



# Symplasmic protein and RNA traffic: regulatory points and regulatory factors

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Plasmodesmata and the phloem form a cytoplasmic network that permits direct cell–cell communication in plants. This network can mediate the trafficking of selective proteins and RNAs that may have important developmental functions. Recent work has provided evidence that protein and RNA traffic across specific interfaces of this network is regulated in a distinct manner. Progress has been made in identifying potential cellular factors that confer such regulation. These advances should promote further investigations into the mechanisms and functions of protein and RNA traffic using biochemical, cellular, genetic and molecular tools.

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## Abbreviations

|               |   |
|---------------|---|
| <b>CC</b>     | companion cell                                |
| <b>CmPP16</b> | 16-kDa <i>Cucurbita maxima</i> phloem protein |
| <b>CMV</b>    | <i>Cucumber mosaic virus</i>                  |
| <b>GFP</b>    | green fluorescent protein                     |
| <b>MP</b>     | movement protein                              |
| <b>NCAPP1</b> | NON-CELL-AUTONOMOUS PATHWAY PROTEIN1          |
| <b>PME</b>    | pectin methyl esterase                        |
| <b>PP2</b>    | phloem lectin2                                |
| <b>PSTVd</b>  | <i>Potato spindle tuber viroid</i>            |
| <b>SAM</b>    | shoot apical meristem                         |
| <b>SE</b>     | sieve element                                 |
| <b>TGB1</b>   | TRIPLE GENE BLOCK1                            |
| <b>TMV</b>    | <i>Tobacco mosaic virus</i>                   |

## Introduction

The cell differentiation that underlies development and morphogenesis results from differential gene expression. Cell–cell communication is essential for proper differentiation [1,2]. There is increasing evidence that specific proteins and RNAs play an important role in cell–cell communication in plants [3–5]. In one mechanism, a cell secretes a protein ligand that migrates within the cell walls. After reaching the surface of the target cell, the ligand interacts with a plasma-membrane-localized recep-

tor to regulate gene expression. This apoplastic pathway is best characterized by CLAVATA-mediated signaling, which regulates the stem cell population in the shoot apical meristem (SAM) of *Arabidopsis* [2].

Proteins and RNAs can also traffic symplasmically via plasmodesmata and the phloem to regulate gene expression in neighboring or distant cells. Readers are referred to recent excellent reviews (e.g. [3–9]) for comprehensive information. In this review, we focus on recent studies that have addressed the mechanisms that regulate symplasmic protein and RNA traffic. We emphasize the integration of specific cellular and molecular findings to derive an understanding of traffic regulation at the whole-plant level.

## Symplasmic protein and RNA traffic: versatile roles in a plant's life

Several transcription factors that play important roles in plant development move intercellularly [10–13]. Numerous RNAs are likely transported in the phloem in pumpkin [14]. Although the precise function of the traffic of these transcription factors and RNAs remains to be determined, two elegant studies have provided experimental evidence that the trafficking of specific proteins and RNAs is crucial for plant function. The *Arabidopsis* transcription factor SHORT-ROOT is produced in the stele of root, and its regulated trafficking into an adjacent cell layer is necessary for the proper development of the endodermis [15]. A tomato homeobox fusion transcript can move long distances through the phloem from a rootstock into the SAM of a grafted scion to alter leaf developmental patterns [16]. As yet unidentified RNAs can traffic through the phloem to induce systemic RNA silencing in a whole plant (reviewed in [17]).

It is almost certain that many proteins and RNAs traffic symplasmically over short or long distances to regulate fundamental plant processes. Novel experimental approaches need to be developed to allow the high-throughput identification of such proteins and RNAs for subsequent functional studies.

## Communication between symplasmically isolated cells: macromolecules breezing through the 'closed' doors

A young plant embryo constitutes a symplasmic continuum, as demonstrated by the presence of plasmodesmata [18,19] and the free flow of fluorescent dyes [20] among all cells. During development, however, groups of cells become symplasmically isolated from their

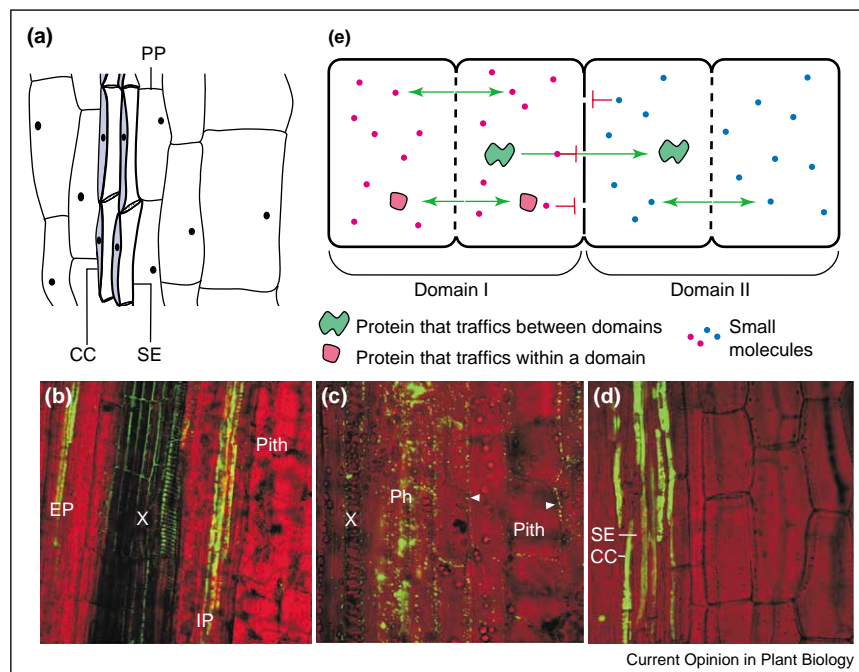
neighbors, as revealed by the decrease or complete blockage of dye-/electrical-coupling and by reduced plasmodesmal frequencies. This isolation leads to the formation of various symplasmic domains or fields within a plant [21–24]. Symplasmic isolation may be permanent or transient. It is closely correlated with specific developmental or cellular processes, such as guard cell maturation [25], meristem function [24], flowering [26,27], elongation of cotton fibers [28] and synchronization of mitosis among cultured cells [29].

Symplasmic isolation may create a condition that allows a group of cells to pursue its unique developmental pathway [30–32]. On the other hand, communication is vital for cells to coordinate differentiation to form a functional plant body. How symplasmically isolated cells communicate with one another is unknown. They could communicate apoplastically via secreted ligands or symplasmically through the trafficking of selective molecules. To test the latter possibility, Itaya *et al.* [33] investigated the potential for protein traffic between the symplasmically isolated sieve element–companion cell (SE–CC) complex and neighboring cells in the phloem tissue. The *Cucumber mosaic virus* (CMV) 3a movement protein (MP) fused with

the green fluorescent protein (GFP) was transgenically expressed in the CC of tobacco. In the absence of viral replication, the fusion protein trafficked from CC to SE, and further from the symplasmically-isolated SE–CC complex into neighboring cells (Figure 1a–d).

Evidently, dye-coupling and plasmodesmal frequency studies offer an important but only partial view of the transport capacity of plasmodesmata between symplasmically isolated cells. Plants have evolved powerful mechanisms that restrict the diffusion of small molecules while facilitating the trafficking of selective macromolecules through the same plasmodesmata between symplasmic domains (Figure 1e). Such macromolecular traffic may not be unique to viral MPs. Rather, a cellular machinery exists to traffic selective endogenous macromolecules between cells so that they can perform important functions [33]. The identification and functional characterization of the endogenous molecules that traffic between symplasmic domains should provide a critical test of this hypothesis. To elucidate how a plasmodesma can be closed for the diffusion of small molecules and at the same time open for the trafficking of macromolecules is an exciting challenge for biologists.

Figure 1



Communication between symplasmic domains. **(a)** Diagrammatic longitudinal view of the cell types in a phloem bundle in a tobacco stem. CC, companion cell; PP, phloem parenchyma cell; SE, sieve element. **(b)** Confocal microscopic image of a mature tobacco stem in longitudinal view. Transported fluorescein (334 Da; green) is confined in the SE–CC complex of the external (EP) and internal phloem (IP). The cell walls of xylem (X) show green autofluorescence. **(c)** A CMV 3a MP::GFP fusion protein that is expressed in the CC of the stems of transgenic tobacco traffics out of the symplasmically isolated SE–CC complex. Arrowheads indicate the localization of the fusion protein to plasmodesmata. Ph, Phloem. **(d)** Dimeric GFP is confined in the CCs of transgenic tobacco. **(e)** Symplasmic domains and the regulated traffic of molecules between them. Green arrows indicate traffic. Red T-bars indicate no traffic. (b–d) are reproduced from [33] with permission.

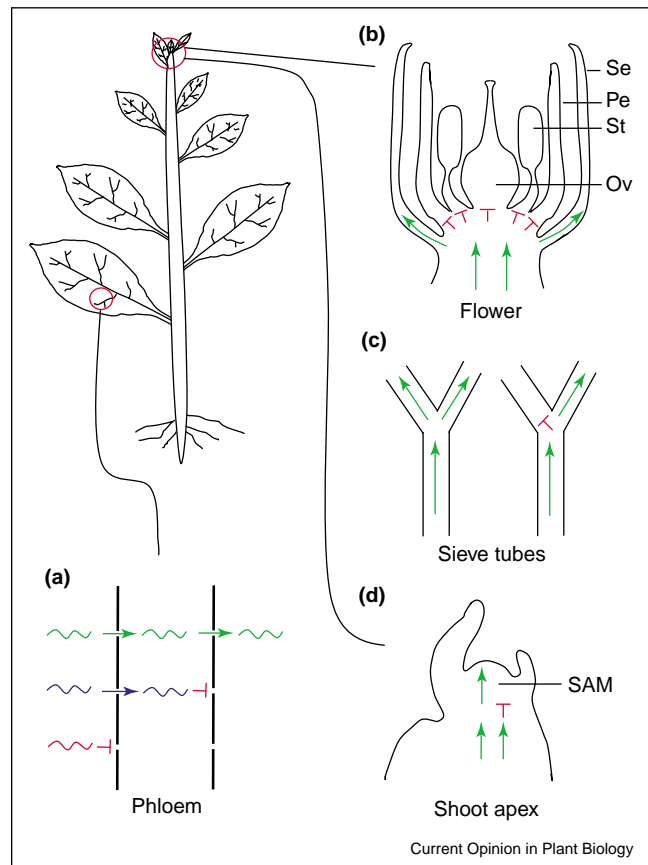
## RNA systemic traffic: passing through multiple checkpoints

RNAs with diverse functions traffic systemically. How an RNA enters, moves within and exits the phloem to reach its final destination is poorly understood. Nevertheless, recent studies using plant pathogens as model systems have begun to unveil the inner-workings of the traffic control system.

Analyses of the systemic infection of tobacco by *Potato spindle tuber viroid* (PSTVd), a small non-coding and self-replicating RNA, suggest that different mechanisms regulate the phloem entry and exit of an RNA. As a viroid RNA does not encode proteins, its trafficking pattern should provide direct insights into the cellular mechanisms that control RNA trafficking. PSTVd variants have been identified that can enter and move long distances through the phloem but cannot exit the phloem to invade surrounding cells in systemic leaves of tobacco [34<sup>\*</sup>]. This suggests that phloem entry and exit require different mechanisms (Figure 2a). Furthermore, PSTVd movement into sepals but not into the other floral organs of infected tomato and *Nicotiana benthamiana* suggests that a phloem-based mechanism transports PSTVd into selected floral organs (Figure 2b; [34<sup>\*</sup>,35]). If this mechanism operates within sieve tubes, we need to consider the possibility that macromolecular movement within sieve tubes does not simply mimic the passive mass-flow of photoassimilates and other small molecules. Rather, macromolecules would be sorted within sieve tubes for delivery to their respective destination organs (Figure 2c; [34<sup>\*</sup>,35]). This is an important issue in phloem biology that warrants further analysis.

The SAM produces all of the above-ground plant organs [36]. Given its vital function, the SAM is likely safeguarded against the penetration of detrimental elements. For instance, most viruses, viroids and RNA-silencing signals are excluded from the SAM by unknown mechanisms. Foster *et al.* [37<sup>\*\*</sup>] reported convincing evidence that a surveillance mechanism operates to control selective RNA trafficking into the SAM. Transgenic *N. benthamiana* plants that overexpressed the TRIPLE GENE BLOCK1 (TGB1) protein encoded by the *White clover mosaic virus* displayed abnormal developmental patterns. Such plants allowed the entry of this virus and RNA-silencing signals into the shoot apex. Significantly, the use of virus- or *Agrobacterium*-mediated RNA silencing to repress the expression of the *TGB1* transgene restored the normal development of the transgenic plants. These findings support the hypothesis that an RNA surveillance mechanism prevents viruses and RNA-silencing signals from entering the SAM. This mechanism may also control the selective entry of other RNAs into the SAM (Figure 2d; [37<sup>\*\*</sup>]). Exciting future studies might determine which endogenous RNAs traffic into the SAM to regulate its functions, whether the expression of TGB1

Figure 2



Checkpoints that control systemic RNA traffic. (a) Entry into and exit from the phloem are distinct checkpoints. Owing to distinct molecular interactions between RNA motifs and cellular factors, some RNAs may enter and exit the phloem (green), some may enter but not exit the phloem (blue) and others may not enter the phloem at all (red). (b) The traffic of PSTVd RNA into sepals (Se; green arrows) but not into other floral organs (red T-bars) implies the existence of a phloem-based mechanism that sorts RNAs for delivery into selective organs [34<sup>\*</sup>,35]. Ov, ovary; Pe, petal; St, stamen. (c) Possible mechanisms for the flow of different types of molecule within sieve tubes. On the left, small molecules move by passive mass-flow within all sieve tubes, driven solely by pressure gradient. On the right, macromolecules within a sieve tube are sorted by molecular recognition at a junction between sieve tubes so that they may move further into one branch (green arrows) but not into another (red T-bars). (d) A surveillance mechanism controls the selective trafficking of RNAs into the SAM [37<sup>\*\*</sup>]. Similar mechanisms may control systemic protein traffic.

in the shoot apex alters the trafficking of certain endogenous RNAs, and whether such altered traffic is directly responsible for the abnormal development of plant organs. Equally exciting will be the determination of where the surveillance mechanism operates in the shoot apex: traffic within the phloem, exit from the phloem, or post-phloem cell–cell traffic.

Altogether, these studies provide compelling experimental evidence to support a model in which systemic

trafficking of an RNA is regulated at multiple checkpoints including phloem entry, transport and exit, and targeting to specific organs (Figure 2). Thus, different cellular factors and RNA motifs may interact at different checkpoints to promote or inhibit trafficking of the same RNA. Within the framework of this model, it will be interesting to determine at which checkpoint a specific mRNA is selected for trafficking into the shoot apex or elsewhere [14]. Furthermore, it will be most desirable to elucidate the potential RNA motifs and cellular factors that interact at each checkpoint.

### Molecular basis of traffic regulation: emerging traffic motifs and cellular mediators

The regulation of the selective intercellular trafficking of a protein or RNA conceivably involves interaction between a motif that resides in the protein or RNA and cognate cellular factors. The identification of such motifs and cellular factors has been a challenge. Point mutation and deletion have identified amino acid residues or sequences that are crucial for the trafficking of several proteins (reviewed in [4,38]). However, a motif that is both necessary and sufficient to mediate the trafficking of a protein has not been identified. Using mutational analysis, Aoki *et al.* [39•] showed that a subclass of Hsp70 chaperones from pumpkin contain a motif that mediates their intercellular trafficking. Furthermore, fusion of this motif to a human Hsp70 chaperone potentiates the latter to move from cell to cell. This motif does not, however, mediate the trafficking of the fused GFP. It appears, therefore, that other domains of the chaperone proteins are also essential for their trafficking. Several possibilities may explain the difficulty in delineating a protein traffic motif that can direct the intercellular trafficking of any heterologous reporter protein. First, the three-dimensional structure of the protein might function as the motif; and second, more than one discrete motif, in the form of peptide sequences or three-dimensional structures, might interact directly with more than one cellular factor. Either possible explanation is extraordinary in comparison with protein traffic into intracellular organelles, which is mediated by simple peptide sequences [40–42]. The most intriguing question is why the motifs for intercellular trafficking need to be so complex and how they have evolved so distinctly.

Initial clues about cellular factors that facilitate intercellular protein trafficking came from studies on the *Tobacco mosaic virus* (TMV) MP. This protein interacts with microtubules [43,44] and actin filaments [43]. This association has been suggested to play a role in the MP-mediated intracellular trafficking of TMV RNA to the plasmodesmata [43–46]. A recent study showed, however, that disruption of microtubules by pharmaceutical drugs or RNA silencing does not inhibit the trafficking of TMV MP or RNA through plasmodesmata [47••]. Furthermore,

a mutant version of the TMV MP that does not associate with microtubules still potentiates the cell–cell movement of TMV [47••]. Thus, the role of microtubules in intercellular viral protein and RNA trafficking remains equivocal. TMV MP also interacts with pectin methyl esterase (PME; [48,49]), which appears to be important for viral movement [49]. PME also interacts with the MPs of *Turnip vein clearing virus* and *Cauliflower mosaic virus* (CaMV) but not with the maize transcription factor KNOTTED1 (KN1), which can traffic intercellularly [10,13], suggesting a certain level of specificity in the interactions [49]. The mechanism of PME's involvement in TMV movement remains unclear.

Very recently, Lee *et al.* [50••] made a major advance in dissecting putative components of the cellular machinery that regulates protein trafficking. They used CmPP16, a protein previously isolated from phloem exudates of pumpkin as a trafficking protein [51], as a bait to probe the plasmodesmal-enriched cell wall protein fraction from tobacco BY2 (Bright Yellow2) cells. This led to the isolation of a protein, termed *Nicotiana tabacum* NON-CELL-AUTONOMOUS PATHWAY PROTEIN1 (NtNCAPP1), that interacts with CmPP16. Microinjection experiments showed that the cell–cell trafficking of CmPP16 and TMV MP, but not of CMV 3a MP or KN1, was blocked when a dominant mutant of NtNCAPP1 (with its amino terminus deleted) was co-injected. The same selective inhibition of protein trafficking was observed in transgenic tobacco plants that expressed the mutant NtNCAPP1 or antisense NtNCAPP1 cDNA. These results suggest that NtNCAPP1 plays a role in mediating the cell–cell trafficking of selective proteins. The altered developmental patterns of the NtNCAPP1 transgenic plants suggest the possibility that NtNCAPP1-mediated trafficking of certain endogenous proteins is crucial for development. These transgenic plants could be useful in elucidating the specific mechanism of NtNCAPP1-mediated trafficking and the precise role of this trafficking in development.

Two proteins may play a role in viroid RNA trafficking. The phloem lectin2 (PP2) binds *Hop stunt viroid* RNA and other RNAs [52,53]. PP2 can move long distances through the phloem [54]. Therefore, HSVd and other RNAs may piggyback on PP2 to move within the sieve tubes. VirP1 from tomato binds to a domain of PSTVd that may be critical for phloem trafficking [55]. Whether PP2 and VirP1 have a genuine role in RNA trafficking remains to be tested.

It is important to note that cell- and developmental stage-specific factors are involved in the regulation of signal-directed protein trafficking [33••,56]. Thus, the specific cell or tissue type in which a regulator is localized should be identified so that the function of the regulator can be understood within the organismal context.

### Molecular structure of the plasmodesma: the dark tunnel being lit up by a glowing protein

At the heart of regulated protein and RNA trafficking is the molecular structure of plasmodesmata. Efforts to reveal the secret of this structure face unique technical challenges because it is embedded in the cell walls. Immunolabeling has identified several putative plasmodesmal components, including actin, tubulin, myosin, centrin and calreticulin (see [9] for summary). Whether these are true components of plasmodesmata awaits biochemical and functional testing. Kim *et al.* [57\*] have identified *Arabidopsis* mutants that have an increased plasmodesmal size exclusion limit in the developing embryo. It will be interesting to see whether the genes that are affected in these mutants encode components of the plasmodesmata or regulatory elements that control the pore size.

In a very recent and exciting development, Escobar *et al.* [58\*\*] used a TMV vector to express libraries of random, partial cDNAs fused to the *GFP* gene in infected *N. benthamiana*. Confocal microscopic examination of the infection sites revealed the subcellular localization of specific fusion proteins. This screening isolated 12 proteins that are localized to plasmodesmata. This approach could lead to further large-scale identification of plasmodesma-localized proteins. Further studies will reveal whether a particular protein is a structural component of plasmodesmata or a trafficking protein in transit through the plasmodesmata.

### Conclusions and perspectives

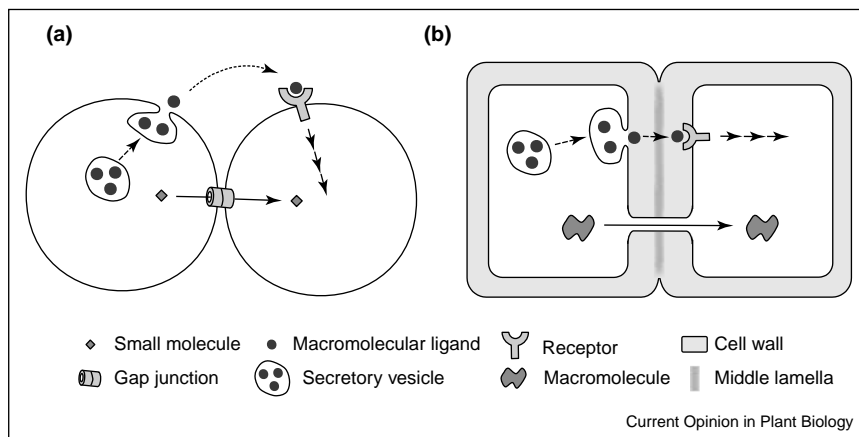
Future research on protein and RNA trafficking needs to focus on mechanisms and functions. This work will

include the extensive identification of proteins and RNAs that traffic symplasmically, the elucidation of protein or RNA motifs that mediate the traffic, the identification of cellular factors (including plasmodesmal components) that mediate the trafficking, and the determination of the role played by such traffic in plant growth, development and responses to the environment.

Meanwhile, it is important to place cell communication via symplasmic protein and RNA traffic within the 'big picture' of cell–cell communication (Figure 3). In animals, direct cell–cell communication via gap junctions is restricted to the passive diffusion of small molecules [59]. Protein signals are secreted into extracellular space. Such signals then act on neighboring cells or are transported to target distant cells through the blood stream. They regulate target cells upon interaction with receptors that are localized in the plasma membrane of those cells [59]. The peptide–ligand–receptor signaling pathway discovered in plants mimics this mode of communication between animal cells. On the other hand, communication via direct symplasmic protein and RNA trafficking is unique to plants.

Many questions surface when we think about the big picture of cell–cell communication. Why do plants communicate by both apoplastic and symplasmic traffic? What are the evolutionary or developmental advantages gained by using both mechanisms? Do these pathways operate between two cells simultaneously or sequentially during development? Does a common switchboard exist to coordinate these pathways? Do some cells use one mechanism more than the other? Solving these pieces of the big puzzle will be a challenging and yet most rewarding step

Figure 3



Cell–cell communication based on macromolecular traffic in animals and plants. (a) In animal cells, signaling proteins that are secreted by a cell interact with plasma-membrane-localized receptors of a target cell to regulate cellular processes in the target cell. Direct cell–cell transport is limited to the diffusion of small molecules (smaller than 1.5 kDa) through gap junctions. (b) In plant cells, cell–cell communication can occur via the apoplastic pathway. In the apoplastic pathway, signaling proteins secreted by a cell interact with plasma-membrane-localized receptors in a target cell to regulate cellular processes in the target cell. This is essentially similar to what happens in animals. In the symplasmic pathway, which is unique to plants, signaling proteins (and RNAs) traffic through plasmodesmata to achieve direct cell–cell communication.

in our adventure into the inner-workings of the entire intercellular communication network, which has probably played a major role in shaping the course of plant evolution and certainly in dictating the developmental pattern of an individual plant.

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