) the peritrophic matrix, a protective layer within the insect midgut that is composed of chitin and protein. Mir1-CP accumulates in the thick-walled sieve elements of maize vascular tissue 24 hours following foliar feeding by the fall armyworm (*Spodoptera frugiperda*) (Lopez et al., 2007). The herbivory-induced accumulation of Mir1-CP in leaves was prevented by removal of the roots, suggesting that the protease could be transported through the vascular system to the site of action.

Defense Strategies and Specialized Phloem Anatomy

Laticifers

Many plants have evolved specialized phloem cell types to address specific nutritional needs or in response to environmental stresses, including defense responses toward microbial pathogens and insects. Laticifers are highly specialized cells defined by their characteristic anatomy and distinct cytoplasm (Hagel et al., 2008). About 10% of all flowering plant species exude latex upon tissue damage, yet latex has no known function within primary metabolism (Agrawal and Konno, 2010). Based on development and overall morphology most laticifers belong to one of two major categories: nonarticulated and articulated.

Nonarticulated laticifers are multinucleate and develop from single cells. These initials elongate extensively during plant growth to form either unbranched vessels or intricately branched networks. The initials of nonarticulated, branched laticifers are detectable in immature embryos at the time of cotyledon initiation (Hagel et al., 2008). As the seedling grows, the laticifer initials elongate and undergo karyokinesis without the formation of a cell plate. How these laticifers form branches, which themselves undergo extensive elongation and branching, is poorly understood. Although initials of nonarticulated, unbranched laticifers have not been recognized in the embryo, new initials arise repeatedly beneath apical meristems and elongate into unbranched tubes. Nonarticulated laticifers exhibit invasive growth, and are generally present throughout the vasculature of the plant, including the phloem.

Initials of articulated laticifers have been observed in the embryos of some species but not others (Evert, 2006). Articulating members are first recruited in the protophloem, and later from phloem initials derived from the vascular cambium. Laticifer differentiation occurs simultaneously with surrounding phloem cells, leading to the formation of discrete longitudinal rows. At maturity, articulated laticifers are composed of a series of superimposed cells with perforated end walls that often generate contiguous vessels. In some species, the perforation of lateral walls leads to the formation of anastomoses between laticifer elements, creating highly branched networks (Hagel et al., 2008). Unlike nonarticulated laticifers that are found generally throughout vasculature tissue (including the phloem), articulated laticifers are usually restricted to the phloem.

Despite their unusual nature, the contents of laticifers have long been deployed as toxins and valuable bioproducts. Aboriginal cultures of Southeast Asia have used exudates of *Antiaris spp.* containing cardiac glycosides as a poison for arrow tips (Bisset, 1966; Carter et al., 1997). Opium poppy (*Papaver somniferum*) owes its fame to the opiates found in phloem-derived latex (Facchini and De Luca, 2008) and para rubber tree (*Hevea brasiliensis*) is a commercial source of natural rubber, a product of phloem laticifers (van Beilen and Poirier, 2007). However, it is the capacity

of latex for plant defense that has aroused the most interest, especially with respect to its role in plant-insect interactions (Agrawal and Konno, 2010). Many studies have linked the unique biochemistry of laticifers with the defense of plants against herbivores. The glue-like consistency of latex also seems to play a defensive role by plugging the mouthparts of foraging insects and protecting damaged plant parts from infection by pathogens (Langenheim, 2003; Helmus and Dussourd, 2005). Latex is usually under positive turgor pressure and exudation upon tissue damage is often followed by rapid coagulation that poses a threat to herbivores (Zalucki and Brower, 1992; Dussourd, 1993, 1995). Plants that accumulate rubber in their latex are thought to have evolved this trait for defensive purposes (Agrawal and Konno, 2010). The sticky emulsion produced by exuding latex at wound sites can entrap whole insects (Dussourd, 1993, 1995) and act as a gummy adhesive within their mouthparts (Dussourd and Eisner, 1987).

Defensive Metabolites of Laticifers

Examples of defensive compounds present in latex include catecholamines, alkaloids, cardenolides, and sesquiterpene lactones (Table 12.1). Metabolite profiling of opium poppy latex (Hagel et al., 2008) revealed the presence of γ -amino butyric acid that acts as a neurotransmitter in animals and is thought to have a defensive role in plants (Bouché and Fromm, 2004). In addition, the latex of Persian poppy (*Papaver bracteatum*) and opium poppy accumulate the neurotransmitter dopamine (Roberts et al., 1983), a precursor to benzylisoquinoline alkaloids. Benzylisoquinoline alkaloids such as codeine and morphine, which are abundant in opium poppy latex, have not yet been studied with respect to possible defensive properties against feeding insects and other pests. Sugar-mimicking alkaloids such as 1,4-dideoxy-1,4-imino-D-arabinitol (D-AB1) can comprise up to 18% of latex dry mass in mulberry (*Morus spp.*) (Konno et al., 2006). Mulberry latex reduces the feeding of generalist caterpillars, but not the silkworm *Bombyx mori*, demonstrating that this specialist insect can circumvent the toxicity of D-AB1. The complex relationship between mulberry and *B. mori* has important economic implications, as mulberry trees have long been used to rear the economically valuable larvae as a source of silk proteins. Evidence suggests that the presence of β -fructofuranosidase genes in *B. mori* may provide a molecular basis for alkaloid tolerance (Daimon et al., 2008). Unlike the more common α -glucosidases used for digesting sugars, β -fructofuranosidases are not inhibited by high concentrations of pyrrolidine alkaloids, thus permitting the silkworm to feed.

The piperidine alkaloid lobeline accumulates in the latex of *Lobelia spp.* such as Indian tobacco (*Lobelia tupa*) and cardinal flower (*Lobelia cardinalis*). The application of lobeline to cabbage loopers (*Trichoplusia ni*) induces leaf-trenching (Dussourd, 2003), a behavior similarly displayed by a plusiine caterpillar *Enigmogramma basigera* when feeding on cardinal flower leaves (Oppel et al., 2009). Leaf-trenching is a behavioral adaptation of many insects toward the defensive canal architecture of many plants. The invading insect severs leaf veins or cuts trenches, allowing latex flow prior to feeding distal to the cut sites. A recent study revealed that leaf-trenching reduces alkaloid levels by ~50% both distal and proximal to the trench, with the trench site itself becoming greatly enriched in alkaloids due to the draining of latex from surrounding areas. In effect, the cutting serves not only to drain latex from the prospective feeding site, but also to prevent further latex influx due to the severing of latex conduits (Oppel et al., 2009). Interestingly, although latex might act as glue or entrapping resin thereby defending the plant via physical measures, it is the chemical arsenal of latex that elicits trenching behavior in insect herbivores (Helmus and Dussourd, 2005).

Cardenolides (cardiac glycosides) are found in the laticifers of many plants in the Apocynaceae family, comprising up to 30% of the dry mass of the latex (Seiber et al., 1982; Malcolm, 1991).

These sterol-derived compounds inhibit Na^+/K^+ -ATPases that are critical for maintenance of the electrical potential in most animal cells. As such, cardenolides are remarkably toxic to a wide range of animals (Malcolm, 1991). Plants producing these compounds often accumulate a diverse array of differentially substituted cardenolide structures. Cardenolides of varying polarity are passed through the cell membranes of animals with different efficiencies, thereby rendering some compounds much more toxic than others. Milkweed (*Asclepias sp.*) cardenolides are toxic to generalist caterpillars (Dussourd and Hoyle, 2000), and insect feeding can increase the cardenolide content of the latex (Martel and Malcolm, 2004). Larvae of Monarch butterflies (*Danaus plexippus*) exhibit trenching behavior to overcome the toxic effects of ingesting latex, although consumption of cardenolides when the insects are still caterpillars renders adult butterflies distasteful or toxic to predators.

Other isoprenoid metabolites found in latex include sesquiterpene lactones that are feeding deterrents to both vertebrates (Peters and van Amerongen, 1998) and invertebrates (Doskotch et al., 1981; Passreiter and Isman, 1997). Sesquiterpene lactones are toxic to a wide variety of organisms due to a relatively nonspecific mode of action attributed to the presence of specific electrophilic groups, such as the α -methylene- γ -lactone, that react with electron-rich atoms such as sulfur and oxygen in proteins and DNA (Frankfater et al., 2005). Garden lettuce (*Lactuca sativa*) possesses articulated, anastomosing laticifers harboring a number of different sesquiterpene lactones, which together contribute to the overall bitter taste of this popular crop. The production of some lactones such as lettuvenin A are induced in response to bacterial and fungal challenges (Sessa et al., 2000), whereas lactucin is constitutive in lettuce and triggers leaf-trenching by the caterpillar *Trichoplusia ni* (Dussourd, 2003). The presence of lactucopicrin and 8-deoxylactucine in chicory latex was found to deter locust (*Schistocerca gregaria*) feeding (Rees and Harborne, 1985). Some evidence has also pointed toward the defensive activity of diterpenes in latex (Noack et al., 1980; Gershenson and Croteau, 1991).

Certain phenolic compounds known to accumulate in the laticifers of sweet potato (*Ipomoea batatas*) might also function to deter herbivory. For example, the concentration of 4-coumaric acid derivatives was inversely correlated with acceptability by weevils (Snook, 1994), suggesting that such compounds might play defensive roles in the latex.

Defensive Proteins of Phloem-Associated Laticifers

Laticifers serve not only as a repository for natural products, but also possess unique proteomes. Many proteins are thought to play defensive roles in plants, including cysteine and serine proteases, protease inhibitors, lectins, chitinases, hydrolases, oxidases, and pathogenesis-related polypeptides (Hagel et al., 2008; Agrawal and Konno, 2010). Some latex proteins might be also involved in stress-induced signaling cascades, such as Ca^{2+} /calmodulin-binding peroxidase in *Euphorbia characias* (Mura et al., 2005) and allene oxide synthase from para rubber tree (Norton et al., 2007). Certain peroxidases and polyphenol oxidases are thought to have antifeedant properties, since they can decrease the bioavailability of leaf protein by initiating a cascade of events involving reactive *ortho* quinones (Felton et al., 1992; Zhu-Salzman et al., 2008).

The cysteine protease papain was identified as a crucial component in the protection of papaya trees (*Carica papaya*) against Eri silkworm (*Samia ricini*), cabbage moth (*Mamestra brassicae*), and tobacco cutworm (*Spodoptera litura*) (Konno et al., 2004). However, with the exception of Mir1-CP in maize (Zhu-Salzman et al., 2008) the mechanisms of protease toxicity toward insects are not well understood. Conversely, protease inhibitors are purported to function by inactivating the endogenous proteases of insects, thereby impeding protein digestion. Serine protease inhibitors

have been reported to occur in *Ficus carica* (Kim et al., 2003) and *C. papaya* (Azarkan et al., 2004) latex, whereas the expression of genes encoding protease inhibitors has been reported in the latex of para rubber tree (Han et al., 2000).

Chitinases are often regarded as defense proteins since chitin is an integral component of insect exoskeleton and gut linings (Kramer and Muthukrishnan, 1997) and also an important constituent of fungal cell walls. Chitinase expression in *F. carica* and *C. papaya* increases in response to wounding or treatment with jasmonic acid (Kim et al., 2003; Azarkan et al., 2004) and recently two chitinase-like proteins from mulberry latex were shown to possess insecticidal activity (Kitajima et al., 2010). Mulberry latex also accumulates the protein mulatexin (MLX56) that is comprised of an extensin domain, two hevein-like chitin-binding domains, and an inactive chitinase-like domain. Mulatexin exhibits toxicity toward lepidopteran caterpillars including cabbage cutworm (*M. brassicae*) and Eri silkworm, although the specialist silkworm *B. mori* was unaffected (Wasano et al., 2009). Mulatexin has strong chitin-binding activity and is highly resistant to proteolysis, suggesting that the protein could remain active in the midgut of feeding insects. The application of *Calotropis procera* latex proteins, including chitin-binding, chitin-degrading, and cysteine proteases to various crop pests reduced their growth and survival (Ramos et al., 2007).

It is generally assumed that certain ubiquitous latex proteins play a defensive role by contributing to latex coagulation upon exposure to air, creating a protective “seal” over wounded plant tissues or acting as “glue” on insect mouthparts (Agrawal and Konno, 2010). For example, hevein, a major latex protein of *H. brasiliensis*, is important for the agglutination of rubber particles (Gidrol et al., 1994). Furthermore, hevein gene expression is induced upon wounding (Broekaert et al., 1990). Conversely, other latex proteins have been recruited for highly specific defensive activities. For example, linamarase in cassava latex catalyzes the hydrolysis of the cyanogenic glycoside linamarin, leading to the production of toxic hydrogen cyanide (HCN) (Santana et al., 2002; Jørgensen et al., 2005).

Constitutive and Inducible Defenses in Phloem Parenchyma and Phloem-Associated Resin Ducts of Conifers

Conifers are long-lived trees that face attack by a range of organisms throughout their lives. Particularly threatening pests include bark beetles, which enter the bark where they breed, create tunnels, and lay their eggs within the secondary phloem/cambial zone. The penetration of bark beetles provides an opportunity for the subsequent entry of pathogenic fungi that can rapidly destroy the phloem, cambium, and even disrupt the flow of water in the xylem by causing embolisms in the tracheids (Sperry and Tyree, 1988), all of which can cause the death of the tree in severe cases. To defend themselves against the attacks of boring insects and other threats, conifers have evolved multifaceted, integrated defense strategies involving both constitutive and inducible responses (Franceschi et al., 2005). Constitutive defenses can be physical (e.g., structural elements providing toughness or thickness) or chemical (e.g., pooling of phenolics and terpenoids that can be released upon insect attack). Chemicals produced by the plant that provide constitutive defense are usually dispersed in anatomical stores throughout the various tissues of the periderm and secondary phloem. The periderm provides the first mechanical line of constitutive defense, with the secondary phloem providing an additional line of constitutive defense, owing to sclerenchyma, calcium oxalate crystals, phenolic bodies, and in certain taxa, resin-producing structures such as resin ducts, resin blisters, and resin cells.

Polyphenolic Phloem Parenchyma Cells

In all conifers studied to date, phloem has been found to contain specialized axial parenchyma cells that produce and accumulate defensive phenolic compounds within their vacuoles (Franceschi et al., 2000, 2005). Termed polyphenolic parenchyma (PP) these cells are important elements to both constitutive and inducible defense responses in conifers since wounding or bark invasion has been shown to activate PP cells through cell expansion and the increased accumulation of phenolics (Klepzig et al., 1995; Franceschi et al., 2000; Kusumoto and Suzuki, 2003). Although the chemistry of phloem parenchyma cells in conifers is poorly studied, it is known that the composition of the phenolic contents varies on a seasonal basis and in response to stress (Krekling et al., 2000).

Production of phenolics is induced in phloem in response to pathogen infection, mechanical wounding, bark beetle attack, or treatment with methyl jasmonate (Franceschi et al., 2005; Krokene et al., 2008) resulting in a rapid amassment of aromatic compounds whose chemical composition is quite different from that of constitutively present phenolics (Brignolas et al., 1995; Lieutier et al., 1996; Viiri et al., 2001; Bonello et al., 2003). It has been suggested that induced phenolics are more toxic or perhaps more specific to invading organisms compared with constitutive phenolics (Franceschi et al., 2005). It is also possible that the toxic effects of phenolics are enhanced by the conversion of polyphenolics to soluble phenolics. This hypothesis is indirectly supported by the reduction of polyphenolics in vacuoles of intact phloem parenchyma cells close to the region of attack (Franceschi et al., 1998). Little is known about the *in vivo* signaling events leading to the induction of polyphenolic parenchyma cell formation (and phenolic accumulation) in secondary phloem. In a study of the anatomical responses of Norway spruce to fungal infection (Krekling et al., 2004), a large number of “extra” polyphenolic parenchyma cells were observed among the sieve cells postinoculation. These extra cells were evident at a considerable distance from the treatment site, prompting speculation that either such a change represented a strategy of enhancing long-term resistance, or that the normal developmental program of the cambium relative to phloem formation was disrupted upon traumatic resin duct formation. A few studies have reported genes with potential function in induced PP cell defenses (Nagy et al., 2004; Ralph et al., 2006a, 2006b). For example, aminocyclopropane carboxylic acid synthase, which catalyzes a regulated step in ethylene-dependent induced conifer defense, was found to be highly abundant in polyphenolic parenchyma cells of spruce (*Picea sitchensis*, *P. glauca*) and Douglas fir (*Pseudotsuga menziesii*) (Ralph et al., 2007). A study of induced responses to fungal infection in Norway spruce phloem established a possible correlation between polyphenolic parenchyma size and the regulation of at least two genes (Nagy et al., 2004). It was shown that a spruce line exhibiting enlarged polyphenolic parenchyma, with denser polyphenol bodies, in response to infection also exhibited increased chalcone synthase and peroxidase transcript levels.

Phenolics can possess antifungal and antifeedant activities and bind hydrolytic enzymes secreted by invading insects, thus slowing their advancement into tissues (Hunter, 1974; Nicholson and Hammerschmidt, 1992; Appel, 1993). Moreover, phenolics and related aromatic compounds bind amino acids and proteins in disrupted plant tissue, thereby reducing the nutritional reward for herbivores. The protein binding capacity of phenolics might also apply to the digestive enzymes of insects by diminishing their digestive abilities. Beyond phenolics, calcium oxalate crystals are commonly found in the secondary phloem of conifers and likely provide mechanical defense against bark-boring insects owing to their tough physical nature (Hudgins et al., 2003; Franceschi et al., 2005). In the Pinaceae, calcium oxalate crystals are embedded within vacuolar phenolic bodies of modified PP cells. Unlike normal PP cells, those containing such crystals are no longer living at

maturity. Other, non-Pinaceae conifers deposit calcium oxalate crystals within or along the edges of the walls of various secondary phloem cell types (Hudgins et al., 2003).

Phloem Resin Ducts and Related Anatomical Features

Phloem-associated secretory cells, ducts (canals), and glands are widely distributed among vascular plants (Fahn, 1979, 1988). These cell types often contain secondary metabolites that participate in defensive functions against insects and microbial pathogens. The resin-producing tissues of conifers, such as resin ducts, resin blisters, and resin cells are well-characterized examples of secretory systems with important roles in plant defense. Resin ducts and blisters are schizogenous secretory cavities lined with plastid-enriched epithelial cells that synthesize terpenoid resins and secrete them into the luminal space where they accumulate under pressure. Conversely, resin cells amass resin internally, and expand into relatively large structures (Franceschi et al., 2005). Although resin-producing structures are found constitutively within Pinaceae family members, they do not occur in the secondary phloem of all conifers (Hudgins et al., 2003, 2004). A distinction is made between constitutive axial and radial resin ducts, since the latter extend into the xylem. A further distinction is made between these structures and traumatic resin ducts that form axially and *de novo* in the xylem as part of the induced defense response in Pinaceae and certain other conifer families. Traumatic resin ducts of the xylem often form interconnections with radial resin ducts of the phloem (Nagy et al., 2000), and some conifer species form traumatic resin ducts in the phloem rather than the xylem (Hudgins et al., 2004).

Oleoresin is secreted by the epithelial cells of resin ducts and blisters, and represents a key line of physical and chemical defense of conifers against herbivore and pathogen attack. This viscous, odoriferous substance is a complex and dynamic mixture of terpenoid compounds. Terpenoids, perhaps the most diverse family of natural products synthesized by plants, are biosynthesized from dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) precursors through two distinct metabolic pathways (Roberts, 2007). The assembly of these basic isoprenoid (C_5) units into prenyl diphosphates (C_{10} , C_{15} , and C_{20}) provides the substrates for terpene synthases, which initiate the assembly of the structurally diverse and often highly complex hydrocarbon molecules. The resulting mono- (C_{10}), sesqui- (C_{15}), di- (C_{20}) and tri- (C_{30}) terpenes are required for primary and secondary biochemistry in plants, including the production of a plethora of defense metabolites.

Oleoresin in conifers is comprised of volatile mono- and sesquiterpenes, in addition to nonvolatile diterpene resin acids (Zulak and Bohlmann, 2010). Monoterpenes and diterpene resin acids are predominant in oleoresin and usually occur in approximately equal proportions, whereas sesquiterpenes represent a minor component. The terpenoid ratio of oleoresin can vary depending on environmental stress factors such as herbivory (Tomlin et al., 2000; Miller et al., 2005), fungal infection (Raffa and Smalley, 1995), air pollution (Kainulainen et al., 1993), fertilizer application (Turtola et al., 2002), and drought stress (Turtola et al., 2003). Oleoresin can “pitch out,” effectively pushing a trespassing insect out of entry site and possibly entombing it in sticky sap. The wound site is simultaneously sealed off from infection by microorganisms. Over time, the volatile components of oleoresin evaporate leaving a hardened mass of polymerized diterpenoid acids (Langenheim, 2003).

Some monoterpenoids exert toxic effects on insects (Cook and Hain, 1988; Raffa and Smalley, 1995; Werner, 1995), whereas other terpenoids act as feeding deterrents (Lindgren et al., 1996) or distract pests away from the feeding site (Nordlander, 1990). Conversely, many insects exhibit a capacity to cope with the chemical cocktails of conifers, as evidenced by their abilities to tolerate and even benefit from the presence of oleoresin terpenoids (Keeling and Bohlmann, 2006). Host

specialization is an important strategy of insects for exploiting the defensive terpenoids of conifer resin.

The unique resin compositions of specific trees might actually guide specialists to the correct feeding location at the right time. Insect population dynamics also play a role. For example, pine beetles from eruptive populations become more tolerant of high α -pinene levels as population density increases (Wallin and Raffa, 2004). Some host terpenes are known to synergize with bark beetle pheromones in eliciting mass attack of host trees (Seybold et al., 2000), while predatory insects use these terpenoids—produced in synergy with the semiochemicals of invading insects—to locate their herbivorous host (e.g., Pettersson, 2001). In an interesting tritrophic interaction, Scots pine (*Pinus sylvestris*) produces volatiles in response to egg deposition by the pine sawfly (*Diprion pini*) that in turn attracts the egg parasitoid *Chrysonotomyia ruforum* (Hilker et al., 2002). A comparison of the volatiles from oviposition-induced twigs and controls (e.g., mechanically wounded twigs) revealed that only amounts of (*E*)- β -farnesene were significantly elevated in response to insect invasion (Mumm et al., 2003). While conifer terpenoids undoubtedly play critical defensive roles toward insects, most plant–insect interactions involving these resin-born metabolites are complex and depend on other factors within the environment.

Multicell Models for the Biosynthesis and Accumulation of Defense Compounds

The accumulation of defensive metabolites in sieve-tube sap, laticifers, resin ducts, or other cell types exposed to insects and microbial pathogens is often the final step of a complex series of biochemical events. These events usually involve the participation of numerous biosynthetic enzymes localized to different cell types, thus requiring the transport of intermediates between cells, tissues, or whole organs. Gene transcripts and enzymes involved in the production of phenylpropanoids (Gang et al., 2001), flavonoids (Saslawsky and Winkel-Shirley, 2001), terpenoids (Lange et al., 2000; Turner and Croteau, 2004), glucosinolates (Andréasson et al., 2001; Halkier and Gershenson, 2006), and alkaloids (Ziegler and Facchini, 2008) have been localized to a variety of cell types that are often associated with the phloem.

Glucosinolates

As described previously, glucosinolates are important defense metabolites that accumulate at high levels in members of the Brassicaceae, including *Arabidopsis*. Bioactive hydrolysis products of glucosinolates form upon tissue damage, when the sulfur-rich compounds are exposed to the enzyme myrosinase. The cellular location of myrosinase remains an unresolved issue, although it is presumed to be sequestered from glucosinolates at either a cellular or subcellular level (Halkier and Gershenson, 2006). Several studies have localized myrosinase to idioblasts of phloem parenchyma (i.e., myrosin cells) in the leaves, stems, flowers and guard cells (Andréasson et al., 2001; Husebye et al., 2002; Thangstad et al., 2004) of *A. thaliana*. However, the location of myrosinase might be species specific, as myrosin cells of *Brassica* spp. are widespread outside the vascular system. In fact, in *Brassica juncea* seeds and seedlings, myrosinase appears to colocalize with glucosinolates in aleurone-type cells, suggesting their storage in different subcellular compartments (Kelly et al., 1998).

In *Arabidopsis*, the location of myrosin cells in phloem parenchyma places them proximal to the elongated, sulfur-rich “S-cells” situated just distal to the phloem (Koroleva et al., 2000, 2010) (Figure 12.1A). Glucosinolates accumulate in S-cells that are ideally placed for the defense of phloem against

herbivores and serve as a temporary storage site before the compounds are transported to developing seeds via the sieve-tube sap. The specific anatomy of S-cells, together with their ability to accumulate specialized metabolites, has been interpreted as an indication of their common evolutionary origin with nonarticulated laticifers (Koroleva et al., 2010).

Alkaloids

Benzylisoquinoline Alkaloids

Benzylisoquinoline alkaloids (BIAs) comprise a family of approximately 2500 compounds and possess potent pharmacological activities (Ziegler and Facchini, 2008). Well-known BIAs include the narcotic analgesic morphine, the cough suppressant codeine, the muscle relaxant codeine, and the antimicrobial agents sanguinarine and berberine. Morphine, a major alkaloid of opium poppy latex, might play a defensive role in the plant by forming bismorphine in response to stress, which is incorporated into the cell walls (Morimoto, 2001). Sanguinarine is a major root alkaloid in opium poppy and related species, and affects neurotransmission by inhibiting choline acetyltransferase, various neuroreceptors, and DNA synthesis in both vertebrates and invertebrates (Schmeller et al., 1997). However, the specific localization of sanguinarine in roots has yet to be established.

BIA biosynthesis begins with the condensation of two tyrosine derivatives by the enzyme norcoclaurine synthase (Lee and Facchini, 2010), followed by a multitude of enzymatic steps to form either sanguinarine or morphine. In opium poppy, BIA accumulation occurs in laticifers, which are found adjacent or proximal to sieve elements of the phloem (Ziegler and Facchini, 2008). Although latex was long thought to be the site of synthesis of these alkaloids, it has been shown that BIA biosynthetic enzymes and gene transcripts occur in different cell types. At least eight biosynthetic enzymes en route to morphine and/or sanguinarine have been localized to sieve elements in opium poppy, whereas the transcripts encoding these enzymes were found in companion cells (Figure 12.1B) (Bird et al., 2003; Samanani et al., 2006; Facchini and De Luca, 2008; Lee and Facchini, 2010). These results imply that BIA biosynthetic enzymes are assembled in companion cells and transferred to sieve elements where they function in association with the sieve element reticulum to produce myriad alkaloids (Alcantara et al., 2005), which are subsequently transported to laticifers for storage.

The process of alkaloid transport could involve a symplastic route via plasmodesmata that connect sieve elements and laticifers in opium poppy (Facchini and De Luca, 2008) or an intercellular transporter. Two membrane-bound transporters accepting alkaloid substrate have been identified in cell cultures of Japanese goldthread (*Coptis japonica*). One of these is an ATP-binding cassette (ABC) transporter localized to the plasma membrane (Shitan et al., 2003). Characterized as an influx transporter, this multidrug resistance (MDR) protein is capable of translocating berberine into the cytoplasm from surrounding media. A second transporter, a tonoplast-localized H⁺/berberine antiporter, is known to be involved in the vacuolar transport of endogenous berberine (Otani et al., 2005). Together, the localization of these proteins has supported a model where cytotoxic berberine taken into the cytoplasm is rapidly imported into the vacuole for safer storage (Otani et al., 2005). Nonetheless, how (or if) this model applies to intact plants has yet to be established.

Although BIA metabolism is thought to have a monophyletic origin in angiosperms (Liscombe et al., 2005), at least two very different pathway localization strategies have been identified. Cell types of the phloem have been recruited for the biosynthesis and accumulation of BIAs in opium poppy and other members of the Papaveraceae. In contrast, yellow meadow rue (*Thalictrum flavum*) produces and accumulates the BIA berberine in the endodermis and pericycle of roots (Samanani

et al., 2005). Yellow meadow rue and Japanese goldthread are members of the Ranunculaceae, a family related to the Papaveraceae.

Monoterpene Indole Alkaloids

Monoterpene indole alkaloids (MIAs) comprise a family of structurally diverse compounds, many of which have important pharmaceutical relevance. These alkaloids are condensation products of a nitrogen-containing indole moiety derived from tryptamine and a monoterpeneoid component derived from the iridoid glucoside secologanin (Ziegler and Facchini, 2008). Vinblastine and related vinca alkaloids from *Catharanthus roseus* are widely used for the treatment of various cancers, whereas ajmaline from *Rauvolfia serpentina* is used to treat antiarrhythmic heart disorders. Catharanthine, which is thought to react with vindoline to form vinblastine upon leaf wounding of *C. roseus*, has recently been found to inhibit the growth of fungal zoospores and exhibits toxicity toward insects (Roepke et al., 2010).

Biosynthesis of catharanthine and vindoline in *C. roseus* requires the participation of multiple enzymes, and is only partially elucidated at the molecular level (O'Connor and Maresh, 2006; Facchini and De Luca, 2008). Genes encoding enzymes involved in the formation of the isoprenoid precursors used for MIA biosynthesis are expressed in specialized internal phloem parenchyma cells (Burlat et al., 2004; Oudin et al., 2007). The subsequent biosynthesis of catharanthine occurs entirely in leaf epidermal cells to facilitate secretion to the cuticle (Figure 12.1C). In contrast, vindoline biosynthesis only partially occurs in epidermal cells and requires additional transport of a pathway intermediate to mesophyll idioblasts and laticifers where the final two enzymes are localized (St-Pierre et al., 1999; Roepke et al., 2010). The spatial separation of catharanthine and vindoline (i.e., in the exterior cuticle and the interior idioblasts and laticifers, respectively) at least partially explains the low levels in *C. roseus* of the dimeric anticancer drug vinblastine, which is formed by the condensation of these monomers. The presence of catharanthine on the leaf surface appears to serve an important defensive function. The role of vindoline in mesophyll idioblasts and laticifers is not known.

Transport of Alkaloids from Root to Shoot

Numerous defensive compounds are synthesized in the roots of plants and transported through vascular tissues to shoots, where they can most effectively exert antiherbivory effects (Erb et al., 2009). For example, nicotine is produced in *Nicotiana tabacum* roots and subsequently translocated via the xylem to the leaves for storage (Dawson, 1941; Shoji et al., 2000; Morita et al., 2009). Xylem is also the mode of transport for tropane alkaloids such as scopolamine and hyoscyamine in *Atropa belladonna* and *Hyoscyamus niger* (Ziegler and Facchini, 2008). Calystegines, a class of tropane alkaloids found in potato (*Solanum tuberosum*) accumulate in tubers (i.e., modified stem tissues) and are biosynthetically related to scopolamine and hyoscyamine. However, the biosynthesis and transport of calystegines appears to be different from other tropane alkaloids since tropinone reductase II, a key branchpoint enzyme in calystegine metabolism, has been localized to phloem parenchyma and companion cells in various potato organs (Kaiser et al., 2006).

The transport of other alkaloids from roots to shoots may occur via the phloem. Pyrrolizidine alkaloids, for example, are purported to be transported mainly, if not exclusively, through the phloem in plants such as *Senecio vulgaris* (Hartmann et al., 1989; Ober and Kaltenegger, 2009). This conclusion is based on the tissue distribution of ¹⁴C-labelled alkaloid following feeding to either

detached steam-girdled shoots, or to intact roots of plants with steam-girdled shoots (Hartmann et al., 1989). It was found that if the phloem route along the stem is blocked by a steam-girdle, translocation of alkaloid from root to leaves is blocked as well. Homospermidine synthase (HSS), the first pathway-specific enzyme of pyrrolizidine alkaloid biosynthesis, has been localized in *Senecio vernalis* to specialized root cortex parenchyma and endodermis cells directly across from the phloem (Moll et al., 2002). This proximity has been interpreted as evidence that these HSS-expressing cells harbor the whole biosynthetic pathway for pyrrolizidine alkaloids (Ober and Kaltenegger, 2009). Quinolizidine alkaloids were detected in both xylem and phloem exudates of bitter (cv. Lupini) and sweet (cv. Ultra) varieties of *Lupinus albus*, suggesting transport of these alkaloids between different tissues organs (Lee et al., 2007). However, despite several studies aimed at establishing the site of quinolizidine alkaloid biosynthesis, no clear resolution has been established although reproductive organs appear to be a major site of product accumulation.

Future Directions

Considerable effort has been invested to harness the potential of phloem defense mechanisms for the improvement of agriculture. For example, *Lupus angustifolius* was bred to exhibit the highest level of aphid resistance by selecting plants with high quinolizidine alkaloid content in leaves and lower quinolizidine alkaloid content in seeds (Gladstones, 1994).

Plant compounds that alter insect behavior have attracted attention as potential commercial pesticides (Unelius et al., 2006; Bohman et al., 2008; Eriksson et al., 2008). Cyanogenic glucosides confer resistance to insects that feed on leaves (Tattersall et al., 2001), but pose significant health problems to humans that ingest plant material accumulating these cyanide-releasing toxins. For example, the storage and processing of cassava tubers leads to the hydrolysis of linamarin and other cyanogenic glucosides into cyanohydrins, hydrogen cyanide, and ketones (Conn, 1980). Cassava has been engineered using RNAi technology to reduce the cyanogenic glucoside content of tubers and leaves (Jørgensen et al., 2005), yielding a safer food source at the expense of a potentially more vulnerable crop. Continued research focused on the biosynthesis and transport of cyanogenic glucosides in cassava could facilitate the organ-specific accumulation of toxic compounds in appropriate cellular locations. Our current understanding of how and where defense compounds are produced and deployed is limited. Continued research in this area will not only expand our ability to develop new technologies aimed at crop enhancement and forest protection, but will advance our understanding of plant chemical ecology.

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13 Viroids and Phloem-Limited Viruses: Unique Molecular Probes of Phloem Biology

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Viruses and viroids are intracellular, obligate parasites that spread through the phloem causing systemic infection of a host plant. Systemic infection, in simplistic terms, increases the number of cells within a host that are infected, and results in a greater mass of infectious material, increasing the probability for subsequent spread to other plants. Plant viruses have DNA or RNA genomes that replicate and encode proteins within infected cells and are encapsidated by proteins in their extracellular forms (virions). Some virus capsids contain additional viral proteins or are enveloped with host-derived membranes. Most plant viruses spread among plants by means of phloem-feeding insect vectors.

Viroids comprise small single-stranded circular RNAs (250–400 nt) that replicate in host cells but do not encode proteins. Plants are the only known hosts for viroids. Viroids are spread by vegetative propagation of infected plants and by wounding with contaminated objects such as farm implements. Because the phloem is critical for the survival and propagation of viruses and viroids, these parasites and their biotic vectors have evolved mechanisms to interact with and utilize the components of the phloem transport system.

Nearly all plant viruses utilize sieve tubes for systemic movement. However, some plant viruses remain confined to or are primarily associated with phloem cells and as such are considered to be “phloem-limited.” These viruses are usually found only in phloem parenchyma cells, companion cells, and sieve elements. The reasons for phloem-limitation of some but not all plant viruses are not well understood. While viroid infection is not phloem-limited, the noncoding nature of viroids raises the important question of how viroid RNA genomes interact with the phloem to move systemically. Viroids and phloem-limited viruses are important plant pathogens that also provide unique insights into the basic biology of phloem.

Abbreviations: ASBVd, avocado sunblotch viroid; BYDV, barley yellow dwarf virus; BYV, Beet yellows virus; BWYV, beet western yellows virus; CP, coat protein; CPm, minor coat protein; CTV, Citrus tristeza virus; GLRaV-3, grapevine leafroll-associated virus 3; Hsp70h, heat shock protein 70 homolog; HSVd, hop stunt viroid; LIYV, lettuce infectious yellows virus; L-Pro, leader proteinase; ORF, open reading frame; PLMVd, peach latent mosaic viroid; PLRV, potato leafroll virus; PSTVd, potato spindle tuber viroid; RdRP, RNA-dependent RNA polymerase

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This chapter will review the current knowledge of phloem interactions and systemic movement of phloem-limited viruses in the families *Closteroviridae* and *Luteoviridae* and of viroids. Special emphasis will be placed on discoveries and unresolved issues that raise fundamental questions in phloem biology. The goal is to bridge the gap between virology and phloem biology and to stimulate development of innovative conceptual and technical tools to accelerate discoveries.

Plant Viruses: Parasites of the Host Plant Translational Machinery

Plant viruses co-opt existing host cell translational machinery to express virus-encoded proteins. Studies of viruses have revealed a great deal about the virus-encoded proteins as well as host factors that interact to achieve replication and movement (Benitez-Alfonso et al., 2010; Harries and Ding, 2010; Harries et al., 2010; Ishibashi et al., 2010; Verchot-Lubicz et al., 2010). Specific virus-encoded proteins modify subcellular compartments to allow virus replication, while others facilitate movement within the host plant. Many plant-infecting viruses initially infect epidermal cells by wounding or surface probing by vectors and move cell-to-cell until they reach phloem cells where they are transported systemically through the sieve tubes. Much more is currently understood about how viruses move cell-to-cell in mesophyll and epidermal cells than how they enter phloem parenchyma cells and traverse phloem parenchyma-companion cell and companion cell-sieve element boundaries to mediate systemic movement in the phloem. The majority of viruses in two taxonomic families, *Closteroviridae* and *Luteoviridae*, are primarily phloem-limited in their host plants. These phloem-limited viruses will be the major focus of the virus portion of this chapter. Many virus species in the family *Geminiviridae* are also phloem-limited, but these will not be discussed herein. Since phloem-limited viruses are adapted to move cell-to-cell and long-distance only in phloem cells, they may provide unique insights into the biology of phloem transport. Studies on these viruses begin to address fundamental questions. For example, how do viruses get into the phloem and then interact with phloem cells during infection to establish systemic infection? Virus-encoded proteins that are required for or implicated in long-distance movement will also be addressed.

Closteroviridae and Luteoviridae: Phloem-Limited Viruses

Closteroviridae

Viruses in the family *Closteroviridae* (referred to collectively as “closteroviruses”) are either strictly confined to phloem cells (phloem parenchyma, companion cells, and sieve elements) or are mostly phloem located with limited egress into nonphloem cells during infection. Members of this family are single-stranded (+) RNA viruses with large genomes of approximately 15–20 kb containing 9–12 open reading frames (ORFs; Figure 13.1). Viruses are classified into families (designated by the—*viridae* suffix) and genera (designated by the—*virus* suffix). Viruses in the family *Closteroviridae* are grouped into three genera based on genome organization and biological properties (Martelli et al., 2002; Dolja et al., 2006). The genus *Closterovirus* comprises aphid-transmitted viruses having a single large genomic RNA, and includes *Citrus tristeza virus* (CTV) and *Beet yellows virus* (BYV). The second genus, *Ampelovirus*, comprises mealybug- and soft-scale-transmitted viruses including *Grapevine leafroll-associated virus 3* (GLRaV-3), also with a single large genomic RNA. The third genus, *Crinivirus*, comprises whitefly-transmitted viruses including *Lettuce infectious yellows virus* (LIYV) with two smaller genomic RNAs (Table 13.1).

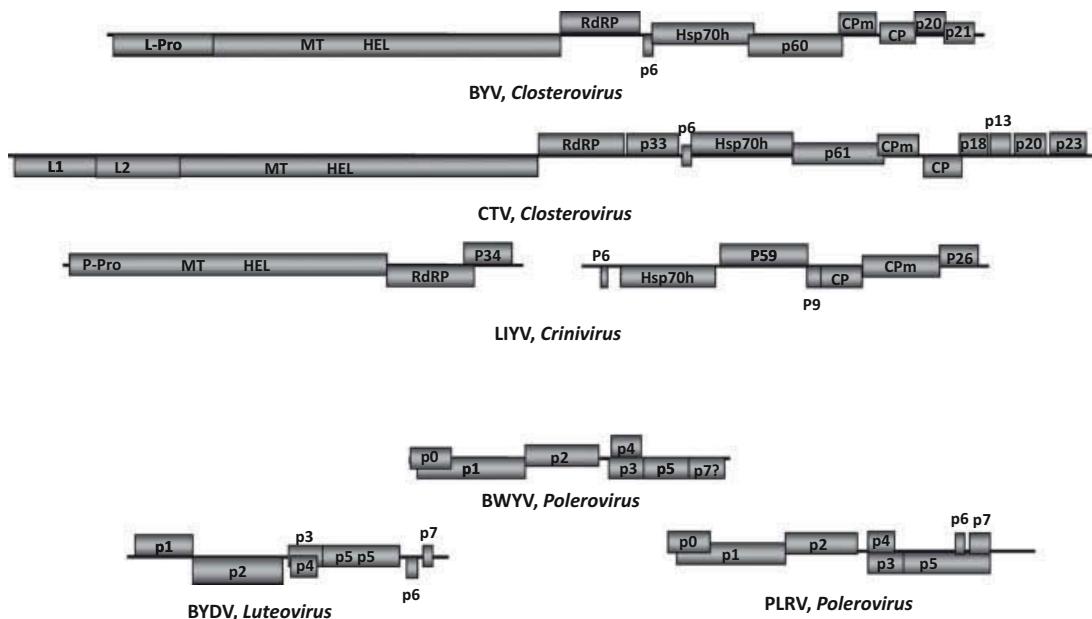


Figure 13.1 Selected virus genome maps. Genomes are based on GenBank sequence for: *Beet yellows virus* (BYV) NC_001589, *Citrus tristeza virus* (CTV) NC_001661.1, *Lettuce infectious yellows virus* (LIYV) NC_003717 RNA 1 (corrected for additional 3' adenine residue in deposited sequence and corrected protein predicted as P34 rather than P32) and NC_003618.1 RNA 2, *Beet western yellows virus* (BWYV) NC_004756, *Potato leafroll virus* (PLRV) NC_001741.1, with p6 and p7 ORFs predicted within these sequences based on Ashoub et al. (1998); and *Barley yellow dwarf virus-PAV* (BYDV) NC_004750.1. L-Pro, leader proteinase; MT, methyltransferase; HEL, helicase; RdRP, RNA-dependent RNA polymerase; Hsp70h, heat shock protein 70 homolog; CPm, minor coat protein; CP, coat protein; L1, leader proteinase 1; L2, leader proteinase 2. Numerical designations with p- or P- prefixes (according to published nomenclature for each virus) indicate ORFs encoding proteins of the numbered molecular weight in kilodaltons.

Table 13.1 Virus and viroid names

Abbreviation	Species name	Genus	Family	Transmission
VIRUSES				
BYV	<i>Beet yellows virus</i>	<i>Closterovirus</i>	<i>Closteroviridae</i>	Aphid
CTV	<i>Citrus tristeza virus</i>	<i>Closterovirus</i>	<i>Closteroviridae</i>	Aphid
LIYV	<i>Lettuce infectious yellows virus</i>	<i>Crinivirus</i>	<i>Closteroviridae</i>	Whitefly
GLRaV-3	<i>Grapevine leafroll-associated virus 3</i>	<i>Ampelovirus</i>	<i>Closteroviridae</i>	Mealybug
BYDV	<i>Barley yellow dwarf virus</i>	<i>Luteovirus</i>	<i>Luteoviridae</i>	Aphid
BWYV	<i>Beet western yellows virus</i>	<i>Polerovirus</i>	<i>Luteoviridae</i>	Aphid
PLRV	<i>Potato leafroll virus</i>	<i>Polerovirus</i>	<i>Luteoviridae</i>	Aphid
VIROIDS				
PSTVd	<i>Potato spindle tuber viroid</i>	<i>Pospiviroid</i>	<i>Pospiviroidae</i>	Vegetative; aphid with PLRV only
ASBVd	<i>Avocado sunblotch viroid</i>	<i>Avsunviroid</i>	<i>Avsunviroidae</i>	Graft, seed
PLMVd	<i>Peach latent mosaic viroid</i>	<i>Pelamoviroid</i>	<i>Avsunviroideae</i>	Mechanical, graft, seed, rarely aphid
HSVd	<i>Hop stunt viroid</i>	<i>Hostuviroid</i>	<i>Pospiviroidae</i>	Mechanical

BYV is transmitted by several aphid species, and has been a significant agricultural problem for many decades in Europe and the United States (Bennett, 1960). It has a single genomic RNA with nine ORFs. ORF 1a encodes a cleaved leader proteinase (L-Pro) and has methyltransferase and helicase domains. ORF 1b is expressed as a fusion protein with the ORF1a product by frame-shifting and encodes the viral RNA-dependent RNA polymerase (RdRP). The methyltransferase/helicase and RdRP proteins are required for viral RNA replication. The remaining ORFs encoding p6, a heat shock protein 70 homolog (Hsp70h), p64, the minor coat protein (CPm), coat protein (CP), p20, and p21 proteins are expressed from 3' coterminous subgenomic RNAs (Figure 13.1). Phloem-limitation for BYV is less strict than for other viruses described as phloem limited and can be somewhat host dependent. BYV has several local lesion hosts including *Chenopodium* species, in which mechanical or rub inoculation will result in infection foci in the inoculated leaves, but infection typically does not become systemic. These features have enabled cell-to-cell movement studies of BYV in mesophyll cells, identifying five proteins involved in this process: the four capsid proteins CP, CPm, Hsp70h, p64, and the transmembrane protein p6 (Figure 13.1; Peremyslov et al., 1999; Alzhanova et al., 2000).

CTV has been an economically important pathogen shaping the citrus production industry since at least 1836 (Moreno et al., 2008). It is transmitted by several species of phloem-feeding aphids, with brown citrus aphid (*Toxoptera citricida*) being one of the most efficient and important vectors. CTV has an overall genome organization similar to BYV. However, the CTV genome is 25% larger than the BYV genome and encodes four unique proteins. The CTV genome contains 12 ORFs encoding at least 18 proteins: two leader proteinases (L1 and L2) and the methyltransferase/helicase protein from ORF 1a, the RdRP translated from ORF1b as a frameshift/fusion product, p33, p6, p65, p61, CPm, CP, p18, p13, p20, and p23. Virions accumulate in phloem cells and four proteins, CPm, CP, p65, and p61, are involved in virion encapsidation. CTV encodes at least three proteins with silencing suppressor activity: CP, p23, and p20 (Lu et al., 2004). p20 is a homolog of BYV p21 that also forms amorphous inclusion bodies in infected cells (Gowda et al., 2000) and p23 is important in regulating RNA accumulation during replication (Satyanarayana et al., 2002). Functions have not been identified for CTV p33, p18, or p13 proteins, and infectious clones lacking these genes were able to replicate, assemble, and move long distance in host plants without apparent deficiencies (Tatineni et al., 2008). CTV p33, p18, p13, and p23 do not have homologs in other members of the genus *Closterovirus*. CTV infects trees and can therefore be used to study movement and phloem interactions in woody species where long-term and superinfections are common.

LIYV is transmitted by whiteflies (*Bemisia tabaci* biotype A) and caused devastating disease of lettuce (*Lactuca sativa*) and cucurbits in the 1980s (Duffus et al., 1986). LIYV has similar genome organization to BYV and CTV, but is divided into two genomic RNAs and encodes two proteins unique to the genus *Crinivirus*, P9 and P26. LIYV P26 is responsible for a plasmodesmata-associated cytopathology. No functions have yet been identified for P9. An RNA 1-encoded LIYV protein, P34, is an RNA-binding protein that is essential for efficient replication of RNA 2 but not RNA 1 (Yeh et al., 2000; Wang et al., 2010).

Luteoviridae

Members of the *Luteoviridae* (luteovirids) are widespread and economically important viruses. Within their plant hosts they invade and are found in only phloem parenchyma cells, companion cells, and sieve elements (D'Arcy and DeZoeten, 1979). Luteovirids are spread among host plants by phloem-feeding aphids with most members showing a high degree of aphid vector specificity. The

majority of viruses in the family *Luteoviridae* fall within two genera, *Luteovirus* and *Polerovirus* (Figure 13.1; Table 13.1); while a third genus, *Enamovirus*, has only one member. Luteovirids have genomic RNAs of ~6 kb, and the genera are differentiated by distinct genome organizations, particularly in the genomic RNA 5' half that encodes proteins involved in RNA replication. By contrast, the 3' halves of the genomic RNAs for members of the *Luteovirus* and *Polerovirus* genera show conservation in gene composition and organization. Within this region are ORFs 3, 4, and 5. The luteovirid p3 and p5 ORFs are separated by an in-frame amber termination codon (UAG), and the ORF encoding p4 is contained completely within the ORF 3 nucleotide sequence, but in a different reading frame register. These ORFs are translated from the same subgenomic mRNA, but specific translation events give rise to the different proteins. When translation initiates at the ORF 3 start codon, if translation terminates at the in-frame UAG the result is the ~22 kD p3. However, if translation read-through occurs at the ORF 3 UAG codon, a ~72 kD p3–p5 fusion protein results, thus ORF 5 is not translated independently. Furthermore, if ribosomes bypass the ORF 3 start codon, translation initiates at the ORF 4 start codon yielding p4. The p3 and p3–p5 proteins are structural components of the luteovirid capsid while p4 is a nonstructural protein. These three proteins all have critical roles in aphid transmission and *in planta* phloem biology.

Luteovirid virions (virus particles) are isometric, ca. 30 nm in diameter with each particle containing a single molecule of the infectious genomic RNA. The capsid shell contains 180 molecules of protein, but is composed of a mixture of p3 (in majority) and a truncated form of p3–p5 (in minority). This is believed to result in a capsid with a truncated form of the p3–p5 protein protruding from the virion surface. The biological significance of the virions and their structural proteins is explained below (see “Long-Distance Movement Factors of Luteoviruses”).

Virus Interaction with Phloem Cells

How Do Viruses Get into Phloem Cells?

Initial delivery of viruses into plant cells depends upon their mode of transmission to the host. Some non-phloem-limited viruses can be transmitted by mechanical inoculation to epidermal or mesophyll cells, from which these viruses can move cell-to-cell into the phloem where they are moved systemically. However, other organisms, or vectors, that include fungus-like organisms, nematodes, and arthropods, transmit most plant viruses. Vectors usually acquire encapsidated viruses (virions) from virus-infected host plants. Viruliferous vectors then disperse and inoculate new host plant cells with infectious virions. Arthropods are the most common vectors of plant viruses, the majority being phloem-feeding hemipteran insects such as aphids. These can also deliver viruses to epidermal or mesophyll cells by probing behavior, and nonphloem-limited viruses can move from epidermal and mesophyll cells into phloem. Phloem-limited viruses, on the other hand, must be delivered directly into phloem cells by their insect vectors.

Hemipteran vectors of clostero- and luteoviruses are piercing-sucking insects that primarily inoculate virions directly into phloem cells to establish infection. Exactly which phloem cells are inoculated is less clear. Unlike nonphloem-limited viruses, phloem-limited viruses have limited or no capacity to move from nonphloem epidermal or mesophyll cells into the phloem. Presumably because of this, phloem-limited viruses are not usually transmissible by rub-inoculation of infected sap on most hosts. Longer vector feeding times can also be required for vectors to acquire and transmit phloem-limited viruses as compared to non-phloem-limited viruses. For example, BYV transmission rates are improved when aphid feeding times are increased from 5–20 minutes to

1–2 hours, providing evidence that sieve element feeding is more effective in virus acquisition and delivery than insect probing of nonphloem cells (Bennett, 1960).

As with the closteroviruses, luteovirid virions are deposited directly into the phloem by viruliferous aphids, and systemic infections commence. Also like closteroviruses, luteovirids are not mechanically transmissible to plants except under select conditions. All natural luteovirid infections result from inoculation by their aphid vectors.

How Do Viruses Interact with Phloem Cells During Infection?

Ultrastructural analyses have been valuable to study the interactions between viruses and phloem. The earliest thorough transmission electron microscopy (TEM) studies for the closteroviruses were performed by Katherine Esau and colleagues. They traced infection by observing two characteristic cytopathologies induced by closterovirus infection: the flexuous rod-shaped virions and membrane vesiculation (BYV-type vesicles) associated with replication in infected cells (Esau et al., 1967). BYV virions were present primarily in the phloem parenchyma or companion cells (indistinguishable in beet) and to variable extents in mature sieve elements of beet (*Beta vulgaris*) plants (Esau et al., 1967). Virus-induced inclusions were also observed in mesophyll cells adjacent to the phloem and sometimes in epidermal cells late in infection; this loss of strict phloem limitation accounts for mechanical delivery of this virus in some hosts. Based on presence of virions in plasmodesmata between phloem parenchyma cells, between companion cells and sieve elements, and in sieve plate pores they postulated that BYV replicated in phloem parenchyma cells and moved cell-to-cell through plasmodesmata and long-distance in sieve tubes as virions. Their observations also suggested that sieve elements were not sites of BYV replication at any time. Immature sieve elements showed no evidence of infection and mature sieve elements lacked essential components such as ribosomes and Golgi, from which BYV-type vesicles observed in infected cells were postulated to arise. Thus, virions observed in mature sieve elements are likely transported there from adjacent cells.

Plants infected by another member of the family *Closteroviridae*, the genus *Crinivirus*, have also been subjects of extensive ultrastructural studies. Unlike BYV, LIYV is strictly phloem-limited in all studied hosts (Medina et al., 2003). LIYV induced BYV-type vesiculation is associated with replication in phloem parenchyma and companion cells, and long filamentous virus particles accumulated in those cell types and in sieve elements (Hoefert et al., 1988; Medina et al., 1998). In addition to the characteristic cytopathologies associated with infection by members of the family *Closteroviridae*, LIYV-infected plants including *Nicotiana* spp., lettuce (*L. sativa*), and lamb's quarter (*Chenopodium murale*) exhibit a unique cytopathology: plasmalemma deposits. Distinct electron-dense conical plasmalemma deposits (Hoefert et al., 1988; Pinto et al., 1988; Medina et al., 2003) are located over plasmodesmata pit fields in phloem parenchyma cells and companion cells adjacent to sieve elements in all host plants observed, including plants in families Malvaceae, Asteraceae, Chenopodiaceae, Brassicaceae, and Solanaceae (Pinto et al., 1988; Medina et al., 2003) (Figure 13.2A–D). Virions oriented perpendicular to the plasma membrane at these plasmalemma deposits are also frequently observed (Medina et al., 2005; Stewart et al., 2009b). LIYV P26, encoded by the 3' ORF of RNA 2 is associated with and responsible for plasmalemma deposit formation (Medina et al., 2005; Stewart et al., 2009b). Heterologous expression of LIYV P26 from *Tobacco mosaic virus* (TMV) showed that P26 in the absence of other LIYV proteins is sufficient to induce plasmalemma deposit formation in phloem and nonphloem cells (Stewart et al., 2009b). Based on the location of plasmalemma deposits and the orientation of virions near them, they may have some function in virus movement between phloem parenchyma and companion cells and from

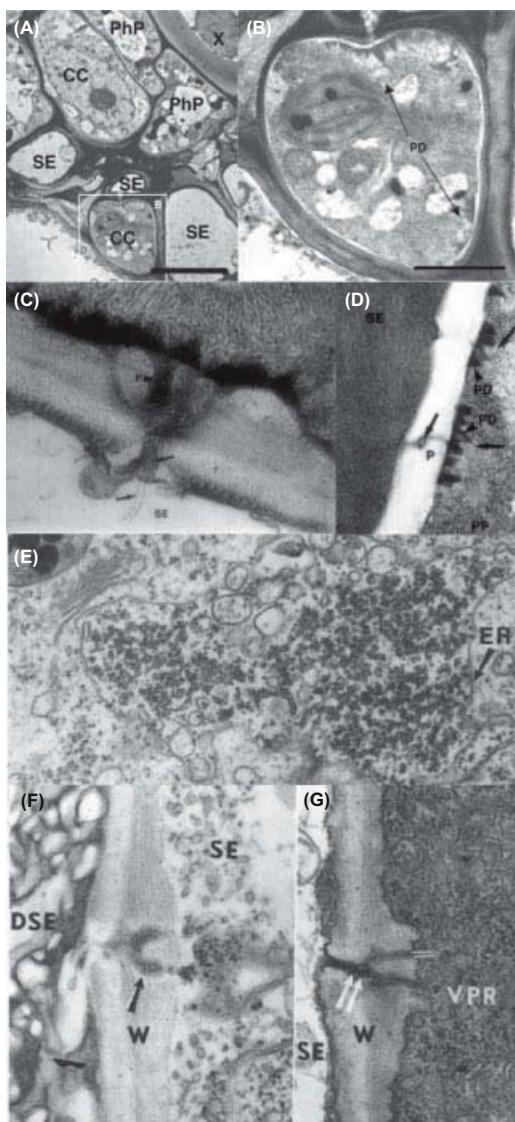


Figure 13.2 Virus-induced phloem cytopathologies. (A–B) TEM images of *Nicotiana benthamiana* infected with LIYV. Adapted from Medina et al., 2003 with permission from Wiley & Sons. (A) Cross section through leaf minor vein. PhP = phloem parenchyma, CC = companion cell, SE = sieve element, PD = plasmalemma deposits. Bar = 3.63 μm . (B) Inset from A showing plasmalemma deposits (PD) in companion cell. Bar = 1.25 μm . C–D. TEM images of LIYV-infected *Chenopodium murale*. Adapted from Pinto et al. (1988) with permission from Elsevier. (C) Pore–plasmodesma connecting sieve element (SE) and phloem parenchyma cell, showing flexuous rod virions (black arrows) within plasmodesma and in both cells, and plasmalemma deposits on the phloem parenchyma cell membrane above plasmodesmal pore (P, black arrowhead). (D) Virus particles filling sieve element (SE) with plasmalemma deposits (PD; black arrowheads) over pore-plasmodesma in adjoining phloem parenchyma cell (PP). Virions are aligned perpendicular to plasmalemma over deposits (black arrows). Virions in cross section are indicated in center cavity of plasmodesma (P; arrow). (E–G) TEM images of BWYV-infected pennycress (*Thlaspi arvense* L.). Adapted from Hoeffert (1984) with permission from Elsevier. (E) Phloem parenchyma cell containing cytoplasmic virions and membrane vesiculation. Black arrow indicates endoplasmic reticulum (ER). (F) A sieve element (SE) and degenerating sieve element (DSE) traversed by a modified plasmodesma containing spherical virus particles (black arrow, W). (G) A phloem parenchyma cell (VPR) and sieve element (SE) showing virions (W) inside connecting plasmodesma (white arrows) still containing intact desmotubule (white parallel lines) on the parenchyma cell side.

companion cells into sieve elements (Pinto et al., 1988), but such a role remains to be experimentally demonstrated.

Other criniviruses studied so far do not exhibit the plasmalemma deposit cytopathology, even though a 3' RNA 2 ORF encoding a protein of approximately 26 kDa is present in all sequenced members of the genus (Medina et al., 2003; Stewart et al., 2009b). Unfortunately, sequence analysis does not provide an unequivocal indication of function, and primary amino acid sequence for this protein is highly divergent even among the criniviruses. The predicted protein secondary structure is more conserved, although the LIYV P26 is an outlier, and crinivirus P26 orthologs share properties such as subcellular compartmentalization (determined by biochemical fractionation) patterns and self-interaction in a yeast-two-hybrid system (Stewart et al., 2009a, 2009b). It is possible that either some P26 functions are conserved or that similar biochemical properties function in different pathways among related viruses.

Ultrastructural studies have also demonstrated important features of luteovirid-plant interactions. For different luteovirids infecting different host plants, virions could be readily observed in phloem parenchyma, companion cells, and sieve elements; only in rare or specific circumstances were virions seen outside of the bundle sheath (Figure 13.2E; Esau and Hoefert, 1972a, 1972b; Gill and Chong, 1975; Gill and Chong, 1976; D'Arcy and DeZoeten, 1979). In addition, virions were detected in modified plasmodesmata lacking their "tubular core and (are) not covered by endoplasmic reticulum (ER) cisternae, features distinguishing them from normal plasmodesmata" (Figure 13.2F–G) (Esau and Hoefert, 1972b; Hoefert, 1984). This suggested that virions moved between cells through modified plasmodesmata.

An intriguing observation from earlier studies (Esau and Hoefert, 1972a, 1972b; Gill and Chong, 1976; Gill and Chong, 1979) was accumulation of virions in and around the nuclei, even though luteovirids replicate cytoplasmically such that no direct association with nuclei is expected. It should be noted that interactions between specific viral proteins and nucleolar components have recently been shown to be critical for systemic movement of other cytoplasmically-replicating RNA viruses including *Groundnut rosette virus* (genus *Umbravirus*) (Kim et al., 2007a, 2007b; Canetta et al., 2008) and *Potato mop-top virus* (genus *Pomovirus*) (Wright et al., 2010).

Together, ultrastructural studies of closteroviruses and luteovirids indicate that these viruses replicate in phloem parenchyma and companion cells, from which they are transported into sieve elements for long-distance movement. The mode of transport through plasmodesmata between phloem cells for each of these groups of viruses is distinct.

How Do Phloem-Limited Viruses Establish Systemic Infection?

Once phloem-limited viruses have been delivered to phloem cells, they must replicate and spread systemically throughout the host plant. Evidence exists for two different scenarios after vector inoculation. Virions might be inoculated directly into sieve elements by vectors and immediately move long distance to establish replication sites distant from the inoculation point. Alternatively, vectors may inoculate virions into phloem parenchyma and companion cells where they establish local replication sites and accumulate prior to transport into sieve elements for long-distance movement. Clever biological assays have been used to address these issues.

To estimate rates of BYV movement in aphid-inoculated beet plants, viruliferous aphids were caged on the distal end of a single leaf over 10 centimeters long. After inoculation times ranging from 10 minutes to 72 hours, aphids were removed and the inoculated leaf was cut off 10 centimeters from the inoculation site. Plants were subsequently observed for symptom development to determine

the rate at which inoculated viruses traversed the ten centimeters of leaf tissue that was excised after inoculation periods (Bennett, 1960). The shortest inoculation interval after which plants with excised leaves became infected was 20 minutes, corresponding to a movement rate of 0.5 cm/min immediately after inoculation (Bennett, 1960). This velocity of virus displacement immediately after delivery is consistent with virion delivery into and rapid transport within sieve elements. Microscopic observations suggesting that virions are the long-distance movement entities for BYV also support this idea, since aphids acquire and inoculate encapsidated virions.

Aphid transmission bioassays using monocot- and dicot-infecting luteovirids revealed patterns that differed with time. Only a 3% infection rate was observed for BYDV in oat plants when aphid inoculated leaves were removed at 6 hours after inoculation, and the rate increased to 47% at 72 hours after inoculation (Gill, 1968). For the dicot-infecting PLRV, no systemic infection was observed for any *Physalis floridana* plants when inoculated leaves were removed at 6 hours after inoculation. Only 2.5% of plants were infected at 24 hours after inoculation, whereas 63% of plants were infected if leaves were not removed (Mackinnon, 1960). One possible interpretation of these data is that viruses replicated at the vector delivery sites until they had accumulated to sufficient levels to begin systemic movement.

Microscopic analyses gave support to the luteovirid inoculation assays. TEM examination of BYDV-infected oat plants showed very few phloem-associated cells infected at 2 to 3 days after inoculation. These cells were found only in the aphid-inoculated leaves and interpreted to be primary infection foci (Gill and Chong, 1975). More recently, Nurkiyanova et al. (2000) utilized PLRV replication-driven green fluorescent protein (GFP) reporter gene expression and whole plant aphid inoculation to assess infection development. When plants were examined as early as 5 days after inoculation, GFP fluorescence was observed only in cells of the inoculated leaves. Approximately 75% of GFP expressing cells were phloem-associated in foci consisting of one to three cells. These foci did not continue to expand with time and were interpreted as sites of primary infection.

Molecular Biology of Virus Movement

Complementary DNA cloning and mutagenesis studies of plant virus genomic RNAs have allowed for reverse genetics approaches to identify virus-encoded proteins that facilitate *in planta* cell-to-cell and systemic spread. This has proven to be difficult for studying phloem-limited closteroviruses and luteovirids. Inoculating cloned viruses to obtain systemic infection of host plants is much more challenging for phloem-limited viruses than for mechanically transmissible viruses. Nevertheless, a variety of approaches have been effectively employed to generate new and important information about the viral factors critical for systemic infection by these viruses.

Long-Distance Movement Factors of Closteroviruses

In the local lesion host, *Claytonia perfoliata*, all of the capsid components of BYV (CP, CPm, Hsp70h, and p64) and p5, a conserved small hydrophobic protein, were found to be involved in virus cell-to-cell movement (Agranovsky et al., 1998; Peremyslov et al., 1999; Alzhanova et al., 2000). RNA protection assays indicated that the movement-deficient mutants were deficient in encapsidation of viral RNA (Alzhanova et al., 2000). These results suggest that encapsidated virions are the entities that move cell-to-cell, consistent with observation of virions in plasmodesmata and in sieve elements in ultrastructural studies.

Some BYV components, such as the capsid proteins, are required for both cell-to-cell and long-distance movement of BYV. Not surprisingly, however, there are BYV components that are specifically required for long-distance movement. These include BYV p20, a virion-interacting protein, and the leader proteinase protein.

Mutations in the BYV gene encoding p20 slightly reduced but did not abolish cell-to-cell movement (Alzhanova et al., 2000), but did negatively impact systemic infection. GFP-tagged BYV was used to test p20 mutants for long-distance movement defects following *Agrobacterium*-infiltration delivery into *Nicotiana benthamiana* plants. Unloading of the p20 mutants at distal uninoculated leaves was delayed (4 weeks vs. 2 weeks) and fluorescence was only observed in 13% of distal leaves compared to 93% for the wild-type GFP-tagged virus (Prokhnevsky et al., 2002). Further experiments revealed interaction between p20 and the host encoded heat shock protein 70 homolog (Hsp70h), and cofractionation of p20 with virions (Prokhnevsky et al., 2002). In yeast-two-hybrid experiments, p20 also interacted with both the N-terminal ATPase and C-terminal divergent domains of Hsp70h as well as with itself, so two molecules of p20 could interact with each Hsp70h molecule (Prokhnevsky et al., 2002). Hsp70h is part of the BYV virion that also localizes to plasmodesmata (Medina et al., 1999; Napuli et al., 2000; Avisar et al., 2008) and is required for mesophyll cell-to-cell movement of BYV in the local lesion host *C. perfoliata* (Alzhanova et al., 2000). When expressed alone in *N. benthamiana* cells, p20 showed diffuse cytosolic localization, but when co-expressed with Hsp70h, it relocalized to plasmodesmata as punctae (Prokhnevsky et al., 2002). This suggests that p20 interaction with virions via Hsp70h association may serve to direct long-distance transport of the virions.

How p20 functions to facilitate long-distance movement of BYV is not known, but several possibilities have been proposed: (1) it permits either entry or exit from the phloem by modifying the Hsp70h movement function, (2) it prevents virus uncoating in the alkaline phloem, (3) it protects virions from phloem defense proteins, or (4) it functions as an RNA silencing suppressor (Prokhnevsky et al., 2002). Since p20 is only marginally similar to other *Closterovirus* proteins, it is uncertain whether p20 functions are conserved among other viruses in the family.

Alanine scanning mutagenesis showed that the leader proteinase (L-Pro) of BYV was also important for long-distance movement (Peng et al., 2003). L-Pro is a 66-kDa protein with a variable N-terminus and a conserved papain-like protease region at the C-terminus required for autoproteolytic cleavage of the leader from the precursor protein. Mutations throughout L-Pro diminished or abolished long-distance movement and establishment of systemic infection in *Agrobacterium*-infiltrated *N. benthamiana* plants. Therefore, no specific region of the protein could be linked to the long-distance movement function. As for p20, the mechanism by which L-Pro functions in virus systemic movement remains unclear.

Unlike BYV, there are no data regarding viral genetic requirements for cell-to-cell movement for CTV. However, CTV genomic regions involved in long-distance movement have been identified. Recombinant CTV constructs with deletions in p6 or p20 were unable to systemically infect plants (Tatineni et al., 2008). These proteins might be involved in long-distance transport directly or indirectly by participating in upstream processes or by suppressing host RNA silencing against viral infection. Using stable GFP-tagged CTV mutants, it was shown that CTV only established infections in a subset of phloem cells, the identity of which is not clear (Folimonova et al., 2008). The fraction of cells infected increased with susceptibility of the host citrus species and the severity of the CTV isolate tested (Folimonova et al., 2008). Some CTV sequences were more virulent and moved longer distances than others that remained confined to sectors of the plants, indicating that movement efficacy and virulence are related (Folimonova et al., 2008).

Long-Distance Movement Factors of Luteoviruses

The cDNA forms of genomic RNAs for several luteovirids have been used for mutagenesis and bioassays in protoplasts and whole plants to identify the viral components associated with specific functions. *In vitro* transcribed RNAs can be introduced into protoplasts where virus replication and virion formation take place. Virions recovered from infected protoplasts are then used to feed aphids, which are in turn used to inoculate plants (Sanger et al., 1994; Chay et al., 1996). *Agrobacterium tumefaciens*-mediated delivery of T-DNAs containing luteovirid cDNAs directly into whole plants also has been developed to establish systemic infections in some plant hosts (Leiser et al., 1992; Commandeur and Martin, 1993).

ORF 0, found in all members of the genus *Poletovirus* but absent from other luteovirid genera, encodes the nonvirion protein p0. Mutations to eliminate production of p0 had debilitating effects on *in planta* accumulation of PLRV (Sadowsy et al., 2001), but not on protoplast replication and virion formation of BWYV (Veidt et al., 1992). Thus, p0 seems to be needed for establishing whole plant infections. Recent analyses identified the p0 protein as a potent suppressor of the RNAi-mediated antiviral defense in plants (Pfeffer et al., 2002). Whether p0 plays a role in systemic infection through its silencing suppressor function is unknown.

The luteovirid-encoded proteins p4, p3, and p5 (p3–p5) have more direct roles as phloem-specific movement proteins. The p4 protein, like p0, is not incorporated in virions. It was originally suggested to be a luteovirid movement protein based on amino acid sequence comparisons with movement proteins from other nonphloem-limited viruses and based on its binding of ssRNA *in vitro* (Tacke et al., 1991). Subsequent studies have confirmed a movement role for p4 of different luteovirids in some plant hosts. A BYDV mutant unable to produce p4 replicated well in protoplasts and formed progeny virions, but failed to systemically infect oat plants, even though it was aphid-transmissible. The authors argued that p4 is a systemic movement protein (Chay et al., 1996). However, the PLRV p4 was not required for systemic infection in *N. benthamiana* and *Nicotiana clevelandii* plants, but was required for systemic infection of *P. floridana* and *Solanum tuberosum* plants (Lee et al., 2002). Thus, p4 seems to facilitate systemic movement but might not be needed in all host species.

Recent work has revealed some host components important for intracellular movement of PLRV p4. Using transgenic *Arabidopsis* expressing a PLRV p4:GFP fusion protein, Vogel et al. (2007) showed microfilament and ER-Golgi-dependent p4:GFP movement to branched plasmodesmata in source leaves, whereas Hofius et al. (2001) showed microtubule and proteasome-dependent degradation in sink leaves. This supports previous localization data suggesting that p4 facilitates luteovirid movement from source leaves.

Because luteovirid virions are the viral entities for movement, it is expected that mutations disrupting virion formation also would affect *in planta* movement. For several luteovirids, mutations in p3 that prevent virion formation abolish systemic infection, but do not affect replication in protoplasts (Brault et al., 2003). However, as described earlier, the virion capsid also contains a form of the p3–p5 readthrough protein. The p3–p5 protein is critical for *in planta* systemic movement, but perhaps not when it is a component of the virion capsid.

The predicted size of the p3–p5 readthrough protein is ca. 72 kD. Even though the p3–p5 72 kD protein can be found in extracts of luteovirid-infected plants, a 55 kD proteolytically processed truncated form of p3–p5 containing only the intact p3 and N-terminal region of p5 is found in luteovirid virions isolated from infected plants. Virions with this 55 kD protein can be transmitted by aphids back to plants to establish systemic infections, and amino acids within the virion-incorporated p5 region of the p3–p5 protein determine aphid transmission specificity (Peter et al.,

2008). Even though p3–p5 is a biologically important component of the luteovirid virion capsid, p5 knockout mutants that make only p3 can still assemble infectious virions (Chay et al., 1996; Brault et al., 2000). However, these are non-aphid transmissible to plants.

In attempts to determine whether or not p3–p5 has a role in systemic plant invasion, a series of point mutation constructs in the conserved, N-terminal region of the BWYV p5 portion of p3–p5 was inoculated to *N. clevelandii* plants using *A. tumefaciens* (Brault et al., 2000). Mutants developed slower systemic infection than did wild-type BWYV, or they reverted to wild-type genotypes resulting in restored systemic movement *in planta*. These observations suggest that virions composed of only p3 were at least partly debilitated, and p3–p5 has a critical role in systemic movement.

Detailed analysis of a series of three amino acid deletions in the PLRV p5 portions of a related *Poletrovirus* showed additional roles of the p3–p5 proteins (Peter et al., 2008). Following *Agrobacterium*-inoculation of whole plants, progeny viruses were analyzed for p3–p5 incorporation into virions and for *in planta* systemic infection in four plant species: *N. benthamiana*, *N. clevelandii*, *P. floridana*, and *Solanum sarrachoides*. In some species, the p3–p5 mutants were slower and less efficient in developing systemic infections. For example, a DRT mutant (producing only p3 proteins, but no p5 proteins) failed to develop systemically in *P. floridana* plants, but did so to some degree in the other three species. Other mutants (QSS, GHPE and ERD) did not incorporate p3–p5 into virions, but did generate the mutant p3–p5 proteins within infected plants. These mutants were competent, at least to some degree, for establishing systemic infections in all host species tested. This indicates that nonvirion incorporated p3–p5 can facilitate movement of PLRV virions in plants. Furthermore, both N- and C-terminal regions of the p3–p5 protein were shown to contribute to systemic movement within the plant host, suggesting that full-length p3–p5 is necessary, and not just the truncated p3–p5 that is incorporated into virions. Further, it has been shown that p5 also is important in confining PLRV infections to the phloem (Peter et al., 2009).

A simple model based on current evidence can be constructed in support of the scenario that luteovirids replicate in cells prior to moving into the sieve tube translocation stream. During plant inoculation, aphids introduce intact luteovirid virions into the phloem parenchyma or companion cells, and these virions establish local infections that cannot move far from their point of introduction until p4, full-length p3–p5, and possibly p0 are produced. Production of these viral proteins that are not part of the aphid-delivered particles may be required to enable virions to move through modified plasmodesmata into sieve tubes through modified plasmodesmata for systemic transport. If aphids do deliver virus particles to sieve elements, it is possible that they do not move out of these cells to establish infection in uninoculated leaves without p4, full-length p3–p5, or p0.

Little is known about the host components that modulate or interact with viral proteins to accomplish specific functions. In a recent study, gel overlay studies followed by protein sequencing were employed to identify cucumber sieve-tube sap proteins bound to virions of *Cucurbit aphid-borne yellows virus* (CABYV, genus *Poletrovirus*) (Bencharki et al., 2010). Two of the identified proteins are lectins of the phloem protein 2 (PP2) family. Whether or not these lectins have a role in luteovirid movement is unknown, but it is of interest to note that several independent studies have shown that these lectins bind viroids and some plant virus RNAs *in vitro* and *in vivo* (Gomez and Pallas, 2001; Owens et al., 2001; Gomez and Pallas, 2004; Bencharki et al., 2010).

Phloem-Limited Viruses and Symptom Development

Phloem-limited viruses have been grouped as “yellows” viruses because of similar symptoms of either vein clearing while leaf blades stay green or chlorosis of leaf blades except for veins

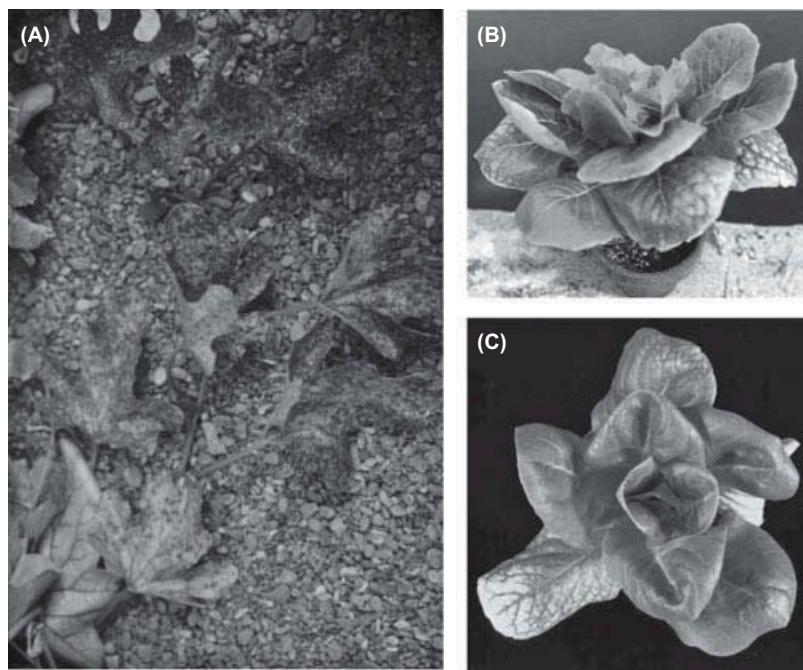


Figure 13.3 Symptoms of phloem-limited viruses. (A) A melon plant infected by *Cucurbit yellow stunting disorder virus* (CYSDV), a *Crinivirus*. (B) A lettuce “Summer Bibb” plant infected by *Beet western yellows virus* (BWYV). (C) A lettuce plant infected by *Lettuce infectious yellows virus* (LIVY). Note yellowing in brittle older leaves with bright green veins, while symptoms are lacking in young leaves. (For a color version of the figure, please see Plate 13.1.)

(Figure 13.3). Since phloem-limited viruses replicate in and are transported within the phloem, an obvious question is whether these interactions result in specific responses by the cells of the phloem tissue or in modifications of normal phloem activity that lead to symptom development. At present however, the molecular basis of symptom development is not well understood for phloem-limited viruses and may be quite complex.

Disruption in vascular transport of host miRNAs has been implicated in CTV symptom development. CTV-like symptoms are observed in transgenic lime (*Citrus aurantifolia*) plants expressing the CTV p23 gene (Fagoaga et al., 2005), which has been shown to have silencing suppressor activity in *Nicotiana* spp. (Lu et al., 2004). Protein accumulation in lime was required for this symptom development. Sweet (*Citrus sinensis*), sour (*Citrus aurantium*), and trifoliolate orange (*Poncirus trifoliata*) plants transgenic for the p23 gene also developed CTV-like symptoms, but not when a truncated version of the p23 gene was transformed into the plants. Symptom intensity in citrus species and relatives other than Mexican lime was related to transcript abundance, while protein was almost undetectable. This indicates that very little p23 protein is required for symptom development in more sensitive hosts. Similar symptoms were not induced by p23 expression in *Nicotiana* spp., which suggests that p23 disrupts miRNA-regulated development only in citrus and citrus relatives (Fagoaga et al., 2005). Thus for CTV, there is evidence that phloem expression of the p23 silencing suppressor protein disrupts endogenous silencing signal transport or receipt and accounts for some of the virus disease symptoms.

Strong symptoms of closterovirus, crinivirus, and ampelovirus infection occur interveinally, in tissues outside of where the phloem-limited viruses replicate and reside. For example, infection by the *Crinivirus* LIYV results in some interveinal chlorosis as well as leaf thickening and brittleness in entire older leaf blades. Like some of the closteroviruses, the majority of luteovirids cause yellowing symptoms on older leaves of virus-infected plants. These economically important plant viruses have been referred to as causal agents of the “yellow plague” (Duffus, 1977). Notably, the older, yellow leaves at least superficially resemble leaves showing nutritional deficiencies, but the leaves are brittle, not senescent, and their veins remain bright green. One speculation is that yellows symptoms are the result of reduced phloem transport of nutrients, since fertile soil can sometimes reduce symptom severity, but more data are needed to support this idea.

Viroids, Parasites of the Host Plant Transcriptional Machinery

Viroids are single-stranded circular RNAs that infect plants. Like viruses, viroids initiate replication in single cells and then spread into neighboring cells and distant organs to establish systemic infection in compatible host plants. They can also cause plant diseases depending on viroid-host combinations. Unlike viruses, viroids do not have protein-coding capacity and are not encapsidated, nor do any viroids have membranous envelopes. Unlike other subviral agents such as defective interfering RNAs and satellite RNAs/viruses (Simon et al., 2004), viroids do not depend on helper viruses for certain aspects of the life cycle. Viroid genomes contain all the genetic information necessary to direct replication in single cells and systemic movement throughout a plant to establish infection. Viroids are not phloem-limited during infection, but their systemic movement occurs through the phloem.

Viroid Interaction with Phloem Cells

How Do Viroids Replicate?

With genome sizes ranging from 250 to 400 nucleotides, viroids are the smallest nucleic acid-based infectious agents and also the smallest self-replicating genetic elements known to date. The approximately 30 species of known viroids are classified into two families, *Pospiviroidae* (type member *Potato spindle tuber viroid*, PSTVd) and *Avsunviroidae* (type member *Avocado sunblotch viroid*, ASBVd) (Flores et al., 2005; Tsagris et al., 2008; Ding, 2009). The two families are distinguished by several characteristics including structure, intracellular replication compartment, and mechanism of replication. Members of *Pospiviroidae* fold into a rod-like secondary structure (Figure 13.4) whereas members of the *Avsunviroidae* fold into a quasi-rod-like secondary structure with branches. The replication of *Pospiviroidae* takes place in nuclei, whereas members of *Avsunviroidae* replicate in chloroplasts. While all members of the *Avsunviroidae* possess ribozyme activities for self-cleavage during replication, those of the *Pospiviroidae* do not.

Viroids in both families replicate by means of an RNA–RNA rolling circle mechanism without the involvement of a DNA intermediate. The circular genomic RNAs are initially transcribed by cellular DNA-dependent RNA polymerases into concatemeric, linear RNA molecules. These molecules undergo cleavage to produce unit-length molecules that are circularized. The detailed aspects of this replication process, including the RNA structures and cellular factors involved as

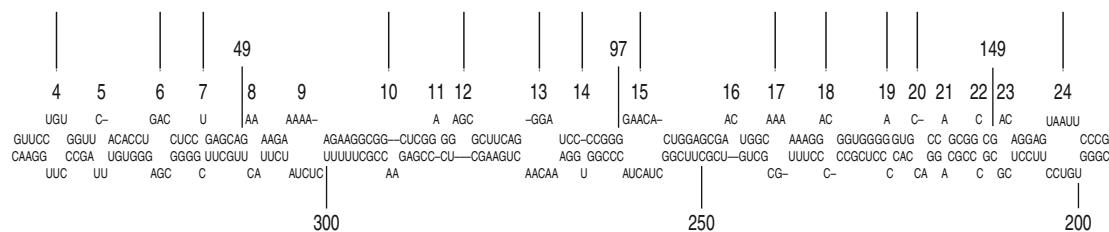


Figure 13.4 Secondary structure showing functional motifs of *Potato spindle tuber viroid* (PSTVd). Motifs (i.e., loops in this secondary structure) critical for systemic movement (M) in a whole plant or for replication (R) in single cells of *Nicotiana benthamiana* are marked. All loops are numbered 1–27 from left to right. The numbers 1 to 359, indicated by thinner lines, indicate reference nucleotide positions. (Reprinted, with modifications, from Zhong and Ding (2008) with permission from the American Society of Plant Biologists).

well as the common and unique features for the two families have been recently reviewed (Ding, 2009; Flores et al., 2009).

How Do Viroids Move in Plants?

To establish systemic infection, viroids must move from initially infected cells into neighboring cells and then systemically to distant organs. Cell-to-cell movement occurs through plasmodesmata. The first evidence of this came from microinjection experiments, in which fluorescently labeled *in vitro* transcripts of PSTVd were injected into tobacco leaf mesophyll cells interconnected by plasmodesmata or mature guard cells lacking such connections. The transcripts injected into a mesophyll cell moved rapidly into neighboring cells but those injected into a mature guard cell did not, supporting the notion that PSTVd moved through plasmodesmata (Ding et al., 1997). PSTVd transcripts could mediate movement of plasmid-derived RNA sequences which otherwise did not move between cells, suggesting that PSTVd has sequence or structural elements to mediate macromolecular cell-to-cell movement (Ding et al., 1997).

Long-distance movement of PSTVd occurs through the phloem. The first evidence of this was provided by a study in which the systemic spread of PSTVd was followed from an inoculated tomato leaf into different tomato organs (Palukaitis, 1987). RNA blot data showed PSTVd accumulated in roots as well as leaves above the inoculated leaf, but not in leaves below. Because this pattern is similar to that of photoassimilate distribution, it was proposed that PSTVd moved long distances through the phloem. In support of this hypothesis, *in situ* hybridization localized PSTVd in the phloem parenchyma and companion cells of infected tomato plants (Zhu et al., 2001). Because the hybridization signals were largely derived from viroid populations in the nuclei, they do not allow confident visualization of cytoplasmic viroid signals in the enucleate sieve tubes.

Mutational analysis identified more than ten loops in PSTVd as being critical for systemic infection in *N. benthamiana* plants (Figure 13.4) (Zhong and Ding, 2008). Detailed studies identified specific PSTVd loops that are required for trafficking from the bundle sheath into spongy mesophyll cells in tobacco leaves (Qi et al., 2004), from the bundle sheath into the phloem in *N. benthamiana* plants (Zhong et al., 2007), and from the palisade mesophyll into spongy mesophyll in *N. benthamiana* plants (Takeda et al., 2011). In the latter two studies, the three-dimensional structures of the loops, called trafficking motifs, were determined. Because similar or identical three-dimensional motifs in rRNAs are protein-binding sites, these PSTVd motifs may also act as sites for protein binding to potentiate PSTVd movement.

Do Viroids Move into Shoot Apices?

After long-distance movement into the shoot apex, it is unclear whether or not viroids enter the shoot apical meristem (SAM). *In situ* hybridization experiments failed to localize PSTVd in the SAMs of infected tomato and *N. benthamiana* plants (Zhu et al., 2002; Qi and Ding, 2003), whereas *Peach latent mosaic viroid* (PLMVd), a member of the family *Avsuniviroidae*, was present in the SAMs of infected peach trees (Rodio et al., 2007). The mechanistic basis for these differences is unknown. One possibility is that PLMVd has a particular structural motif to allow entry into the SAM, whereas PSTVd lacks an equivalent motif. Alternatively, RNA silencing could prevent movement of PSTVd into the SAM (Di Serio and Flores, 2008). Recent reports provide experimental evidence that RNA silencing, as demonstrated for some viruses (Foster et al., 2002; Schwach et al., 2005), plays a

role in preventing PSTVd from infecting SAM of *N. benthamiana* plants (Di Serio et al., 2010). PSTVd accumulated in the SAMs of infected transgenic *N. benthamiana* plants that had reduced expression of RNA-dependent RNA polymerase 6 (RDR6), an enzyme that has a prominent role in RNA silencing against viral infection (Foster et al., 2002; Qu et al., 2005, 2008; Schwach et al., 2005; Vaistij and Jones, 2009). An unresolved question is why RDR6 represses PSTVd infection in the SAM, but not in other sink organs. Furthermore, it remains to be determined whether RNA silencing inhibits the replication of incoming viroids in the SAM or blocks movement of viroid RNAs into the SAM by a yet-to-be understood mechanism. How PLMVd overcomes the RNA silencing system in peach to invade the SAM warrants further investigation.

In infected *N. benthamiana* and tomato plants, PSTVd was detected by *in situ* hybridization in sepals but not in the other floral organs such as petals, stamens, and pistils (Zhu et al., 2001; Zhu et al., 2001). Despite this result, PSTVd could replicate in all floral organs when they were directly “inoculated” by transgenic expression of a PSTVd cDNA that produced primary transcripts to initiate RNA–RNA replication. The presence or absence of PSTVd in the floral organs in mechanically inoculated plants was best explained by the selective movement of PSTVd into sepals that did not occur in the other floral organs (Zhu et al., 2002). However, reverse transcriptase-polymerase chain reaction (RT-PCR) experiments detected presence of PSTVd in all floral organs of infected tomato plants (Singh, 2006). Whether such differences resulted from the different sensitivities of analytical methods or growth conditions remain to be resolved. Clearly, further studies are necessary to address these issues.

Interactions between Phloem Proteins and Viroids

The host factors involved in viroid movement, whether from cell to cell through plasmodesmata or long distance in the sieve tubes, remain to be elucidated. Several sieve-tube exudate proteins interact with viroids, raising the question of whether they play a role in viroid movement within the sieve tubes. The phloem lectin PP2 from cucumber (CsPP2) binds *Hop stunt viroid* (HSVd) *in vitro* (Owens et al., 2001; Gomez and Pallas, 2004). Immunoprecipitation experiments demonstrated that CsPP2 and HSVd interact *in vivo* in infected cucumber plants (Gomez and Pallas, 2004). Furthermore, both CsPP2 (Golecki et al., 1999; Gomez and Pallas, 2004) and HSVd (Gomez and Pallas, 2004) can move long distances from rootstocks into scions in heterografts. The CsPP2 has an RNA-binding motif (Gomez and Pallas, 2004). Gomez et al. (2005) identified two proteins that bind ASBVd. These include the previously characterized PP2-like CmmLec17 that can move long distance from rootstocks to scions in heterografts (Dinant et al., 2003) and an uncharacterized 14-kDa protein. The functions of these proteins in viroid movement or other aspects of infection remain to be experimentally determined.

Future Directions

Many fundamental questions remain to be answered regarding how viruses and viroids move between various phloem cells and through the phloem. Viruses and viroids are intracellular parasites that co-opt host components to complete their life cycles, including replication in phloem parenchyma and companion cells and long-distance movement through sieve tubes. Viroid RNA structural motifs and virus-encoded proteins can facilitate long-distance movement, and viruses can induce alterations of host phloem cells. However, technical hurdles impede progress in understanding the interactions of these parasites with plant tissues. TEM studies rely on fixation methods that can wound or alter the

structures observed. Although microscopy can beautifully show virus particles and cytopathologies, it is not simple to generate a sequence of events or explain mechanisms. Viroids and plant viruses use phloem for long-distance transport, but the regulation of phloem entry and egress are variable in different host plants. General rules that apply to plant virus movement in regard to the nature of each barrier are not well described. Plasmodesmata regulate the transport of macromolecules in phloem cells, but have been recalcitrant to study. Recent work has begun to identify plasmodesmatal components that relate to viral movement (Benitez-Alfonso et al., 2010). A future focus of research is to elucidate the components of plasmodesmata interconnecting the different phloem cell types, in order to come to the next level of understanding of virus and viroid interactions with the phloem in cell-to-cell and systemic movement.

Plant viruses and viroids, because of their intimate association with and co-opting of host phloem, have been and will continue to be valuable tools to understand the biology of phloem function and mechanisms for transport of macromolecules under healthy conditions as well as during infection. Viroids in the family *Avsunviroidae* pose a unique question about the biology of phloem. Do the plastids in the sieve tubes support replication of these viroids in order to sustain long-distance movement? If so, what is the function of these plastids for basic phloem function? Further extending this question, do plastids in other phloem cells such as phloem parenchyma and companion cells support viroid replication?

Many virus-encoded proteins that were originally identified as long distance movement proteins have been discovered to be silencing suppressors. What is the role of RNAi in the phloem, and how does RNAi affect the ability of viruses and viroids to move systemically? Is silencing stronger outside the phloem, confining viruses with weaker silencing suppression capabilities? Alternatively, is there a selective advantage for phloem limitation? These questions and others that will be addressed in future work will significantly contribute to knowledge about the basic biology of phloem. Further work and improving approaches to identify viral long-distance movement factors and host factors involved in virus and viroid long-distance movement in the phloem will enable discovery of mechanisms behind these intracellular parasites that utilize plant phloem systems for replication and transport.

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14 Phytoplasmas and Spiroplasmas: The Phytopathogenic Mollicutes of the Phloem

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Spiroplasma and “*Candidatus Phytoplasma*” comprise two genera of insect-transmitted plant pathogens that belong to the class *Mollicutes* (trivial name, mycoplasmas) within the Gram-positive bacteria. Spiroplasmas and phytoplasmas are small bacteria (200 nm to 2 μM diameter) that lack cell walls and have limited genome sizes (530–2220 kbp), all of which are common characteristics of mollicutes. Unlike other mollicutes, however, spiroplasmas and phytoplasmas have a unique life cycle that encompasses hosts from two distinct kingdoms, namely insects (Animalia) and plants (Plantae). The ability of these bacterial pathogens to successfully invade and colonize two such dissimilar host environments is remarkable and implies the evolution of mechanisms that enable these bacteria to modulate processes in both eukaryotic hosts.

Biology of Spiroplasmas and Phytoplasmas

Phytoplasmas and spiroplasmas share similar habitats and common environmental niches, although phylogenetic analyses reveal these bacterial pathogens to be only distantly related within the class *Mollicutes*. Within this class, two clades diverged at an early stage of evolution, to give rise to the AAA (*Asterolesplasma*, *Anaeroplasma*, and *Acholeplasma*) and the SEM (*Spiroplasma*, *Entomoplasma*, and *Mycoplasma*) branches of *Mollicutes*; the genera *Spiroplasma* and *Mycoplasma* fall within the SEM branch whereas “*Ca. Phytoplasma*” fall within the AAA branch (Woese, 1987). The term “spiroplasma” was first employed in 1973 to describe the causative agent of corn stunt disease (*Spiroplasma kunkelii*) and this species has been successfully cultivated *in vitro* (Liao and Chen, 1977). The term “phytoplasma” was proposed in 1994; *Candidatus* is a prefix that reflects the inability to cultivate phytoplasmas *in vitro*. Within “*Ca. Phytoplasma*”, members are assigned to the designated groups according to such parameters as 16S rRNA sequence homology, vector specificity, and host range.

Abbreviations: AY-WB, “*Candidatus Phytoplasma asteris*” strain witches’ broom; *Ca*, *Candidatus*; NLS, nuclear localization signal; OY, “*Candidatus Phytoplasma asteris*” strain onion yellows; SAP, secreted AY-WB protein

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Life Cycle of Phytopathogenic Spiroplasmas and Phytoplasmas

The life cycles of plant pathogenic spiroplasmas and phytoplasmas are very similar, and the infection patterns of these organisms exhibit common characteristics. Both groups of bacteria are limited to colonizing the phloem of an infected plant, whereas they can invade multiple organs and tissue-types within an insect host. Phloem-feeding insects acquire the spiroplasmas and phytoplasmas while ingesting the sieve-tube sap of a diseased plant (Figure 14.1).

Upon ingestion by the insect, the bacteria traverse the gut epithelial cell layer to access the circulatory system (hemolymph) of the host. During the course of infection, the bacteria gain access to the salivary glands of the insect, at which stage the insect becomes competent to transmit the bacteria to a healthy plant (Ammar and Hogenhout, 2006). An infected insect inoculates a healthy plant during feeding by injecting saliva containing the phytoplasma or spiroplasma into the sieve tube of the host plant via the stylets. Both spiroplasmas and phytoplasmas remain limited to the phloem within the plant, replicating within this environment and enabling transmission of the bacteria to naive insects that feed upon the host. Once infected by either spiroplasma or phytoplasma, insects and plants remain infected for life and serve as reservoirs from which the infection can be disseminated. This is particularly relevant with respect to the insect vector, as phytoplasmas and spiroplasmas can “overwinter” within these hosts and establish a new outbreak of disease in agricultural crops following spring planting.

Spiroplasmas and Phytoplasmas: Expert Colonizers of the Phloem

Spiroplasmas and phytoplasmas are injected by their insect vectors directly into sieve tubes and become distributed systemically throughout the plant via the phloem (Christensen et al., 2004; Wei et al., 2004; Saracco et al., 2005). Microscopic examination revealed the presence of phytoplasmas and spiroplasmas in mature and immature sieve elements (Lherminier et al., 1994; Christensen et al., 2004; Ammar and Hogenhout, 2006) and phytoplasmas in phloem parenchyma and companion cells (Siller et al., 1987; Rudzinska-Langwald and Kaminska, 1999). Phytoplasmas have been observed to move through sieve plate pores (Rudzinska-Langwald and Kaminska, 1999; Chung et al., 2001; Christensen et al., 2005) suggesting this is the method used to migrate along the longitudinal length of the sieve tube. The pleomorphic nature of phytoplasma cells enables these bacteria to squeeze through sieve plate pores in the form of fingerlike projections (Rudzinska-Langwald and Kaminska, 1999). The mechanism that allows these prokaryotes to move from sieve elements to the companion cells and phloem parenchyma cells is unknown, although presumably involves passage via plasmodesmata. Nonetheless, the diameter of the bacterial cell is too large to permit free passage through the plasmodesmata-pore unit and plasmodesmata, and examination of infected plants fails to reveal any alterations or damage to these structures (Rudzinska-Langwald and Kaminska, 1999; Christensen et al., 2005). Possibly, the bacteria could produce proteins, analogous to viral movement proteins that enable the modification of plasmodesmata to facilitate cell-to-cell transfer. Alternatively, phytoplasmas might degrade or damage the cell walls of infected sieve elements and subsequently phloem parenchyma or companion cells, initiating the formation of cavities in which the pathogens could be located (Rudzinska-Langwald and Kaminska, 1999).

Once inoculated by a phloem-feeding insect, phytoplasmas and spiroplasmas spread via the symplasm throughout the plant over a period of days to weeks, with movement typically occurring from the point of inoculation to the sink tissues. The titer of chrysanthemum yellows phytoplasma is greatest within the sink tissues of developing leaves and particularly roots when inoculated from the

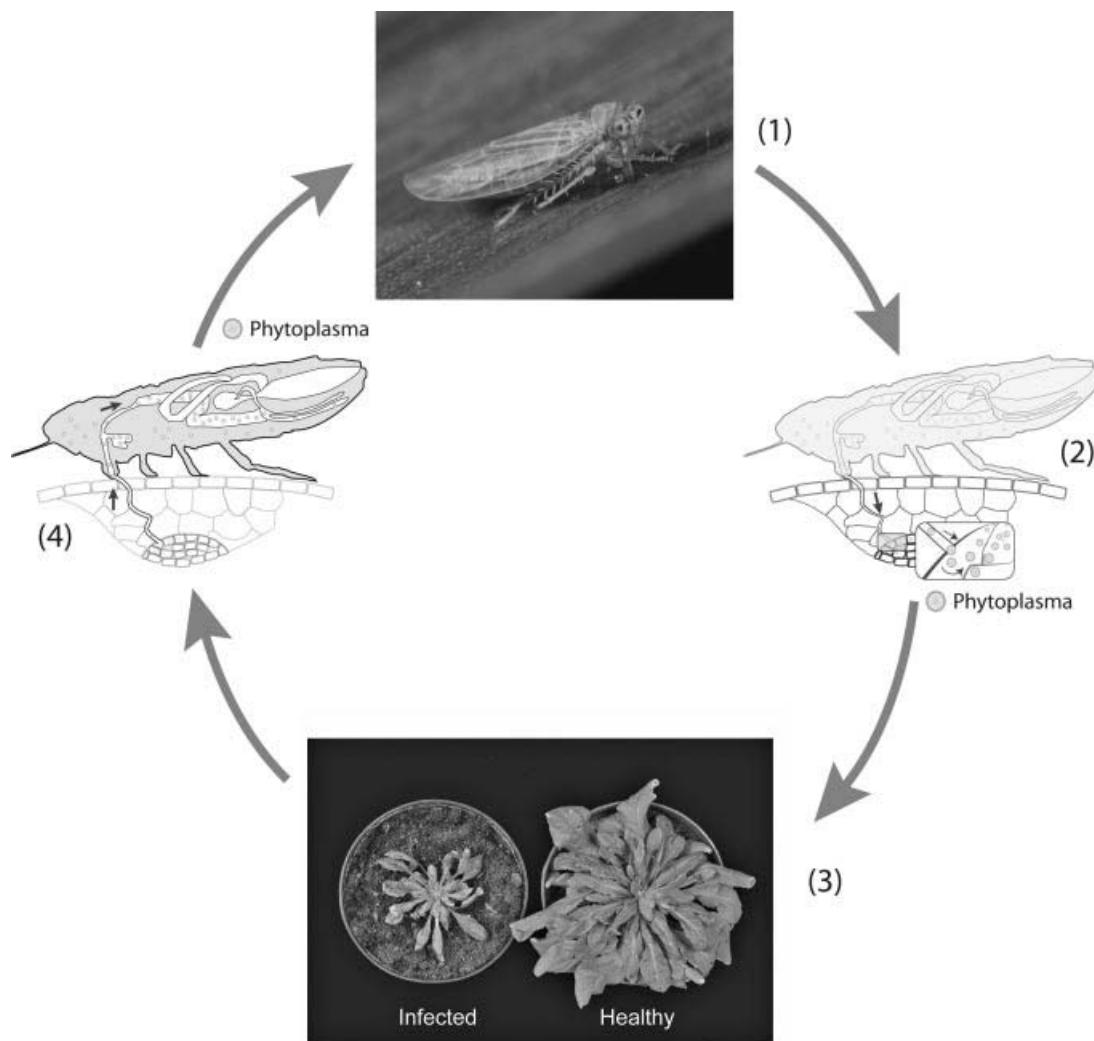


Figure 14.1 Phytoplasmas and spiroplasmas have a unique dual host life cycle: (1) Phytoplasmas and spiroplasmas are plant pathogenic bacteria that can be transmitted to plants by a phloem-feeding insect vector such as the aster leafhopper (*Macrostelus quadrilineatus*); (2) A phytoplasma-infected leafhopper injects the mollicutes into the phloem of a healthy plant, a process referred to as inoculation feeding; (3) The bacteria colonize the new host, inducing a variety of symptoms such as yellowing and stunting; and (4) An uninfected leafhopper acquires the plant pathogenic bacteria while feeding from the sieve tube of an infected plant, a process referred to as acquisition feeding. The bacteria are ingested into the midgut of the insect, and must cross the epithelial cell layer to access the hemolymph of the host. Following replication in the hemolymph, phytoplasmas and spiroplasmas migrate to the salivary glands of the insect, which subsequently becomes competent to transmit the infectious bacteria to a plant while feeding from the phloem. (For a color version of the figure, please see Plate 14.1.)

basal source leaves of chrysanthemum (*Chrysanthemum carinatum*), leading to the proposal that the translocation of the microbial cells occurs via passive diffusion alongside photoassimilates (Saracco et al., 2005). Likewise, onion yellows phytoplasma was reported to spread from a localized point of inoculation to the stem and then young apical leaves and roots, with older leaves showing evidence of infection only at a later stage (Wei et al., 2004). In this particular study, leafhopper access was

restricted to a portion of the third or fourth leaf of an eight-leaf plant, thus allowing researchers to track the dissemination of the phytoplasma within the host from a single point of origin.

Other researchers have described contradictory results. A study that monitored the distribution of phytoplasmas in poinsettia (*Euphorbia pulcherrima*) reported the greatest titer of phytoplasma to occur in source tissues (photosynthetic leaves), with a lower titer of the bacteria present in sink tissues (roots and developing leaves) (Christensen et al., 2004), leading to the suggestion that the bacteria might adhere to and colonize the length of the sieve tube, allowing movement against the mass flow (Christensen et al., 2005). Alternatively, the phytoplasmas might have become lodged or retained within the sieve elements of source leaves, which might also serve to support a greater rate of phytoplasma replication than sink tissues.

Spiroplasmas are well known for their characteristic twitching movements and these motile bacteria exhibit a chemotactic response towards attractants such as carbohydrates and amino acids (Daniels et al., 1980). Spiroplasma motility is catalyzed by a unique cytoskeleton comprising fibril and actin-like proteins that assemble to form parallel filaments whose coordinated movements translate to cellular motion (Kurner et al., 2005). *Spiroplasma citri* G540 was isolated in a screen implemented to identify motility mutants following transposon mutagenesis, and is unable to generate the rotational movement that would otherwise permit the cells to migrate across a viscous solution. A culture of the *S. citri* mutant microinjected into the leafhopper vector of the spiroplasma (*Circulifer haematoceps*) demonstrated that the mutant was able to migrate beyond the hemolymph to access the salivary glands of the insect (Duret et al., 2005). Moreover, the insect readily transmitted the strain to periwinkle (*Catharanthus roseus*), whereupon the phytopathogen induced symptoms that were equivalent to those induced by the wild-type parental strain. These studies indicate that motility might not be essential to the pathogenicity of *S. citri* in either insects or plants, as a strain that was unable to initiate active rotational movement nonetheless aggressively colonized both eukaryotic hosts. This conclusion is supported by the report of a nonmotile natural isolate of *S. citri* (strain ASP-1) that was cultured from a symptomatic sweet orange (*Citrus sinensis*) tree (Townsend et al., 1977).

Genomic Analyses Yield Insight into the Metabolism of Phloem Colonizing Bacteria

The genome sequences of four phytoplasmas have been reported (Oshima et al., 2004; Bai et al., 2006; Kube et al., 2008; Tran-Nguyen et al., 2008) revealing that these representative species lack intact metabolic pathways for amino acid and nucleotide biosynthesis, ATP synthesis, and fatty acid metabolism (Table 14.1), the result of a reductive evolution that is characteristic of obligate intracellular symbionts and pathogens. Indeed, “*Ca. Phytoplasma asteris*” strain onion yellows (OY) was the first reported biological organism to lack a canonical ATP-synthase system, which in other prokaryotes (including spiroplasmas) couples the biosynthesis of ATP to the proton motive force (Oshima et al., 2004). Subsequent genome sequencing has revealed this to be a common trait among the phytoplasmas, and the mechanism by which these bacteria synthesize ATP and generate a membrane potential is presently unknown. P-type ATPases are used in animal and plant cells as a means of pumping charged substrates across the cell membrane, and genes predicted to encode this system are present in the phytoplasma genomes. Possibly, phytoplasmas employ P-type ATPases to create an electrochemical gradient across the membrane (Christensen et al., 2005). Phytoplasmas also appear to lack cytochrome genes that encode products essential to oxidative phosphorylation, and it has been proposed that substrate-level phosphorylation (i.e., glycolysis) might be used to generate ATP (Tran-Nguyen et al., 2008).

Table 14.1 Characteristics of bacterial model organisms that form close associations with plants and animals

Strain	<i>Ca. phytoplasma</i> asteris AY-WB	<i>Spiroplasma citri</i> GII3-3X	<i>Ca. liberibacter</i> <i>asiaticus</i> PsY62	<i>Sinorhizobium</i> <i>melliloti</i> Rm1021	<i>Xylella fastidiosa</i> 9aSc	<i>Escherichia coli</i> K12 MG1655
Gram stain	Not applicable	Positive	Negative	Negative	Negative	Negative
Class	<i>Mollicutes</i>	<i>Mollicutes</i>	α-proteobacteria	α-proteobacteria	γ-proteobacteria	γ-proteobacteria
Host(s) or habitat	Insects and plants	Insects and plants	Insects and plants	Plants and rhizosphere	Insects and plants	Animals
Cultivated <i>in vitro</i> ?	No	Yes	Yes	Yes	Yes	Yes
Genome size (kbp) ^a	707	1820	1227	6691	2679	4639
Sugar uptake via PTS?	No	Yes	No	No	Enzyme I and HPr present	Yes
Generation of membrane potential	Undetermined	F ₁ F ₀ type ATP synthase	F ₁ F ₀ type ATP synthase	F ₁ F ₀ type ATP synthase	F ₁ F ₀ type ATP synthase	F ₁ F ₀ type ATP synthase
Tricarboxylic cycle	Absent	Absent	Present	Present	Present	Present
Amino acid biosynthetic capacity	Limited	Limited	Limited	Extensive	Extensive	Extensive
Secretion system(s)	Sec	Sec	Sec, Type I	Sec, Type I, Type IV	Sec, Type I, Type IV	Sec, Type I, Type II
Total number of transport proteins ^b	28	84	137	387	78	354

^aPlasmids were not included in calculations of genome size.^bTotal number of transport proteins as annotated by TransportDB (Ren et al., 2007) or determined in genome sequencing initiatives (Duan et al., 2009; Carle et al., 2010).

Glycolysis is likely the main energy-yielding pathway in phytoplasmas, although the glycolytic pathway appears to be incomplete in the apple proliferation phytoplasma “*Ca. Phytoplasma mali*” (Kube et al., 2008). Maltose, malate, and citrate are abundant within the sieve-tube sap of apples (*Malus domestica*, host plant of “*Ca. Phytoplasma mali*”) and have been hypothesized to serve as the main source of energy for this phytoplasma, however, the metabolic pathway by which these sugars and organic acids are degraded remains obscure (Kube et al., 2008). Thus far, only “*Ca. Phytoplasma australiense*” has been inferred to catabolize sucrose via a sucrose phosphorylase (Tran-Nguyen et al., 2008); the genomes of AY-WB and OY phytoplasma do not appear to encode this enzyme (Oshima et al., 2004; Bai et al., 2006).

The genome sequence of only one spiroplasma, *Spiroplasma citri*, has been partially completed to date (Carle et al., 2010). Similar to phytoplasma, the genome of *S. citri* lacks many genes associated with an autonomous lifestyle. Interestingly, despite sharing similar biological habitats and environmental niches as insect-transmitted and phloem-colonizing microorganisms, the gene content of the *S. citri* genome is more similar to that of *Mycoplasma mycoides* and *Mycoplasma pulmonis* (related species that cause disease in mammals) than that of the phytoplasmas (Carle et al., 2010). Thus it would appear that the genome composition reflects the phylogeny more than biology among these microorganisms.

To enable the efficient uptake of amino acids, inorganic ions, sugars, nucleotides, cofactors and vitamins, from the phloem, these intracellular parasites employ a number of transport systems which actively import these essential biomolecules. Unlike many bacteria, phytoplasmas do not appear to make use of a phosphoenolpyruvate-dependent phosphotransferase system (PTS) to import and phosphorylate sugars such as glucose, fructose, and sucrose. While unknown at present, it is possible that phytoplasmas utilize ABC-type transport systems as a mechanism to import sugars into the cell (Bai et al., 2006; Tran-Nguyen et al., 2008). This is in direct contrast to the spiroplasmas, in which a gene encoding the permease of a fructose PTS has been implicated in the pathogenicity of these microbes in plants (discussed in Molecular Mechanisms Underlying the Virulence of Spiroplasmas and Phytoplasmas in Plants).

A comparison of the genome content of the insect-transmitted phloem-inhabiting plant pathogens “*Candidatus Phytoplasma asteris*” strain witches’ broom (AY-WB), *Spiroplasma citri*, and “*Ca. Liberibacter asiaticus*”, with other bacteria that form close associations with plants and animals reveals trends in genome composition that reflect the lifestyles of each species (Table 14.1). “*Ca. L. asiaticus*” and *Sinorhizobium meliloti* are both members of the family *Rhizobiaceae*, and yet *S. meliloti* can exist as either an intracellular legume microsymbiont or a free-living soil saprophyte. *E. coli* is a common commensal inhabitant of the mammalian digestive tract, however, this species can persist in the external environment as well. Unlike the phloem-colonizing bacteria, the genomes of *S. meliloti* and *E. coli* are richly endowed with genes relevant to the biosynthesis of amino acids, nucleotides, cofactors and vitamins (Blattner et al., 1997; Galibert et al., 2001). Furthermore, *E. coli* and *S. meliloti* encode a greater number of regulatory, transport, and metabolic pathways that enable these species to adapt to a variety of environmental conditions. In contrast, the conditions encountered while inhabiting the interior of a host, such as sieve-tube sap, are relatively constant.

For an intracellular pathogen to survive, the host must complement essential metabolites that the parasite lacks the capacity to produce endogenously. Thus, the phloem-inhabiting mollicutes must derive various amino acids, sugars, nucleotides, and many additional metabolites, directly from the plant. Although enucleate sieve elements have limited metabolic activity, companion cells likely provide metabolites necessary to sustain the growth and replication of phytoplasmas and spiroplasmas. Sieve-tube sap contains an abundance of sugars and amino acids, as well as lower levels of micronutrients that are undoubtedly utilized by the endocellular parasites (Dinant et al.,

2010). That the phloem-inhabiting bacteria are provided a rich source of nutrients by their host is reflected by the relative disparity that exists between the metabolic profiles encoded in the genomes of AY-WB, *S. citri*, and “*Ca. L. asiaticus*,” when compared to that of *Xylella fastidiosa*. Unlike the phloem-limited species, *X. fastidiosa* is xylem-inhabiting bacterium and has an extensive array of genes related to the biosynthesis of amino acids, fatty acids, purines and pyrimidines, cofactors, and vitamins (Simpson et al., 2000) that likely reflects the nutrient-poor qualities of xylem sap.

Host Responses to Infection—the Plants Fight Back

Plants colonized by phytoplasmas and spiroplasmas remain infected for life, however the host can nonetheless mount a defensive response that might limit the progression of disease. For example, apple trees infected with “*Ca. Phytoplasma mali*” can recover from the symptoms induced by the phytoplasma, although the trees still test positive for the presence of phytoplasmas (Musetti et al., 2010). Recovery occurs following the disappearance of phytoplasmas from the crown of the tree during the winter months. In most infected trees, phytoplasmas that overwinter in the tree roots emerge to recolonize the tree in spring, while a few trees manage to block the spread of the pathogen beyond the roots. Ultrastructural examinations of leaves obtained from healthy, diseased, and recovered apple trees revealed that callose deposition and phloem-protein aggregates might serve as defensive mechanisms to impede phytoplasma movement between sieve elements via sieve pore occlusion (Musetti et al., 2010). Whereas the blockage of sieve pores by phloem-proteins may be considered a reversible and rapid process (Knoblauch et al., 2001), callose deposition is thought to have a longer-term effect (van Bel, 2003), raising the issue of whether callose deposition on a large-scale might impair photoassimilate movement within the recovered plant.

The treatment of tobacco with oomycete elicitors similarly stimulates the aggregation of phloem-proteins and callose deposition as a defense response elicited by the plant against the potential of invasion by the oomycete (Lherminier et al., 2003). Plants treated with the elicitors also failed to develop symptoms of phytoplasma infection when exposed to this pathogen, presumably due to the impaired migration of the phytoplasma beyond the occluded sieve pores (Lherminier et al., 2003). There is also evidence that auxin might induce recovery of phytoplasma-infected periwinkle; however, recovery was artificially induced in these studies by the exogenous application of the phytohormone and did not result from an endogenous response mounted by the plant (Curkovic Perica, 2008).

From the Phloem to Beyond—Making the Host Transition

The life cycle of spiroplasmas and phytoplasmas includes the colonization of two very distinct environments, requiring the ability to rapidly adapt following the transition from one host to another. The capacity to genetically manipulate *S. citri* has allowed researchers to decipher the role of carbohydrate metabolism in facilitating the successful transition to a new host. *S. citri* has the ability to catabolize three sugars: glucose, fructose, and trehalose (Bové et al., 2003). Glucose and fructose are utilized by spiroplasmas *in planta*, and mutation of a gene involved in the regulation of fructose uptake affects the phytopathogenicity of *S. citri* (Gaurivaud et al., 2000a). Trehalose is present in high concentrations within the hemolymph of insects, including those organisms that vector spiroplasmas, and thus the ability to catabolize this sugar is likely most relevant to the successful colonization of these hosts.

Glucose, fructose, and trehalose are imported into the bacterial cell via the activity of phosphoenolpyruvate:sugar phosphotransferase systems (PTS). PTS is a multiprotein transport system of which the permease is comprised of three domains. In *S. citri*, one of these domains is shared between the glucose and trehalose transport systems. It has been proposed that this organization has evolved as a means of enhancing the adaptability of *S. citri* during host transition (Andre et al., 2003; Bové et al., 2003). For example, when moving from the phloem of a plant (in which fructose and glucose are catabolized) to the hemolymph of an insect (in which trehalose predominates), or vice versa, *S. citri* may adapt more rapidly to the new host environment by using a permease domain that can function immediately in the presence of either a glucose-specific or trehalose-specific uptake system.

Insect Host Range of Spiroplasmas and Phytoplasmas

As a genus, *Spiroplasma* infects a wide range of insects including beetles, butterflies and moths, mosquitoes, fruit flies, wasps and bees, as well as other arthropods such as ticks, spiders, and crustaceans (Gasparich, 2010). Only a few species of spiroplasmas infect plants and are the causal agents of diseases such as corn stunt disease (*Spiroplasma kunkelii*) (Williamson and Whitcomb, 1975) and citrus stubborn disease (*S. citri*) (Markham et al., 1974). A third spiroplasma has also been isolated from periwinkle (*Spiroplasma phoeniceum*) (Saillard et al., 1987). In general, arthropods are considered to be the main host or reservoir for spiroplasmas (Gasparich, 2002, 2010) and the association between the organisms is typically commensal. However, spiroplasmas can act as insect pathogens as well (Sakaguchi and Poulson, 1961; Majerus et al., 1999; Jiggins et al., 2000), and there are also well-documented cases of symbiotic associations formed between spiroplasmas and insects (Jaenike et al., 2010).

Phytoplasmas infect a narrower range of insects, comprising members of the phloem-feeding order Hemiptera such as leafhoppers, planthoppers, and psyllids, however, these insect vectors enable phytoplasmas to infect a broad range of plants, including high-value horticultural crops (Ammar and Hogenhout, 2006). The plant and insect host ranges of phytoplasmas and spiroplasmas occasionally overlap, and it is possible that one host can be infected with both mollicutes. For example, the maize leafhopper (*Dalbulus maidis*) can act as a host to both corn stunt spiroplasma and maize bushy stunt phytoplasma (Madden and Nault, 1983).

Phytoplasmas Influence Plant–Insect Interactions

There is evidence that phytoplasmas manipulate the phenotype and behavior of their hosts to facilitate plant–insect interactions. For example, “*Ca. Phytoplasma mali*” (the causative agent of apple proliferation disease) alters the odor of infected apple trees to attract the insect vector, a psyllid *Cacopsylla picta* (Mayer et al., 2008). AY-WB interferes with plant defense against the aster leafhopper (*Macrostelus quadrilineatus*) during infection of *Arabidopsis*, an alteration in the plant that enhances the reproduction of the leafhopper and thus provides increased opportunities for phytoplasma transmission by this vector (Sugio et al., 2011). In these ways, the bacterial pathogens alter the dynamics of the plant–insect interface to the benefit of the microbe, by manipulating their plant hosts to become more attractive to phloem-feeding insects.

Molecular Mechanisms Underlying the Virulence of Spiroplasmas and Phytoplasmas in Plants

A number of mechanisms have been proposed in recent years to explain the range of symptoms elicited by spiroplasmas and phytoplasmas in plants. The production of bacterial toxins, impaired

carbohydrate transport, an accumulation of lactic acid in infected plants, alterations to phytohormone levels, and various other hypotheses have been proposed as potential mechanisms by which spiroplasmas and phytoplasmas stunt the growth and alter the development of their plant hosts. Recently, two primary pathogenicity mechanisms have emerged to account for the symptoms induced in phytoplasma- and spiroplasma-infected plants.

Impaired Carbohydrate Partitioning

The requirement of sugar catabolism to the virulence of spiroplasmas in plants was first proposed following the discovery of a fructose-minus mutant of *S. citri* that elicited only mild symptoms upon infection of a host plant (Gaurivaud et al., 2000a, 2000b). *S. citri* preferentially metabolizes fructose over glucose, although both sugars occur at only low concentrations within the sieve tubes of most plants. It has been proposed that spiroplasmas acquire fructose and glucose from companion cells (Gaurivaud et al., 2000a; Machenaud et al., 2007). In many plants, sucrose is loaded into phloem via the activity of companion cells, which rely upon the metabolism of a small proportion of the sucrose to provide the energy necessary to enable phloem loading (Lerchl et al., 1995; Geigenberger et al., 1996). The fructose and glucose generated by the cleavage of sucrose within the companion cells may diffuse through the plasmodesmata-pore units into the sieve elements.

The *fruRAK* operon, a gene cluster encoding a transcriptional regulator (FruR) (Gaurivaud et al., 2001), transport permease (FruA), and catabolic enzyme (FruK), is required for the metabolism of fructose. Mutation of a gene within this operon is sufficient to abolish fructose catabolism and simultaneously to impair the pathogenicity of *S. citri*, as reflected by a reduction in bacterial cell titer and development of symptoms (Gaurivaud et al., 2000a, 2000b). FruA is a permease protein that forms a part of a phosphoenolpyruvate:fructose phosphotransferase system (or fructose PTS) that is dedicated to the import of fructose into the bacterial cell. During transport, fructose is also phosphorylated by the activity of fructose PTS to yield fructose-1-phosphate, which is acted upon by the metabolic enzyme 1-phosphofructokinase (FruK). FruK catalyzes the phosphorylation of its substrate, fructose-1-phosphate, to yield fructose-1,6-bisphosphate, which is further catabolized by *S. citri* as a means of providing a carbon and energy source (Gaurivaud et al., 2000b). Fructose clearly represents an important energy source to spiroplasmas that inhabit a plant, and fructose mutants of *S. citri* are rapidly replaced by their fructose-positive counterparts if reversion occurs within a population (Gaurivaud et al., 2000a). Housekeeping genes, such as those encoding products involved in carbohydrate catabolism are not usually considered as being relevant to pathogenicity *per se* and thus, the model proposed is novel. Often, a decrease in a pathogen's virulence upon mutation of such a gene can simply be attributed to a decreased growth rate or reduced ability to survive, particularly in a stressful environment such as within an antagonistic host. However, a mildly pathogenic *S. citri* fructose mutant has been described that can achieve a high titer within a plant host (1×10^4 CFU per mg leaf midribs), and therefore this strain is not likely to simply be compromised in its rate growth or survival (Gaurivaud et al., 2000a; Bové et al., 2003).

In contrast, *S. citri* mutants that can no longer metabolize glucose remain highly pathogenic in periwinkle (Andre et al., 2005). For example, mutation of a gene encoding a component of the glucose PTS (*ptsG*) creates a strain of *S. citri* that is unable to import glucose, and such a mutant can no longer grow at the expense of this sugar. Nonetheless, the *S. citri ptsG* mutant readily infects and induces symptoms of a severe infection when inoculated into periwinkle, indicating that the inability to metabolize glucose does not seriously compromise the virulence of this phytopathogen (Andre et al., 2005).

The observation that fructose and glucose utilization contribute unequally toward the pathogenicity of *S. citri* has led to the proposal of a model in which the preferential catabolism of fructose by *Spiroplasma* results in an accumulation of glucose and a corresponding repression of photosynthesis in infected plants (Andre et al., 2005). There is support for this model from phytoplasma-based research. Coconut trees (*Cocos nucifera*) infected with coconut lethal yellows phytoplasma show an accumulation of carbohydrates and starch in newly developed and mature leaves at early and intermediate stages of the lethal yellows disease and decreased concentration of sugars in root of infected plants (Maust et al., 2003). These events also coincide with decreased rates of photosynthesis, leading the authors to propose that phytoplasmas induce disease symptoms by impairing sugar translocation from source to sink tissues, possibly by occluding the sieve tubes via callose deposition or physical blockage of the elements by the phytoplasmic cells themselves. Nevertheless, many of the symptoms exhibited by phytoplasma-infected plants cannot be explained solely as resulting from an impaired sugar translocation. Infection of *Arabidopsis* with phytoplasma strain AY-WB induces virescence (greening of nongreen plant tissues, such as petals) and phyllody (conversion of floral organs into leaf-like structures) and the development of a witches' broom phenotype that results from increased production of axillary stems (Figure 14.2). These symptoms are consistent with alterations in plant development as opposed to carbohydrate metabolism and have been documented as arising from many plant-phytoplasma interactions (Firrao et al., 1996; Lee et al., 2004; Zamharir and Mirabolfathi, 2011). Such observations have led to a hypothesis that phytoplasmas actively release factors that target specific processes in plant development and growth, a model that is described more fully in the following section.

Effector-Based Pathogenicity

Prokaryotic plant pathogens typically produce proteinaceous virulence factors or effectors that are secreted into host cells via a complex and highly specialized secretory apparatus that is referred to as a Type-III secretion system (Ghosh, 2004). Central dogma dictates that these effectors have a primary role in suppressing the host immune system or host defense mechanisms to enable colonization by the phytopathogen. However, this definition has recently been broadened to include bacterial factors that modulate host development as well as modulating host defense (Hogenhout et al., 2009), primarily in recognition of research relating to phytoplasma phytopathogenicity.

Arabidopsis as a Model to Probe Phytoplasma/Plant Interactions

AY-WB infects a broad range of plants and is vectored by the generalist phloem-feeding aster leafhopper that can utilize *Arabidopsis* as a host for feeding and reproduction. When exposed to leafhoppers that carry AY-WB, *Arabidopsis* typically exhibits symptoms of infection within 2–3 weeks postinoculation (MacLean et al., 2011). Infected plants show evidence of stunted growth, as indicated by reductions in plant height and mass. Furthermore, phytoplasma-infected plants have a bushy appearance due to the growth of an increased number of stems referred to as a witches' broom phenotype. Strikingly, infected plants exclusively produce flowers with green petals in lieu of white, and the flowers themselves occasionally produce shoots from the center of the organ, which is never observed in healthy plants. In effect, the reproductive organs of infected plants appear to revert to vegetative tissues.

In addition to the obvious alterations in plant morphology, phytoplasmas such as AY-WB also interfere with the plant's ability to mount a defense against leafhoppers (Sugio et al., 2011b). Aster leafhoppers caged upon AY-WB infected *Arabidopsis* have a greater degree of fecundity, producing



Figure 14.2 Phytoplasmas induce symptoms consistent with alterations in plant development when infected with AY-WB. (A) *Arabidopsis* plant with the witches' broom phenotype. (B) Infected plants form green flowers as compared to the white flowers of a healthy plant. (C) The witches' broom phenotype produces an increased number of axillary stems in infected as compared with healthy plants. (For a color version of the figure, please see Plate 14.2.)

about 60% more nymphs than insects exposed to healthy plants (Sugio et al., 2011 and Sugio et al., 2011b). The maize leafhopper (*Dalbulus maidis*) also survives longer and produces progeny upon AY-WB infected *Arabidopsis*. This observation is remarkable because the maize leafhopper is a specialist leafhopper that cannot utilize healthy *Arabidopsis* as a feeding or reproductive host. Thus, it appears that certain strains of phytoplasmas have the ability to interfere with plant defense mechanisms against a third organism, the phytoplasma vector.

Phytoplasma Effectors Modulate Plant Development

The genome of AY-WB has been sequenced (Bai et al., 2006) and genome mining has led to the identification of 56 secreted AY-WB proteins (or SAPs) (Bai et al., 2009). As a means of identifying the function of these potential effectors *in planta*, each effector gene was expressed in *Arabidopsis* and the transgenic lines were examined for alterations in plant phenotype (MacLean et al., 2011). Three effector proteins were identified that affected morphological and developmental traits in *Arabidopsis*.

Expression of the effector gene *SAP11* in transgenic *Arabidopsis* induces the formation of severely crinkled leaves and an increased production of axillary stems that is reminiscent of witches' broom (Sugio et al., 2011 and Sugio et al., 2000b). Moreover, the aster leafhopper produces 20–30% more progeny when reared upon *SAP11* expressing lines (Sugio et al., 2011 and Sugio et al., 2011b), a trait that is reminiscent of AY-WB infected *Arabidopsis*. Western blotting and immunocytology experiments indicate that *SAP11* is abundant in the salivary glands of AY-WB infected leafhoppers (Bai et al., 2007). Thus, *SAP11* might be injected directly into the phloem of a plant together with the phytoplasma cells through the stylets of a feeding leafhopper, in addition to being secreted by phloem-dwelling bacteria. *SAP11* has a nuclear localization signal (NLS) that targets this prokaryotic protein to the nuclei of plant cells (Bai et al., 2009). The presence of an NLS encoded within this effector is intriguing, as it implies that the bacterial protein likely targets cells beyond the enucleate sieve elements wherein the phytoplasma primarily reside. The localization of *SAP11* to the plant cell nucleus in conjunction with the phenotype exhibited by *SAP11* expressing lines of *Arabidopsis* implies that the effector might interact with a plant transcription factor that is involved in regulating the growth of axillary stems or plant defense mechanisms against phloem-feeding insects.

A second AY-WB effector protein (*SAP54*) appears to target floral development in *Arabidopsis* (MacLean et al., 2011). Expression of *SAP54* in transgenic plants induces the formation of highly unusual flowers (Figure 14.3).



Figure 14.3 Expression of a phytoplasma effector protein induces the formation of atypical flowers in *Arabidopsis*. (A and B) Flowers produced by transgenic *Arabidopsis* plants expressing the phytoplasma effector gene *SAP54* have leaf-like petals, enlarged sepals, and produce secondary flowers from the center of the flower. (C) Wild-type *Arabidopsis* flower. (For a color version of the figure, please see Plate 14.3.)

Stolbur phytoplasma have also been reported to alter the morphology of flowers in infected plants, including tomato (*Solanum lycopersicum*) (Pracros et al., 2006). An analysis of gene expression in the affected tomatoes reveals that several genes encoding products relevant to the regulation of floral meristem maintenance and floral organ identity are misregulated in these plants. Particularly, expression of *LeWUS*, *LeCLV1*, and *LeDEF*, is decreased compared to that observed in healthy plants, whereas expression of *FA* is upregulated. These genes are orthologs of *Arabidopsis WUS*, *CLV1*, *AP3*, and *LFY*, respectively. Furthermore, expression of *TAG1* (an ortholog of *AG*) was induced at an earlier time-point during flower development but failed to reach wild-type levels at later stages in flower development. It was hypothesized that the regulation of these genes was altered in phytoplasma-infected plants as the result of impaired carbohydrate partitioning (Pracros et al., 2006), primarily because sugars such as sucrose can act as important signaling molecules that could modulate the initiation of reproductive growth (Blazquez et al., 1998; Blazquez, 2000). Nevertheless, the identification of SAP54 as a phytoplasma effector protein that alters flower development raises the possibility that these bacteria might directly target this process.

A third AY-WB effector has been identified that alters plant phenotype when expressed in transgenic *Arabidopsis*. Expression of *SAP05* induces the formation of smooth young rosette leaves that lack the serration along the leaf margin that is present in wild-type plants. Furthermore, transgenic plants flower earlier than wild-type plants when grown in a short-day photoperiod, however, the flowers that do emerge have a normal appearance. The mechanism by which *SAP05* acts to effect this alteration in phenotype is currently under investigation.

OY likewise secretes at least one effector protein (Tengu) that induces alterations in plant architecture when expressed in *Nicotiana benthamiana* and *Arabidopsis* (Hoshi et al., 2009). This tiny protein (<5 kDa) is sufficient to cause dwarfism, witches' broom, and infertility, when produced in OY-infected and transgenic plants. Microarray analysis of transgenic *Arabidopsis* expressing *Tengu* indicates that auxin signaling is perturbed in these plants, however, the mechanism by which Tengu acts has yet to be determined.

Movement of Phytoplasma Effectors beyond Sieve Tubes

Unlike the majority of Gram negative plant pathogens, phytoplasmas are true intracellular inhabitants and do not require a Type-III secretion system as a means of delivering effector molecules into a host cell (Hogenhout and Loria, 2008). Rather, phytoplasmas can secrete proteins directly into the cytoplasm of sieve elements. The small size of these effector proteins, typically less than 30 kDa (Bai et al., 2009), raises the issue of whether these molecules access tissues beyond the phloem. While the translocation of proteins that are synthesized in companion cells and move into sieve elements by way of plasmodesmata-pore units has been well documented (Fisher et al., 1992; Imlau et al., 1999; van Bel, 2003; Stadler et al., 2005), less is known regarding the potential for the opposing movement of macromolecules produced from within sieve elements to companion cells, phloem parenchyma cells, and beyond (van Bel et al., 2011). There is evidence that at least two phytoplasma effectors (SAP11 and Tengu) migrate beyond the phloem to access distal tissues (Bai et al., 2009; Hoshi et al., 2009), including parenchyma and mesophyll cells. Viral proteins have been shown to alter the permeability of the symplasmic barrier that restricts movement between the sieve tube and neighboring cells (Peleg et al., 2007; Omid et al., 2008). Similarly, phytoplasmas might produce a protein(s), such as a chaperone, that facilitates effector movement from the sieve element–companion cell complex to the phloem parenchyma cells. This could occur, for example, by interfering with the selectivity of protein trafficking, if such a mechanism regulates macromolecular movement in this direction. Whether many phytoplasma proteins penetrate beyond the phloem in

infected plants, and whether the pathogens themselves play an active role in facilitating or regulating effector protein movement remains to be addressed.

Future Directions

Spiroplasmas and phytoplasmas are fascinating microorganisms that can offer unique insight into the biology of phloem. A number of questions, however, remain to be resolved in this area of research. It is currently unclear whether the movement of the plant pathogenic bacteria along the sieve tube is the result of an active process or whether the bacteria passively translocate within the assimilate stream. Similarly, the mechanism by which these bacteria move from the sieve elements to access companion cells and even phloem parenchyma cells has yet to be elucidated, although most likely involves passage through the plasmodesmata–pore unit. It is likewise uncertain how the pathogens avoid mounting a plant defense response within the phloem of susceptible hosts. One possibility is that the host simply does not perceive the intracellular pathogens, or conversely, these bacteria could be actively suppressing plant defense mechanisms that would otherwise eliminate the pathogens. The absence of an effective defense system within phloem might be the reason these bacteria are limited to these tissues. Furthermore, plant defenses against insect vectors might influence host resistance or susceptibility towards the bacterial pathogens. The nature and characteristics of phytoplasma effectors also raises interesting problems in understanding the etiology of phytoplasma-induced diseases. Phytoplasma effectors might primarily interact with targets within the phloem or may require access to distal tissues to influence host processes. If so, the role the pathogen plays in facilitating the movement of the effector proteins beyond the phloem becomes significant.

Spiroplasma and phytoplasma research has advanced considerably in the last decades, and the continual development of molecular technologies offers considerable promise in further understanding these unique plant and insect inhabitants. Initiatives that focus upon addressing these outstanding issues promise to advance both our understanding of phloem biochemistry and the biology of these fascinating plant pathogens.

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15 Breaching the Sieve Element—The Role of Saliva as the Molecular Interface between Aphids and the Phloem

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Aphids (order Hemiptera; suborder Sternorrhyncha) are fast becoming an excellent system for studying molecular interactions between sap feeding insects and their host plants. This has been facilitated, in part, by the recent completion of the pea aphid (*Acyrtosiphon pisum*) genome project (IAGC, 2010) and the development of high throughput genomic resources such as preliminary genomes and large-scale EST libraries for a number of other aphid species. Coupled with a strong tradition as a model system for investigating insect–plant interactions more generally, the recent increase in the number of studies involving aphids has lead to greater insights into the complexity of plant–aphid interactions and has provided a tantalizing preview of the intricate molecular mechanisms that govern this intimate association.

Aphids and other sap-feeding hemipterans (e.g., whiteflies) have evolved specialized mouthparts, the stylets, which penetrate through plant tissues to feed directly from a single sieve element within the phloem. The feeding pathway, mechanism of penetration, and insect behavior associated with feeding have been well described (reviewed by Powell et al., 2006), but the role of the copious amounts of saliva that is continuously secreted during feeding remains obscure. Two types of saliva are recognized: a “gelling” saliva that constitutes the salivary sheath that is left *in situ* following stylet withdrawal from plant tissues, and a “watery” saliva that is secreted intracellularly either when the stylets briefly puncture cells during probing or immediately before and during sap ingestion (Martin et al., 1997). The gelling saliva is thought to provide mechanical support to the delicate stylets and to offer a degree of molecular concealment from plant defenses (Miles, 1999; Tjallingii, 2006; Will et al., 2007), although the assignment of function to the various types of saliva remains speculative, largely because of difficulties with collection and analysis of aphid saliva in general.

Abbreviations: ACE, angiotensin converting enzyme; Avr, avirulence; EPG, electrical penetration graph; GMC, glucose-methanol-choline oxidoreductase; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; NBS-LRR, nucleotide-binding site leucine-rich repeat; PI, protease inhibitor; PAMPs, pathogen associated molecular patterns; PP1, phloem protein 1; PP2, phloem protein 2; PTI, PAMP-triggered immunity; Redox, reduction-oxidation; RNAi, RNA interference; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SMP-30, senescence marker protein

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Nevertheless, several authors have suggested that aphid saliva mediates the insect–plant interaction by overcoming plant defenses before and after the sieve elements have been located (e.g., Miles, 1999; Tjallingii, 2006; Will et al., 2007). In contrast to the multitude of studies involving leaf-chewing insects (reviewed by Wu and Baldwin, 2010) direct evidence of a role for individual salivary proteins in the aphid–plant interaction is only slowly becoming available (Will et al., 2007; Mutti et al., 2008; Bos et al., 2010).

This chapter will discuss the potential role of aphid saliva within the sieve element, focusing largely on the watery saliva because this component is known to be actively secreted both prior to and during ingestion of phloem sap. However, it should be noted that the division of aphid saliva into gelling and watery components is based solely on physical properties, in particular the hardening of the saliva during tissue penetration that is not observed within the sieve element. Although protein profiles of the two types of saliva when analyzed by SDS-PAGE appear different (e.g., Cherqui and Tjallingii, 2000; Harmel et al., 2008) the protein composition of the different types of saliva has not been comprehensively compared, and the processes that govern the change in saliva conformation remain unknown.

Aphid Saliva—Production, Secretion, and Composition

The primary components of aphid saliva originate in the salivary glands, a pair of organs located in the dorsal metathorax (Ponsen, 1972). Each half of the salivary gland pair consists of two components, a large principal gland that is often bilobed and a smaller accessory gland. The two subunits join in a common duct that unites with the other partner to form the salivary canal that leads to the stylet tips. The principal gland is innervated and includes eight secretory cells, whereas the accessory gland has no nerve connections and the cells are not differentiated. The contribution of the two subunits to the saliva has been suggested largely through plant virus transmission studies (Gray and Gildow, 2003). Persistent and circulative viruses that infect the phloem are transferred from the aphid hemolymph into saliva through the accessory gland, indicating that this structure might be responsible for the production of some watery saliva. However, the relative contribution of the accessory and principal glands to saliva production remains unclear.

The salivary gland tissue of the pea aphid has recently been investigated in detail using transcriptomic and proteomic methodologies to generate a catalogue of putative effector proteins of the salivary secretome (Carolan et al., 2011). Effectors are generally defined as proteins and small molecules that alter host cell structure and function (Hogenhout et al., 2009). Over 300 proteins with secretion signals (N-terminal peptide sequences) indicating extracellular localization and possible association with saliva were identified from the salivary gland, approximately half of which had no homolog outside the aphids and were of unknown function. A significant number of the candidate effectors that could be assigned identity through homology were previously characterized in other phytopathogenic organisms, particularly plant-parasitic nematodes, perhaps indicating the evolution of common mechanisms to the plant-parasitic habit.

The composition of both the gelling and watery saliva has been investigated widely and is summarized in Table 15.1. Early studies primarily used enzymatic assays to determine protein activity of saliva and the salivary sheaths. The gelling process is thought to be mediated by an oxidation/reduction reaction involving phenoloxidases (Cherqui and Tjallingii, 2000; Tjallingii, 2006; Will et al., in press). In addition, phospholipids and conjugated carbohydrates have been detected in the sheath saliva (Miles, 1999). A candidate for the principal protein component of the sheath has been identified by mass spectrometry (Carolan et al., 2009). The amino acid composition

Table 15.1 Proteins identified from or associated with aphid saliva

Protein/enzyme type	Protein	Method of determination	Species	Fraction	Putative functions	References
Glucosidases	Cellulase	SSA	<i>S. graminum</i>	WO	Degrad cellulose	Campbell and Dreyer, 1990
	ND	SSA	<i>S. graminum</i>	Saliva	Degrad phenophilic glycosides	Urbanska et al., 1994; Cherqui and Tjallingii, 2000
GMC-oxidoreductase family	Glucose dehydrogenase	MS, TP	<i>A. pisum, M. persicae</i>	Saliva, SGs	Disrupt JA-regulated defense responses, secondary metabolite detoxification, salivary sheath solidification	Harmel et al., 2008; Carolan et al., 2009, 2011
	Glucose oxidase	SSA	<i>M. persicae</i>	Saliva	Disrupt JA-regulated defense responses, secondary metabolite detoxification, salivary sheath solidification	Harmel et al., 2008
Olfactory segment D2 family	<i>Mp10</i> (OS-D2-like)	TP	<i>M. persicae</i>	SGs	Chemosensory protein, potential effector	Bos et al., 2010
Pectinases and pectinesterases	ND	SSA	<i>S. graminum</i>	Saliva	Cell wall degradation during probing and stylet penetration	Ma et al., 1990; Madhusudhan and Miles, 1998; Cherqui and Tjallingii, 2000
Polyphenoloxidases & peroxidases	ND	SSA	<i>S. graminum, S. avenae, A. pisum, M. persicae</i>	Saliva, Salivary Sheath	Phytochemical detoxification, ROS scavenging to maintain redox potential	Urbanska et al., 1994; Madhusudhan and Miles, 1998; Miles and Peng, 1988; Urbanska et al., 1998; Cherqui and Tjallingii, 2000

(Continued)

Table 15.1 (Continued)

Protein/enzyme type	Protein	Method of determination	Species	Fraction	Putative functions	References
Polygalacturonase	ND	SSA	<i>A. pisum</i> , <i>M. persicae</i>	Saliva	Cell wall degradation	Campbell and Dreyer, 1990
Metalloproteases	M1 metalloprotease, M2 metalloprotease	MS	<i>A. pisum</i>	Saliva, SGs	Regulate or process signaling peptides through cleavage of di- or tri-peptides	Carolan et al., 2009
SMP-30 family	Regucalcin	MS	<i>A. pisum</i>	Saliva	Calcium chelation, regulate sieve element occlusion	Carolan et al., 2009
Proteins of unknown function	Protein C002	TP	<i>A. pisum</i>	SGs	Unknown function.	Mutti et al., 2006, 2008
	ACYPI009881	MS, TP	<i>A. pisum</i>	Saliva, SGs	Component of the salivary sheath	Carolan et al., 2009, 2011
	ACYPI008224	MS, TP	<i>A. pisum</i>	Saliva, SGs	Unknown function	Carolan et al., 2009, 2011
	ACYPI006346	MS, TP	<i>A. pisum</i>	Saliva, SGs	Unknown function	Carolan et al., 2009, 2011
	40 and 43 kDa calcium binding proteins	⁴⁵ Ca ₂ labeling	<i>M. viciae</i>	Saliva	Calcium chelation, regulate sieve element occlusion	Will et al., 2007
	<i>Mp42</i>	TP	<i>M. persicae</i>	SGs	Potential effector	Bos et al., 2010

ND: not determined; SSA: substrate specific assay; MS: mass spectrometry; TP: transcript profiling; SGs: salivary glands; WO: whole organism (homogenates).

of this “sheath protein,” a novel protein unique to aphids, suggests an abundance of disulfide bridges can form between cysteine residues consistent with a gelling function. Disulfide bridge formation is reversibly catalyzed by protein disulfide isomerases and several disulfide isomerases were identified in the pea aphid salivary gland secretome (Carolan et al., 2011). Watery saliva appears to have a more complex composition. Substrate-specific assays have demonstrated the presence of two pectinases, pectin methylesterase, polygalacturonase, and oxidation/reduction enzymes such as phenoloxidases and peroxidases (Urbanska et al., 1998; Madhusudhan and Miles, 1998; Miles, 1999). More recently, mass spectrometry and proteomics of aphid salivary proteins secreted into artificial diets identified metalloproteases, glucose dehydrogenases and oxidases, regucalcin, NADH dehydrogenase, and several novel proteins with no homolog outside aphids (Harmel et al., 2008; Carolan et al., 2009).

In addition to structural and enzymatic functions, novel molecular roles for constituents of aphid saliva are being recognized. For example, it is becoming increasingly evident that a gene-for-gene interaction exists between the aphid and its plant host, analogous to the interactions that occur between plants and phytopathogens such as nematodes, oomycetes, fungi, and bacteria. As with these phytopathogenic organisms, it is expected that aphids secrete analogous pathogen-associated molecular patterns (PAMPs) that are recognized by the plant, and subsequently salivary effectors that in some way negate the response to such recognition. Recent functional genomic (Bos et al., 2010) and RNA library prospecting (Mutti et al., 2006, 2008) approaches have yielded candidate proteins (Table 15.1) that could potentially function at the molecular interface between aphid and plant.

Calcium chelating proteins have also been demonstrated in aphid saliva through calcium affinity studies (Will et al., 2007) and mass spectrometry of proteins isolated from concentrated saliva (Carolan et al., 2009). The presence of such proteins might explain the ability of aphids to contend with calcium-regulated sieve element occlusion mechanisms and calcium-based defense signaling pathways. Thus, it seems that aphid saliva is diverse both in protein composition and function.

Stylet Penetration toward the Sieve Elements: The Role of the Salivary Sheath

The route taken by the stylets as they penetrate plant tissues has been reconstructed from serial sections of salivary sheaths viewed by both light and electron microscopy (Tjallingii and Esch, 1993). After initially secreting a salivary “flange” on the plant surface, the stylet tips penetrate between cells and through airspaces and briefly puncture individual cells as they progress to the sieve elements. The route can be circuitous, commonly involving dead-ends, direction reversal, and aborted sieve-element punctures. Overall, it can take from 30 minutes to several hours for the aphid to locate a suitable feeding site. Analysis by electrical penetration graph (EPG) (McLean and Kinsey, 1965; Prado and Tjallingii, 1994; Walker, 2000), a technique to observe aphid behavior during plant probing and ingestion, has added considerable detail to these directly unobservable events (see Chapter 16). Often the frequency of cell punctures increases immediately before the final feeding site is attained, and salivary secretions seem to be important in the decision of the insect to accept or reject a sieve element that could be mediated by gustatory receptors in the pharynx (Hewer et al., 2010). However, the cell puncture that leads to sustained phloem ingestion appears indistinguishable from other previous cell punctures.

The puncturing of plant cells, either in the mesophyll or the sieve elements, presents aphids with a serious problem since loss of internal turgor pressure and the influx of calcium ions results in the initiation of defensive cascades that could interrupt feeding behavior. In this regard, the salivary sheath plays an important role since it can effectively seal puncture sites irrespective of the plant

cell type. Nevertheless, the continuous secretion of gelling saliva during penetration to the sieve element presents a considerable input of “foreign” material into the plant that has the potential to activate plant defense responses. Early reports in the literature suggested that the gelling saliva contained enzymes such as pectinases and cellulases that are capable of digesting plant cell walls to aid penetration (Table 15.1). Although the stylets predominantly follow the secondary cell walls and thus encounter cellulose fibers (Tjallingii and Esch, 1993) and a pectin layer, it has yet to be determined whether the products of their digestion (pectin fragments for example) act as elicitors of plant defense (Cherqui and Tjallingii, 2000). Obviously, teasing apart the seemingly conflicting roles of cell wall digesting enzymes present in aphid saliva requires considerably more attention.

Penetration of the Sieve Element: The Role of Watery Saliva

Upon puncturing the sieve element, the aphid initiates a period of sieve-element salivation that involves the injection of watery saliva directly into the sieve element lumen (Tjallingii, 2006). Secretion of saliva coincides with the closing of the precibarial valve in the food canal in the aphid head, so that saliva remains in the mouthparts rather than being drawn through the stylet and into the gut as occurs during ingestion (Figure 15.1). It is assumed that saliva is secreted into the lumen of the sieve element and transported within the sieve tube by mass flow. It is unclear how far the stylet tip progresses into the sieve element and it is feasible that saliva could accumulate in the stationary mictoplasm at the inner periphery of the sieve element. However, early experiments with ³²P-labeled aphids detected radioactivity in unlabeled aphids (and plant roots) downstream of the feeding site, indicating that saliva enters the sieve element lumen and is carried with the mass flow within sieve tubes (Forrest and Noordink, 1971). The secretion of watery saliva prior to ingestion has long been considered a significant factor in the conditioning of the sieve element prior to ingestion. Such conditioning most likely involves secretion of effector molecules to overcome or negate plant defenses such as protein occlusion mechanisms, although the identification of such effectors is still at an early stage (Bos et al., 2010). Irregularities in sieve element salivation have been observed in aphids feeding from resistant plant cultivars (van Helden and Tjallingii, 1993; Garzo et al., 2002; Alvarez et al., 2006; Tjallingii, 2006) and in aphids experimentally deprived of an endogenous supply of essential amino acids through elimination of their bacterial endosymbionts (Wilkinson and Douglas, 1995). In both cases, incompatibilities between the sieve element and protein composition of the saliva are thought to underlie the irregular feeding behavior exhibited by the aphids.

To initiate ingestion, the precibarial valve opens and the aphid imbibes sieve-tube sap passively due to the positive pressure associated with mass flow. The opening of the salivary canal is situated just behind the tip of the stylets (Figure 15.1) such that once ingestion starts, saliva is swept back into the aphid with the stream of sieve-tube sap (e.g., Tjallingii, 2006). The functional implications for the ingestion of saliva are unclear, but one likely explanation involves prevention of protein plug formation in the narrow food canal or aphid gut. The intermittent secretion of watery saliva is critical for sustained feeding, and the precibarial valve will close periodically to allow the emission of watery saliva back into the sieve element. Severing of aphid stylets by stylectomy (Fisher and Frame, 1984; van Helden et al., 1994) during ingestion interrupts the secretion of watery saliva into the sieve element and in eudicots, sieve-tube sap eventually ceases to flow presumably due to the plugging of the stylet tips or sieve element. It is clear that saliva plays a major role in the interaction between the aphid and the sieve-tube from which it feeds.

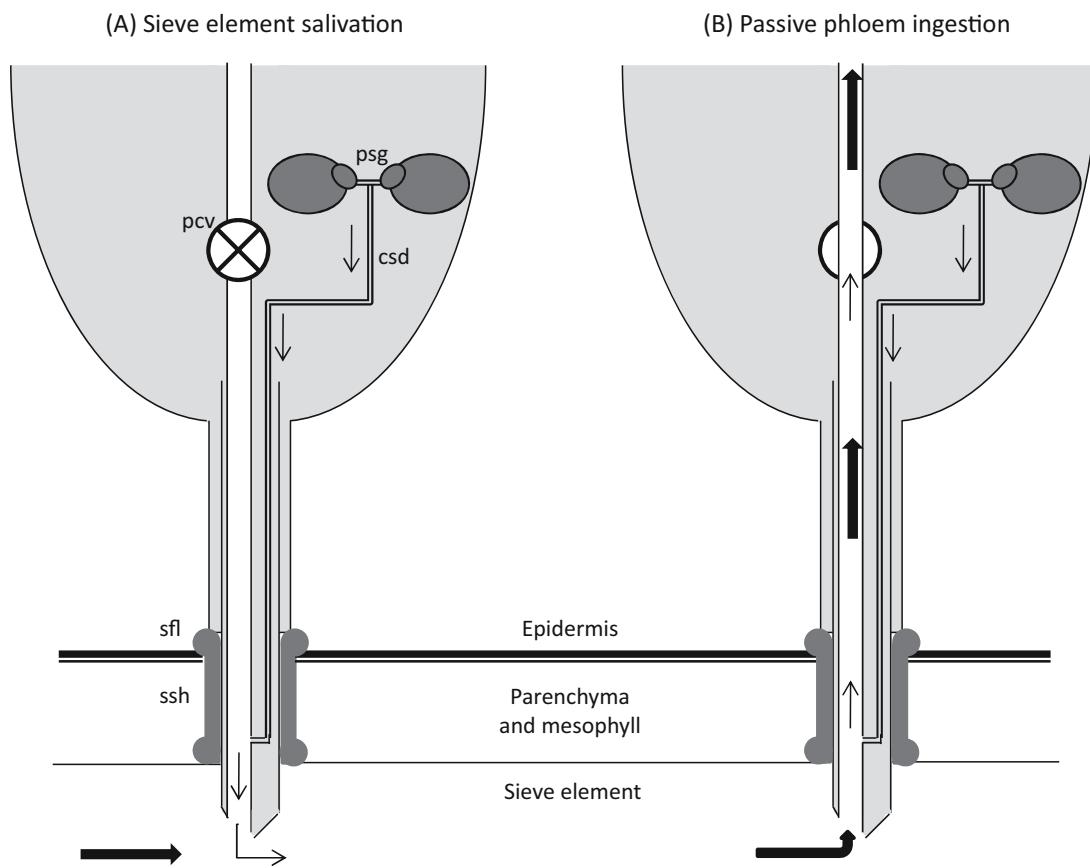


Figure 15.1 Schematic representation of the movement of saliva and sieve-tube sap during sieve element salivation and passive phloem ingestion. (A) Sieve element salivation originates from saliva in the paired salivary glands (psg; each comprising a principal and accessory gland) that is continually secreted via the common salivary duct (csd) from an opening in the stylet bundle distal to the tip. During penetration of pathway tissues to the sieve element the saliva hardens to form an initial salivary flange (sfl) and salivary sheath (ssh) surrounding the stylets. During penetration and brief intracellular punctures as well as initially entering the sieve element, the precibarial valve (pcv) in the head remains closed and saliva exits from the tip of the stylet bundle. (B) Passive phloem ingestion occurs if the stylet-sieve element interaction is compatible. The precibarial valve opens and sieve-tube sap together with secreted saliva is imbibed passively due to the positive pressure in the sieve element. Small arrows indicate the direction of saliva flow; large arrows indicate the direction of sieve tube sap flow.

Aphids Manipulate Sieve Element Occlusion

Sieve Element Occlusion Mechanisms

Sieve elements are particularly sensitive to injury and under extreme conditions will completely collapse. More commonly, mechanical damage or feeding damage by herbivores leads to a blockage or occlusion of the sieve elements to prevent leakage of the sieve tube contents (see Chapter 7). Occlusion mechanisms can act relatively slowly (minute range) in case of callose deposition or rapidly (second range) by plugging with proteins (Furch et al., 2007, 2010). Both occlusion

mechanisms can act concurrently to effectively block mass flow through sieve pores between adjacent phloem cells (see Chapter 7). Callose, a 1,3- β -glucan polymer (Aspinall and Kessler, 1957), is synthesized in the cell wall outside the plasma membrane by the enzyme callose synthase, which is activated by increased levels of intracellular calcium. Because of the site of synthesis, callose plugs are actually more like collars that constrict the sieve pores.

Protein-based sieve pore occlusion mechanisms can occur more rapidly and involve a wide variety of putative protein structures (Evert, 1990). The types of protein plugging mechanisms that are present depend upon the plant family but all mechanisms lead to blockage of the sieve plate by formation of a plug. Sieve elements of plants in the Fabaceae contain forisomes, which are unique protein structures with a spindle-like appearance that disperse in response to fluctuations in turgor pressure or damage (Knoblauch et al., 2001). These physical changes to sieve elements also lead to increased levels of intracellular calcium. Isolated forisomes *in vitro* can be induced to disperse by the addition of calcium and contract by the addition of calcium chelators (Knoblauch et al., 2003) or crude extracts of concentrated aphid saliva (Will et al., 2007). This rapid structural conversion of forisomes suggests that an unidentified component of aphid saliva plays a vital role in mitigating defense responses in this particular plant family.

How Aphids React to Sieve Element Occlusion

Secretion of watery saliva into sieve elements directly after penetration by the aphid stylets, but prior to sieve-tube sap ingestion is a common behavior that has been observed for all aphid species studied to date. Furthermore, detailed analysis of aphid feeding behavior has demonstrated that aphids respond to artificial induction of sieve element occlusion with prolonged periods of watery saliva secretion (Will et al., 2007, 2009). This observation was made for a number of different aphid species on highly divergent plant taxa, suggesting that salivation into sieve tubes is a general mechanism to suppress sieve element occlusion (Will et al., 2009).

Factors that can trigger the previously described occlusion mechanisms include calcium fluxes (Knoblauch et al., 2001), turgor loss (Ehlers et al., 2000), and variations in redox potential (Leineweber et al., 2000).

Occlusion Associated Factors: Calcium

Calcium plays a central role in the induction of sieve element occlusion, either as a trigger for a variety of mechanisms (Furch et al., 2007) or as a second messenger in defense-associated signal cascades (Lecourieux et al., 2006), leading to the hypothesis that proteins within the watery saliva limit or modulate calcium influx (Will and van Bel, 2006). Such proteins appear to be a key factor in the ability of aphids to imbibe phloem sap by preventing sieve element occlusion (Caillaud and Niemeyer, 1996; Chen et al., 1997). Whether aphids prevent occlusion of sieve elements as opposed to directly disintegrating the protein and callose plugs is as yet undetermined.

Occlusion Associated Factors: Hydrostatic Pressure

Mass flow inside the sieve tubes is driven by a hydrostatic pressure difference between source and sink (Münch, 1930; Gould et al., 2005). The high turgor pressure of sieve tubes results in passive ingestion of sieve-tube sap (Prado and Tjallingii, 1994; Tjallingii and Cherqui, 1999; Miles, 1999), and the flow of ingesta is regulated by the preciberial valve that opens and closes the food canal

(McLean and Kinsey, 1984). Loss of turgor in sieve tubes would therefore lead to a reduction in the rate of ingestion or cessation of feeding altogether.

Sieve tube occlusion leads to a decrease of pressure downstream of the occlusion-inducing stimulus (Gould et al., 2004) and a reduction in flow velocity inside the sieve tubes (Peuke et al., 2006). Using a flow chamber system in which a single aphid penetrates a stretched parafilm membrane with its stylet and feeds on artificial diet, it was demonstrated that aphid behavior changes from ingestion to secretion of watery saliva after a rapid decrease of system pressure (Will et al., 2008), while an increase of calcium had no effect. This suggests that aphids respond to a drop in hydrostatic pressure following sieve element occlusion rather than calcium influx (Will et al., 2008).

Occlusion Associated Factors: Redox State

The redox state represents the sum of the reducing and oxidizing potentials of the molecules in plant cells (Potters et al., 2010) and is involved in plant stress biology and pathogen responses (Foyer and Noctor, 2005). In addition to calcium, the redox state inside sieve tubes represents a further trigger factor for protein based occlusion mechanisms in cucurbits. Solubilized phloem protein monomers (PP1) together with phloem lectin (PP2) form filaments due to oxidation (Read and Northcote, 1983; Leineweber et al., 2000) leading to sieve-tube occlusion. Furthermore variation of the redox state has been implicated in conferring disease resistance in plants (Van Camp et al., 1998).

The redox state of a cell is influenced by phenolic compounds and antioxidants, such as ascorbate, glutathione, and NADH/NADPH (Queval and Noctor, 2007; Potters et al., 2010). Reactive oxygen species (ROS) are toxic byproducts of aerobic metabolism, which in addition to contributing to cellular redox state (Potters et al., 2010) can adversely affect plant cell health leading to programmed cell death (de Pinto et al., 2002). ROS are suspected to play an integrative role in aphid–plant interactions. For example, sieve element feeding by aphids results in the production of the ROS hydrogen peroxide in the apoplast of aphid resistant plants (van der Westhuizen et al., 1998), indicating that aphids directly induce or trigger plant defenses under certain circumstances. Thus, aphids are confronted with antioxidant-based defense systems localized in the sieve tubes (Walz et al., 2002). It is likely that aphids and other phytophagous insects secrete a suite of scavenging proteins that contend with the ROS associated with biotic attack or cellular damage.

One potential strategy aphids can use to influence ROS levels in the plant is to secrete oxidoreductase enzymes in the saliva. Many oxidoreductases have been identified in or are associated with saliva from different aphid species (reviewed by Miles, 1999). This group of enzymes include copper-dependent oxidoreductases (Madhusudhan and Miles, 1998), phenoloxidases (Cherqui and Tjallingii, 2000; Ma et al., 2010), glucose oxidase (Harmel et al., 2008), glucose dehydrogenase (Carolan et al., 2009) and peroxidase (Urbanska et al., 1998; Cherqui and Tjallingii, 2000).

With respect to ROS scavenging the identification of glucose–methanol–choline (GMC) oxidoreductases in the saliva of the pea aphid and green peach aphid (Harmel et al., 2008; Carolan et al., 2009) is particularly contradictory, as GMC-oxidoreductases convert glucose, a component of sieve element sap (van Bel and Hess, 2008) to gluconic acid and hydrogen peroxide both of which are ROS. Therefore, salivary enzymes may actually result in an increase in ROS content.

Besides functioning in plant defense responses, ROS are increasingly implicated as key signaling molecules involved in cell-to-cell communication (reviewed by Suzuki et al., 2012). It is therefore not unreasonable to assume that aphid modulation of ROS and ROS scavengers could confer an ability to control ROS-based signaling pathways.

Suppression of Sieve Element Occlusion by Calcium-Binding Saliva Components

An *in vitro* approach to investigate whether components of aphid watery saliva suppress sieve element occlusion mechanisms was conducted using isolated forisomes from broad bean (*Vicia faba*) and concentrated aphid saliva from the vetch aphid (*Megoura viciae*). In translocating sieve elements of Fabaceae, forisomes exist in a contracted spindle-like shape that permits free mass flow through the sieve element lumen. Physical disruption or membrane depolarization of sieve elements (effected by electropotential waves) result in a calcium influx that induces forisome transition from the fusiform into a larger, dispersed globular conformation that prohibits mass flow through sieve-plate occlusion (Knoblauch and van Bel, 1998). Aphid saliva changes forisomes from their dispersed state back into their contracted state. The absence of forisome transformation in the presence of proteolytically digested salivary proteins indicates an active role for salivary proteins in sequestering calcium to prevent occlusion. Seven proteins of different molecular weights with putative roles in calcium binding were obtained from watery saliva collected from several thousand individuals of the vetch aphid feeding from artificial diets (Will et al., 2007). Large volumes of artificial diets were also used for the mass spectrometry-based identification of other putative calcium-binding candidates (Carolan et al., 2009).

Stabilization of Sieve Element Calcium-Homeostasis by a Secreted Regucalcin-Like Protein?

Proteomic characterization of secreted saliva from pea aphids identified a regucalcin-like protein, a candidate calcium-binding protein (Carolan et al., 2009). This salivary protein has a molecular mass of 43 kDa, which is comparable in size to a calcium-binding protein that was previously detected in aphid saliva (Will et al., 2007). Regucalcin is a member of the senescence marker protein-30 (SMP-30) family that functions by sequestering signaling molecules such as calcium (Fujita et al., 1992; Shimokawa and Yamaguchi, 1993). In addition, regucalcin plays a pivotal role in maintaining intracellular calcium homeostasis by activating calcium-pumps in the plasma membrane, endoplasmic reticulum, and mitochondria of many animal cell types (Yamaguchi, 2000). Moreover, regucalcin has an inhibitory effect on the activation of calcium/calmodulin-dependent enzymes and protein kinase C and has been demonstrated to regulate nuclear function in liver cells through the inhibition of calcium-activated DNA fragmentation, DNA and RNA synthesis, protein kinase, and protein phosphatase activities in the nuclei (Yamaguchi, 2005). However, since sieve elements do not possess nuclei, the secreted regucalcin might not be particularly relevant for DNA- and RNA-associated functions. Plant homologs of the SMP-30 protein have not been described so far, which adds to the mystery of how aphid SMP-30 proteins function. However, it is evident from the ever-expanding list of aphid saliva proteins that a specific function within the aphid does not necessarily equate to the same function when secreted into the plant (Carolan et al., 2011).

Salivary Proteins: Modifiers of Ingestion and the Sieve Element Environment

The C002 Salivary Protein Is a Relevant Effector for Ingestion

C002, a salivary protein localized to the principal salivary glands of pea aphid, plays a significant role in successful aphid feeding (Mutti et al., 2008). Suppression of C002 transcripts by RNA interference (RNAi) in pea aphids led to a reduction in aphid life span and the ability of the aphid to locate or

reach the sieve elements during probing (Mutti et al., 2006). In the few successful penetrations to the sieve element, the aphids were unable to sustain sieve-tube sap ingestion for longer than 30 minutes (Mutti et al., 2008). The fecundity of the green peach aphid (*Myzus persicae*) increased when reared on *Nicotiana benthamiana* with ectopically expressed C002, suggesting that the protein functions as an effector that is integral to promoting aphid infestation (Bos et al., 2010). Interestingly, pea aphid and green peach aphid C002 are divergent in amino acid sequence (Mutti et al., 2006; Bos et al., 2010), but no biological significance for the divergence has as yet been attributed.

Influence of Salivary Proteins on Ingested Sieve-Tube Sap Content

At the onset of ingestion, secreted saliva is drawn into the gut with the flow of sieve-tube sap (Figure 15.1). Prevention of sieve-element protein coagulation in the digestive track by calcium-binding is most likely one important function of saliva to prevent occlusion of the narrow food canal of the stylet (Tjallingii, 2006). The presence of specialized cells that accumulate calcium in the form of calcium sulfate and calcium carbonate in the midgut (Ehrhardt, 1965) could indicate that reingested calcium binding salivary proteins are less relevant. Ingested saliva could also function to detoxify sieve-tube sap components that are harmful to aphids including protease inhibitors (PIs), which are known constituents of the sieve-tube sap. Sieve tube PIs could potentially target proteases within the insect midgut and thus, prevent digestion of nutritive proteins and consequently restrict insect growth and development by limiting nitrogen supply. Cysteine and trypsin PIs have been shown to reduce aphid performance (Casaretto and Corcueria, 1998; Rahb   et al., 2003; Pyati et al., 2011), but the response is often species-specific, and in some host plants, accumulation of the PI is induced by aphid infestation.

A long-held assumption argues against a role for PIs in the gut because of the belief that aphids were incapable of digesting protein, relying instead on free amino acids in the sieve-tube sap as the primary source of nitrogen-based nutrition (e.g., Sandstrom, 2000). However, active proteases have been found in the midgut of the pea aphid (Cristofolietti et al., 2006), and two proteases were identified in pea aphid saliva (Carolan et al., 2009). Taken together with evidence of PI activity against a variety of aphid species, some proteolytic activity must be present in the aphid gut (Pyati et al., 2011). Plant PIs could potentially cross the midgut epithelium into the hemolymph and negatively influence aphid metabolism or processes related to reproduction (Rhab   et al., 2003).

The two proteases identified in the saliva of the pea aphid are a M1 zinc metalloprotease and angiotensin converting enzyme-like enzyme (ACE-like), which is a member of the M2 metalloprotease family (Carolan et al., 2009). The ACE homolog was previously described in insect hemolymph, but its association with saliva and the salivary glands has recently been confirmed (Carolan et al., 2011). Both M1 and M2 zinc metalloproteases are involved in the digestion of small peptides and similar proteases are present in the saliva of other fluid-feeding insects such as mosquitoes (Arca et al., 2005) and ticks (Decrem et al., 2008). In addition to a putative role in the digestion of proteins within the sieve elements (Carolan et al., 2009), these salivary metalloproteases could also function within the insect gut to deactivate plant PIs in the diet. M1 and M2 metalloproteases cleave one or two amino acids from the C- and N-terminal ends, respectively, from short peptides such as hormones or neuropeptides indicating a potentially direct role in negating or modulating various defensive signaling mechanisms.

Phytochemicals in the ingested sieve element sap such as phenolic compounds would have to be detoxified by the aphid either in the plant or in the aphid midgut. This could be achieved by oxidoreductases (Miles and Oertli, 1993) such as those identified in the saliva of green peach

aphid and pea aphid (Harmel et al., 2008; Carolan et al., 2009). Salivary GMC-oxidoreductases could fulfill a potential function within the aphid if reingested. However, further identification and characterization of the salivary components along with functional assays are clearly necessary before the role of salivary proteins in pre- and post-ingestion events is fully understood.

Potential Recognition of Salivary Proteins by Intracellular Receptors

There is a growing appreciation that the relationship between sap-feeders and their host plants is much more intricate than the relatively indiscriminate feeding of many polyphagous-chewing insects. The mouthparts of an aphid locate and penetrate a single sieve element from which they can feed for days, often without causing significant damage to the plant. By doing so, aphids and whiteflies induce plant defense signaling pathways that are also activated by bacterial or fungal pathogens (de Vos et al., 2005; Kempema et al., 2007), indicating remarkable functional analogy among these phylogenetically diverse phytopathogenic organisms (Walling, 2000; Goggin, 2007). As discussed in Chapter 16, the only cloned aphid resistance genes, *Mi-1.2* in tomato (*Solanum lycopersicum*) (Martinez de Ilarduya et al., 2003) and *Vat* in melon (*Cucumis melo*) (Dogimont et al., 2008), are members of the NBS-LRR superfamily of resistance proteins that confer resistance to both eukaryotic and prokaryotic pathogens (Staal and Dixelius, 2007). As with the secretions of other plant pathogens, aphid saliva most likely comprises pathogen-associated molecular patterns (PAMPs) and proteins that result in plant-derived products that are analogous to pathogen-induced molecular patterns. Whether or not the plant mounts a successful defense, resulting in pathogen-triggered immunity (PTI), for example, depends on the presence of additional salivary agents such as avirulence (Avr) gene products that function as effectors that essentially interfere with PTI.

Recent results suggest that the expression of *Mi-1.2* and *VAT* is not restricted to sieve elements (Sarria-Villada et al., 2009; Pallipparambil et al., 2010) and, since both gene products are located in the cytoplasm in common with most plant NBS-LRR proteins (McHale et al., 2006), plants are likely to recognize aphid infestation at an early stage of probing before sieve element penetration. A potential candidate protein that is detected by the plant is MP42 from the saliva of green peach aphid, whose overexpression in *Nicotiana benthamiana* leads to reduction in aphid performance, indicating an induced defense response against aphids (Bos et al., 2010).

Microscopic hypersensitive-like responses have been observed in mesophyll tissues of melon plants possessing the *VAT* gene in response to cotton-melon aphid (*Aphis gossypii*) probing (Sarria-Villada et al., 2009). In contrast, a hypersensitive response was absent in tomatoes with the *Mi-1.2* gene (Martinez de Ilarduya et al., 2003), suggesting that alternative defense mechanisms are mediated by NBS-LRR proteins. Interestingly, the microscopic hypersensitive-like responses in melon plants that express *VAT* is a rapidly induced host response when probed by cotton-melon aphids whereas a significant temporal lag of similar responses occurs after green peach aphid probing (i.e., a nonhost response). It is not unreasonable to postulate that the rapid response is based upon a direct detection of an Avr gene product in cotton-melon aphid saliva while the delayed response can be assigned to innate immunity.

Salivary Proteins Influence Metabolic Pathways in the Phloem

Aphid feeding changes resource allocation within the host plant by altering sink–source relationships (Girousse et al., 2003). Nutrients are suggested to be preferentially allocated toward insect-infested

tissue due to the creation of strong sinks as sieve-tube sap is ingested by the aphids (Girousse et al., 2003). The demand for nutrients in response to a dense infestation of aphids imposes such a strongly competitive sink that normal sink tissues such as primary growth zones have been suggested to convert to source tissues (Mittler and Sylvester, 1961; Dixon, 1998; Girousse et al., 2005). This assumption is questionable because of the need for substantial structural changes involved in a switch from sink to source tissues.

Aphid feeding can also alter the biochemical composition of sieve-tube sap to enhance the nutritional quality of the aphid's diet. Infestation of wheat (*Triticum aestivum*) by greenbug (*Schizaphis graminum*) and Russian wheat aphid (*Diuraphis noxia*), induced an increase in both total amino acid content and the relative amount of essential amino acids in the sieve element sap (Sandstrom, 2000). The increased amino acid content could be caused by degradation of sieve element sap proteins due to secreted salivary proteases. Similar results were obtained following feeding by the bird cherry-oat aphid (*Rhopalosiphum padi*) on maize (*Zea mays*) and barley (*Hordeum vulgare*) (Eleftherianos et al., 2006), but in this study there was no effect on the amino acid composition in response to feeding by greenbugs.

Systemic activation of metabolic genes was observed in the phloem of celery (*Apium graveolens*) in response to feeding by green peach aphid (Divol et al., 2005). In contrast, premature leaf senescence in *Arabidopsis* following feeding by green peach aphid is thought to be a resistance mechanism that limits aphid growth by exporting nutrients from the leaf (Pegadaraju et al., 2005). Irrespective of the final outcome, it seems clear that aphid feeding can alter phloem biochemistry to influence aphid fitness. The involvement of aphid salivary proteins in this process remains speculative, but the secretion of saliva into the sieve element represents a plausible recognition mechanism. However, the plant receptors and signaling mechanisms that ultimately determine phloem composition remain obscure.

Future Directions

The continued identification and characterization of aphid salivary proteins is central to understanding interactions between sapfeeding insects and their host plants. The availability of the pea aphid genome and the imminent completion of genomic projects for other aphid species will result in a greater level of insight into these interactions at the molecular level. The continued collaborative efforts of the IAGC and the dedicated curation of aphid bioinformatic resources have resulted in the availability of extensive genomic resources and databases to the wider scientific community. In addition, methods such as next generation sequencing to investigate the aphid transcriptome, *in planta* expression of aphid salivary proteins, RNA interference, and mass spectrometry-based proteomics are all contributing to a greater understanding of the composition and function of aphid salivary proteins.

The continued identification of salivary proteins will lead to two main areas of research. The first of these will involve assessing the variability within the salivary proteome itself. For example, it is unknown whether there are differences between the principal and accessory gland proteome or whether the watery and gelling saliva differ in protein composition. Wide-scale interspecific and intraspecific comparisons are required to determine the effect of host plant and phylogeny on the salivary proteome. Such studies will involve investigating salivary proteome variability among specialist and generalist aphid species or monocot and eudicot feeders, leading to a better understanding of the evolution of the plant-aphid interaction. It is also important that attempts are made to attribute the different levels of plant resistance or susceptibility and aphid virulence

or avirulence to variability within the salivary proteome or within the individual salivary proteins themselves.

Although proteomic, functional genomic, and comparative pathogenomic approaches will result in the generation of lists of candidate proteins involved in the various levels of the plant–aphid interaction, considerably more effort will be required to deduce their actual function within the plant. This second and probably most important area of research will employ methods such as *in planta* expression of salivary proteins or other salivary protein delivery methods to determine changes in the plant proteome and transcriptome, in the levels of susceptibility and resistance, and in plant physiological responses such as hypersensitive response or chlorosis. The functional characterization of individual salivary proteins will benefit from the continued use of plant near-isogenic lines and plant cultivars (some of which are transgenic) that demonstrate a given phenotype in response to specific functional activities of the foreign protein under study. For many of the plant cultivars used in reporter bioassay experiments, the molecular pathways and cascades that result in a given phenotype are known and thus, the potential to couch the plant–aphid salivary protein interaction in molecular terms is achievable.

Finally, in addition to the continued characterization of the plant–aphid interaction at the molecular level, it is essential that the tradition of excellent plant physiology experiments involving aphids continue. For example, additional *in vivo* and *in vitro* sieve element occlusion experiments are required to further characterize the events that occur within the sieve element itself. Stalwart methods such as the remarkably illuminating EPG technique and aphid stylectomy will undoubtedly remain integral to understanding the mechanisms of feeding before and after the sieve element is located. Future studies intent at measuring changes in plant cell microenvironment in response to aphid feeding or stylet penetration have been used in the past but require significantly more attention. Such experiments measure pH, ROS, and calcium levels of individual cells and tissues before and after aphid feeding and will yield insights into the plant–aphid interaction at the cellular and biochemical levels. Immunodetection studies using antibodies obtained for individual salivary proteins will validate the *in planta* delivery of salivary proteins and can potentially be used to visualize salivary proteins at the cellular level. And finally plant microscopy will continue to provide the direct observation of physical interaction between the stylets (and salivary sheath) and the cells and sieve elements they encounter during the feeding process.

In conclusion, the mechanistic role of individual salivary proteins in processes such as calcium chelation, ROS modulation, structural modification, nutrient enhancement, phytochemical detoxification, avirulence, effector, and PAMP functions is slowly emerging. The postgenomic era for aphids and many of the plants on which they feed is encouraging renewed interest in aphid–plant interactions and will undoubtedly lead to a deeper understanding of this intimate and fascinating relationship.

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16 Behavioral and Molecular-Genetic Basis of Resistance against Phloem-Feeding Insects

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Insects that use a piercing/sucking mode of feeding belong to the Hemipteran suborders Sternorrhyncha (aphids, whiteflies, psyllids, and mealybugs) and Auchenorrhyncha (planthoppers and leafhoppers) and the phytophagous insects of the Heteroptera (lygaeids, pentatomids, and coreids) (Dolling, 1991). Many of these insects recover their nutrients from the phloem by inserting their highly modified mouthparts called stylets directly into the sieve elements. Phloem-feeding insects have physiological adaptations to be able to utilize this high sugar diet and endosymbionts to produce the essential amino acids missing from a phloem diet (Douglas, 2006).

Phloem-feeding planthoppers and leafhoppers, drill through numerous layers of cells to reach the phloem causing considerable cellular damage. In contrast, aphid and whitefly stylet pathways to the phloem are largely intercellular, primarily penetrating through the pectin layer located between cellulose layers of the secondary cell walls. Aphids puncture virtually all mesophyll cells on their way to the phloem to taste the cellular milieu and presumably garner “directions” to the phloem (Tjallingii, 2006). In contrast, whiteflies rarely puncture epidermal cells, and begin cellular punctures only when in close proximity to phloem sieve elements (Johnson and Walker, 1999). All cellular probing results in cell wall disturbance, damage to the plasma membranes of mesophyll and parenchyma cells, and cellular penetration (Pollard, 1972; Tjallingii and Hogen Esch, 1993).

Abbreviations: AIL, aphid-induced lesions; AIN, *Acyrthosiphon*-induced necrosis; AKR, *Acyrthosiphon kondoi* resistance; AP, apetala; ATF, activating transcription factor; Avr, avirulence; bHLH, basic helix-loop-helix; BPH, brown planthopper; *Bph/bph*, brown planthopper resistance genes; CC-NBS-LRR, coiled-coil nucleotide binding-site leucine rich repeat; EPG, electropenetration graph; EREBP, ethylene-responsive element binding protein; ERF, ethylene-response factor; ET, ethylene; ETI, effector-triggered immunity; HR, hypersensitive response; HSP, heat-shock protein; JA, jasmonic acid; MAPK, mitogen-activated protein kinase; *Mi*, *Meloidogyne incognita* resistance gene; MKK, mitogen-activated protein kinase kinase; NAC, no apical meristem; ATAF, cup-shaped cotyledon; *Ovc*, ovicidal gene; PAMP, pathogen-associated molecular pattern; PR, pathogenesis related; PTI, PAMP-triggered immunity; *qBph*, QTL influencing BPH resistance; QTL, quantitative trait locus; *qWph*, QTL influencing WBPH resistance; *R* gene, resistance gene; RAP, resistance to *Acyrthosiphon pisum*; RIL, recombinant inbred line; *rme*, resistance to *Meloidogyne*; SA, salicylic acid; SERK, somatic embryogenesis receptor kinase; *Sgt1*, suppressor of G-two allele of Skp1; TTR, *Theroaphis trifolii* resistance; Vat, Virus aphid transmission resistance gene; VIGS, Virus-induced gene silencing; *Wbph*, whitebacked plant hopper resistance gene

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Recent studies discussed in Chapter 15 reveal the critical role insect saliva plays in modifying the sieve element environment and eliciting changes in plant gene expression (Will and van Bel, 2006; Will et al., 2007, 2009; Hogenhout and Bos, 2011). Injection and then recovery of saliva from the cuticle and epidermal cells during surface probing represents the first chemical exchange between phloem-feeding insects and plants. Saliva is also injected into the plant tissue during stylet penetration and secreted into the sieve-tube sap and translocated during both nonsustained sieve element probing and sustained feeding (Forrest and Noordink, 1971; Tjallingii, 1994; Madhusudhan and Miles, 1998).

This chapter focuses on the linkages between the behavioral responses of phloem-feeding insects and the genetic mechanisms that are involved in host plant resistance against phloem-feeding insects. Evidence from numerous studies indicates that, while not exclusive, most of the primary mechanisms involved in host-plant resistance occur within the phloem.

Immunity, the Basis of Insect Resistance in Plants

Plant-insect relationships have evolved over millennia (Berenbaum and Zangerl, 2008). Insects that are attracted to plants by visual or volatile cues encounter variable degrees of host-derived resistance or susceptibility. Most plant-insect interactions are considered “nonhost” interactions because the insect has not adapted to the host plant’s defense arsenal and therefore cannot effectively colonize a plant. The plant’s constitutive chemical and structural defenses repel, irritate, trap, kill, or impale nonadapted insects. When a phloem-feeding insect chooses to probe a nonhost plant, it is presumed that these interactions activate innate immune responses similar to nonhost plant-microbe interactions. During nonhost responses, highly conserved molecules called Pathogen-Associated Molecular Patterns (PAMPs) are introduced by the attacker and are recognized by plasma membrane receptors to detect damage-induced and nonself (i.e., a microbe or pest) signals (Jones and Dangl, 2006; Bittel and Robatzek, 2007; De Lorenzo et al., 2011; Thomma et al., 2011).

PAMP-triggered immunity (PTI) is at the core of nonhost resistance and all other levels of plant resistance. PTI is characterized by a rapid influx of protons (H^+) and calcium (Ca^{2+}) ions, activation of MAP kinase signal-transduction cascades, emission of ethylene (ET), and production of reactive oxygen species, which cause rapid changes in defense gene expression profiles and induce resistance to pathogens (Bittel and Robatzek, 2007). The MAP kinase cascades are coordinated with the salicylic acid (SA), jasmonic acid (JA), and ethylene regulated defense-signaling pathways (Glazebrook, 2005; Wang et al., 2008). In addition, this network links to abscisic acid-, auxin-, gibberellin acid-, and reactive oxygen species-mediated signaling, as well as less understood signal-transduction pathways (Robert-Seilantian et al., 2007; López et al., 2008; Spoel and Dong, 2008). Collectively, PTI and the downstream networks control the induced biochemical and physiological changes in the host plant that alters phloem-feeding insect attraction, settling, feeding, fecundity, longevity, and development time (Walling, 2000, 2009; De Vos et al., 2005).

Phloem-feeders that have adapted to or evade the preformed chemical and structural defenses of a plant family, subfamily, or species are able to colonize and feed from these hosts with varying degrees of success. Adapted insects induce PTI by the injection of salivary PAMPs and elicitors that activate defense-signaling pathways (Felton and Tumlinson, 2008; Mithöfer and Boland, 2008; Walling, 2009). To successfully recover nutrients from and colonize plants with these effective surveillance systems, phloem-feeders must introduce virulence factors or effectors to block steps essential for PTI resulting in host plant susceptibility (Zarate et al., 2007; De Vos and Jander, 2009; Bos et al., 2010).

To counteract the virulence factors introduced in the saliva of phloem-feeders, plants have evolved resistance (*R*) proteins that are able to perceive and counteract the effectors. The dynamics of *R* protein and effector evolution in plant–microbe interactions have been extensively reviewed (Hogenhout et al., 2009; Metraux et al., 2009; Postel and Kemmerling, 2009; Zipfel, 2009; Thomma et al., 2011) and the principles are believed to pertain to plant–insect interactions (Walling, 2009). Briefly, *R* proteins enable plants to eradicate the effects of the phloem-feeder's effectors and thereby allow rapid perception of a phloem-feeder and activation of the innate immunity system. The prompt deployment of defenses that adversely affect insect survival confers resistance to the phloem-feeding attacker. This is called effector-triggered immunity (ETI). ETI is often, but not always, associated with a localized cell death called the hypersensitive response (HR). The largest family of plant *R* genes encodes nucleotide binding site–leucine rich repeat (NBS-LRR) proteins. *R* genes can confer resistance to one insect biotype or confer resistance to as many as four animal taxa (DeYoung and Innes, 2006; McHale et al., 2006; Bent and Mackey, 2007).

Electropenetratograph Analysis: A Tool to Define Host Plant Resistance Mechanisms Against Phloem-Feeding Insects

Monitoring life history parameters and population growth in host choice and no-choice experiments provides an assessment of whether the resistance to a phloem-feeding insect is antixenotic, antibiotic or a tolerance (Smith, 2005). Resistance due to antibiosis alters insect life history traits resulting in a variety of responses from rapid insect death to impacting population development by decreasing insect longevity, fecundity, growth, and increasing developmental periods (Painter, 1951). In contrast, antixenosis impacts insect behavior, delaying acceptance, and consequently rejecting the plant (Panda and Khush, 1995; Le Roux et al., 2010). Tolerance is a host plant trait that allows insect populations to develop and reproduce to high levels without causing significant damage to the plant (Smith et al., 1994). These widely used categories of resistance attempt to describe complex biological activities, but are often inadequate to describe resistance in the context of analytical data (Diaz-Montano et al., 2007).

A limitation of host-choice or reproductive assays is the inability to discern whether the resistance determinants are present at the cell surface, in the apoplast, or within epidermal, mesophyll, or phloem cells. Therefore, to determine location of resistance determinants, the feeding behaviors of piercing–sucking insects as they interact with their host must be analyzed. Since phloem-feeding insects insert their mouthparts into plant tissues, these real-time feeding behaviors cannot be readily visualized and therefore are monitored indirectly using an electropenetratograph (EPG) monitoring systems. EPGs record the changes in electrical resistance in the plant and probing insect, which reflect distinct insect feeding behaviors. There are DC, AC, and dual AC/DC EPG systems; the principles of EPG monitoring have been thoroughly reviewed (Tjallingii, 2000, 2006; Walker, 2000; Tjallingii et al., 2010).

Briefly, an EPG monitoring system uses an insect electrode, a plant electrode, a voltage source, and an input resistor (Figure 16.1). The plant electrode is a stiff wire that is placed in damp soil in a pot with the host plant. The insect electrode is a thin, flexible gold or platinum wire (diameter = ~2.5–25 μm) that is attached with conductive adhesive to the insect's dorsum. The electrical current is delivered by a voltage source to the plant electrode. When the insect inserts its stylet into the plant, it connects a circuit and the current flows through the plant electrode to the plant and from the plant through the insect to the insect electrode. Different insect behaviors cause differences in

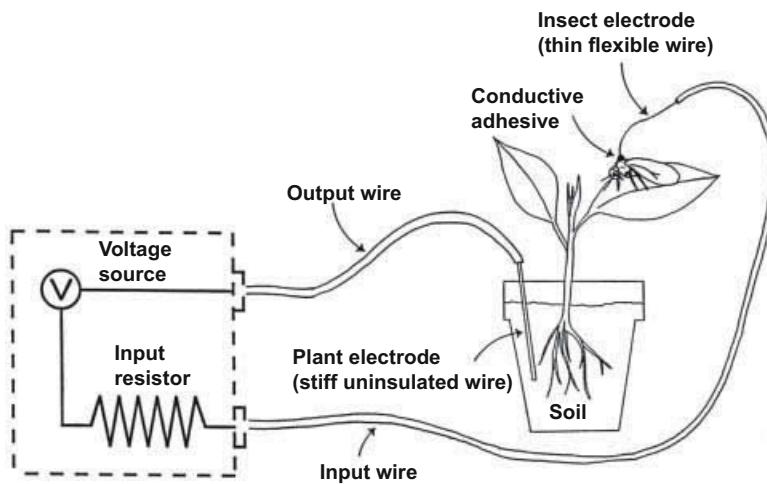


Figure 16.1 Electropenetratograph (EPG) basics. A schematic diagram of an EPG monitoring system is shown. The system includes the insect electrode (a thin flexible wire attached to the insect's dorsum), the plant electrode (a stiff uninsulated wire inserted into the pot or plant), and the EPG monitor that houses a voltage source and an input resistor. The electrical circuit is completed when an insect's stylets probe into plant tissue. This allows the current from the voltage source to flow through the plant electrode, to the plant, through the insect, and to the insect electrode. Insect behaviors are detected as differences in resistance as recorded by the input resistor (see text for further details). (This figure originally appeared in Walker (2000) and is published here with permission.)

resistance, which are monitored as the current travels from the insect electrode to the input resistor on its return to the voltage source. By capturing insects in different behaviors on their host plants and correlating the behaviors with EPG readings, investigators have identified feeding behaviors with distinctive EPG recording patterns (Tjallingii, 2000, 2006; Walker, 2000). EPG systems record the number of cell punctures, the duration of stylet movement through plant apoplast and secretion of saliva, the time and duration of phloem or xylem feeding, and insect salivation in the phloem.

For plants displaying a phloem-localized resistance, it would be expected that behaviors associated with the pathway phase of stylet movement (i.e., passaging between mesophyll cells, puncturing of mesophyll cells, and consumption of xylem sap) would be similar in resistant and susceptible hosts. On a resistant plant, the duration of the phloem-feeding phase could be shortened and insect salivation into the phloem could be lengthened. EPG analysis has been especially informative to relate topology with the behavioral responses that occur during stylet probing, implicating the tissues involved in resistance mechanisms. EPG assays have been useful to demonstrate that resistance genes confer a phloem-mediated resistance to insects as well as identifying the determinants important in the basal resistance that is conferred by the plant's innate immunity system. Plant-insect interactions that have used EPG analyses to demonstrate phloem-localized insect resistance in host plants are summarized in Table 16.1.

Genetic Resistance against Phloem-Feeding Insects

Considerable work has been done to gain insights into the feeding behaviors, genetics, and molecular mechanisms that are involved in plant resistance against phloem-feeding insects. Feeding behaviors

Table 16.1 Phloem-localized resistance in host plants

Plant	Insect	Gene	References ^a
Aphid resistance			
<i>Beta vulgaris</i> (sugar beet)	<i>Myzus persicae</i> (green peach aphid)		Haniotakis and Lange, 1974
<i>Brassica</i> spp.	<i>Brevicoryne brassicae</i> (cabbage aphid)		Cole, 1994
<i>Cucumis melo</i> (melon) ^b	<i>Aphis gossypii</i> (cotton-melon aphid)	<i>Vat</i>	Kennedy et al., 1978
<i>Glycine max</i> (soybean) ^b	<i>Aphis glycines</i> (soybean aphid)	<i>Rag1</i>	Crompton and Ode, 2010
<i>Humulus lupulus</i> (hops)	<i>Phorodon humuli</i> (damson-hop aphid)		Paul et al., 1996
<i>Lactuca sativa</i> (lettuce) ^b	<i>Nasonovia ribisnigri</i> (lettuce aphid)	<i>NR</i>	van Helden and Tjallingii, 1993
<i>Solanum lycopersicum</i> (tomato) ^b	<i>Macrosiphum euphorbiae</i> (potato aphid)	<i>Mi-1.2</i>	Kaloshian et al., 2000
<i>Medicago sativa</i> (alfalfa)	<i>Acyrtosiphon pisum</i> (pea aphid)		Girousse & Bournoville, 1994
<i>Medicago sativa</i> (alfalfa)	<i>Theroaphis maculata</i> (spotted alfalfa aphid)		Nielson & Don, 1974
<i>Medicago truncatula</i> (barrel medic) ^b	<i>Acyrtosiphon kondoi</i> (blue alfalfa aphid)	<i>AKR</i>	Klingler et al., 2005
<i>M. truncatula</i> (barrel medic) ^b	<i>Theroaphis maculata</i> (spotted alfalfa aphid)	<i>TTR</i>	Klingler et al., 2007
<i>M. truncatula</i> (barrel medic) ^b	<i>Acyrtosiphon pisum</i> (pea aphid)	<i>RAP1</i>	Stewart et al., 2009
<i>Prunus persicae</i> (peach) ^b	<i>M. persicae</i> (green peach aphid)	<i>Rm2</i>	Lambert and Pascal, 2011
<i>Rubus idaeus</i> (red raspberry) ^b	<i>Amphorophora idaei</i> (raspberry aphid)	<i>A₁</i>	Sargent et al., 2007
<i>Solanum</i> spp. (wild potato)	<i>Macrosiphum euphorbiae</i> (potato aphid)		Leroux et al., 2008
<i>Solanum</i> spp. (wild potato)	<i>Myzus persicae</i> (green peach aphid)		Leroux et al., 2008
<i>Sorghum bicolor</i> (sorghum)	<i>Schizaphis graminum</i> (greenbug aphid)		Campbell et al., 1982
<i>Triticum aestivum</i> (wheat)	<i>Schizaphis graminum</i> (greenbug aphid)		Ryan et al., 1990
<i>Triticum monococcum</i> (wheat)	<i>Sitobion avenae</i> (grain aphid)		Caillaud et al., 1995
<i>Vigna sinensis</i> (cowpea)	<i>Aphis craccivora</i> (black cowpea aphid)		Mesfin et al., 1992
<i>Malus domestica</i> (apple)	<i>Dysaphis plantaginea</i> (rosy apple aphid)		Marchetti et al., 2009
Plant hopper resistance^c			
<i>Oryza sativa</i> (rice) ^b	<i>Nilaparvata lugens</i> (brown planthopper)	<i>Bph14</i>	Du et al., 2009
<i>O. sativa</i> (rice)	<i>N. lugens</i> (brown planthopper)	<i>Bph1,</i> <i>bph2</i>	Khan and Saxena, 1988; Seo et al., 2010
<i>O. sativa</i> (rice)	<i>N. lugens</i> (brown planthopper)	<i>bph4</i>	Hattori, 2001
<i>O. sativa</i> (rice)	<i>N. lugens</i> (brown planthopper)	<i>Bph3,</i> <i>bph2</i>	Velusamy and Heinrichs, 1986
<i>O. sativa</i> (rice)	<i>N. lugens</i> (brown planthopper)	<i>Bph1</i>	Kimmins, 1989
<i>O. sativa</i> (rice)	<i>Sogatella furcifera</i> (whitebacked planthopper)	<i>Wbph1,</i> <i>Wbph2</i>	Khan and Saxena, 1984

(Continued)

Table 16.1 (Continued)

Plant	Insect	Gene	References ^a
Whitefly resistance			
<i>Solanum lycopersicum</i> (tomato) ^b	<i>Bemisia tabaci</i> B ^{d,e}	<i>Mi-1.2</i>	Jiang et al., 2001
<i>Medicago sativa</i> (alfalfa)	<i>B. tabaci</i> B		Jiang and Walker, 2007

^aAdditional references often support hypotheses regarding specific interactions.

^bMonogenic resistance.

^cBrown planthopper resistance is conferred by over 26 genes that respond differentially to brown planthopper biotypes. Only those that have been studied by EPG are listed. For a complete listing, see Jena and Kim (2010).

^d*Bemisia tabaci* B is part of a cryptic species complex (De Barro et al., 2011). It is also known as Middle East Asia Minor 1 (MEAM1), *Bemisia argentifolii*, *B. tabaci* biotype B or type B.

^eEPGs have shown that *Mi-1.2*-mediated resistance to *M. euphorbiae* is phloem-localized, while resistance to *B. tabaci* is in the plant apoplast (pathway to the phloem).

associated with resistance are often manifested during the initial interactions with the phloem. Aphid resistance mediated by single genes is well characterized in several plant–aphid interactions even though in many cases, the resistance gene has yet to be cloned (Table 16.1). Three resistance genes—*Mi* in tomato (*Solanum lycopersicum*), *Vat* in melon (*Cucumis melo*), and *Bph/bph* in rice (*Oryza sativa*)—that impact phloem-feeding insects are members of the nucleotide binding site–leucine rich repeat (NBS-LRR) family of plant resistance genes. In addition, several aphid resistance genes genetically characterized in the model legume barrel medic (*Medicago truncatula*) map to clusters of resistance gene homologs. The following sections will focus on the complex interactions mediated by resistance genes in plant–insect interactions involving aphids, whiteflies, and planthoppers and discuss new data that is emerging from plant resistance studies against these phloem-feeding insects.

***Mi*-Mediated Resistance in Tomato**

Mi (resistance to *Meloidogyne incognita*: *Mi* = *Mi-1.2*) is a single dominant gene in tomato that confers resistance to taxa that parasitize the phloem tissue. The *Mi* locus was first identified in the wild tomato *Solanum peruvianum* for its role in resistance to three root-knot nematode species: *M. incognita*, *M. arenaria*, and *M. javanica* (Roberts and Thomason, 1986). When introgressed into tomato cultivars, it was discovered that the *Mi* gene also mediates resistance against a wide range of phloem-feeding insects, including the potato aphid (*Macrosiphum euphorbiae*; Rossi et al., 1998), two members of a cryptic whitefly-species complex (*Bemisia tabaci* B and Q; Nombela et al., 2000, 2001, 2003), and the tomato psyllid (*Bactericera cockerelli*; Casteel et al., 2006). While an association with the phloem tissue appears to be a common theme, the overall characteristics of *Mi*-mediated resistance to the different insect taxa are quite different.

Root-knot nematodes initiate feeding sites by inducing the differentiation of giant cells from the provascular tissues, creating the characteristic galls by inducing division of pericycle and parenchyma cells (Jones and Goto, 2011). A hallmark of *Mi*-mediated nematode resistance is a localized HR at the feeding sites (Dropkin, 1969). These characteristic symptoms occur at all stages of plant development when juvenile nematodes penetrate roots of *Mi* plants.

In contrast, *Mi*-mediated resistance to the potato aphid is developmentally regulated and does not involve a HR (Kaloshian et al., 1995; Martinez de Ilarduya et al., 2003). Resistance that strongly affects aphid feeding, development, longevity, and fecundity is initially expressed in fully expanded leaves of four- to 5-week-old tomato plants (Kaloshian et al., 1997; Rossi et al., 1998). Regardless of leaf position, expanding leaves remain susceptible (Kaloshian et al., 1995). *Mi* RNA is present in all developmental stages and is not induced by herbivory suggesting the regulation of *Mi*-mediated resistance occurs at the translational or posttranslational levels (Kaloshian et al., 1997, 2000; Martinez de Ilarduya and Kaloshian, 2001; Goggin et al., 2004). Resistance appears to be tissue autonomous and is not graft-transmissible (Martinez de Ilarduya et al., 2003).

Mi-mediated resistance has antibiotic effects with 100% mortality occurring within 10 days on resistant plants (Hebert et al., 2007). However, potato aphids survive when transferred from resistant to susceptible plants (Kaloshian et al., 1997). Observations of honeydew excretion combined with AC-EPG assays indicate that potato aphids feeding on *Mi* plants readily access the phloem, yet ingest limited amounts of sieve-tube sap (Kaloshian et al., 1997, 2000). Aphid stylets in these initial EPG studies penetrate the epidermal and mesophyll (pathway) tissues equally well on nearly isogenic resistant and susceptible tomato lines, leading to the conclusion that surface or epidermis/mesophyll components are not involved in the resistance mechanism. Rather, the duration of the sieve-element phase is significantly longer on susceptible plants with more frequent and briefer probes on resistant plants, suggesting that the primary mechanism of resistance that limits sieve-tube sap ingestion is phloem localized.

Clonal populations of potato aphids respond differently when feeding on *Mi* resistant plants (Goggin et al., 2001). DC-EPG assays comparing feeding behaviors of aphid populations strongly deterred by *Mi* (avirulent) with those partially deterred by *Mi* (semivirulent) reveal that *Mi*-mediated aphid resistance impacts stylet probing behaviors in both pathway and phloem tissues, implicating either epidermis/mesophyll or mesophyll/phloem resistance mechanisms (Pallipparambil et al., 2010). Sieve-tube sap ingestion and phloem-related behaviors on resistant plants are similar to those observed in the AC-EPG studies confirming the significance of phloem-mediated resistance. Avirulent aphids require significantly more time than the semivirulent aphids to penetrate the sieve element, implicating increased sensitivity of the avirulent aphids to pathway deterrents (epidermis/mesophyll-resistance mechanisms). Penetrating cells along the pathway, however, cannot be fully distinguished from sieve element penetration if sustained phloem ingestion is not established. Bioassays still show that semivirulent aphids perform better on resistant plants in contrast to the rapid rejection of *Mi* plants by the avirulent aphids (Defibaugh-Chavez, 2008). Recent work suggests that the endosymbionts in different aphid populations impact aphid responses to *Mi*-mediated resistance (Francis et al., 2010).

The presence of single gene resistance to both nematodes and phloem-feeding insects raises interesting questions about the mechanistic role of this protein. *Mi* encodes a coiled-coil-nucleotide binding site-leucine rich repeat (CC-NBS-LRR)-receptor kinase-like protein with ATP binding and ATPase activity (Milligan et al., 1998; Tameling et al., 2002) that appears to have an essential role in perceiving biotic elicitors. As implicated by the timing and tissue specificity of nematode and aphid resistance, the resistance to aphids and nematodes can be uncoupled. Transgenic eggplants (*Solanum melongena*) expressing *Mi* are resistant to root-knot nematode (*M. javanica*), but are susceptible to potato aphid (Goggin et al., 2006). Hypotheses to explain differences in nematode and aphid resistance invoke mechanisms that range from differential posttranscriptional regulation in roots and leaves to alternative components in the resistance-signaling pathway (Martinez de Ilarduya and Kaloshian, 2001). Silencing a receptor-like kinase *SERK1* (somatic embryogenesis receptor kinase 1), necessary for *Mi* function, weakens potato aphid but not nematode resistance,

providing further support for the idea of distinct pathways in *Mi*-mediated resistance (Mantelin et al., 2011).

Mutational analyses generating loss-of-function phenotypes identified components of *Mi*-mediated signaling perception and transduction pathways. A recessive mutation in *rme1* (*Resistance to Meloidogyne*) abolishes *Mi*-mediated resistance against the three species of root-knot nematode, potato aphid, and whiteflies (Martinez de Ilarduya and Kaloshian, 2001; Martinez de Ilarduya et al., 2003; Nombela et al., 2003). The functional conservation in the resistance pathway for the different taxa suggests that Rme1 is an essential component for *Mi*-mediated resistance and functions early in the signaling pathway. Furthermore, the inability to experimentally uncouple the HR from nematode resistance in the *rme1* mutant provides further genetic evidence that Rme1 functions early in the *Mi* signaling pathway (Martinez de Ilarduya et al., 2004).

Virus-induced gene silencing (VIGS) has been a useful tool to identify additional components of the *Mi*-mediated signaling perception and transduction pathways. VIGS repression of target genes known to be early participants in resistance gene signaling pathways implicate HSP90-1 and Sgt1 (suppressor of G-two allele of Skp1) in potato aphid and nematode resistance (Bhattarai et al., 2007b). Silencing mitogen-activated protein kinase (MAPK) kinase (LeMKK2) and MAPKs (LeMPK2 and LeMPK1, or LeMPK3) attenuates *Mi*-mediated aphid resistance, implicating MAPK phosphorylation cascades as downstream defense signals in the *Mi*-mediated defense response (Li et al., 2006). Furthermore, silencing WRKY72-type transcription factors in tomato reduces both basal and *Mi*-mediated resistance against potato aphids and root-knot nematodes (Bhattarai et al., 2010).

SA-regulated defenses also appear to be important in *Mi*-mediated aphid resistance (Li et al., 2006). Transcripts of the SA-regulated pathogenesis-related protein gene (*PR-1*) accumulate more rapidly and to higher levels in response to aphid feeding on resistant than susceptible plants (Martinez de Ilarduya et al., 2003). In addition, aphids perform better on SA-deficient *Mi* plants, indicating an important role for SA signaling in aphid resistance (Branch et al., 2004; Li et al., 2006). Neither JA nor ET significantly impact *Mi*-mediated aphid resistance; however, both JA and ET appear to influence host susceptibility to potato aphid feeding (Cooper et al., 2004; Bhattarai et al., 2007a; Mantelin et al., 2009; Anstead et al., 2010).

Two alternative working models for the *Mi* signal transduction pathway are summarized in Figure 16.2 (Bhattarai et al., 2007b). In the first model, Mi forms a signaling complex that includes HSP90-1 and SGT1 to serve as a surveillance mechanism guarding RME1. In the presence of insect effectors, the NBS domain of Mi binds and hydrolyzes ATP causing a conformational change in Mi that activates defense responses. The alternative model proposes that Mi is in an inactive form due to interactions between the NBS and LRR domains. RME1 modifications by the insect Avr factors are detected and Mi is activated by ATP-binding and hydrolysis. Activated Mi recruits HSP90-1 and SGT1 forming a signaling complex that is transduced through SA and MAPK cascades to activate defense gene expression. A specific role for the plasma membrane-localized SERK1 is unknown, but this transmembrane receptor kinase could form a signaling complex with cytosolic Mi as an early component in the signaling pathway following Mi activation (Mantelin et al., 2011).

Vat-Mediated Aphid Resistance in Melon

The single dominant gene *Vat* (*Virus Aphid Transmission*) in melon confers a unique dual resistance against the cotton-melon aphid (*Aphis gossypii*) and *A. gossypii*-mediated transmission of several unrelated viruses (cucumber mosaic virus, zucchini yellow mosaic virus, watermelon mosaic

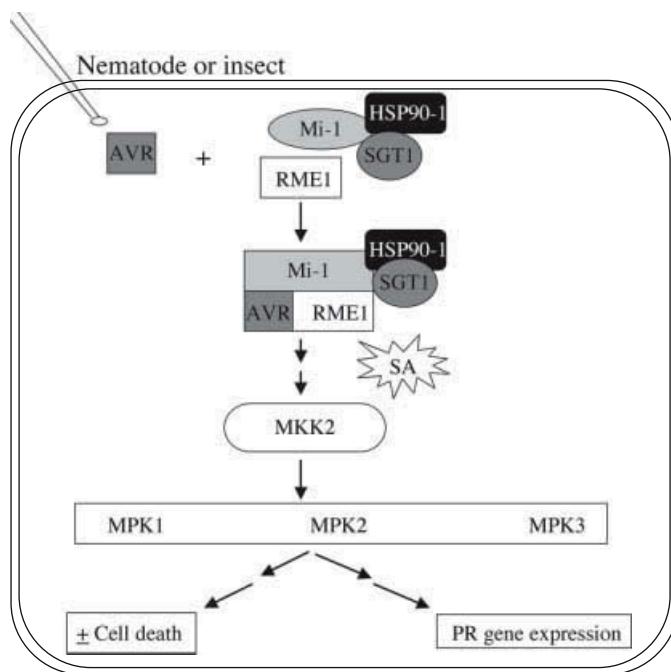


Figure 16.2 A model for Mi signal transduction pathway. The interactions in this model are based on other plant nucleotide binding site-leucine rich repeat (NBS-LRR) R proteins. Mi, HSP90-1, and SGT1 form a R-signaling complex. Mi guards RME1, which possibly represents the host target for the nematode and insect Avr determinants. The animal Avr determinant modifies RME1 and this modification is detected by Mi, which causes a conformational change in Mi and activates R-signaling pathway. Salicylic acid (SA) and mitogen-activated protein kinase (MAPK) cascade follows, resulting in activation of PR genes and presence or absence of cell death in root-knot nematodes and potato aphid interactions, respectively. (This figure originally appeared in Bhattacharai et al. (2007b) and is published here with permission.)

virus-2, and papaya ringspot virus)(Lecoq and Pitrat, 1980; Pitrat and Lecoq, 1980; Soria et al., 2003; Sarria et al., 2008). This resistant trait has been extensively studied in geographically diverse melon germplasm accessions originating from India, Korea, and Zimbabwe (Kishaba et al., 1971; Pitrat and Lecoq, 1980; McCreight et al., 1992; Garzo et al., 2002; Soria et al., 2003). Segregation analyses and allelism tests indicate that aphid-resistance in all three melon accessions is controlled by the *Vat* gene (Kishaba et al., 1976; Pitrat and Lecoq, 1982; Klingler et al., 2001; Sarria et al., 2008).

The *Vat* gene isolated by mapped-based cloning encodes a CC-NBS-LRR protein that is predicted to localize within the cytoplasm (Dogimont et al., 2008). Transgenic melon plants expressing the *Vat* gene confirm the dual role of this single *R* gene in conferring resistance against aphids and virus transmission (Pauquet et al., 2004; Pech et al., 2007; Dogimont et al., 2008). Sequence analyses also confirm the conservation of the *Vat* gene among the melon accessions from India, Korea, and Zimbabwe. In each accession, *Vat* is physically linked to a cluster of *Vat*-like *R* genes of unknown function (Brotman et al., 2002); the number of *Vat*-like genes is a distinguishing feature among the diverse geographical origins of *Vat*-mediated resistance (Dogimont et al., 2008). Comparing LRR domains of *Vat* and *Vat*-like genes reveals differences in the number of a repeated 65 amino acid

motif that could play a role in determining the specificity of *Vat* for cotton-melon aphid (Pauquet et al., 2004).

Melon plants containing the *Vat* gene exhibit three modes of aphid resistance: plant tolerance of colonization, antixenosis, and antibiosis (Bohn et al., 1972). In combination, these resistance mechanisms significantly inhibit aphid population growth through reduced feeding, fecundity, and survival (Kennedy and Kishaba, 1977; Klingler et al., 1998). In contrast to the developmentally regulated aphid resistance in *Mi* plants, aphid resistance in melon is uniformly expressed throughout leaf development regardless of plant age (Klingler et al., 1998). The primary defense response both lengthens the time to sustained phloem feeding and reduces phloem sap ingestion by the cotton-melon aphid, suggesting that resistance acts at least in part by nutrient deprivation. The antibiotic component of resistance appears to strongly influence aphid development by extending the prereproductive period and shortening the reproductive and postreproductive periods causing individual aphids to produce fewer progeny (Klingler et al., 1998). The antixenotic component of resistance can be linked to the rapid interruption of feeding after the stylet penetrates the sieve element (Chen et al., 1997b). Ingestion of phloem sap, quantified indirectly as rates of honeydew excretion, is significantly delayed on resistant plants (Klingler et al., 1998). Variation in resistance levels suggests that modifiers influence the *Vat*-mediated antixenosis and antibiosis to cotton-melon aphid (Kishaba et al., 1976). Two major quantitative trait loci (QTL) influence the acceptance and biotic potential of two aphid genotypes (NM1 and Q9) on melon plants containing the *Vat* gene (Boissot et al., 2008; Thomas et al., 2008). Both of these major QTLs colocalize with the *Vat* gene, and minor additive or epistatic QTLs affect each of the resistance components (Boissot et al., 2010).

EPG analyses provide interesting insights into the dual function of *Vat*-mediated resistance. Histological observations combined with AC-EPG recordings of cotton-melon aphid probing on resistant plants reveal highly branched stylet paths with more sieve element contacts and less time ingesting sieve-tube sap (Kennedy et al., 1978). DC-EPG recordings provide additional insight into the phloem-phase behaviors. Increased sieve element probing and salivation on resistant plants are associated with the inhibition of sustained sieve-tube sap ingestion (Chen et al., 1997a, 1997b; Klingler et al., 1998; Garzo et al., 2002). Multiple studies comparing aphid feeding behaviors on nearly-isogenic susceptible and resistant melon lines provide evidence that phloem-localized resistance is the primary effect of *Vat*-mediated resistance on cotton-melon aphid behavior. Prior aphid infestation does not alter feeding behaviors or enhance aphid resistance (Chen et al., 1997a).

EPG-based behavioral studies also indicate that prephloem factors in the epidermis and mesophyll are likely involved in susceptibility differences among different melon accessions (Chen et al., 1997a, 1997b; Garzo et al., 2002). The association between aphid resistance and resistance to nonpersistent virus transmission by the same aphid complicates simplistic interpretations of the existing data. Nonpersistent viruses are transmitted to epidermal and nonphloem tissues through intracellular punctures along the pathway to the phloem that are reduced in frequency and duration on resistant melon genotypes (Chen et al., 1997a). In another study, the frequency of intercellular punctures did not differ, yet the duration was the same or longer (Garzo et al., 2002). The mechanism explaining the inhibition of viral transmission is enigmatic, as altered probing behavior does not fully explain the inhibition of viral transmission. In resistant melon plants, highly localized cellular responses in epidermal and mesophyll cells along aphid stylet tracks that are indicative of microscopic HRs could limit viral infections to the inoculated cells (Sarria Villada et al., 2009). Temporary blockage of the aphid stylets associated with changes in cellular redox status is also proposed as a mechanism to prevent the release of virions in resistant melon plants (Martin et al., 2003). Other mechanisms could involve *Vat*-mediated inhibition of viral replication in pathway tissues of resistant plants.

The combined results of several studies on the interactions between cotton-melon aphid and melon are consistent with a phloem-localized site of action for aphid resistance and support one or more possible resistance mechanisms within this host-plant tissue (Kennedy and Kishaba, 1977; Kennedy et al., 1978; Chen et al., 1996, 1997a, 1997b; Klingler et al., 1998). A central question focuses on whether resistance is mediated by soluble or structural components of the sieve elements. Soluble compounds in the sieve-tube sap could be detected by the insect during the initial penetration of a sieve element. In resistant plants, this would lead to rejection of that potential feeding site. Resistance could also be due to an inhibition of the aphid's suppression of constitutive or induced defenses. Analysis of sieve-tube sap failed to identify components that significantly differ between resistant and susceptible melon lines (Chen et al., 1997b). Reciprocal grafting experiments between susceptible and resistant varieties demonstrate that resistance factors are not translocated across the graft union, implying that the resistance factor is not a translocated soluble component of the sieve-tube sap (Kennedy and Kishaba, 1977). Mechanisms for localized phloem-specific resistance could include the local synthesis of resistance factors near the sites of stylet penetration or structural features within sieve elements such as callose deposition (Shinoda, 1993) or polymerization of P-proteins that physically prevent sieve-tube sap ingestion at the feeding site (Tjallingii, 2006).

Cotton-melon aphid feeding induces significant transcriptional reprogramming in melon plants with *Vat*-mediated resistance (Samuel, 2008). First, transcripts for several ethylene-responsive genes accumulate in response to cotton-melon aphid feeding on a resistant melon line (Samuel, 2008; Anstead et al., 2010). Second, transcription factor genes associated with early events in the ET-signaling pathway are highly induced within the first 6 hours after infestation of resistant plants, implicating the involvement of ET signaling in *Vat*-mediated host-plant resistance (Anstead et al., 2010). Interestingly, ethylene response factor 1 (ERF1), which is a component of the ET response element-binding protein family and an integrator between the ET and JA pathways shows the strongest response. Finally, numerous other genes encoding transcription factors are activated by aphid feeding in resistant melon plants, including an activating transcription factor 1-like (ATF1-like) gene that is a member of the NAC transcription factor family and a negative regulator of defense responses against fungal and bacterial pathogens (Anstead and Thompson, unpublished data; Wu et al., 2009). Whether responses occur globally throughout the leaves or are involved with molecular events in the phloem have yet to be determined.

Aphid Resistance in Medicago truncatula

Naturally occurring aphid resistance in *M. truncatula* against three pests of forage legumes, blue-green aphid (*Acyrtosiphon kondoi*), spotted alfalfa aphid (*Therioaphis trifolii* f. *maculata*), and pea aphid (*Acyrtosiphon pisum*), represent interactions that occur in a legume model system. Resistance to the bluegreen aphid is mediated by single dominant gene *AKR* (*Acrythosiphon kondoi* resistance) that genetically maps to chromosome 3 within a cluster of NBS-LRR resistance gene analogs (Klingler et al., 2005). *AKR*-mediated resistance expresses as both antixenosis and antibiosis in field and greenhouse settings (Klingler et al., 2005). Although resistance occurs throughout most plant developmental stages, actively growing tissues such as shoot tips appear to be less resistant than mature leaves.

EPG analysis of bluegreen aphid feeding reveals that *AKR* mediates a phloem-specific inducible defense (Klingler et al., 2005). The primary differences in probing activities within susceptible and resistant plants occur at the sieve element. Unlike aphid interactions with *Mi* or *Vat*, prior aphid infestation dramatically primes *AKR* plants for an enhanced defense response upon challenge.

Reduced sieve-tube sap ingestion from resistant plants and enhanced antibiotic defenses slows the expansion of bluegreen aphid colonies (Klingler et al., 2005). Antixenotic behavioral effects could be due to the inhibition of sieve-tube sap consumption encouraging winged morphs to find alternative hosts. Induced resistance is not unique to *AKR*-mediated resistance, but also occurs in other interactions such as green peach aphid (*Myzus persicae*) resistance in peach trees (*Prunus persicae*; Pascal et al., 2002; Sauge et al., 2002, 2006). Plant defense priming by the initial attack allows for a more intense physiological response to the subsequent attack and is likely the basis of induced aphid resistance (Frost et al., 2008).

Transcription profiling of sentinel genes representing different phytohormone-signaling pathways reveals the importance of the JA pathway in *AKR*-mediated responses to bluegreen aphid feeding (Gao et al., 2007). JA biosynthetic genes and JA-responsive genes are specifically induced in resistant plants. In contrast, the SA- and ET-signaling pathways are activated as general responses to bluegreen aphid feeding in both resistant and susceptible plants; this is in marked contrast to the activation of SA signaling during *Mi*-mediated responses in tomato (Li et al., 2006) or the ET-induced *Vat*-mediated responses in melon (Anstead et al., 2010). A subset of transcription factors induced by bluegreen aphid at the early stages of *AKR*-mediated resistance serves as candidate regulators of resistance (Gao et al., 2010). Genes in the AP2/EREBP (Apetala 2/Ethylene-responsive element binding protein) and C2H2 type zinc-finger families are expressed in response to aphid feeding, however, genes from the bHLH (basic helix-loop-helix dimerization) and WRKY gene families are most highly correlated with bluegreen aphid resistance.

Resistance against the spotted alfalfa aphid is mediated by the single dominant gene *TTR* (*Therioaphis trifolii* resistance) (Klingler et al., 2007; Gao et al., 2008). Like *AKR*-mediated resistance against bluegreen aphid, antibiosis is the primary mode of *TTR*-mediated resistance against spotted alfalfa aphid. *TTR* and *AKR* map to independent loci on chromosome 3, and performance of the two aphid species on allelic haplotypes (*akr-ttr*, *akr-TTR*, *AKR-ttr*, *AKR-TTR*) clearly reveals that resistance against the target aphids is independent. Spotted alfalfa aphid feeding on *Medicago* species is noted for a variety of systemic and local physiological responses that are visually obvious on infested and uninfested leaves. In susceptible interactions, spotted alfalfa aphid feeding systemically induces vein chlorosis in the uninfested expanding leaves, possibly due to the translocation of compounds in aphid saliva to sink leaves (Madhusudhan and Miles, 1998; Klingler et al., 2007). Interestingly, the presence of *TTR* attenuates the systemic chlorosis and instead induces a localized chlorosis and purple pigmentation on bluegreen aphid-infested plants that ultimately results in premature leaf senescence. Local chlorosis and necrosis induced by aphid feeding has been explained by the ‘redox hypothesis’, which suggests an imbalance of antioxidants and oxidases, some of which originate from aphid saliva, stimulate overproduction of reactive oxygen species (Miles and Oertli, 1993).

The identification of resistance against pea aphid in *M. truncatula* is notable due to the annotated genome sequence data and other genetic resources that are being developed for both organisms. Phloem-specific antibiosis against pea aphids cosegregates with the *AKR* resistance gene but has four unique characteristics that suggest that pea aphid resistance is tightly-linked but independent from *AKR* (Gao et al., 2008; Guo et al., 2009). First, although the pea aphid and bluegreen aphid are closely related species in the genus *Acyrtosiphon*, the antibiotic and antixenotic resistance to the pea aphids was less effective than resistance to bluegreen aphids (Klingler et al., 2005). Second, preferential expression of JA-biosynthetic or JA-regulated genes is not induced by pea aphid infestation of resistant plants. Although, as previously noted for *AKR*-mediated resistance to the bluegreen aphid, SA- and ET-responsive sentinel genes are induced in both resistant and susceptible cultivars in response to pea aphid feeding. Third, pea aphid resistance is not affected by prior infestation nor influences bluegreen aphid resistance. Fourth, and most compellingly, some

cultivars containing the *AKR* and *TTR* resistance genes are susceptible to pea aphid infestation. Aphid performance on the progenitors of the resistant cultivars reveal that pea aphid and bluegreen aphid resistance originated from different accessions and represent different single, dominant resistance genes.

A semidominant gene *AIN* (*Acyrthosiphon*-induced necrosis) in *M. truncatula* genotype A17 correlates to the necrotic lesion phenotype typical of susceptible interactions and is associated with a moderate level of bluegreen and pea aphid resistance when compared to the highly susceptible A20 genotype (Klingler et al., 2009). However, pea aphid resistance does not cosegregate with the necrotic lesion phenotype.

In a separate study, infestation of A17 by the pea aphid genotype PS01 results in a highly resistant interaction with 100% aphid mortality occurring within 3 days (Stewart et al., 2009). PS01 aphids induce lesions at feeding sites with the hallmarks of a HR that are absent in the susceptible interaction between the virulent LL01 pea aphid genotype. EPG analyses reveal aphid-induced lesions are the result of prolonged multiple cell punctures that occur during pathway probing that are not linked to sieve element penetration. QTL analyses of recombinant inbred lines (RILs) demonstrate that aphid resistance and lesion formation are functionally separate events. A major resistance QTL named *RAP1* (*resistance to Acyrthosiphon pisum 1*) and the *AIL* (*aphid-induced lesions*) locus map to chromosome 3 at different locations from the *AKR* and *TTR* resistance genes. *RAP1*-mediated resistance does not appear to be dependent on plant age, and like many other resistant aphid–plant interactions, EPG analyses of PS01 probing of A17 shows repeated salivation into sieve elements with limited sieve-tube sap ingestion (Stewart et al., 2009).

Whitefly Resistance

Sources of whitefly resistance and tolerance have been identified in several crops or their wild species including soybeans (*Glycine max*), cotton (*Gossypium spp.*), alfalfa (*Medicago sativa*), tomato, and cassava (*Manihot esculenta*) (De Ponti et al., 1990; Teuber et al., 1996; Bellotti and Arias, 2001; Nombela et al., 2003; Walker and Natwick, 2006). However, the mechanisms of resistance are largely uncharacterized. The most well-known genetic resistance to members of a whitefly cryptic species complex (*B. tabaci*) and greenhouse whitefly (*Trialeurodes vaporariorum*) is derived from wild tomato species and are primarily antixenotic. This includes methyl ketone (2-tridecanone)- and acyl sugar-mediated resistance from wild *Solanum spp.* (Williams et al., 1980; Blauth et al., 1998), as well as repellence mediated by volatile terpenoids (Bleeker et al., 2009).

The tomato *Mi* gene provides modest resistance to whiteflies (*B. tabaci* B and Q; Nombela et al., 2003). EPG studies of whitefly interactions with *Mi* tomato plants indicate that whiteflies encounter resistance factors along the pathway to the phloem in epidermal or mesophyll cells (Jiang et al., 2001). If *B. tabaci* stylets puncture the phloem, the whitefly can develop in an unimpeded manner. Therefore, the *Mi*-controlled phloem resistance factors are either ineffective against whiteflies, are not expressed, or are suppressed during whitefly infestation of tomatoes.

Alfalfa lines that express a strong resistance to *B. tabaci* B (Teuber et al., 1996) causes a decrease in whitefly fecundity and high egg/1st-instar mortality (Jiang and Walker, 2007). EPG evaluations of resistant and susceptible alfalfa lines indicate that resistance is primarily phloem-mediated (Jiang and Walker, 2007). Some resistance determinants are associated with the apoplast of epidermal and mesophyll cells, since the pathway phase is prolonged for adult whiteflies and 1st-instar nymphs feeding on the resistant lines. While stylets proceed more slowly through the pathway tissues, over 90% of the whitefly stylets pierce a sieve element on resistant plants; however, the duration of the

phloem-ingestion is shorter, phloem-ingestion is terminated more frequently, and less honeydew is produced. In contrast, whitefly salivation into the phloem is similar in all lines.

EPG has been used extensively to characterize host acceptance by the greenhouse whitefly (Lei et al., 1998, 1999, 2001). Greenhouse whitefly behaviors on plants spanning the spectrum of insect-plant interactions from a preferred host cucumber (*Cucumis sativus*) to putative whitefly-resistant tomato lines to a nonhost sweet pepper (*Capsicum annum*) are documented. Feeding behaviors on the nonhost are similar to the behaviors of insects on plants expressing phloem-mediated resistance; the number of probes to first phloem phase is increased, phloem-ingestion is severely reduced, and the duration of xylem feeding is lengthened. EPG data indicate that the nonhost plants also express resistance factors in the epidermis, mesophyll, and other parenchyma cells.

Little is known about the signaling pathways that control phloem or pathway-phase resistance to whiteflies (Walling, 2008). Transcriptional profiling and *B. tabaci* performance on six defense mutants of *Arabidopsis* provides insight into the defense-signaling pathways important in PTI (Kempema et al., 2007; Zarate et al., 2007). Feeding of whitefly nymphs, stimulate expression of SA-responsive genes, while JA- and ET-responsive gene expression decreases or remains unchanged. Analysis of whitefly performance on five mutants that alter SA- and JA-defense signaling and JA treatments show that JA controls defenses slow whitefly nymph development (Zarate et al., 2007). Collectively, these data indicate that whiteflies suppress the defenses antagonize whitefly performance on its host. Current EPG studies are determining the location of the resistance determinants in whitefly-*Arabidopsis* interactions. Preliminary evidence indicates that resistance is located at the surface, along the pathway and within the phloem (Zhou, Walker, and Walling, unpublished).

The ability of *B. tabaci* to suppress JA-regulated defenses also occurs in Lima bean (*Phaseolus lunatus*) where whiteflies suppress the release of volatiles that attract natural enemies to spider mite-infested plants (Zhang et al., 2009). Like whiteflies, the importance of JA in regulating basal resistance to aphids occurs in many susceptible plant interactions including *Arabidopsis*, sorghum (*Sorghum bicolor*), and *M. truncatula* (Moran and Thompson, 2001; Ellis et al., 2002; Zhu-Salzman et al., 2004; De Vos et al., 2005; Gao et al., 2007). Collectively, these data suggest that in many, but not in all hosts, both whiteflies and aphids are well adapted to their hosts and suppress or avoid the JA-regulated defenses that antagonize insect performance (Thompson and Goggin, 2006; Zhu-Salzman et al., 2006; Walling, 2008).

Planthopper Resistance

With up to 26 resistance genes and additional quantitative trait loci that enhance resistance (Jairin et al., 2010; Jena and Kim, 2010) and five brown planthopper (BPH; *Nilaparvata lugens*) biotypes (Khush, 1984; Chelliah and Bharathi, 1993), rice BPH interactions provide a robust genetic system to assess mechanisms of defense to phloem-feeding pests. With the introduction of BPH susceptible, high-yielding rice varieties during the green revolution and the rapid development of insecticide-resistant BPHs, the BPH is elevated to primary pest status. Single dominant (*Bph*) or recessive (*bph*) *R* genes and quantitative resistance trait loci (*qBph*) are deployed in rice cultivars (Jena and Kim, 2010). The durability of BPH resistance genes in the field varies from 2 years for *Bph1* to 14 years for *bph2* varieties (Alam and Cohen, 1998). Marker-assisted breeding enables the incorporation of new *R* genes either singly or in pyramiding strategies into elite rice varieties in an attempt to avoid the selection of virulent planthopper populations (Jena and Kim, 2010).

Although the mechanisms of BPH resistance are largely unknown, it is clear that BPH-resistant plants have differential responses to BPH biotypes and can express antixenosis, antibiosis, and

tolerance (Cohen et al., 1997; Alam and Cohen, 1998; Yamasaki et al., 2000; Du et al., 2009; Jena and Kim, 2010; Qiu et al., 2010). BPH resistance is complex since the *R* gene-mediated resistance is strongly influenced by its genetic context. QTLs that enhance or inhibit BPH resistance are known and recessive *R* genes (*bps2* and *bps4*) can become dominant once incorporated into different genetic backgrounds (Murata et al., 1998; Murai et al., 2001; Jairin et al., 2010). In addition, multiple BPH resistance genes are clustered on chromosomes 4 and 12 (Jena and Kim, 2010). For this reason, elucidation of the role of individual *R* genes in antibiosis, antixenosis and tolerance will need to be resolved by studying single *R* genes in multiple rice backgrounds.

Only one of the 26 BPH resistance genes has been cloned. Similar to the aphid-resistance genes *Mi* and *Vat*, *Bph14* encodes a CC-NBS-LRR protein (Du et al., 2009). *Bph14* RNAs are detected in ten BPH-resistant genotypes and 11 BPH-susceptible genotypes; therefore it is thought that the divergence of the *Bph14* LRR protein domain is critical for *Bph14*-mediated resistance. Transgenic rice expressing *Bph14* show decreases the rate of BPH population expansion and nymph survival and does not influence insect settling or oviposition (Du et al., 2009).

DC-EPG studies with inbred or transgenic *Bph14* lines demonstrate that *Bph14* impacts BPH feeding behaviors in three ways (Hao et al., 2008; Du et al., 2009). First, *Bph14* increases in the total duration of nonprobing activity. Second, *Bph14* prolongs the pathway phase indicating that BPH takes a longer time to search for a suitable target cell for feeding. Third, while the duration of the total phloem phase increases, the duration of phloem ingestion is decreased. Finally, the duration of xylem feeding is increased. Collectively these data indicate that on *Bph14* resistance is mediated by resistance factors in along the path to the vasculature and within the phloem.

While the exact identity of the phloem- and pathway-resistance factors controlled by *Bph14* has not been discovered, the role of callose deposition is implicated (Hao et al., 2008; Du et al., 2009). Increases in callose at the sites of sieve element punctures are observed during *Bph14*-mediated resistance. Consistent with this, several callose synthase RNAs are elevated and several β -1,3-glucanase RNAs are suppressed in *Bph14* transgenic plants. Finally, SA-regulated transcripts were elevated in resistant plants, while JA- and ET-regulated RNAs were at lower levels, suggesting that SA-regulated defenses mediate *Bph14* resistance.

The feeding behaviors of virulent and avirulent BPH on plants with other BPH resistance genes have also been monitored using either AC- or DC-EPGs (Khan and Saxena, 1984, 1988; Velusamy and Heinrichs, 1986; Youn and Chang, 1993; Hattori, 2001; Hao et al., 2008; Du et al., 2009; Seo et al., 2010). These studies allow the comparison of lines expressing dominant (*Bps1*, *Bps3*, *Bps14* and *Bps15*) or recessive (*bps2*, *bps4*) *R* genes. In addition, comparisons of *R* genes that confer resistance to a single BPH biotype versus broad-based *R* genes that confer resistance to four BPH biotypes can be made (Jena and Kim, 2010).

While there are different BPH-feeding behaviors that occur on *Bph1*, *bph2*, *Bph3*, *bph4*, and *Bph15* plants, phloem-mediated resistance is clearly evidenced based on the larger number of probes, longer phloem phase with reduced phloem ingestion, and longer xylem phase (Velusamy and Heinrichs, 1986; Khan and Saxena, 1988; Kimmins, 1989; Hattori, 2001; Hao et al., 2008; Du et al., 2009; Seo et al., 2010). At the present time, it is not clear if the phloem-mediated resistance is due to sieve element occlusion, antibiotic factors, and/or plant physiological mechanisms that allow plants to better tolerate BPH damage.

Like *Bph14*-mediated resistance, *Bph15*, *Bph1*-, *bps2*- and *Bph3*-mediated resistance is also partially due to factors along the path to the phloem, since the time to the first phloem puncture and duration of the pathway phase is prolonged (Velusamy and Heinrichs, 1986; Khan and Saxena, 1988; Kimmins, 1989; Hao et al., 2008; Du et al., 2009; Seo et al., 2010). However, although it takes longer to reach the vasculature, BPH stylets reach phloem sieve elements with similar frequencies in

both resistant and susceptible varieties. This pathway-phase resistance mechanism is not detected in *bph4* plants (Hattori, 2001). In addition, antixenosis and egg mortality are additional BPH-resistance mechanisms (Cohen et al., 1997; Soundararajan et al., 2004; Qiu et al., 2010).

Bph19 was recently cloned and encodes a CC-NBS domain protein but it lacks the LRR domain (Jena and Kim, 2010). Fine-mapping of *Bph3*, *Bph15*, *Bph18(t)*, *bph19*, *Bph20(t)*, and *Bph21(t)* indicates that the identity of these *R* genes is on the horizon (Yang et al., 2004; Chen et al., 2006; Rahman et al., 2009; Jairin et al., 2010; Jena and Kim, 2010).

Finally, seven dominant *Wbph* genes [*Wbph1*, 2, 3, 5, 6, 7(*t*), 8(*t*)], one recessive *wbph* gene (*wbph4*), and additional QTLs (*qWph*) provide resistance to the white-backed planthopper (*Sogatella furcifera*; Geethanjali et al., 2009; Chen et al., 2010). Markers tightly linked to *Wbph1*, *Wbph2*, *Wbph6*, *Wbph7(t)*, and *Wbph8(t)* are known (Tan et al., 2004). Interestingly, *Wbph8(t)* is linked to four BPH resistance genes on chromosome 4: *Bph15*, *Bph12(t)*, *Bph17*, and *Bph 20(t)* (Tan et al., 2004; Jena and Kim, 2010). In addition, *Wbph7(t)* maps to the *Bph14* locus on chromosome 3 (Tan et al., 2004); however, transgenic *Bph14* plants are not resistant to white-backed planthopper. Therefore, *Bph14* and *Wbph7(t)* could be alleles or members of a complex resistance locus.

EPG studies indicate that *Wbph1* and *Wbph2* resistance is expressed in the phloem (Khan and Saxena, 1984); on *Wbph1* and *Wbph2* plants, *S. furcifera* increases its number of probes, shortens its phloem-ingestion period, and spends more time salivating. Additionally, QTL analysis indicates that a single dominant gene *Ovicidal* (*Ovc* or *qOVA-6*) and four QTLs increase egg mortality (Suzuki et al., 1996; Yamasaki et al., 2003). *Ovc*-controlled egg mortality can be as high as 80%, is active against both WBPH and BPH, and is correlated with high levels of benzoyl benzoate (Yamasaki et al., 2000, 2003).

Future Directions

Genetic resistance against phloem-feeding insects has been identified in many crop plants; however, little is still known about the biochemical and molecular basis for this resistance, especially those mechanisms that occur within the phloem tissue. A better understanding is needed of the genes, regulatory pathways, and metabolic components that contribute to resistance. The best understood resistance traits that affect phloem-feeding insects involve the recognition of genotype-specific taxa by canonical *R* genes. Studies of *R* gene-mediated resistance in plants are beginning to reveal that phloem-feeding insects induce specific signaling pathways and defense genes that are unique to the individual plant-insect interactions.

The diversity of responses emerging from the detailed studies of individual plant-insect interactions clearly demonstrates that individual genotypes within an insect species respond differently to a single *R* gene. This explains how resistance can be easily overcome in the field by simply selecting for insect populations that fail to interact with an individual *R* gene. Such differential recognition is almost certainly dependent upon specific compounds in insect saliva. While great strides are being made to identify effectors in the saliva of phloem feeding insects, specific molecules have yet to be characterized that correspond with *R* genes. Furthermore, the role of endosymbionts in resistance has only just begun to be investigated. Further characterization of effector-*R* gene recognition and effective signal transduction cascades will continue to be a fruitful area of investigation.

Studies focusing on both the plant and insect components of the interaction are essential to fully understand resistance. Phloem-feeding insects are exposed to bioactive molecules ranging from regulatory RNAs to secondary metabolites in their diet. However, the physiological consequences of resistance in insects that derive nutrition from sieve-tube sap are poorly understood. Host plant

genetics strongly influence resistance against phloem-feeding insects. The identification of QTLs affecting the levels and modes of *R* gene-mediated resistance suggest that different suites of genes impact the interaction. Furthermore, host plant resistance is developmentally regulated in some systems. Genomic studies ranging from global transcription to metabolic networks will continue to contribute to our understanding, but are most often hampered by the paucity of genome-level data for both plant and insect systems. Genome sequencing of both components of the plant-insect interaction, such as recent sequencing of pea aphid and *M. truncatula* genomes, will greatly facilitate genomic studies. Genome sequencing projects have yet to be completed for many other interactions that will provide the depth of information required for a global understanding of these complex relationships.

Revealing the role of phloem in these interactions will be an active area of research in the upcoming years. It is apparent throughout the chapters in this book that our fundamental understanding of the phloem has greatly increased in the past decade alone. Physiological concepts are being revealed and revised by the development of powerful analytical data that are rapidly being generated at all levels from the genome to the metabolome. A new generation of visualization tools, both for microscopy and electrophysiology, are increasingly providing in-depth views in real-time of interactions that occur within this recalcitrant tissue.

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