Long distance transport and movement of RNA through the phloem

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Abstract
Cell-to-cell communication is essential for plant development and adaptation to environmental changes. As a strategy for efficient intercellular communication, plants have evolved a plant-specific symplasmic network connected via plasmodesmata that allows a locally restricted information exchange from cell to cell. A rapid information transfer over long distances is enabled via the phloem transport tubes that pervade the complete plant and thus connect even the most distant organs. While communication by small molecules like metabolites and phytohormones is comparably well studied, the intercellular trafficking of proteins and RNAs has only recently emerged as a novel mechanism of cell-to-cell and long-distance signalling in plants. In particular the non-cell-autonomous and systemic transport pathway for specific RNAs seems to play a key role in the coordination of important physiological processes, including virus defence, gene silencing, regulation of development, and nutrient allocation. This review is a summary of the current knowledge on RNAs contained in the phloem long-distance transport system, their transport mechanism, and their potential functions.

Key words: Macromolecules, phloem transport, plasmodesmata, post-transcriptional gene silencing, RNA signalling, RNA silencing, systemic signalling, virus transport.

The phloem transmits information over long distances
Plants viability requires a close and balanced co-ordination between environmental conditions, growth and development, and pathogen defence. Information exchange between cells is an important prerequisite for such co-ordination, and all multicellular organisms have evolved different strategies to allow an efficient intercellular exchange of information molecules. In plants, plasmodesmata (PD) build a cytoplasmic continuum throughout the plant body (Ruiz-Medrano et al., 2004). PD allow a selective and directed transport of molecules over short distances. In addition, higher plants contain a specialized tube system for long-distance transport of nutrients, water, and signalling molecules, consisting of xylem and phloem. While the xylem tubes are attributed to the apoplast, the phloem sieve elements (SEs) are interconnected with the cytoplasmic continuum by specialized secondary PD, the pore plasmodesmal units (PPUs). PPUs have been shown to allow the passage of molecules larger than 60 kDa (Stadler et al., 2005), and therefore should also allow the passage of macromolecules like proteins or smaller RNAs.

The phloem interconnects even the most distant parts of a plant and thus provides an ideal route not only for the allocation of photoassimilates from source to sink organs,
but it also enables a fast and directed systemic information transfer. To co-ordinate development, defence, and nutrient allocation, plants have evolved a complex battery of signalling molecules that can be translocated via the phloem. For example, the elusive ‘florigen’ has long been known as a phloem-mobile, essential trigger for flower induction of unknown chemical nature (Chailakhyan, 1936). In addition, a variety of phloem-mobile molecules are potentially involved in defence-related signalling leading to responses like systemic-acquired resistance (van Bel and Gaupels, 2004). Finally, gene regulation by RNA silencing can spread systemically what is most likely mediated via the phloem (Voinnet and Baulcombe, 1997).

In the last decade it has been established that not only small molecules like phytohormones or metabolites, but also macromolecules like proteins and RNAs, can be considered as potential long-distance signalling substances (Ruiz-Medrano et al., 1999). The apparent non-cell-autonomous nature of some RNAs is receiving particular attention at present, since RNA molecules were traditionally believed to act in the same cell in which they are synthesized (Ueki and Citovsky, 2001b). However, accumulating evidence indicates that RNA can move locally between cells, potentially as a regulator of gene expression. The homeodomain protein KNOTTED1, for example, has been shown to facilitate the transport of its own messenger RNA (mRNA) from cell-to-cell in the meristem in a locally restricted manner (Lucas et al., 1995). A more recent study demonstrated that the KNOX homeodomain of KNOTTED1 is necessary and sufficient to confer trafficking ability to the cell-autonomous protein GLABROUS1 and that KNOTTED1 sense RNA is translated after its translocation (Kim et al., 2005). In addition, it is now known that specific RNAs can not only move locally, but even enter the long-distance transport route to spread systemically between different organs (Chen and Kim, 2006). Recent studies suggest that RNA transport through the phloem is no rare phenomenon, but that it is a rather common process in higher plants (Ruiz-Medrano et al., 1999; Lucas et al., 2001; Yoo et al., 2004).

Types and functions of phloem RNAs

Although RNA had been found in phloem exudates of pumpkin (Cucurbita maxima) more than 30 years ago (Kollmann et al., 1970; Ziegler, 1975), it was long regarded to be contamination from the sample collection procedure rather than a true component of the transport stream. Since then it has become accepted that RNAs in the phloem are no artefact but are an authentic constituent of the transport stream. It is known that three major types of RNAs can be systemically transported: RNA genomes of viruses, endogenous cellular mRNAs, and small non-coding RNAs (Voinnet and Baulcombe, 1997; Citovsky and Zambryski, 2000; Mlotshwa et al., 2002). The phloem tubes seem to be an ideal transport route for RNA given that, unlike other tissues, phloem sap contains no detectable RNase activity (Sasaki et al., 1998; Doering-Saad et al., 2002).

Viral RNAs

Long-distance movement of RNA was first observed during the spread of viral infections. The majority of plant viruses are single-stranded RNA viruses that are replicated via double-stranded RNA intermediates produced by an RNA-dependent RNA polymerase. These unusual double-stranded RNAs allow plants to recognize viral RNA as being foreign (Eckardt, 2002). The movement of viruses is thought to occur in two phases: local cell-to-cell and systemic movement through the phloem (Santa Cruz, 1999). After infection, viruses normally spread locally from cell to cell through PD until they reach the vascular tissue. Viruses contain special movement proteins (MPs) that have the ability to bind and unfold single-stranded RNAs (Citovsky et al., 1990; Citovsky and Zambryski, 1991), and facilitate the intercellular translocation of viral nucleic acids by building protein–RNA transport complexes (Lucas and Gilbertson, 1994; Ghoshroy et al., 1997; Lucas, 2006). Additionally, MPs have the ability to increase the SEL of PD up to 10-fold in mature leaves (Ding et al., 1992), allowing the movement of MP–RNA complexes between host cells (Wolf et al., 1989). Another important factor for virus movement is the coat protein (CP) and viruses can be categorized into three groups depending on whether the CP is not required (type I) or required (type II) for movement, or virus particles are translocated from cell to cell (type III) (Scholthof, 2005).

Interestingly, some viral mutants exist that are inhibited in systemic but not in local spread (Ding et al., 1996), indicating that the mechanism of phloem import is different from that of cell-to-cell movement. Whether MPs are also involved in the phloem-dependent movement is currently unknown (Sareila et al., 2004). The MP of tobacco mosaic virus, for example, is essential for cell-to-cell movement but not for vascular transport of the virus, while replicase, functional CP, and the origin-of-assembly are critical for phloem long-distance transport of tobacco mosaic virus in a host-dependent manner (Gera et al., 1995; Derrick et al., 1997). While CPs are dispensable for cell-to-cell trafficking in some virus types, CPs seem to constitute a factor widely required for virus long-distance translocation (Callaway et al., 2004; Scholthof, 2005). However, virus RNAs from specific virus families also seem to be able to traffic from cell-to-cell and even over long distances without the presence of MPs or CPs (Gopinath and Kao, 2007).

Also endogenous phloem proteins, namely different phloem lectins (CmPP2, CsPP2, CmmLeC17) and
the phloem protein CmPP16, have been found to interact with viral RNAs as well as endogenous mRNAs (Gomez and Pallas, 2004; Gomez et al., 2005) and could thus be involved in virus import or translocation within the phloem stream.

Interestingly, cadmium at non-toxic concentrations specifically blocks systemic but not local movement of tobanoviruses, suggesting that plant factors, impaired by cadmium, are involved in the control of systemic virus movement (Citovsky et al., 1998; Ghoshroy et al., 1998). Consistently, a cadmium-induced glycine-rich protein (cdiGRP) that can negatively regulate viral systemic spread was discovered in the cell walls of vascular tissue of cadmium-treated tobacco plants. Over-expression of cdiGRP inhibited viral movement in transgenic plants, while its suppression allowed virus transport even in the presence of cadmium (Ueki and Citovsky, 2002). This effect is probably caused by the ability of cdiGRP to induce callose deposits in phloem tissue. Such callose deposits are thought to restrict intercellular transport by reducing the SEL of PD (Iglesias and Meins, 2000) and thus could hinder virus entry into the phloem.

Once inside SEs, evidence from electron microscopic studies suggests that several viruses travel as virions (Blackman et al., 1998). However, they probably do not cross the CC–SE boundary as virus particles but as ribonucleoprotein complexes (Santa Cruz, 1999). Viruses are then passively transported from source to sink tissues along with the photoassimilates at a rate similar to the rate of nutrient movement (Schneider, 1965; Santa Cruz, 1999). Vascular movement of most viruses is thus dependent on the source–sink relationship and seems to be restricted only by the as yet unknown mechanisms by which viruses are loaded and unloaded from the phloem transport system (Silva et al., 2002). However, results from viroid trafficking suggest a potential specific component of phloem virus transport, as the potato spindle tuber viroid was selectively transported into sepals but not into other floral organs in Nicotiana benthamiana and tomato plants, although all floral organs were strong sinks for photoassimilates (Zhu et al., 2001, 2002).

**Cellular mRNAs**

Among the systemically transported RNAs, the presence of endogenous plant mRNAs in the phloem was most surprising, because functional SEs do not contain nuclei or ribosomes that could synthesize proteins from such templates. The first convincing evidence for an endogenous plant mRNA inside SEs came from a study that localized the mRNA of sucrose transporter 1 (SUT1) in Solanaceae in SEs and the PD connecting SEs to CCs (Kühn et al., 1997). Subsequently, mRNAs from thioredoxin h, cystatin, and actin have been detected by RT-PCR in rice phloem samples obtained with the minimal-invasive aphid stylet technique (Sasaki et al., 1998) and, more recently, thioredoxin h mRNA was found in phloem exudate of Brassica napus (Giavalisco et al., 2006) and several other transcripts, including SUT1, aquaporin, and a proton ATPase, could be amplified from phloem sap of barley (Doering-Saad et al., 2002). Other, more comprehensive, approaches have resulted in several functionally unrelated mRNAs from Cucurbita maxima exudate (Ruiz-Medrano et al., 1999, 2007) and a phloem-enriched library from Ricinus (Doering-Saad et al., 2006).

Since mature phloem SEs are not equipped for transcription and translation, it is likely that endogenous mRNAs function as systemic long-distance signals that can influence gene expression in the target tissues and can thus control diverse processes in plant development and morphogenesis (Ueki and Citovsky, 2001b) (see list in Table 1). Accordingly, phloem transport of a CmNACP mRNA from pumpkin could be directly demonstrated by heterograft experiments between pumpkin and cucumber plants, in which CmNACP transcripts could be detected in cucumber scion phloem and apical tissues (Ruiz-Medrano et al., 1999). Also by grafting studies, Kim et al. (2001) could show that the transcript of a KNOTTED1-like homeobox gene can cross graft junctions and induce phenotypic changes in the scions in tomato, indicating a functional relevance of mRNA long-distance movement. Another example comes from potato, where over-expression of a BEL1-like transcription factor led to a marked increase of tubers per plant and this phenomenon was graft-transmissible (Banerjee et al., 2006).

The same was observed for another gene, GIBBERELLIC ACID INSENSITIVE (GAI), in Cucurbita maxima and Arabidopsis gain-of-function mutants. The transcripts of GAI could move over long distances and cross graft unions. This long-distance translocation led to a change of the phenotype of scion leaves in grafting experiments (Haywood et al., 2005). The trafficking of GAI RNA seemed to be selective, since a control enhanced GFP (EGFP) RNA could not enter the SEs from their expression site in CCs and could thus not enter the long-distance trafficking pathway. Interestingly, sink strength seems not to be the only influence on transport of GAI as transgenically expressed pumpkin and Arabidopsis GAI RNAs could traffic in non-transgenic scion shoot apex and floral organs but were restricted from developing fruits, pedicles, and peduncles (Haywood et al., 2005), suggesting that phloem transport of mRNAs might not be regulated by simple diffusion but that the phloem has a mechanism to selectively deliver macromolecules to specific plant organs (Ding et al., 2005).

**Small, non-coding RNAs**

Small non-coding RNAs have recently emerged as important transcriptional and post-transcriptional regulators of
gene expression. Interestingly, plants seem to have developed different, partially overlapping small RNA biosynthesis pathways resulting in two major classes of small regulatory RNAs, short interfering and micro RNAs (siRNAs and miRNAs, respectively), that can be distinguished by their way of biogenesis and their mode of function.

siRNAs mediate a process called post-transcriptional gene silencing (PTGS), an innate plant defence mechanism that is a widespread defence mechanism against the activity of transposable elements and viruses (Waterhouse et al., 2001). Early work using grafting experiments on transgenic plants showed that a signal from silenced rootstocks is transmitted to non-silenced scions expressing the respective transgene, leading to PTGS in scions (Palauqui et al., 1997). The observed silencing signal was always sequence specific. Since the first discovery of gene silencing, RNAs were believed to be involved in the transmission of the trigger for silencing (Hamilton and Baulcombe, 1999; Lucas et al., 2001; Tournier et al., 2006). Aberrant RNAs or small RNA molecules were proposed as potential candidates for the mobile signal (Mlothsaw et al., 2002). Recently, siRNA originating from a transgene or a virus infection could be detected in the phloem of silenced but not in unsilenced plants, suggesting that siRNAs could be the signalling molecules themselves (Yoo et al., 2004). Meanwhile, it is known that siRNAs can spread from their site of production, probably in the form of 21 nt RNAs. Larger siRNAs (24–26 nt), on the other hand, are supposed to be involved in propagating the systemic signal (Himber et al., 2003).

In accordance with virus transport, non-toxic levels of cadmium also lead to an uncoupling of local and systemic spread of PTGS (Ueki and Citovsky, 2001a). This indicates that long-distance transport of PTGS signals and viruses relies on common mechanisms.

Once inside the phloem, the silencing signal is transported from source to sink, following the direction of phloem flow. Tournier et al. (2006) demonstrated that systemic spread of silencing could be altered by manipulating the source–sink relationships in Nicotiana benthamiana plants. However, the spread of the silencing signal could be further regulated by spatially restricted phloem connections, as silencing can obviously be directed to single plant parts while the bulk phloem stream to these sites is disconnected (Tournier et al., 2006). Concerning the transport form of siRNAs, recent results indicate that, although sense and antisense strands of siRNAs occur in phloem sap at similar levels, no double-stranded siRNA molecules are present (Yoo et al., 2004). This suggests that siRNAs are transported through the phloem as single strands.

The other small modulatory RNA species found in the phloem are micro RNAs (miRNAs). miRNAs are endogenous 21–24 nt long molecules that are universal negative regulators of gene expression in eukaryotes. They exert their function by targeting mRNAs for degradation or by repressing their translation. The regulation of gene

### Table 1. List of endogenous mRNAs and miRNAs found in phloem samples in alphabetical order

<table>
<thead>
<tr>
<th>RNA</th>
<th>Function</th>
<th>Plant species</th>
<th>Transport demonstrated</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>Cytoskeleton</td>
<td>Oryza sativa</td>
<td>No</td>
<td>Sasaki et al., 1998</td>
</tr>
<tr>
<td>Aquaporin</td>
<td>Water transport</td>
<td>Hordeum vulgare</td>
<td>No</td>
<td>Doering-Saad et al., 2002</td>
</tr>
<tr>
<td>BEL-1</td>
<td>Tuber development</td>
<td>Solanum tuberosum</td>
<td>Yes</td>
<td>Banerjee et al., 2006</td>
</tr>
<tr>
<td>CmCYCLINP</td>
<td>Cell cycle</td>
<td>Cucurbita maxima</td>
<td>No</td>
<td>Ruiz-Medrano et al., 1999</td>
</tr>
<tr>
<td>CmAIP</td>
<td>Leaf development</td>
<td>Cucurbita maxima</td>
<td>Yes</td>
<td>Haywood et al., 2005</td>
</tr>
<tr>
<td>CmAACP</td>
<td>Meristem maintenance</td>
<td>Cucurbita maxima</td>
<td>Yes</td>
<td>Ruiz-Medrano et al., 1999</td>
</tr>
<tr>
<td>CmPP16</td>
<td>Regulation of glycolysis</td>
<td>Cucurbita maxima</td>
<td>No</td>
<td>Ruiz-Medrano et al., 1999</td>
</tr>
<tr>
<td>CmPP16</td>
<td>RNA transport</td>
<td>Cucurbita maxima</td>
<td>Yes</td>
<td>Xoxonostle-Cazares et al., 1999</td>
</tr>
<tr>
<td>CmRINGP</td>
<td>Intracellular vesicular trafficking</td>
<td>Cucurbita maxima</td>
<td>No</td>
<td>Ruiz-Medrano et al., 1999</td>
</tr>
<tr>
<td>CmSTMP</td>
<td>Meristem cell fate</td>
<td>Cucurbita maxima</td>
<td>No</td>
<td>Ruiz-Medrano et al., 1999</td>
</tr>
<tr>
<td>CmSUTP1</td>
<td>Sucrose transport</td>
<td>Cucurbita maxima</td>
<td>No</td>
<td>Ruiz-Medrano et al., 1999</td>
</tr>
<tr>
<td>CmWRKYP</td>
<td>Defence response</td>
<td>Arabidopsis thaliana</td>
<td>Yes</td>
<td>Haywood et al., 2005</td>
</tr>
<tr>
<td>DELLA-GAI</td>
<td>Leaf development</td>
<td>Arabidopsis thaliana</td>
<td>Yes</td>
<td>Ruiz-Medrano et al., 1999</td>
</tr>
<tr>
<td>H+ ATPase PPA1</td>
<td>Energy transformation</td>
<td>Hordeum vulgare</td>
<td>No</td>
<td>Doering-Saad et al., 2002</td>
</tr>
<tr>
<td>miR156</td>
<td>Transcriptional regulation</td>
<td>Cucurbita maxima</td>
<td>No</td>
<td>Yoo et al., 2004</td>
</tr>
<tr>
<td>miR159</td>
<td>Transcriptional regulation</td>
<td>Cucurbita maxima</td>
<td>No</td>
<td>Yoo et al., 2004</td>
</tr>
<tr>
<td>miR167</td>
<td>Transcriptional regulation</td>
<td>Cucurbita maxima</td>
<td>No</td>
<td>Yoo et al., 2004</td>
</tr>
<tr>
<td>miR171</td>
<td>Transcriptional regulation</td>
<td>Cucurbita maxima</td>
<td>No</td>
<td>Yoo et al., 2004</td>
</tr>
<tr>
<td>Oryzacystatin-I</td>
<td>Protease inhibition</td>
<td>Oryza sativa</td>
<td>No</td>
<td>Sasaki et al., 1998</td>
</tr>
<tr>
<td>PFP-LeT6</td>
<td>Leaf development</td>
<td>Lycopersicon esculentum</td>
<td>Yes</td>
<td>Kühn et al., 1997</td>
</tr>
<tr>
<td>SUT1</td>
<td>Sucrose transport</td>
<td>Hordeum vulgare, Solanum tuberosum</td>
<td>No</td>
<td>Doering-Saad et al., 2002; Kühn et al., 1997</td>
</tr>
<tr>
<td>Thioredoxin h</td>
<td>Redox regulation</td>
<td>Oryza sativa, Brassica napus</td>
<td>No</td>
<td>Sasaki et al., 1998; Giavalisco et al., 2006</td>
</tr>
</tbody>
</table>
expression by miRNAs therefore represents another pathway besides siRNA-mediated PTGS. miRNAs have many diverse roles in the regulation of different developmental processes, mostly by regulating transcription factors (Guo et al., 2005; Wang et al., 2005; Zamore and Haley, 2005), and mutants in the miRNA biosynthesis pathway exhibit a number of developmental abnormalities (reviewed in Duan et al., 2006; Jones-Rhoades et al., 2006; Zhang et al., 2006).

In comparison with siRNAs, it is not yet clear if miRNAs can be translocated locally between cells and also systemically via the phloem, or if they are completely cell-autonomous (Voinnet, 2005). It has, however, been established that miRNAs occur in the phloem. Homologues of several miRNAs have been identified by cloning and sequencing experiments in pumpkin (Yoo et al., 2004), lupin (Atkins and Smith, 2007), and oilseed rape (A. Buhtz and J. Kehr, unpublished results). From in situ hybridization and grafting studies, two miRNAs, miR166 and miR399, have been implicated with being phloem-mobile signals, although direct proof for their presence in the phloem is as yet missing. miR166 is expressed during leaf development and its accumulation in the phloem of maize led to the suggestion that miR166 is a mobile signal (Juarez et al., 2004). Bari et al. (2006) provided evidence, by grafting experiments using miR399 target gene mutants, that miR399 could be involved in phloem-mediated signalling of the plant phosphate status. In addition, promoter::reporter studies have shown that this specific miRNA localizes to the vascular central cylinder of Arabidopsis plants under phosphate starvation (Aung et al., 2006). This indicates that miRNAs in the phloem might be directly involved in information transfer over long distances, potentially by being the signalling molecules themselves. However, direct evidence for the biological relevance of long-distance transport of miRNAs is still missing.

If systemic miRNA movement takes place, how are miRNAs transported? Microinjection experiments demonstrated that pure diffusion through PD is not sufficient to move miRNA species between cells (Yoo et al., 2004). It is therefore assumed that cell-to-cell movement, as well as the entrance to the long-distance transport tubes of the phloem, is enabled by interaction of miRNAs with proteins (see below). As for siRNAs, miRNAs occur in the phloem as sense and antisense strands (A. Buhtz and J. Kehr, unpublished results; Fig. 1), but phloem transport also seems to occur in the single-stranded form (Yoo et al., 2004). This suggests that components of the phloem transport mechanism for siRNAs and miRNAs are at least partially conserved.

**Import of phloem sap RNAs into SEs**

As already mentioned above, the PPUs connecting CCs with SEs have an unusually large SEL, opening the question whether macromolecular transport is really specific or only restricted by the size of the molecules. Non-plant proteins like GFP (green fluorescent protein) (Imlau et al., 1999) can move between CCs and SEs through PPUs, indicating a non-selective trafficking of macromolecules between these two cell types. A more recent study reports that GFP fusion proteins as large as 67 kDa can move between CCs and SEs and are only retained in CCs when targeted to membranes or the endoplasmatic reticulum (Stadler et al., 2005). Indeed it seems as if all cytrosolic proteins can enter SEs when coupled to GFP. This suggests that proteins (and probably also nucleic acids) reach the SEs by unspecific loss from CCs rather than by specific import mechanisms.

Because of their small size, it has been assumed that especially small RNAs are able to move between cells and reach SEs through PD by simple diffusion. Microinjection
experiments in pumpkin have demonstrated, however, that specific miRNAs cannot move between cells by diffusion alone but need the presence of a specific 27 kDa small RNA-binding protein that facilitates the passage between cells (Yoo et al., 2004), perhaps by acting as an RNA chaperone. Also other studies have identified several phloem sap proteins in different species that have the capacity to bind and translocate RNA (Barnes et al., 2004; Gomez and Pallas, 2004; Giavalisco et al., 2006). These proteins are thought to be part of a specific phloem import and transport machinery. One endogenous plant protein, CmPP16, shows sequence homology to viral MPs, is able to bind several RNA molecules including its own mRNA, and facilitates RNA transport from cell to cell by increasing the SEL of PD (Xoconostle-Cazares et al., 1999). Interestingly, most phloem sap proteins seem to be able to enlarge the SEL of PD in microinjection experiments (Balachandran et al., 1997; Ishiwatari et al., 1998), while isoforms not found in the phloem are incapable of such an interaction (Aoki et al., 2002). This indicates that phloem proteins use a similar mechanism like viral MPs to facilitate the entry of nucleic acids into SEs.

Conclusions

During the last few years it has been shown that RNAs, in addition to being a DNA copy for protein synthesis, can exert a range of important regulatory functions.

Increasing evidence accumulates that systemic transport of different classes of RNA via the phloem might constitute an essential route for plant communication. Different RNA species have been found to occur in phloem samples and this, together with grafting studies, suggests that miRNAs as well as miRNAs and siRNAs might be involved in a complex network that transmits information (Fig. 1), probably in addition to protein and small molecule signalling. This long-distance communication allows plants to react efficiently to changes in growth conditions, abiotic stresses, or viral attacks, and to coordinate these reactions with the resources needed for growth and development.

A high specificity and a tight regulation of phloem entry and exit seem essential, given the diverse and important physiological effects that the transported RNAs can exert. Thus, the main future challenge will be to understand the mechanisms of RNA import and transport, and to identify the internal and external triggers that induce systemic RNA signals in the phloem.

References


