COVER PAGE

Final Report

FUNCTION OF PHLOEM-BORNE INFORMATION MACROMOLECULES IN INTEGRATING PLANT GROWTH & DEVELOPMENT

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ABSTRACT

Studies on higher plants have revealed the operation of cell-to-cell and long-distance communication networks that mediate the transport of information macromolecules, such as proteins and RNA. Based on the findings from this DOE-funded project and results from other groups, it is now well established that the enucleate sieve tube system of the angiosperms contains a complex set of proteins including RNA binding proteins as well as a unique population of RNA molecules, comprised of both mRNA and small RNA species. Hetero-grafting experiments demonstrated that delivery of such RNA molecules, into the scion, is highly correlated with changes in developmental phenotypes. Furthermore, over the course of this project, our studies showed that plasmodesmata and the phloem are intimately involved in the local and systemic spread of sequence-specific signals that underlie gene silencing in plants. Major advances were also made in elucidating the underlying mechanisms that operate to mediate the selective entry and exit of proteins and RNA into and out of the phloem translocation stream. Our pioneering studies identified the first plant protein with the capacity to both bind specifically to small RNA molecules (si-RNA) and mediate in the cell-to-cell movement of such siRNA. Importantly, studies conducted with support from this DOE program also yielded a detailed characterization of the first phloem-mobile RNP complex isolated from pumpkin, namely the CmRBP50-RNP complex. This RNP complex was shown to bind, in a sequence-specific manner, to a set of transcripts encoding for transcription factors. The remarkable stability of this CmRBP50-RNP complex allows for long-distance delivery of bound transcripts from mature leaves into developing tissues and organs. Knowledge gained from this project can be used to exert control over the long-distance signaling networks used by plants to integrate their physiological and developmental programs at a whole plant level. Eventually, this information will aid in the engineering of elite plant lines with optimal traits for plant growth under non-ideal conditions, enhanced biomass and/or seed yield, and directed carbon allocation for efficient and sustainable biofuels production.

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EXECUTIVE SUMMARY

During the course of this project, considerable progress was made in terms of building an understanding of the role of the plant vascular system, and the phloem in particular, in coordinating physiological and developmental processes at the whole-plant level. In the angiosperms, the plants that provide the major source of food and fiber to our global population, the phloem underwent a major developmental change that gave rise to an enhanced capacity for delivery of sugars and amino acids to heterotrophic tissues and organs. This phloem translocation stream moves through files of specialized cells that form individual sieve tubes, comprised of millions of sieve elements (SEs) connected end-to-end. To achieve this high delivery capacity, angiosperm phloem development utilizes the unique properties of plasmodesmata (PD), the intercellular organelles that connect the cytoplasm of neighboring plant cells.

During their developmental progression, individual sieve elements lose their nuclei and vacuoles; other cytoplasmic constituents also become greatly reduced. This reduction in cellular complexity, along with the formation of open sieve plate pores located in the cross walls between adjoining cells, established an hydraulic system with functional properties greatly improved over those present in the gymnosperms. However, the critical question that remained to be resolved was how could such mature, enucleate, sieve elements maintain their functional integrity? As SEs are connected to neighboring companion cells (CCs), via PD, we initiated this project by testing the hypothesis that proteins present in the sieve tube system are synthesized in CCs and then gain entry into the SEs by trafficking through the CC-SE PD.

Members of the cucurbit family, such as pumpkin and cucumber, can be used to collect phloem sap from the stem and so our model system for these studies was the Halloween pumpkin, *Cucurbit maxima*, cv. Big Max. Our biochemical studies performed on pumpkin phloem sap established the presence of a wide array of proteins, ranging in molecular mass from ~5 kDa to greater than 200 kDa. A combination of protein fractionation and microinjection techniques provided direct evidence that a significant number of these proteins have indeed acquired the capacity to interact with PD to mediate their trafficking into the sieve tube system. In addition, our findings provided the first insight into the fact that such phloem proteins have very high affinities for the PD macromolecular trafficking machinery. This raised the possibility that phloem-mobile proteins could well have evolved the capacity to function as long-distance signaling agents.

Molecular studies conducted on the pumpkin phloem sap also identified the presence of a unique population of RNA, including mRNA and small interfering (si)-RNA. This seemed incongruent with the knowledge that SEs lack nuclei, and, further, it was generally assumed that SEs also lack the capacity for protein synthesis. These findings allowed us to develop a model in which the phloem serves as an RNA-based information superhighway in plants. Support for this notion was provided by our studies in which we identified a novel RNA binding protein, the CmPP16, whose properties allowed it to bind non-sequence-specifically to mRNA and mediate the trafficking of the CmPP16-RNA complex through PD. In addition, we were able to show that specific transcripts, bound by CmPP16, could enter the phloem translocation stream for delivery into distantly-located tissues. As some of these mRNAs encode transcription factors, such as CmNACP, our studies provided the first insights into the operation of a novel mechanism used by angiosperms to integrate developmental processes at the whole-plant level.

Direct experimental support for the involvement of this phloem long-distance control system came from studies on phloem-mobile members of the GRAS gene family, the GIBBERELLIC ACID INSENSITIVE PHLOEM (CmGAIP), known to function as transcriptional regulators of GA

signaling in plants. Experiments conducted on transgenic tomato plants expressing a dominant negative mutant form of *CmGAIP* provided incontrovertible evidence that delivery of these transcripts into a wild-type tomato shoot can cause emerging leaves to acquire the morphological phenotype exhibited by the dominant negative mutant plant. Importantly, these studies established the principle that phloem delivery of mRNAs can allow for flexibility in fine tuning of specific developmental programs to ensure that, for example, emerging leaves have morphological characteristics that are optimal for physiological performance under the prevailing environmental conditions.

Insights into the nature of the mRNA-protein complexes that can assemble within the sieve tube system were provided by our biochemical studies performed on the 50 kDa pumpkin phloem RNA binding protein, CmRBP50. This protein is a member of the polypyrimidine tract binding (PTB) protein family whose members function in alternate mRNA splicing and delivery of mRNA to ribosomes. Biochemical-based experiments led to the identification of the core set of phloem proteins that interact either directly or indirectly with CmRBP50 to form a super-stable ribonucleoprotein (RNP) complex. An important requirement for assembly of this stable RNP complex is that a set of serine residues located within the carboxy-terminus of CmRBP50 must be phosphorylated. Co-immunoprecipitation studies, using an anti-CmRBP50 antibody, allowed us to identify six mRNA species that were contained within this RNP complex. Interestingly, five of the six transcripts encoded transcription factors (TFs), and grafting experiments confirmed that these bound TF mRNAs are delivered into the plant apex. The role of these TFs in orchestrating organ development will be the subject of future studies.

Another important achievement made during this project was the identification and characterization of the first plant protein that can bind, specifically, to small (21-24 nucleotide) RNA species. This protein, PHLOEM SMALL RNA BINDING PROTEIN 1 (PSRP1), functions in the phloem translocation stream to bind and deliver siRNA into target tissues. Our studies established that the phloem siRNA is comprised largely of 24 nucleotide species, and thus, CmPSRP1 likely functions in mediating transcriptional gene silencing within meristematic tissues. As with CmRBP50, phosphorylation of serine residues in the carboxy-terminus is required for assemble of this PSRP1-based RNP complex.

A long-standing question in plant biology has been the nature of the phloem-mobile agent that signals the change in day length to the shoot apex in order to activate the change from vegetative to reproductive phase. This agent, long identified as “florigen” has escaped identification for many, many decades. With support from this DOE grant, we were successful in providing direct experimental evidence that a small 20 kDa protein, FLOWERING LOCUS T (FT), serves as the florgenic signal in the cucurbits. Findings from other labs based on Arabidopsis and rice offered indirect evidence in support of this very important discovery.

Finally, using advanced mass spectroscopy methods, we established a phloem proteome database for the cucurbits containing information on the identities for some 1,500 proteins present in the phloem sap collected from pumpkin. Our analysis of this phloem proteome indicated that the enucleate sieve tube system likely has retained the capacity for protein synthesis, along with the machinery for protein degradation via the 26S proteasome. In addition, these studies identified more than 80 RNA binding proteins, which are likely important for the formation of RNP complexes associated with the more than 1000 mRNAs that appear to enter the sieve tube system. Future studies aimed at characterizing the roles of these potentially long-distance signaling complexes should offer novel opportunities for engineering of novel agricultural traits.

REPORT DETAILS

1. Phloem Proteins have the Capacity to Traffic Cell to Cell through Plasmodesmata

In the angiosperms, the functional enucleate sieve tube system of the phloem is maintained by the surrounding companion cells. During this project, we established that polypeptides present within the phloem sap can traffic cell to cell from the companion cells, where they are synthesized, into the sieve tube. This trafficking occurs through plasmodesmata (PD), the unique intercellular organelle of the plant kingdom that established cytoplasmic continuity between neighboring cells. Co-injection of fluorescently labeled dextrans along with size-fractionated pumpkin phloem proteins, ranging in size from 10 to 200 kDa, as well as injection of individual fluorescently labeled phloem proteins, provided unambiguous evidence that these proteins have the capacity to interact with mesophyll plasmodesmata in cucurbit cotyledons to: (a) induce an increase in size exclusion limit (SEL) and (b) traffic cell to cell. During this interaction, plasmodesmal size exclusion limit underwent and increase from ~ 1 kDa, in the resting stated, to greater than 20 kDa, but less than 40 kDa, as the injected phloem protein trafficked into the neighboring cells. This increase in PD SEL occurred irrespective of the size of the injected protein, establishing that partial protein unfolding is a requirement for transport.

A threshold protein concentration in the 20–100 nM range was required for this cell-to-cell transport, indicating that phloem proteins have a very high affinity for plasmodesmal binding site(s). Microinjection-based experiments performed with glutaredoxin and cystatin, two phloem sap proteins from castor bean, established that they too could traffic through cucurbit mesophyll PD. These findings provided the first evidence that proteins produced in companion cells and located in the phloem translocation stream have the capacity to interact with and move through PD to function as non-cell-autonomously acting proteins. Insight was also provided in terms of the requirements for regulated protein trafficking between companion cells and the sieve tube system. Furthermore, as the threshold value for plasmodesmal transport of phloem proteins fell within the same range as many plant hormones this finding raised the possibility that some of these proteins may act as long-distance signaling agents (Balachandran et al. 2007).

2. Role for Plasmodesmal Companion Cell-Mesophyll Communication in the Control Over Carbon Metabolism and Phloem Transport

To explore the role of PD in regulating carbon metabolism in the mesophyll and phloem loading that takes place in the companion cell-sieve element complex studies were performed on transgenic plants expressing viral proteins. Many plant viruses encode for a protein(s) that is essential for movement from the site of replication to surrounding, uninfected cells. These proteins have the ability to interact with PD to increase the SEL. Interestingly, in an earlier study we showed that, when expressed in transgenic tobacco plants, the movement protein of tobacco mosaic virus (TMV-MP) caused an increase in mesophyll PD SEL. During the present project, we discovered that expression of the TMV-MP also alters the biomass partitioning and carbon allocation within these plants. During the day, source leaves of transgenic plants that express the TMV-MP accumulated sugars and starch and biomass partitioning into roots was reduced when compared with vector control plants.

Experiments carried out with transgenic tobacco plants expressing various mutant forms of the TMV-MP, as well as plants expressing the MP of cucumber mosaic virus, established that the effect on biomass partitioning and carbon allocation was independent of its effect on PD SEL. Grafting studies and analysis of transgenic tobacco and potato plants expressing the TMV-MP under tissue-specific promoters established that mesophyll cells were the site of TMV-MP action. These finding provided evidence that PD within the leaf can establish a special communication network between the companion cells and the mesophyll. Based on these studies, we proposed that output signals from the companion cells to the mesophyll and input signals from the mesophyll to the companion cells are involved in regulating photosynthesis occurring within the mesophyll and loading/export that takes place in the CC-SE complex. The TMV-MP-mediated influence on PD trafficking of these signal molecules alters this endogenous control mechanism, resulting in a shift in biomass partitioning and carbon allocation. This work provided the first insight into the operation of such a regulatory system to coordinate the processes of photosynthesis and phloem loading (Lucas et al. 1996).

3. An RNA-based Information Superhighway in Plants

Transgenic plants that overproduce useful products project an appealing image: grains with increased protein content, fruits and vegetables with enhanced nutritional value to flowers with deeper colors. But in trying to create such plants by overexpressing the plant's own proteins, geneticists often inadvertently caused the opposite result. Instead of producing large quantities of new protein, high-expressing transgenes introduced into the plant were found to inhibit the expression of the plant's own genes by triggering sequence-specific destruction of similar transcripts. Thus, the transgene ends up silencing both its own expression and that of similar endogenous genes when the concentration of transgene transcript (mRNA) becomes too high in the cytoplasm.

Cosuppression can affect the entire plant, but more often it silences genes in ordered patterns that follow features of plant morphology, believed to reflect underlying prepatterns of transgene transcription. Some patterns, however, suggest that cosuppression *per se* might not be cell-autonomous, that is, it can be transmitted between cells, perhaps throughout the entire plant. Confirmation of this hypothesis was obtained by experiments in which a normal, nonsuppressed scion (upper vegetative tissues) was grafted onto a cosuppressed stock (lower vegetative tissues and the root system). Here, cosuppression was induced in the scion (5). This transmission of cosuppression through a graft union was gene-specific and required that a transcriptionally active, nonsuppressed transgene be present in the scion (see Figure 1A).

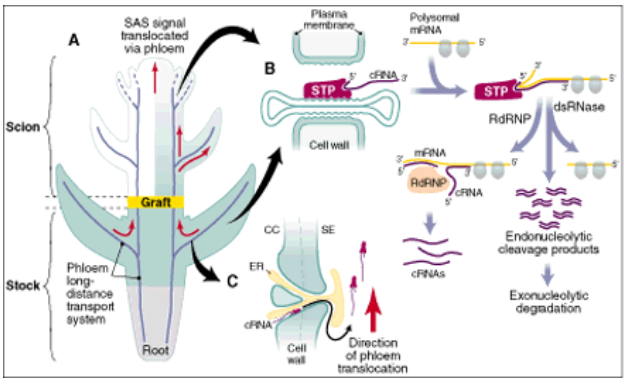

Figure 1. Information transfer through the plant. (A) Long-distance (phloem) transmission of the co-suppression state. (B) Model for PD trafficking and propagation of an RNA surveillance signal within tissues expressing the transgene. STP, surveillance translocation protein (fac-ilitates cRNA cell-to-cell & long-distance trafficking); RdRNP, RNA-dependent RNA polymerase; dsRNase, double-stranded ribonuclease. (C) cRNA-STP complex entering from the companion cell (CC) to the sieve element (SE) of the phloem (ER, endoplasmic reticulum).

Based on these results and our studies on the contents of the angiosperm phloem translocation stream (including many proteins and RNA molecules), we developed a model for a gene-specific, mobile signal molecule that transmits the cosuppression state through the plant's long-distance transport system, the phloem, and from the phloem into the surrounding tissues (Figure 1B and 1C). The precise identity of the molecule that carries the signal for cosuppression was unknown, but we proposed that a likely candidate is an RNA surveillance signal derived from the suppressed gene or its transcripts and transported from cell-to-cell through PD (Jorgensen et al. 2007). This RNA surveillance model served as the basis for our later studies on systemic gene silencing.

4. Rice Phloem Thioredoxin h Mediates its Own Cell-to-Cell Transport Through PD

Rice (Oryza sativa L.) phloem sieve tubes contain RPP13-1, a thioredoxin h protein that moves around the plant via the translocation stream. Such phloem-mobile proteins are synthesized in the companion cells prior to being transferred, through PD, to the enucleate sieve-tube members. In this study, in-situ hybridization experiments confirmed that expression of RPP13-1 is restricted to companion cells within the mature phloem. To test the hypothesis that RPP13-1 enters the sieve tube, via PD, recombinant RPP13-1 was expressed in *Escherichia coli*, extracted, purified and fluorescently labeled with fluorescein isothiocyanate (FITC) for use in microinjection experiments into tobacco (*Nicotiana tabacum* L.) mesophyll cells. These studies confirmed that FITC-RPP13-1 moved from the injected cell into surrounding cells, whereas the *E. coli* thioredoxin, an evolutionary homolog of RPP13-1, when similarly labeled and injected, failed to move in this same experimental system. In addition, co-injection of RPP13-1 and FITC-dextrans established that RPP13-1 can induce an increase in PD SEL to a value greater than 9.4 but less than 20 kDa.

Nine mutant forms of RPP13-1 were constructed and tested for their capacity to move from cell to cell; two such mutants were found to be incapable of movement. Crystal-structure prediction studies performed on wild-type and mutant RPP13-1 identified the location of structural motifs required for protein trafficking through PD (Ishiwatari et al. 1998). These studies, in conjunction with our earlier work on phloem proteins from pumpkin and castor bean, are consistent with the notion that protein trafficking through the companion cell-sieve element PD is a common feature of the angiosperm sieve tube system.

5. Identification of a Phloem Protein that Potentiates Transport of mRNA into the Sieve Tube System

Based on our finding that plant viruses use the endogenous PD pathway to move their infectious RNA/DNA, we reasoned that viral MPs likely contained motifs held in common with plant RNA-binding proteins which also engage in cell-to-cell trafficking of mRNA. This hypothesis was tested by probing the pumpkin phloem sap with an antibody directed against a well-characterized viral MP. This assay identified a 16 kDa phloem protein, that was named CmPP16. Cloning of this protein was followed by gel mobility-shift assays; these studies established that CmPP16 possessed properties similar to those of viral MPs.

The *CmPP16* messenger RNA (mRNA) was detected in phloem tissue, whereas protein appeared to be confined to sieve elements. Microinjection and grafting studies revealed that

CmPP16 moves from cell to cell, mediates the transport of sense and antisense RNA, and moves together with its mRNA into the sieve elements of scion tissue. These studies provided the first characterization of a phloem-mobile RNA binding protein with the characteristics required to mediate RNA delivery into the long-distance translocation stream. Furthermore, this work provided strong support for the notion that RNA molecules move within the phloem as a component of a plant information superhighway (Xoconostle-Cázares et al. 1999).

6. Phloem Long-distance Transport of CmNACP mRNA & Supracellular Regulation of Plant Development

Direct support for the concept that RNA molecules circulate throughout the plant, via the phloem, was provided through the characterization of mRNA from phloem sap of mature pumpkin leaves and stems. One of these mRNAs, *CmNACP*, is a member of the NAC domain gene family, some of whose members have been shown to be involved in apical meristem development. Our in situ RT-PCR analysis revealed the presence of *CmNACP* mRNA in the companion cell-sieve element complex of leaf, stem and root phloem. Longitudinal and transverse sections showed continuity of transcript distribution between meristems and sieve elements of the protophloem, consistent with *CmNACP* mRNA transport over long distances and accumulation in vegetative, root and floral meristems. In situ hybridization studies conducted on *CmNACP* confirmed the results obtained using in situ RT-PCR.

Phloem transport of *CmNACP* mRNA was established directly by heterograft studies between pumpkin and cucumber plants, in which *CmNACP* transcripts were shown to accumulate in cucumber scion phloem and apical tissues. Importantly, similar experiments were conducted with 7 additional phloem-related transcripts. This pioneering study established the existence of a system for the delivery of specific mRNA transcripts from the body of the plant to the shoot apex. These findings provided insight into the presence of a novel mechanism used by higher plants to integrate developmental and physiological processes at the whole-plant level (Ruiz-Medrano et al. 1999).

7. Peptide Antagonists of the Plasmodesmal Macromolecular Trafficking Pathway

In plants, cell-to-cell transport of endogenous and viral proteins and ribonucleoprotein complexes (RNPCs) occurs via PD. Specificity of this transport pathway appears to involve interaction between such proteins/RNPCs and PD chaperones/receptors. The nature of this interaction was further explored using KNOTTED1 (KN1), a non-cell-autonomous transcription factor (Lucas et al. 1995), and the *Cucumber mosaic virus* MP (CMV-MP). A modified phage-display screening system was used to identify peptides capable of interacting with proteins present in a PD-enriched cell wall fraction. Binding/competition assays and microinjection experiments revealed that these phage-displayed peptides and homologous synthetic oligopeptides function as ligand-specific antagonists of macromolecular trafficking through PD.

This study identified a KN1 peptide antagonist having the capacity to interact with a motif involved in the dilation of PD microchannels. Although KN1 could still achieve limited movement through PD when this SEL motif was blocked, KN1-mediated transport of KN1-sense RNA was fully inhibited. These findings provided direct support for the hypothesis that KN1 requires, minimally, two physically separated signal motifs involved in the dilation of, and

protein translocation through, PD microchannels. In addition, this work established, for the first time, that PD dilation is a prerequisite for the cell-to-cell transport of an RNPC. A model for the trafficking of proteins and RNPCs was developed based on these findings (Figure 2) (Kragler et al. 2000).

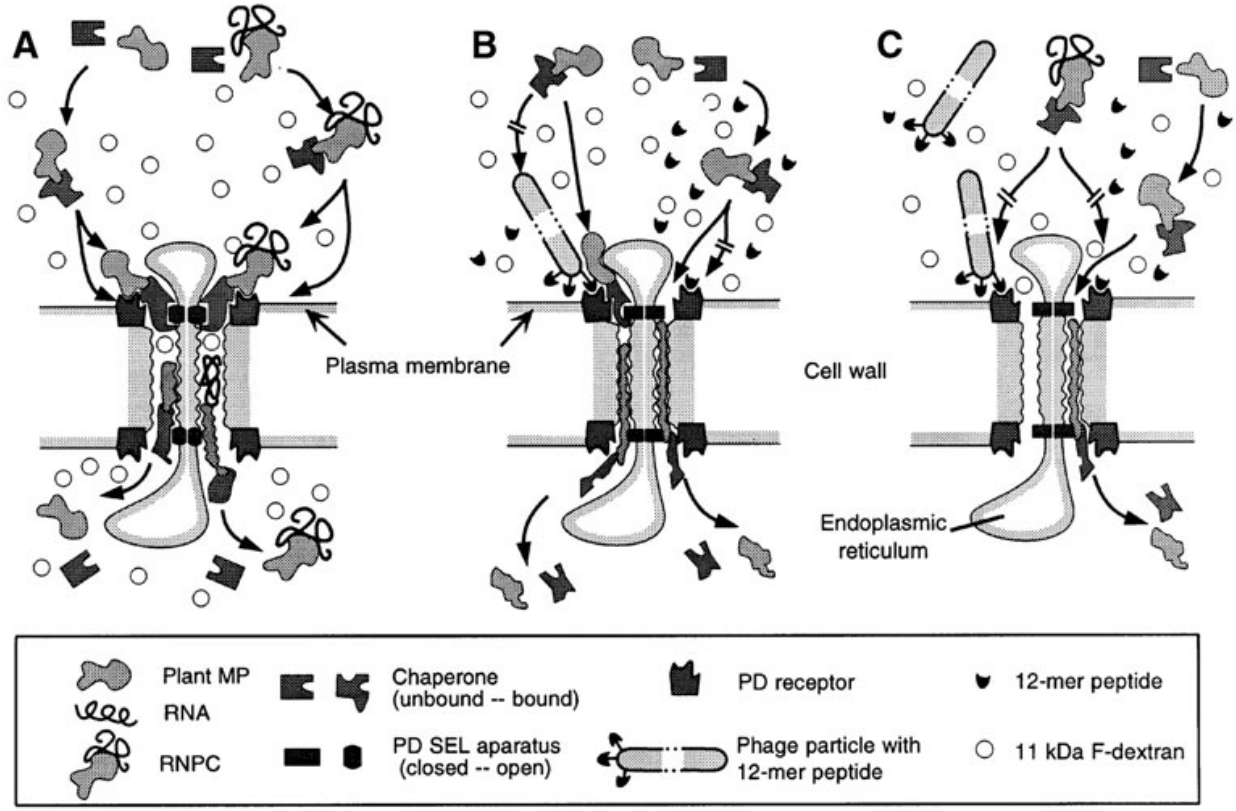

Figure 2. Model illustrating the manner in which 12mer peptide-displaying phage particles, or synthetic oligopeptides, interact with the constituents of the plasmodesmal macromolecular (endogenous MP/RNPC) translocation system. (A) Control scenario in which an endogenous MP/RNPC binds to its cognate chaperone for delivery to the plasmodesmal receptor; binding fully activates the SEL motif on the chaperone, leading to dilation of the microchannel. The chaperone–endogenous MP/RNPC then engages the translocation machinery for transport along the length of the microchannel and eventual release into the cytoplasm of the adjacent cell. Note that, during each translocation event, 11 kDa F-dextran molecules enter the microchannel, by diffusion, and are eventually released into the adjacent cell. (B) Ligand-specific competition between the 12mer peptide-displaying phage particles, or the synthetic oligopeptide, and the endogenous MP, for the plasmodesmal receptor site, inhibits the normal docking and microchannel dilation steps; under these conditions, although retarded, the chaperone–endogenous MP can still engage the translocation machinery. With the SEL apparatus in the closed condition, 11 kDa F-dextran cannot enter the microchannel and the endogenous MP must undergo more extensive unfolding to allow movement along the microchannel; upon release into the cytoplasm of the adjacent cell, both the chaperone and the endogenous MP are inactive due to misfolding. (C) Phage-displayed peptides and synthetic oligopeptides prevent the chaperone–RNPC activation of the SEL apparatus, which blocks further transport of the RNPC. Under these conditions, chaperone–endogenous MP behaves as in (B).

8. Characterization of Phloem Serpin-1 (CmPS-1): a Developmentally Regulated Elastase Inhibitor

Phloem-feeding insects cause major damage to agricultural crops, especially through their delivery of viral pathogens. Many plants are more susceptible to aphids during the early stages of their life cycle. To explore the underlying molecular determinants for this change in resistance, phloem proteins from pumpkin were studied in terms of their expression level and ability to negatively affect aphid feeding behavior. Molecular, biochemical, and functional characterization of CmPS-1, a novel 42-kDa serine proteinase inhibitor displaying anti-elastase properties, revealed that its expression was developmentally regulated. Interestingly, CmPS-1 displayed no detectable inhibitory properties against chymotrypsin, trypsin, or thrombin. The elastase cleavage sites within the reactive center loop of CmPS-1 were determined to be Val347-Gly348 and Val350-Ser351 with a 3:2 molar ratio. Importantly, in vivo feeding assays conducted with the aphid, *Myzus persicae*, established a close correlation between the developmentally regulated increase in CmPS-1 within the phloem sap and the reduced ability of these insects to survive and reproduce on pumpkin (Yoo et al. 2000). These studies suggested that early expression of CmPS-1 might contribute to increased resistance to aphid infection.

9. Protein Kinases within the Phloem Sieve Tube System

Protein kinases play central roles in both signaling pathways and activation of enzyme complexes. A mass spectrometry approach was employed to search for the presence of protein kinases within the pumpkin phloem. Based on these studies, five putative protein kinases (three calcium-independent and two calcium-dependent protein kinases) were detected within the phloem sap extracted from stem tissues. Biochemical methods were used to purify one such calcium-dependent protein kinase. The gene for this calmodulin-like domain protein kinase 1 (CmCPK1), was cloned using obtained peptide sequences. A combination of mass spectrometry, peptide fingerprinting, and amino-terminal sequencing established that, in the phloem sap, CmCPK1 exists as an amino-terminally cleaved protein. A second highly homologous isoform, CmCPK2, was identified, but although transcripts could be detected in the companion cells, peptide fingerprint analysis suggested that CmCPK2 does not enter the phloem sap. Potential substrates for CmCPK1, within the phloem sap, were also detected using an on-membrane phosphorylation assay. As many phloem proteins are phosphorylated, CmCPK1 and other such proteins within the sieve tube system likely function in the posttranslational modification of specific phloem proteins (Yoo et al. 2002).

10. A Systemic Small RNA Signaling System in Plants

Systemic translocation of RNA can exert non-cell-autonomous control over plant development and defense. Long-distance delivery of mRNA has been proven, but transport of small interfering RNA and microRNA remained to be demonstrated. Analyses performed on phloem sap collected from a range of plants identified populations of small RNA species. The dynamic nature of this population was reflected in its response to growth conditions and viral infection. The authenticity of these phloem small RNA molecules was confirmed by bioinformatics analysis; potential targets for a set of phloem small RNA species were identified.

Heterografting studies, using spontaneously silencing coat protein (CP) plant lines, established that transgene-derived si-RNA moved in the long-distance phloem and initiated CP

gene silencing in the scion. Biochemical analysis of pumpkin phloem sap led to the characterization of Phloem SMALL RNA BINDING PROTEIN1 (PSRP1), a unique component of the protein machinery probably involved in small RNA trafficking. Equivalently sized small RNA binding proteins were detected in phloem sap from cucumber and lupin. PSRP1 binds selectively to 25-nucleotide single-stranded RNA species. Microinjection studies provided direct evidence that PSRP1 mediates the cell-to-cell trafficking of 25-nucleotide single-stranded, but not double-stranded, RNA molecules. PSRP1 was the first plant protein shown to have the capacity to traffic si-RNA through PD. In the cucurbits, PSRP1 likely plays an important role in long-distance transmission of silencing signals involved in systemic control over developmental and physiological processes (Yoo et al. 2004).

11. Regulation of Leaf Development by Phloem Long-distance Delivery of mRNA

The phloem translocation stream contains a population of RNA molecules, suggesting plants use RNA to integrate developmental processes, at the whole-plant level. Support for this notion was provided by studies conducted on two members of the GRAS family, namely C. maxima GIBBERELLIC ACID INSENSITIVE PHLOEM (CmGAIP) and GAI. These two homologs were chosen because of their involvement as transcriptional regulators in GA signaling and because they were expressed in companion cells and present in the sieve elements (Figure 3).

A combination of pumpkin, tomato and Arabidopsis was employed to examine the processes involved in long-distance delivery, to sink tissues, of RNA for engineered dominant gain-of-function pumpkin (*Cmgaip*) and Arabidopsis (*ΔDELLA-gai*) genes. Our studies demonstrated that *gai* RNA entry into functional sieve elements occurred via a selective process. Both engineered mutant *gai* transcripts were able to exit the scion phloem and traffic cell to cell into the shoot apex. Delivery of *Cmgaip* and *ΔDELLA-gai* RNA mediated highly reproducible changes in leaf phenotype in transgenic tomato lines grown under greenhouse conditions. Phenotypic analysis indicated that tomato leaflet morphology was influenced quite late in development. In addition, tissue sink strength did not appear to dictate *gai* RNA delivery, suggesting complexity in the process underlying macromolecular trafficking (Figure 4). These results established that the molecular properties of the *Cmgaip* and *ΔDELLA-gai* transcripts are compatible with the tomato cell-to-cell and long-distance macromolecular trafficking systems. An important conclusion, based on these findings, was that control over *GAI* RNA delivery, via the phloem, may be regulated by sequence motifs conserved between plant families. We proposed a model in which RNA delivery, via the phloem, allows for flexibility in fine tuning of developmental programs to ensure newly developing leaves are optimized for performance under the prevailing environmental conditions (Haywood et al. 2005).

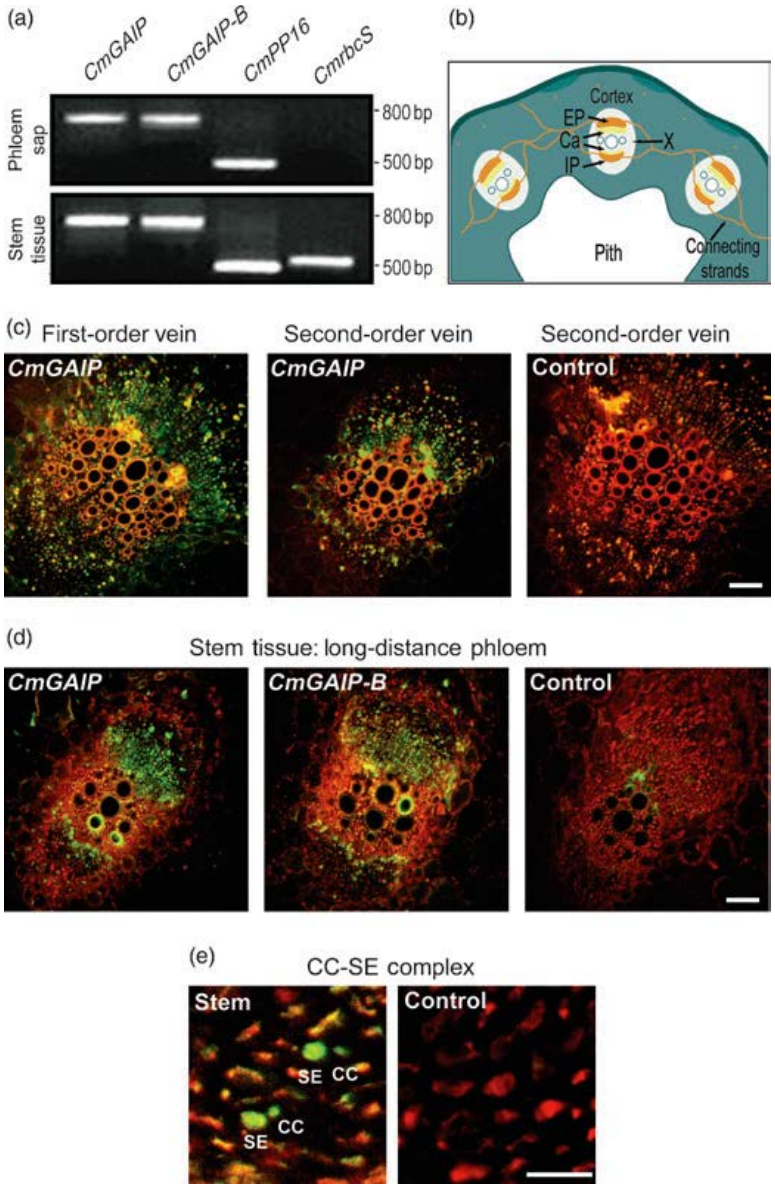

Figure 3. Both CmGAIP and CmGAIP-B transcripts accumulate in phloem tissues and are present in the pumpkin phloem translocation stream.

(a) Gene-specific primers for CmGAIP and CmGAIP-B were used in RT-PCR performed on RNA extracted from pumpkin phloem sap (upper panel); note that both mRNA species were amplified. CmPP16 RNA was amplified as a positive control (see Ruiz-Medrano et al., 1999). Absence of CmrbcS transcript amplification demonstrated the lack of wound-induced contamination during the phloem sap collection process. RT-PCR was performed on RNA extracted from pumpkin stem tissue (lower panel), as a positive control to demonstrate that the CmrbcS primers do amplify the expected product.

(b) Diagram of a cross-section through the pumpkin petiole/stem illustrating the arrangement of the bicollateral vascular bundles: tissues are indicated by the following symbols; Ca, cambium; EP, external phloem; IP, internal phloem; X, xylem.

(c) Pumpkin source leaf first-order and second order veins analyzed by in situ RT-PCR. Note that CmGAIP transcripts (green fluorescent signal) accumulated to high levels in the phloem. Control: RT-PCR performed in absence of CmGAIP gene-specific primers. Red signal; tissue autofluorescence used to illustrate cellular architecture.

(d) Pumpkin stem tissues analyzed by in situ RTPCR. Both CmGAIP and CmGAIP-B transcripts were detected in the external and internal components of the long-distance phloem. Control as in (c). Scale bars: 500 µm [common for (c) and (d)].

(e) Localization of CmGAIP transcripts to sieve elements (SE) and their companion cells (CC) in stem vascular bundles. Control as in (c). Scale bar: 100 µm (common for both images).

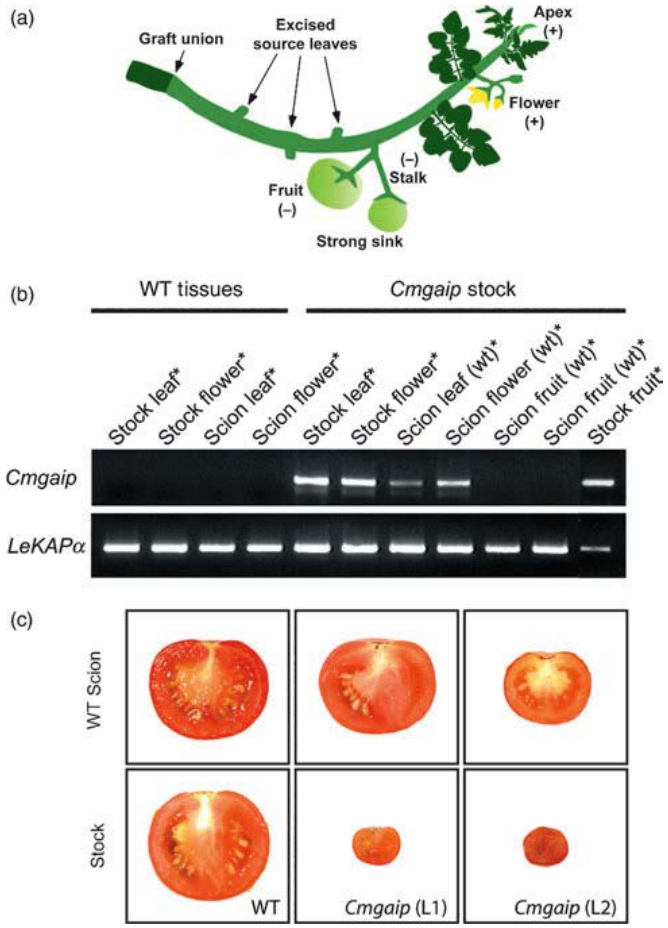

Figure 4. Tissue developmental status can affect accumulation of *Cmgaip* transcripts in grafted wild-type tomato scion tissues.
(a) Schematic illustration of a wild-type tomato scion depicting the position of the tissues analyzed with respect to the location of the *P35S:Cmgaip* (stock):wild-type (scion) graft union. Upon graft union establishment, all scion source leaves were excised to prolong translocation of stock phloem sap into the scion. Note that fruits were located closer to the graft union than the apical or floral tissues. Dark green: scion leaves displaying *Cmgaip*-induced leaflet phenotype.
Light green: wild-type scion tissues. (b) RT-PCR analyses performed on RNA extracted from leaves, floral buds and fruits of the indicated plant materials. *Cmgaip* transcripts were amplified with the appropriate primer set. Amplification of *LeKAPa* RNA served as a positive control. (c) Comparative analysis of fruit development that occurred with the following graft combinations: wild-type:wild-type (stock:scion), transgenic *Cmgaip* (L1):wild-type (stock:scion) and *Cmgaip* (L2):wild-type (stock:scion). Note the absence of developing seeds in fruits of the *Cmgaip* (L1) and *Cmgaip* (L2) tomato lines, but normal fruit and seed development in wild-type scions.

12. Plasmodesmal-Associated Protein Kinases in Tobacco and Arabidopsis Recognize a Subset of Non-Cell-Autonomous Proteins

Cell-to-cell communication in plants involves the trafficking of macromolecules through PD. This exchange of proteins and RNA is likely regulated, and a role for protein phosphorylation had been implicated, but specific components remained to be identified. A 34-kDa protein, isolated from a tobacco PD preparation, exhibited calcium-independent kinase activity and displayed substrate specificity in that it recognized a subset of viral and endogenous non-cell-autonomous proteins. This PD-ASSOCIATED PROTEIN KINASE (PAPK) specifically phosphorylated the C-terminal residues of TMV-MP; this posttranslational modification had been shown to affect MP function.

Molecular analysis of purified protein established that this tobacco (*Nicotiana tabacum*) PAPK was a member of the casein kinase I family. Subcellular localization studies identified a possible *Arabidopsis thaliana* PAPK homolog, PAPK1. TMV-MP and PAPK1 were co-localized within cross-walls in a pattern consistent with targeting to PD. Moreover, *Arabidopsis* PAPK1 also phosphorylated TMV-MP *in vitro* at its C terminus. These findings strongly suggested that *Arabidopsis* PAPK1 is a close homolog of tobacco PAPK. Thus, PAPK1 represents a novel plant

protein kinase that is targeted to PD and may play a regulatory role in macromolecular trafficking between plant cells (Lee et al. 2005). Figure 5 illustrates a model for the likely mechanisms involved in cell-to-cell trafficking of proteins and RNA between companion cells and the sieve tube system (Lough and Lucas, 2006).

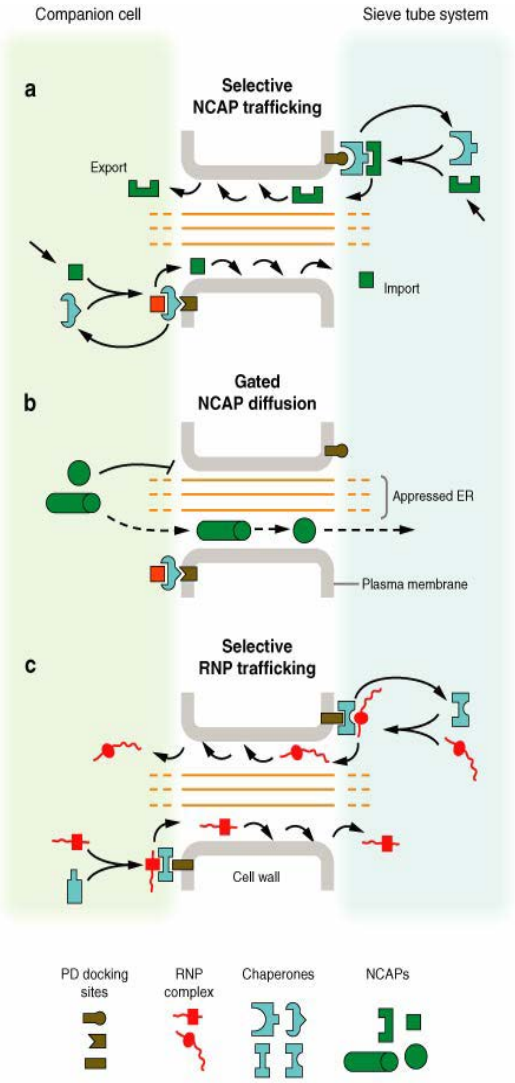

Figure 5. Potential mechanisms for the cell-to-cell trafficking of macromolecules through the companion cell-sieve element PD. (a) Selective delivery of non-cell-autonomous proteins (NCAPs) to specific PD docking sites is mediated by chaperones. Docking of the NCAP-chaperone complex induces dilation in a PD microchannel that allows NCAP translocation along the length of the PD. Mutations within the NCAP can prevent formation of the NCAP-chaperone complex, thereby inhibiting delivery to and/or trafficking through the PD. The model illustrates NCAP import into and export from the sieve tube system. (b) Dilated PD microchannels can act as a size-exclusion limit barrier to NCAP diffusion. Mutations within NCAPs that move in this manner are not expected to affect their capacity for movement. (c) In a process analogous to (a), cell-to-cell trafficking of RNA molecules into and out of the sieve tube system is mediated by RNA binding proteins in conjunction with specific chaperones.

13. FLOWERING LOCUS T Protein Acts as the Long-distance Florigenic Signal

Cucurbita moschata, a cucurbit species responsive to inductive short-day (SD) photoperiods, and a *Zucchini yellow mosaic virus* (ZYMV) vector were used to test whether long-distance movement of FLOWERING LOCUS T (FT) mRNA or FT is required for floral induction. Ectopic expression of FT by ZYMV was highly effective in mediating floral induction of long-day (LD)-treated plants. Moreover, the infection zone of ZYMV was far removed from floral meristems, suggesting that FT transcripts do not function as the florigenic signal in this system. In addition, heterografting experiments demonstrated efficient transmission of a florigenic signal from flowering *Cucurbita maxima* stocks to LD-grown *C. moschata* scions. Real-time RT-PCR performed on phloem sap collected from *C. maxima* stocks detected no *FT* transcripts, whereas

mass spectrometry of phloem sap proteins revealed the presence of Cm-FTL1 and Cm-FTL2 (Figure 6A-6D).

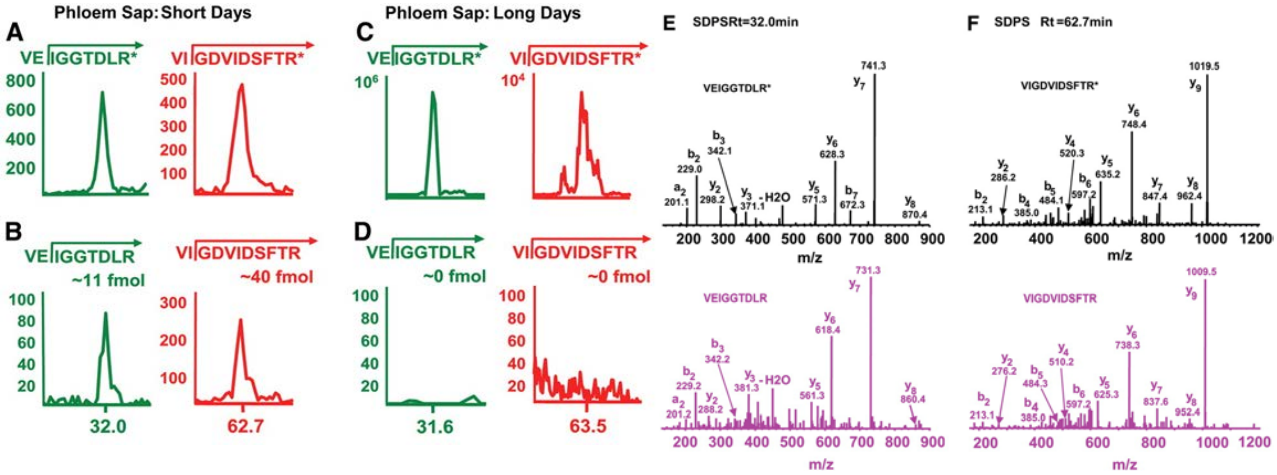

Figure 6. Photoperiodic Control of Cmo-FTL1/FTL2 Entry into the Phloem Translocation Stream.

Phloem sap was collected from mature stems of C. moschata grown under inductive SD or noninductive LD conditions. Proteins contained within the 18- to ~22-kD region of SDS-PAGE gels were in-gel digested and total peptides extracted for analysis by MS. Peptide a liquots (7 µg) for each treatment were subjected to LC-single reaction monitoring (SRM). Arrows indicate the reaction monitored for each trace ion chromatogram. All LC-SRM traces are from the same data set. The x and y axes in LC-SRM traces are retention times, in minutes, and ion counts, respectively.

(A) LC-SRM traces of isotopically labeled synthetic peptides (AQUA peptides; 100 fmol; R* represents Arg[13C615N4]) added to the 7-µg aliquot of peptides derived from phloem sap collected from SD-grown plants. The AQUA peptides served as internal standards for measuring the levels of VIGDVIDSFTR (TR) and VEIGGTDLR (LR) peptides derived from Cmo-FTL2.

(B) LC-SRM traces of Cmo-FTL2 native peptides, VIGDVIDSFTR and VEIGGTDLR, derived from phloem sap collected from SD-grown plants. Peptide quantification was made by comparing peak abundances at the same retention times between the traces presented in (A) and (B).

(C) LC-SRM traces of AQUA peptides (100 fmol) added to the 7-µg aliquot of peptides derived from phloem sap collected from LD-grown plants.

(D) LC-SRM traces of Cmo-FTL2 native peptides derived from phloem sap collected from LD-grown plants. Note the absence of peaks at the same retention times illustrated for the traces in (C).

(E) Tandem MS spectra of AQUA (top) and native (bottom) peptides of VEIGGTDLR from SD-grown plant phloem sap (SDPS) at the retention time of 32 min. Native peptides have the same tandem MS spectra as the AQUA peptides, except for a 10-D shift for y series fragments.

(F) Tandem MS spectra of AQUA (top) and native (bottom) peptides of VIGDVIDSFTR from SDPS at the retention time of 62.7 min.

Importantly, studies on LD- and SD-treated *C. moschata* plants established that Cmo-FTL1 and Cmo-FTL2 are regulated by photoperiod at the level of movement into the phloem and not by transcription. Finally, mass spectrometry of florally induced heterografted *C. moschata* scions revealed that *C. maxima* FT, but not FT mRNA, crossed the graft union in the phloem translocation stream (Figure 6E and 6F). Collectively, these studies are consistent with FT functioning as a component of the florigenic signaling system in the cucurbits (Lin et al. 2007)(Figure 7).

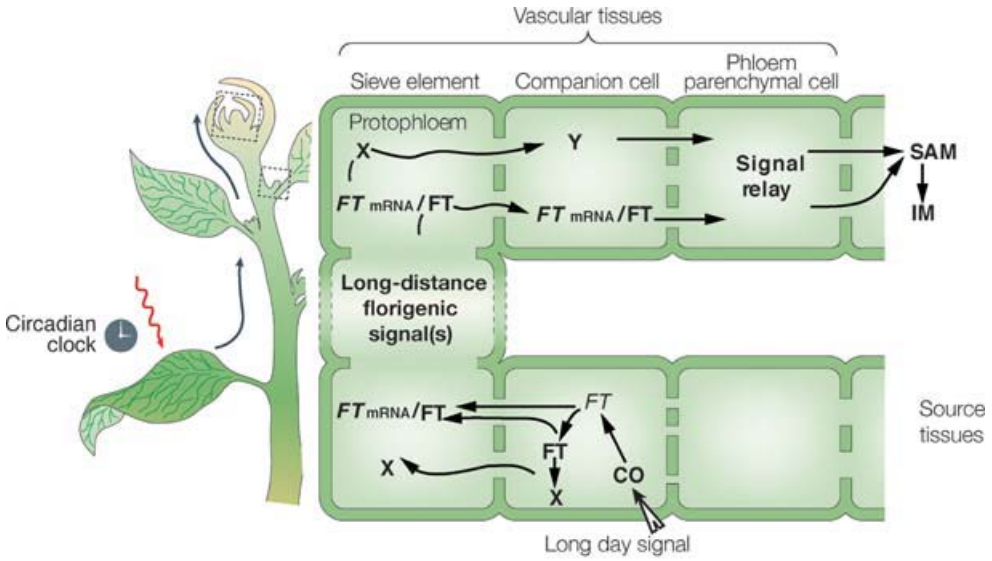

Figure 7. Photoperiodic control over floral induction requires phloem-mediated delivery of florigenic signal(s). CONSTANS (CO) expression is under photoperiodic control and occurs in the companion cells (CCs) of source leaves where it activates FLOWERING LOCUS T (FT) expression. FT and/or FT mRNA may serve as the florigenic stimulus that controls the shoot apical meristem (SAM) to inflorescence meristem (IM) transition. Also, FT may exert control over floral induction through a kinase signaling cascade and/or a transcriptional event within the CCs that regulates entry into the phloem of an as yet unidentified molecule (X) (Lough and Lucas, 2006). As shown by Lin et al. (2007), FT protein, not the mRNA enters and moves long-distance through the phloem. Arrival of FT into specific target cells of the SAM allows an interaction with a bZIP protein, FD, that triggers IM formation and initiates organ development by transcriptional activation of APETALA1, a floral meristem identity gene.

14. A Phloem Cyclophilin Functions in Protein Folding

A special population of proteins that are produced in the companion cells subsequently are transported into the sieve tube system through the companion cell-sieve element PD. During this process, these non-cell-autonomous proteins appear to undergo partial unfolding. A mass spectroscopy approach was used to search for potential proteins that might function in the refolding of these phloem proteins. One such candidate, a cyclophilin, CYP1 was cloned and shown to have high peptidyl-prolyl cis-trans isomerase activity. Equivalent enzymatic activity was detected with phloem sap or purified recombinant (His) 6-tagged CYP1. Mass spectrometry analysis of proteolytic peptides, derived from a 22 kDa band in HPLC-fractionated phloem sap, immunolocalisation studies and Western analysis of proteins extracted from plant tissues/organs indicated that CYP1 is an abundant protein in the companion cell-sieve element complex. Microinjection experiments established that purified recombinant (His) 6-CYP1 can interact with PD to both induce an increase in SEL and mediate its own cell-to-cell trafficking. Collectively, these findings supported the hypothesis that this phloem CYP1 plays a role in the refolding of non-cell-autonomous proteins after their entry into the phloem translocation stream (Gottschalk et al. 2008).

15. Characterization of the First Phloem-Mobile Ribonucleoprotein Complex

RNA binding proteins (RBPs) are integral components of ribonucleoprotein (RNP) complexes and play a central role in RNA processing. In plants, some RBPs function in a non-cell-

autonomous manner. The angiosperm phloem translocation stream contains a unique population of RBPs, but little is known regarding the nature of the proteins and mRNA species that constitute phloem-mobile RNP complexes. In this project we identified and characterized a 50-kD pumpkin phloem RNA binding protein (RBP50) that is evolutionarily related to animal polypyrimidine tract binding proteins. In situ hybridization studies indicated a high level of RBP50 transcripts in companion cells, while immunolocalization experiments detected RBP50 in both companion cells and sieve elements. A comparison of the levels of RBP50 present in vascular bundles and phloem sap indicated that this protein is highly enriched in the phloem sap. Heterografting experiments confirmed that RBP50 is translocated from source to sink tissues. Collectively, these findings established that RBP50 functions as a non-cell-autonomous RBP. Protein overlay, coimmunoprecipitation, and cross-linking experiments identified the phloem proteins and mRNA species that constitute RBP50-based RNP complexes. Gel mobility-shift assays demonstrated that specificity, with respect to the bound mRNA, is established by the polypyrimidine tract binding motifs within such transcripts. A model was developed for RBP50-based RNP complexes within the pumpkin phloem translocation stream (Figure 8) (Ham et al. 2009).

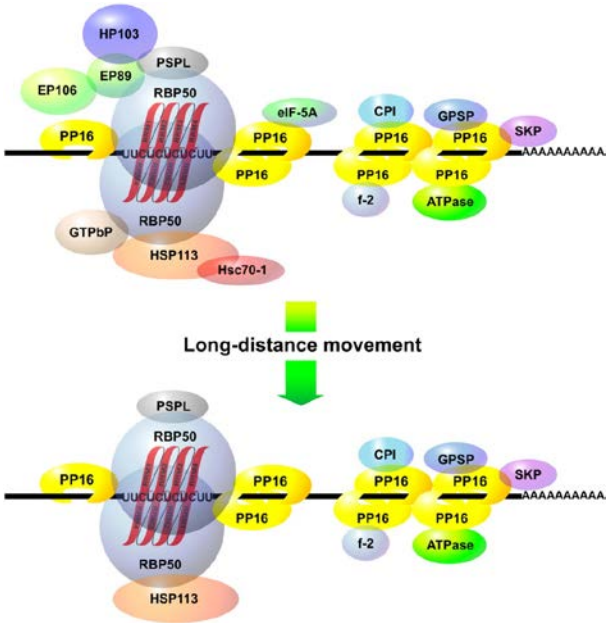

Figure 8. Schematic Illustration of a Phloem RBP50-Based Ribonucleoprotein Complex.

RBP50 binds to PTB motifs (UUCUCUCUCUU) present within a subclass of phloem-mobile, polyadenylated transcripts, and this interaction leads to the binding of additional RBP50 (shown as a homodimer). PP16-1/-2 interact with both the RBP50 and the target mRNA, thereby forming the core of the RNP complex. RBP50 interacts directly with a GTPbP–HSP113–Hsc70-1 complex that may chaperone the RNP complex to and through the companion cell–sieve element plasmodesmata. 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 PSPL are shown interacting with the RBP50. eIF-5A is a component of the RBP50 core and binds directly to PP16-1/-2. Regions outside the PTB motifs are bound by PP16-1/-2 along with five additional proteins: CPI, the Csf-2 related protein (Cmf-2), the 44-kD putative ATP binding protein (ATPase), the glutathione-regulated potassium-efflux system protein (GPSP), and the shikimate kinase precursor (SKP). The bottom image shows the composition of the phloem-mobile RBP50-based RNP complex based on co-IP results obtained using cucumber scion phloem sap.

16. Pumpkin Phloem Proteome Provides Insights into Angiosperm Sieve Tube Function

Increasing evidence suggested that proteins present in the angiosperm sieve tube system play an important role in the long distance signaling system of plants. A large scale proteomics approach was employed to analyze pumpkin phloem exudates in order to develop an understanding of the nature of these putatively non-cell-autonomous proteins. Phloem proteins were fractionated by fast protein liquid chromatography using both anion and cation exchange columns and then

either in-solution or in-gel digested following further separation by SDS-PAGE (Figure 9). A total of 345 LC-MS/MS datasets were analyzed using a combination of Mascot and X!Tandem against the NCBI non-redundant green plant database and an extensive pumpkin expressed sequence tag database. In this analysis, 1,209 different consensi were obtained of which 1,121 were annotated from GenBank™ and BLAST search analyses against three plant species, *Arabidopsis thaliana*, rice (*Oryza sativa*), and poplar (*Populus trichocarpa*).

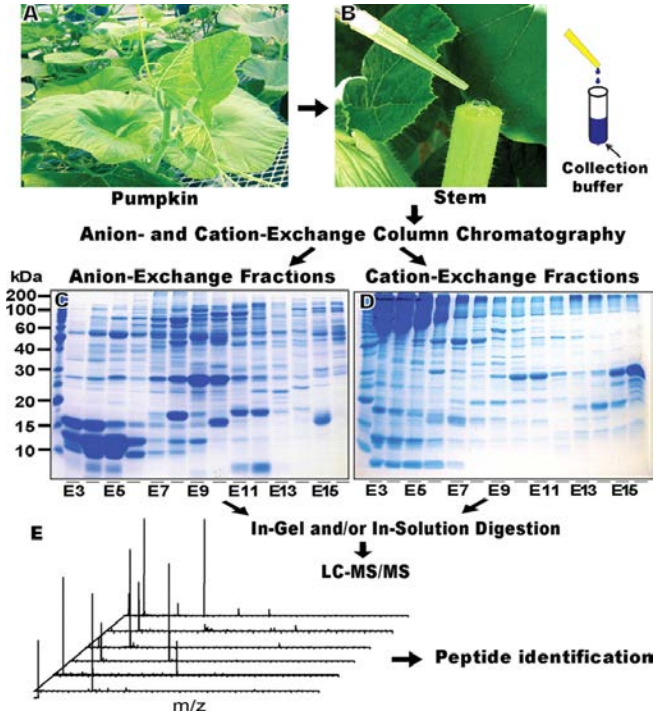

Figure 9. Flow diagram of the procedures used to collect and analyze the protein content of the pumpkin (C. maxima) phloem translocation stream. A, 8-week-old pumpkin plants were used as a source for angiosperm phloem sap. B, mature source leaves (petioles) or stems were excised from the plant, and phloem sap that exuded from the cut surface was collected and transferred into collection buffer. C and D, proteins contained in the pumpkin phloem sap were separated by anion and cation exchange chromatography, respectively. E, in-gel and/or in-solution digestion was used to prepare tryptic peptides that were then subjected to LC-MS/MS analysis. The resultant MS/MS spectra were searched against a proprietary cucurbit EST and the NCBI green plant databases.

Gene ontology (GO) enrichment analysis identified sets of phloem proteins that function in RNA binding, mRNA translation, ubiquitin-mediated proteolysis, and macromolecular and vesicle trafficking. These studies indicated that protein synthesis and turnover, processes that were previously thought to be absent in enucleate sieve elements, likely occur within the angiosperm phloem translocation stream. In addition, our GO studies identified a set of phloem proteins that are associated with the GO term “embryonic development ending in seed dormancy (Figure 10).

Of the 39 *Arabidopsis* genes corresponding to the GO term embryonic development ending in seed dormancy a number encoded enzymes involved in housekeeping functions, such as those that participate in protein turnover (e.g. FUS6, RPN12, SAE2, ASK2, and UBP14). However, other proteins, like TOPLESS-1 (TPL-1), TOPLESS-RELATED 2 (TPR2), and TPR4 are involved in the regulation of apical embryonic fate. Thus, their presence in the enucleate sieve tube system is most certainly intriguing and may implicate the phloem in control over plant embryonic and seed development. The presence of CELL DIVISION CONTROL 2 (CDC2), MULTICOPY SUPPRESSOR OF IRA1 (MSI1), and the transcription activator EMBRYO DEFECTIVE 1374 (EMB1374) adds further weight to the notion that the phloem translocation stream may deliver information macromolecules produced in the source region of the plant that influence the genetic programming of developing tissues and organs, such as seeds. The vascular cambium could also be a target for these regulatory proteins.

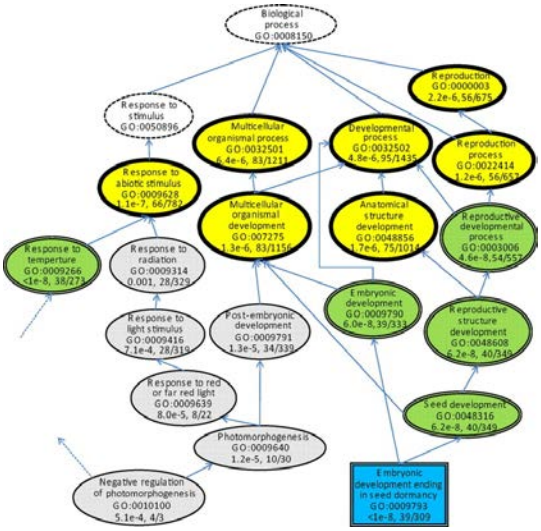

Figure 10. Gene ontology analysis of a pumpkin phloem proteome identified a set of proteins associated with embryonic development ending in seed dormancy. This analysis is presented in the form of a part of a hierarchical directed acyclic graph in the aspect of biological process. The thin, thick, and double lined ovals or box represent enriched GO terms at a FDR of 1e−2 to 1e−5 (gray), 1e−5 to 1e−8 (yellow), and less than 1e−8 (green), respectively. The double lined box indicates the lowest level GO term having a FDR of less than 1e−8; this specific GO term is hierarchically associated with the upper level GOs. Dotted ovals represent non-enriched GOs. Values in each GO represent the FDR and the number of genes in the test and reference groups, respectively. The GO term in blue was reported to be highly enriched in a large scale *Arabidopsis* proteomics study (Baerenfaller et al. 2008).

Finally, the universal significance of this phloem proteome was highlighted by conservation of the phloem proteome in species as diverse as monocots (rice), eudicots (Arabidopsis and pumpkin), and trees (poplar). This phloem proteome database now provides a framework for future studies on the role of individual proteins as integral components of the whole-plant communication system (Lin et al. 2009).

17. Impact to Producers

This project developed important new knowledge concerning the mechanisms utilized by plants to coordinate physiological and developmental processes at the whole-plant level. Our discovery that resource allocation of fixed carbon, produced in source leaves, is controlled by processes acting at the level of cell-to-cell communication offers a means to begin to bioengineer the delivery of fixed carbon to discrete sink tissues. The finding that signaling between the mesophyll and the phloem serves to regulate when and how much photosynthate is loaded into the phloem translocation stream provides further evidence that such control processes can be managed to enhance biomass production. The knowledge gained in terms of the role of the CC-SE PD in contributing to the operation of the phloem as an information superhighway should likewise allow academic and/or agbiotech companies to utilize this signaling pathway to engineer plants to have novel features essential for either biomass production for biofuels or photosynthate partitioning into precursors for biodiesel, well as being able to have optimal growth under non-ideal conditions.

Technology Transfer Efforts

A number of the findings from this project were submitted for development as patent applications. A patent application associated with the role of PD and viral movement proteins to orchestrate the allocation of photosynthate between shot and root tissues was filed. In addition, a patent application was filed regarding the role of PSRP1 and systemic long-distance signaling of small RNA. Finally, a patent was filed on the role of FT as the florigenic agent in the signaling cascade from the mature leaves to the vegetative apex to activate the vegetative-to-reproductive transition in photoperiodically-controlled plants.

Conclusions

It is clear that the plant vascular system acts as a central component in the life cycle of land plants, and in particular, for angiosperms that constitute the food and energy source for both animals and mankind. Implications of how the successes are relevant to technology development in the future. Based on this unchallengeable premise, and the major challenges facing the US in terms of both food and energy security, it is unfathomable as to why the DOE would divert it attention away from this critical area of research. The insights afforded by the research carried our during this DOE-funded project have offered entirely new avenues of research into ways to engineer novel controls over resource allocation of fixed carbon as a resource both for biofuels production and engineering plants suited for growth under non-optimal environmental conditions. Building on this knowledge would provide avenues to ensure a competitive, global advantage to US agbiotech industries.

Recommendations

A sustainable energy supply for the US, that is not reliant on foreign oil, will require diversification into a range of energy resources, including solar, thermal, wind and biofuels (algal and plant). To avoid competition with local and global food and fiber initiatives, plant-based energy systems will need to be developed for production on non-optimal (marginal) agricultural lands. Such systems will require coordination of research programs focused on enhancing plant cellulosic and oil production in conjunction with development of engineered plants that can be cultivated on marginal lands. It is inconceivable that this national objective can be achieved without utilizing the knowledge gained form studies on the role of the plant vascular system as a coordinating network for overall plant growth. Funding basic studies on the role of the phloem as an information superhighway will ensure a successful outcome to the DOE biofuels initiative.

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