Tansley review

Heavy traffic in the fast lane: long-distance signalling by macromolecules

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Summary

The two major vascular conduits in plants, the xylem and phloem, theoretically provide opportunities for the long-distance translocation of almost any type of water-borne molecule. This review focuses on the signalling functions conveyed by the movement of macromolecules. Here, a signal is defined as the communication of information from source to destination, where it modifies development, physiology or defence through altered gene expression or by direct influences on other cellular processes. Xylem and phloem sap both contain diverse classes of proteins; in addition, phloem contains many full-length and small RNA species. Only a few of these mobile molecules have proven functions in signalling. The transduction of signals typically depends on connection to appropriate signalling pathways. Incoming protein signals require specific detection systems, generally via receptors. Mobile RNAs require either the translation or presence of a homologous target. Given that phloem sieve elements are enucleate and lack translation machinery, RNA function requires subsequent unloading at least into adjacent companion cells. The binding of RNA by proteins in ribonucleoprotein complexes enables the translocation of some signals, with evidence for both sequence-specific and size-specific binding. Several examples of long-distance macromolecular signalling are highlighted, including the FT protein signal which regulates flowering time and other developmental switches.

I. Introduction – plant communication systems

Multicellular plants appear to have evolved a judicious balance between containment and communication. The plasma membrane and cell wall present substantial barriers between cells, yet many molecules can pass across the plasma membrane into the apoplastic space and migrate through the cell wall matrix. The potential for movement is related to molecular properties, including charge, mass and shape, and, in many cases, also depends on selective and specific transporter mechanisms. In addition, most adjacent plant cells possess plasmodesmata (PD) – intercellularcytoplasmic bridges – which confer noncell autonomous behaviour.
on a wide array of molecules. There are also examples of symplasmic isolation that have a profound impact on molecular traffic. PD are rare or absent between suspensor and embryo sac during embryogenesis (Schulz & Jensen, 1969), and between mature guard cells and adjacent epidermis or mesophyll (Wilmer & Sexton, 1979). In addition, PD are dynamic structures whose ability to enable or restrict traffic can be regulated during development or in response to environmental signals. Examples include the regulation of size exclusion limits (SEL) during sink–source transition in leaves (Roberts et al., 2001), and modulation of connections within the shoot apical meristem (SAM) related to the transition from the vegetative to the floral state (Gisel et al., 1999; Ormenese et al., 2002).

For the purposes of this review, a complete and effective long-distance signalling system is succinctly defined as one comprising a source, a transmission path and a destination at which the signal causes specific cellular actions. Major features of long-distance signalling by macromolecules are described, including structural and regulatory aspects, highlighting several examples in which there have been significant recent breakthroughs.

1. Making use of vascular highways – the fast lane

Intercellular communications fall into two main categories based on the range of action. Local traffic moves molecules from cell to cell by apoplasmic and/or symplasmic paths. By contrast, long-distance vascular routes connect distant organs. Hitching a ride via mass flow in xylem and phloem enables very rapid translocation between distant parts of the plant, with much higher velocities than any other known transport mechanism. Phloem linear flow is typically estimated at 40–140 cm h⁻¹ (Peuke et al., 2006), providing hour-scale journey times from shoot to root for most small herbaceous species, but several days for photosynthesising plants. Xylem transpiration flow rates can be reduced at night, but is usually much faster under normal diurnal conditions: 3–20 m h⁻¹ (Windt et al., 2006) and up to 70 m h⁻¹ in some vines with wider vessels (Peuke, 2000). In both xylem and phloem, effective molecular signalling systems must be suited to these speeds of transport and must be able to cope with substantial diurnal or seasonal variation in flow rates. Diffusive transport is much slower, and even dedicated systems, such as polar auxin transport, operate at only c. 1 cm h⁻¹, a fraction of phloem velocity.

Although vascular systems provide efficient long-distance transport, not all parts of the plant are able to distribute signals to all other regions (Table 1). Major drivers are transpirational pull for xylem flow, and osmotic push–pull towards sinks for phloem flow. This means that several sink tissues are unlikely to export signalling molecules. In particular, apical meristems, despite their inherent ‘control centre’ organization and local cell–cell communication, cannot directly employ vascular transport to exert the same level of regulatory influence over long distances. Nevertheless, apical meristem loss has dramatic effects on the development of distant organs, especially the initiation of lateral outgrowth. Further subtleties include unequal mass flow to different organs as a result of vascular architecture (Kiefer & Slusarenko, 2003), and destination-specific or selective signal movement (e.g. Schittko & Baldwin, 2003; Aoki et al., 2005).

2. Multiple functional demands on phloem and xylem systems

Xylem and phloem primarily function to distribute water, together with sugars, amino acids, organic acids and inorganic nutrients, throughout the plant to meet its structural and metabolic needs. Many secondary metabolites and hormones also travel in the vascular streams. The highly nutritious composition of phloem is attractive to pathogens and pests, especially aphids and other phloem-feeding insects. Although plants cannot afford to greatly restrict the nutrient content of phloem because of the penalty in potential growth rate, they have evolved a suite of local and systemic defence traits that protect their phloem from attack and parasitism. Abiotic stresses also frequently trigger integrated whole-plant responses involving long-distance signalling via xylem and phloem, for example to regulate the (re)allocation of scarce nutrients or to initiate enhanced uptake from soil. In the macromolecular context of this review, a key question is the degree to which long-distance communication is dependent on mobile proteins and RNA, as opposed to classic small-molecule hormones. An overview of signalling inputs and outputs is depicted in Fig. 1.

II. The importance of method selection and experimental design

1. Phloem and xylem analysis: potential for artefacts

The choice of vascular sap collection method, especially for phloem, greatly influences sample composition, and therefore care is needed in drawing conclusions, especially about signalling functions. Profuse exudation on tissue cutting or puncturing has been reported only for restricted taxa, including Ricinus, cucurbits, Brassica, Yucca and some legumes (Atkins, 1999). For other species, the methods of choice are exudation, either from cut petioles or stems, facilitated by EDTA solutions (King & Zeevaart, 1974; Hoffmann-Benning et al., 2002), or from cut aphid stylets (Fisher & Frame, 1984). EDTA methods yield substantial amounts of exudate, although highly diluted and of only moderate quantitative reproducibility. Styllectomy is technically more demanding and generates nanolitre volumes, but styllet-derived samples are generally considered to approximate ‘true’ in vivo phloem sap. Even so, all sap samples may contain components that would not ordinarily be transported over long distances, including sieve element (SE) plastid contents because of their bursting during sample collection (Knoblauch & van Bel, 1998), and other parietal SE materials dislodged from anchorage on the plasma membrane (van Bel et al., 2002). Equally, some proteins present in the phloem of intact plants may not appear in exudates, because of wound-induced coagulation or aggregation. For example, the proposed principal wound sealing sieve element occluding (SEO) proteins of Arabidopsis (Anstead et al., 2012) do not feature in the phloem proteome.
Batailler et al. (2012). Gaupels et al. (2008) found substantial overlap between protein complements derived by EDTA and stylet methods for barley. However, many two-dimensional gel spots were unique to each method, indicating that maximal coverage requires a combinatorial approach.

Care is also needed with other methods of investigation of phloem macromolecules. Based on immunological and in situ hybridization evidence, it has been reported previously that the sucrose transporter protein SUT1 in tobacco, tomato and potato is localized to SE plasma membranes, whereas its mRNA appears to be targeted to companion cell (CC) PD, suggesting that either mRNA or protein traffics across CC–SE junctions (Kuhn et al., 1997, 2003). Subsequently, however, spurious antibody recognition of SEs was discovered (Schmitt et al., 2008). Modified methods now reveal that SUT1 in Solanaceae is in fact localized to CCs, consistent with data from other families and with most models for apoplastic sugar loading.

Table 1 Directionality of vascular connections for long-distance communication

<table>
<thead>
<tr>
<th>Source</th>
<th>Sink leaf</th>
<th>Source leaf</th>
<th>Stem</th>
<th>Mature root</th>
<th>RAM</th>
<th>Flower</th>
<th>Fruit</th>
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<tr>
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<td>P</td>
<td>P</td>
<td>X+P</td>
<td></td>
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</tr>
<tr>
<td>Mature root</td>
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<td>Flower</td>
<td>P</td>
<td>X</td>
<td>X</td>
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<tr>
<td>Fruit</td>
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<td>X,P</td>
<td>X</td>
<td>revX</td>
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</table>

Only the simplest or most direct paths are indicated, based on annual herbaceous species with potentially more than one of each type of organ, for example, multiple root and shoot meristems, multiple reproductive axes. Additional, more complex re-export and/or xylem-phloem exchange may occur in some instances, or may be artificially induced by experimental conditions that disrupt water relations or osmotic gradients. Perennial woody species may use alternative mechanisms, such as sugar transport in xylem during spring remobilization of trunk and root carbohydrate reserves. Blank cells indicate that it is unlikely that mass flow in xylem or phloem would allow the direct connection of the particular source and destination. *P, phloem transport; P+X, phloem export then phloem-to-xylem exchange; RAM, root apical meristem; revX, diurnal reversed xylem flow; revX+P, reversed xylem flow then re-export from phloem; SAM, shoot apical meristem; X, xylem transport; X+P, xylem-to-phloem exchange, or xylem transport to transpirational sink then re-export in phloem.

(Batailler et al., 2012). Gaupels et al. (2008) found substantial overlap between protein complements derived by EDTA and stylet methods for barley. However, many two-dimensional gel spots were unique to each method, indicating that maximal coverage requires a combinatorial approach.

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Several options are available for xylem sampling, recently reviewed by Alexou & Peuke (2013). Commonly, root xylem sap is collected from decapitated stem stumps. In some cases, exudation is spontaneous as a result of internal root pressure, but flow can be increased by applying a light vacuum. Alternatively, enclosing the root system within a pressure vessel allows the application of root pressure to balance exudation rate to normal transpirational flow. Leaf xylem sap can be obtained by pressurization by enclosing the leaf blade in a similar chamber and allowing the petiole to extend

Fig. 1 Overview of long-distance signalling in plants. Coordination requires communication in all multicellular organisms. In plants, xylem and phloem vascular conduits provide rapid and efficient long-distance routes between distant organs. Abiotic and biotic inputs perceived in one location are transduced into molecular signals that migrate to the rest of the plant, where they induce developmental and physiological responses. In addition to modulation by environmental factors, internal signalling maintains developmental balance, especially between root and shoot. Arrows indicate directions of signal movement in xylem (blue) and phloem (red).
through the sealed port. In general, there seem to be fewer concerns over the ‘quality’ of xylem sap, although the composition of common solutes and proteins can vary with the time of day (Krishnan et al., 2011).

A final caveat for phloem or xylem ‘omics’ experiments is the potential for substantial complements of macromolecules of nonplant origin, even in plants with no disease symptoms. Many fungi, bacteria and viruses exist in intimate contact with plant tissues: in the rhizosphere, as phylloplane species, and internally within the intercellular space or intracellularly. The risk is greatest for plant species with incompletely sequenced genomes, where novel RNAs or proteins may be wrongly attributed to the host plant.

2. Phloem transport in cucurbits – a special case

Although cucurbits are popular model species for phloem biology, they are not necessarily representative of vascular plants in general. The main reason is the unusual phloem anatomy of the Cucurbitaceae family. Most cucurbit phloem is contained within bicollateral vascular bundles with phloem tissue (fascicular phloem; FP) lying both external and internal to the xylem. In addition, there are individual extrafascicular phloem (EFP) strands on the periphery of vascular bundles and scattered through the cortex. Lateral commissural EFP elements connect cortical strands with those adjacent to the vascular bundles, thus potentially generating an anastomosing network.

There is ongoing debate about EFP function. One proposal (e.g. Golecki et al., 1999) is that FP and EFP are interconnected, acting as a unified system. However, recent evidence has indicated that FP and EFP are physically and functionally distinct (Zhang et al., 2010; Zhang et al., 2012). This has major ramifications for our understanding of macromolecule transport and function. A further problem is that most measurements of sugar content of cucurbit exudates are massively at odds with values from other species and with predictions of photosynthate export. Typical exudate content is c. 10–30 mM, comprising a range of monosaccharides and short-chain oligosaccharides (Richardson et al., 1982; Fiehn, 2003; Zhang et al., 2012), many fold less than the typical 1 M found in other species. This paradox has been discussed in the past (e.g. Richardson et al., 1984). Symplasmic vs apoplastic loading mechanism differences do not offer a plausible explanation, nor does dilution by contaminating xylem sap or wounded cell contents. A related assumption is that cucurbit phloem exudate emanates largely from the more substantial FP, with, at most, minor contributions from EFP. Zhang et al. (2010) provided evidence that the opposite may be true: FP becomes rapidly blocked on wounding, whereas EFP continues to exude. Zhang et al. (2012) reported similar findings for pumpkin, but, in cucumber, exudate droplets were deduced to emanate from both EFP and FP. Sugar analyses of dissected FP tissue reveals sugar content totalling 0.6–1.1 M, largely comprising raffinose-family oligosaccharides, very different from the exudate composition (Haritatos et al., 1996; Zhang et al., 2010). Importantly, the FP proteome – assessed from microdissected protein plugs of individual FP SEs – is highly dissimilar to that of phloem (i.e. EFP) exudate. This now explains why the major cucurbit phloem exudate proteins PP1 and PP2 are immunolocalized only to EFP and not to FP (Golecki et al., 1999). Thus, all previous studies of cucurbit phloem exudate have examined largely or exclusively the contents of EFP and not the major FP. An inescapable conclusion is that there is little exchange of metabolites and proteins between the two divergent systems. Sugar transport is a likely primary function of FP, whereas EFP may fulfil other roles, such as signalling or defence. Consistent with the latter notion are the many reports of signalling macromolecules in EFP exudate, for example, 16 kDa Cucurbita maxima phloem protein (CmPP16) (Xoxonostle-Cázares et al., 1999), FLOWER-ING LOCUS T (FT) protein (Lin et al., 2007) and GIBBERELLC ACID INSENSITIVE (GAI) mRNA (Haywood et al., 2005), as well as other potential long-distance signalling molecules, for example, microRNA (miRNA) (Yoo et al., 2004). It remains to be seen whether cucurbit FP also carries signalling macromolecules.

The divergence of FP and EFP anatomy, composition and function suggests early evolutionary separation. Further work is required to resolve whether the sum of FP and EFP composition and functions is equivalent to that found in species with single phloem systems. Nonetheless, the dual cucurbit phloems enable comparative experimentation to associate functions, such as aphid feeding, biotic defences and long-distance signaling, with one or both systems. This may provide widely applicable insights not readily obtained in other species. Finally, there is evidence that laticifers (latex-containing canals) found in several noncucurbit families have much in common with EFP (Pickard, 2008; Gaupels et al., 2012). The current lack of detailed molecular data on laticifer composition prevents precise comparisons, but access to the genomes of cucurbits and laticiferous species, such as papaya, Ricinus, rubber, cassava and banana (Huang et al., 2009; Porter et al., 2009; Chan et al., 2010; Xia et al., 2011; Xu et al., 2011; D’Hont et al., 2012; Garcia-Mas et al., 2012; Prochnik et al., 2012), opens up new research avenues.

3. Grafting – an unambiguous tool for diagnosing long-distance transport and action

Historically, grafting – the connection of two different plants or plant parts – has been of enormous benefit, especially to the horticultural industry. Typically, a shoot piece, known as a scion, is connected to a rootstock. Depending on the method of construction, the rootstock may or may not contain stem and leaves in addition to roots. Commercial applications include fruit tree size control in apple through a range of dwarfing rootstocks, salt-tolerant rootstocks in grapevines and the maintenance of clonal genotypes.

However, grafting has also been instrumental in the discovery of many major concepts in plant science, particularly in relation to long-distance signalling in both phloem and xylem. Normally, root-to-shoot signals follow transpirational paths in xylem, whereas phloem conducts signals from shoot to root, as well as from source leaves to sink leaves (Table 1). In grafts, flow may be from scion to rootstock or vice versa, depending on the net photoassimilate supply and demand in each part of the plant. The analysis of phloem or xylem sap can provide direct evidence for the nature of
transmitted signal molecules. Alternatively, phenotypic or molecular analysis of tissues on the receiving side of the graft indicates primary or secondary consequences of signal transmission.

Early physiological and then forward genetic studies on the long-distance regulation of developmental processes include photoperiodic flowering (Lang et al., 1977), stem height (Reid et al., 1983), legume nodulation (Delves et al., 1986) and shoot branching (Beveridge et al., 1994). However, the absence of sequenced genomes and inefficient transformation protocols in physiological and genetic models, such as pea, hampered the isolation of genes. The development of Arabidopsis grafting, especially for seedlings (Turnbull et al., 2002), but also for later developmental stages (Rhee & Somerville, 1995; Ayre & Turgeon, 2004), has now provided a platform for the rapid screening of genes for potential functions in long-distance signalling processes. One advantage of grafting is that native genes can readily be studied, whereas most other means for spatial or temporal control of gene expression depend on the over-expression or ectopic mis-expression of transgenes.

III. Macromolecules in xylem and phloem

In addition to nutrients and the huge diversity of metabolites, surveys of macromolecule composition of vascular saps reveal very large numbers of different proteins and RNAs (Table 2). In phloem, a total of over 1500 proteins, 1500 mRNAs and 1000s of small RNAs (sRNAs) have been estimated by Lough & Lucas (2006). Over 2400 mRNAs and over 1200 proteins have been reported from Arabidopsis and cucumber phloem exudates, respectively (Deeken et al., 2008; Lin et al., 2009), and several hundred in maize xylem (Alvarez et al., 2006). Great progress is therefore being made towards comprehensive sap proteomes and transcriptomes, although databases, even for Arabidopsis, remain incomplete. Once better consensus datasets are assembled across a greater number of species, ideal models for phloem and xylem studies may emerge. Comparative high-throughput proteomics and RNAseq approaches can greatly accelerate progress in this regard. Whatever the total number of macromolecule species present in moving sap, only a proportion will be involved in long-distance signalling functions.

1. Macromolecule composition of xylem: xylem-based signalling

The xylem transport system is apoplastic, requiring the loading of solutes into the moving stream within the dead vessel and tracheid conducting cells. In roots, the endodermis prevents apoplastic transport from cell layers external to the stele, and thus the cell-to-cell transport route towards the xylem includes at least one segment of symplasmic transport. There is no evidence for endogenous RNA in xylem sap (Buhtz et al., 2008), and it is unlikely that such molecules, even sRNAs, could be effectively loaded, transported and unloaded via this apoplastic route. Some viruses do exist in xylem as intact particles, but not as naked RNA. Functional evidence from Arabidopsis indicates no transport of miRNAs from root to shoot, even when shoots are grafted to over-expressing rootstocks (Pant et al., 2008).

A broader inference is that if RNA cannot move from root to shoot, all long-distance signals in that direction must employ molecular classes other than RNA, that is, proteins, peptides and small molecules. Indeed, there is a wide array of proteins present in xylem, first reported in the early 1990s (e.g. VanCleve et al., 1991; Satoh et al., 1992) and now extended by many detailed proteomics studies (Table 2; also Rep et al., 2002, 2003; Buhtz et al., 2004; Satoh, 2006; Atkins & Smith, 2007; Houterman et al., 2007; Alvarez et al., 2008; Krishnan et al., 2011). Consistent with the predicted secretory origins, xylem sap proteomics indicates a high incidence (>80%) of proteins with N-terminal signal peptide sequences (Djordjevic et al., 2007; Ligat et al., 2011). Many functional classes of xylem protein point strongly to roles in stress protection and biotic defence (Buhtz et al., 2004; Kehr et al., 2005). The dynamic nature of the xylem sap proteome was demonstrated by the presence of several infection-specific proteins in comparative studies of healthy and biotically challenged plants (Houterman et al., 2007; Subramanian et al., 2009). Although proteins loaded into xylem sap may be delivered to shoot destinations, there are hurdles to be overcome if such proteins are to act as true long-distance signals. If their final site of action is intracellular, some uptake mechanism is required, via endocytosis or protein channels, although such evidence is currently lacking for any xylem-borne protein. Alternatively, membrane-bound receptors or other protein targets could exist with extracellular binding domains. Currently, such types of evidence are lacking for putative xylem protein signal systems.

2. Macromolecule composition of phloem

Phloem sap proteins The diversity and large number of proteins identified in phloem exudates (Table 2) implicates them in a wide range of functions, from structure to defence to signalling. However, in vivo transport has been demonstrated directly for only a small proportion of these proteins, and even fewer have confirmed long-distance signalling functions.

Some phloem proteins may function as long-distance signalling molecules only after post-translational modification, for example phosphorylation by calcium-dependent protein kinases in rice SE (Nakamura et al., 1995). The presence in tomato SE of a key enzyme in the jasmonate pathway, allene oxide cyclase, but not its mRNA, implies synthesis in the CC and export to SE (Hause et al., 2003). This enzyme is likely to modulate long-distance jasmonate-dependent signalling. Redox-regulating enzymes in SE may also have signalling functions. For example, the phloem-specific thioredoxin h, RPP13-1, is involved in the reduction of disulfide bridges (Schobert et al., 1998) and the repair of damaged proteins in SE (Ishiwatari et al., 1995). First identified in rice, RPP13-1 accumulates specifically in leaf and stem CC and is then transported into SE (Ishiwatari et al., 1995, 1998). Recently, many other signalling proteins have been identified in Arabidopsis: G-box binding factors, annexins, calreticulins, leucine-rich repeat (LRR) proteins, cyclophilins, components of the 20S proteasome, protease inhibitors, aminopeptidases and the DJ-1 putative peptidase.
However, functions in long-distance transport and signalling remain unclear (Batailler et al., 2012). To date, one very clear example of a phloem-transmitted signal is the flower-inducing protein FT, which is discussed later.

Amongst the most studied phloem sap proteins are the structural P proteins which function in sealing wounded SE, and thus may indirectly affect signalling functions during pathogen or herbivore attack (Clark et al., 1997; van Bel, 2003). Cucurbit exudate which, showed the movement of P proteins (Bostwick et al., 1997). Grafting between Cucurbita maxima and Cucumis sativus, both being synthesized in CC and then transported into SE complex. Therefore, the movement of P proteins (Bostwick et al., 1992; Clark et al., 1997; Dannenhoffer et al., 1997; Golecki et al., 1999). However, PP2-A1, the closest orthologue of CmPP2 protein in Arabidopsis, is present in SE (Dinant et al., 2003; Batailler et al., 2012), but absent from EDTA exudates, suggesting that it may not be mobile in Arabidopsis phloem, instead being anchored to other P proteins or to organelles such as phloem plastids (Batailler et al., 2012).

**Phloem sap RNA** Surveys of the total phloem sap transcriptome by either expressed sequence tag (EST) sequencing or microarrays (Table 2) have identified large numbers of mRNA species in melon (986 Unigenes; Omid et al., 2007) and Arabidopsis (2417 significant signals on microarray; Deeken et al., 2008). One conclusion is that mRNA passage from CC to SE occurs readily, and it is unlikely that specific mechanisms regulate the movement of each of these transcripts. Most phloem sap RNA probably derives from transcription in CCs. Exceptions could include residual RNA retained within SEs during phloem differentiation and RNA delivered from other cell types outside the CC–SE complex. However, over 60% of phloem sap transcripts were not detected in CC or laser-dissected phloem tissue samples (Deeken et al., 2008). A major question is how many transcripts in SE are ultimately by EST sequencing or microarrays.

<table>
<thead>
<tr>
<th>Plant name</th>
<th>Source</th>
<th>Molecule type</th>
<th>Total number detected*</th>
<th>Total identified†</th>
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<td>1209</td>
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<td>18–25-nt sRNA</td>
<td>Several 1000</td>
<td>(4 miRNA)</td>
<td>Yoo et al. (2004)</td>
</tr>
</tbody>
</table>

*Number of spots visible on two-dimensional gels or number of distinct RNA sequences.†Based on sequence homology to annotated nucleotide and/or protein databases, or significant signal on microarray.

–, Not stated or not measured.
et al., 2009). In addition to endogenous mRNAs, foreign RNA and sRNA are transported in the phloem, with substantial numbers of the latter detected in phloem surveys of several species (Yoo et al., 2004; Buhtz et al., 2008; Rodriguez-Medina et al., 2011).

3. Peptide signals in xylem and phloem

Several plant peptide signals, derived from the post-translational cleavage of precursor proteins, have been identified (Wheeler & Irving, 2010), ranging in size from five amino acids for phytosulfokine (Matsubayashi & Sakagami, 1996; Srivastava et al., 2008) to over 100 amino acids for epidermal patterning factor 1 (EPF1; Hara et al., 2007). Although phloem exudate contains several small proteins in the range 2–10 kDa, equivalent to 15–75 amino acids (Marentes & Grusak, 1998), many peptides are instead secreted and typically act as intracellular short-range signals detected by plasma membrane receptors. Even phloem-expressed noncell autonomous peptides, such as TDF, operate locally, in this case suppressing xylem differentiation, rather than acting over long distances (Hirakawa et al., 2008).

However, there is evidence for long-distance peptide signalling in both xylem and phloem. First, Arabidopsis plant natriuretic peptide (PNP) is found in xylem sap (Maryani et al., 2003) and may be a systemic signal in the regulation of cell volume (Morse et al., 2004). Second, the lipid transfer protein XSP10 may be involved in lipid-based defence signalling (Rep et al., 2003; Krasikov et al., 2011), although there is some debate as to its role in systemic defence. Third, radiolabelled systemin supplied exogenously to wounded leaves travels in phloem and, to some extent, in xylem (Narvaez-Vasquez & Ryan, 2004), but there is some doubt as to the necessity of its movement for systemin-dependent signalling (Lee & Howe, 2003; Stratmann, 2003). The significance of the reported binding of systemin to the brassinosteroid receptor SR160/BRI (Scheer & Ryan, 2002) is unclear because systemin-dependent wound signalling, as measured by the systemic induction of proteinase inhibitor expression, is not defective in the curl3 tomato BRI loss-of-function mutant (Holton et al., 2007). Fourth, CLE (CLAVATA3-like) peptides may act as the systemic root-to-shoot signal that initiates autoregulation of nodulation (AON) in legumes. The AON signalling pathway depends on a receptor-like kinase (RLK), identified in soybean as GmNARK (Searle et al., 2003), which is expressed predominantly in phloem (Nontachal-yapoom et al., 2007). GmNARK orthologues LjHAR, PsSYM29 and MtSUNN1 have been isolated from Lotus japonicus, pea and Medicago truncatula, respectively (reviewed by Reid et al., 2011b). The AON RLKs most probably recognize peptide ligands delivered from the roots via the xylem stream. Evidence from several legumes indicates that the mobile factors are two CLE peptides (Okamoto et al., 2009; Mortier et al., 2010; Lim et al., 2011; Reid et al., 2011a). At this stage, direct evidence for their transport in xylem is lacking, but a bioassay-based screen for xylem compounds has been reported (Reid et al., 2012) and transgenic CLE expression affected nodulation in nontransgenic parts of the root system (Okamoto et al., 2009; Mortier et al., 2010). In the second part of the signal relay, the activated AON RLK is predicted to control a second long-distance signal that moves via the phloem from shoot to root, where it down-regulates nodule numbers (Delves et al., 1986). Bioassay evidence indicates that the phloem-borne signal is probably a small molecule, not a protein or RNA (Lin et al., 2010, 2011). Nodulation signals therefore remain an area of intensive research.

Finally, an interesting biotechnology application is the transformation of grapevine rootstocks with the Shiva-1 gene encoding an antimicrobial lytic peptide that is graft transmitted in the xylem, and acts against xylem-limited pathogens (Dutt et al., 2007). Restriction of the transgene to the rootstock precludes risks of transgene escape in pollen or seed, and greatly reduces the number of genotypes that need to be transformed, because many scions are compatible with each rootstock variety.

IV. Plasmodesmata – complex intercellular exchange junctions enabling short- and long-range signalling

Plasmodesmal traffic enables phloem long-distance macromolecular signalling and local symplasmic traffic, and there is now extensive knowledge of PD structure, with several recent reviews (e.g. Lucas et al., 2009; Xu & Jackson, 2010; Bell & Oparka, 2011; Maule et al., 2011).

1. Primary and secondary plasmodesmata

Primary PD form during cytokinesis as a result of incomplete cytoplasmic separation, allowing the retention of continuity between daughter cells (Kragler et al., 1998). On completion of division, an endoplasmic reticulum (ER) strand, known as the desmotubule, is retained, running through the centre of the PD, giving ER membrane continuity between the daughter cell pairs. However, intercellular traffic via the ER lumen may be restricted because, frequently, the membranes are seen to be appressed. Macromolecules, instead, are proposed to pass predominantly via the cytoplasmic sleeve between the desmotubule and the plasma membrane lining of the PD, although movement may also occur through the desmotubule (Barton et al., 2011). In some cases, molecules may move freely, especially if the PD are dilated, but movement may also be facilitated by binding to ER or plasma membrane attachment sites.

Most cell pairs have primary PD, but there are some notable exceptions, such as between mature guard cells and adjacent epidermal or subsidiary cells. The mean number of connections also varies considerably, suggesting a degree of developmental control. Such control may occur at the time of cytokinesis by the regulation of the number of traversing ER strands, or subsequently through closure of PD bridges, as happens with guard cells (Wilmer & Sexton, 1979).

Independent of cell division, secondary PD may also form by plasma membrane and ER extensions into and through the cell wall (Monzer, 1991). Secondary PD can arise de novo, sometimes associated with wall thinning, or may derive from the elaboration of primary PD (Kragler et al., 1998). Successful bridging to the adjacent cell is not guaranteed, as evidenced by blind endings. Secondary PD sometimes exhibit functions not seen in primary PD within the same tissue, such as the localization of viral movement proteins (Ding et al., 1992).
2. What dictates the size exclusion limit of plasmodesmata?

Molecular size, assessed either as molecular mass or, better, as Stokes’ radius, is a major factor in determining the potential passage through PD pores. The idea that PD have a low SEL persisted for many years. However, based largely on viral spread studies from the early 1990s, it became clear that very large molecules, such as viral nucleic acids, could move symplasmically between cells. Seminal discoveries included movement proteins in complexes with single-stranded DNA and RNA (ssDNA and ssRNA) (Citovsky & Zambrayski, 1991; Citovsky et al., 1992; Fujiwara et al., 1993; Noueiry et al., 1994; Ding et al., 1995). Movement proteins interact with PD, increasing SEL and enabling the passage of nucleic acids. Subsequently, functional similarities were found between viral movement proteins and endogenous plant proteins based on immunological cross-reactivity, RNA binding and effects on SEL of PD (Xoconostle-Cázares et al., 1999). However, there is limited sequence identity between the viral and plant paralogues.

More than one set of rules exists for PD traffic, with current models proposing two major classes of movement: passive/diffusive/nonspecific and selective/specific. In diffusive movement, molecular dimensions are the key determinant – very large molecules do not pass across PD. Labelled molecules, such as fluorescent dextrans or proteins, allow the estimation of SEL. The largest proteins with demonstrated movement include green fluorescent protein (GFP)–patatin fusions in roots (67 kDa; Stadler et al., 2005) and triple GFP fusions in embryos (81 kDa; Kim et al., 2005). Free GFP (27 kDa, Sr 2.8 nm; Terry et al., 1995) is much more mobile, and can reasonably be assumed not to have any specific transport mechanism in plants. By contrast, selective movement requires the interaction between the mobile molecule and other components at or within the PD complex, such as chaperones and gating proteins. Selectivity may also relate to mechanisms of delivery to the PD (Thomas et al., 2008). These interacting factors enable passage via co-transport, especially important in the case of RNA movement as ribonucleoprotein (RNP) complexes, or through dilation of the PD pore. In passing, it is worth noting that the molecular mass of mRNA is rarely discussed. Even a modest 500-nucleotide RNA is approximately 160 kDa, substantially greater than the maximum PD SEL for proteins. A much larger 2.7-kb full-length mRNA including untranslated region (UTR) sequences, encoding StBEL5, with a net mass of c. 900 kDa, moved across potato graft unions (Banerjee et al., 2006). Although mRNA does not adopt the same degree of tertiary structure as proteins, it does have predictable secondary structure, and is unlikely to exist as a simple linear molecule. Protein coding and UTR sequences can both enable phloem movement (Banerjee et al., 2006, 2009; Li et al., 2009; Li et al., 2011).

When PD are dilated, other macromolecules may transit opportunistically by simple diffusion. The regulation of dilation is usually referred to as gating, a term originally proposed by Vaquero et al. (1994) based on the dramatic effects of viral movement proteins on increasing nonspecific SEL (Wolf et al., 1989). For example, microinjected fluorescently labelled dextrans or peptides, even as small as 0.85 kDa, were normally unable to exit leaf trichome cells, but, within 5 h of viral inoculation of that same cell, molecules up to 4.4 kDa appeared in adjacent cells, coincident with the start of viral movement (Derrick et al., 1992).

V. Phloem signalling: regulation

1. Phloem loading: leaf and vascular symplasmic domains

A key question is whether PD type and frequency are significant factors in the regulation of long-distance transport of macromolecular signals, either through selective or nonspecific pathways. PD in the form of plasmodesmal pore units (PPU) are typically frequent between SE and CC (Fig. 2). All species examined to date have PD between CC and SE, and thus all can potentially use symplasmic routes for the systemic transport of macromolecules originating from CC or other cell types. However, PD are
infrquent from SE to phloem parenchyma (PP) or bundle sheath (BS) (Ding et al., 1992; Haritatos et al., 2000b), reinforcing the concept of SE isolation from the rest of the leaf, with the great majority of symplasmic transport via CC (Fig. 2). Some variation in PD into CC is attributable to whether a species is classed as apoplastic or symplasmic loading for photosynthetic transport (Kempers et al., 1998), although this is not a perfect relationship, with some apoplastic loaders having relatively high PD frequencies (Turgeon & Medville, 2004). The CC therefore represents the gatekeeper for the great majority of materials delivered symplastically to and from the phloem transport stream (van Bel & Knoblauch, 2000). Many phloem-mobile macromolecule signals are known or predicted to be synthesized in CCs, but some may originate in other leaf cell types. Equally, many signals may have sites of action beyond the SE–CC complex. Therefore, highly regulated pre- and post-phloem intercellular routes may be the norm, whether by selective or nonselective mechanisms, enabling targeted responses in accord with the supracellular concepts originally proposed by Lucas et al. (1993).

It is also clear that, even within phloem, multiple functional domains exist with different SEL, illustrated by the differential mobility of proteins expressed under the AtSUC2 and CmGAS1 promoters. The former expresses in all loading and transport phloem, including substantial sections of roots (Imlau et al., 1999; Stadler et al., 2005), whereas the latter is restricted to leaf minor veins (Haritatos et al., 2000a). Expression of the 20-kDa flowering hormone protein FT (see also later) under either promoter was sufficient to cause acceleration of flowering, but, when FT was expressed as a translational fusion to GFP (net 47 kDa), only the SUC2-driven version retained floral activity, whereas protein expressed from the GAS1 promoter was immobile and florally inactive (Corbesier et al., 2007). The phloem-loading SEL for Arabidopsis minor veins thus appears to be between 20 and 47 kDa, but up to 63 kDa in SUC2-expressing domains (Stadler et al., 2005).

By contrast, although PD connections to epidermal cells are found in immature leaves, mature guard cells become symplasmically disconnected (Wilmer & Sexton, 1979). Based on GFP movement, epidermal cells exchange macromolecules largely with each other (Kim et al., 2002), whereas guard cell isolation is probably a prerequisite for effective turgor-driven changes that regulate stomatal aperture. Epidermal and guard cells thus communicate with the rest of the leaf predominantly by apoplastic routes.

Viral studies have provided further evidence for specific domains within leaves. Li et al. (2001) showed differences in PD transmission of mutated viral movement protein forms, suggesting cell junction-specific regulation. Using a similar approach, Wang et al. (1998) found that cell–cell movement amongst mesophyll, BS and PP was under separate regulation to entry into the CC–SE complex and consequent systemic movement. Parallel studies with mutated noncoding viroid RNA sequences revealed two separate specific motifs associated with the regulation of traffic from BS into phloem and from BS into mesophyll (Qi et al., 2004; Zhong et al., 2007). Intriguingly, reverse movement from mesophyll to BS was not influenced. A general conclusion is that each type of cell junction has specific PD features, and several of these junctions may influence the access of macromolecules to and from the phloem transport system.

2. Phloem signal unloading

Symplasmic delivery to sink leaves, roots, meristems and reproductive organs is vital to many long-distance signalling processes. Detailed studies, especially of the SAM, indicate tightly regulated domains. Although many noncell autonomous signals move only locally, the mechanisms and patterns allow us to postulate how PD influence the potential post-phloem delivery of long-distance signals.

Transmission factors, such as LEAFY and KNOTTED, and sometimes their mRNA precursors, show short-range movement within the SAM, and indicate the high spatial precision of signalling within the meristem domains, reviewed by Wu et al. (2003). LEAFY protein expressed specifically in the outer (L1) layer moved passively through PD into the inner layers. By contrast, KNOTTED1 (KN1) protein resulted in increased SEL between meristem cells, and moved selectively through PD, together with its mRNA. KN1 mRNA was expressed only in L2 and L3, but a normal phenotype required protein presence at least in L1 (Kim et al., 2003). The Antirrhinum APETALA3 orthologue, DEFICIENT, moves from L2 to L1, but not vice versa (Perbal et al., 1996), a selective property analogous to an electronic rectifier. Not all meristem transcription factors can migrate: APETALA1 (AP1) acts cell autonomously, as does the fusion protein AP1::GFP. Intriguingly, GFP:AP1 is nonfunctional in rescuing ap1 phenotypes but does move (Wu et al., 2003). This suggests that, in some cases, intracellular retention is associated with normal function and loss of domain integrity results in failure of floral organ patterning.

Similar domains may exist along the transport phloem and in root sink tissues. For example, transcription factors, such as SHORT ROOT, show noncell autonomous passage between different root meristem cell types (Nakajima et al., 2001). Free GFP expressed in CC under the AtSUC2 promoter is readily transported to sink tissues via phloem and unloaded into all meristem cells (Imlau et al., 1999), suggesting a SEL of at least 27 kDa between protophloem and other cell types within the root meristem. By contrast, larger soluble GFP fusion proteins, up to 67 kDa, were translocated in phloem, but did not significantly exit into meristematic tissues (Stadler et al., 2005). This means that many phloem-mobile macromolecules may not be delivered to all sink tissues because of SEL restrictions.

3. Dynamic domains

In addition to responses to viral infection, plasmodesmal SEL can change during development, often with tight temporal and spatial regulation, giving rise to altered connectivity between different cells and tissues. These dynamic shifts in supracellular domains have major impacts on local communications, but can also influence the dispatch and receipt of molecules to and from long-distance transport in the phloem. In developing leaves during the sink to source transition, there are dramatic changes in PD type and
frequency (Ding et al., 1992; Oparka et al., 1999). Sink leaves with simple PD can traffic GFP extensively to all cell layers, including epidermis, guard cells and trichomes (Oparka et al., 1999), and GFP fusions up to 50 kDa move between cells. This traffic disappears in source leaves. Within the SAM, rapid and precise changes occur during and immediately following floral initiation, which may modulate the access of long-distance signals from leaves into the SAM (see later). Rapid increases in secondary PD frequency are seen between and within SAM layers of Sinapis alba following transfer to inductive long days (Ormenese et al., 2000). Single cell microinjection of fluorescent probes indicates that, in the vegetative apex, the central zone is restricted in symplasmic connectivity to the peripheral zone cells (Ormenese et al., 2002). Symplasmic delivery of similar tracers into the SAM via the vasculature instead showed transient restriction during floral initiation (Gisel et al., 1999, 2002), superficially contradictory to results from the microinjection approach. However, the two experiments can be rationalized because Gisel et al. (1999, 2002) showed import into peripheral zones and leaf primordia of vegetative meristems, but not into the isolated central zone.

4. Selectivity

As stated earlier, long-distance transport of macromolecules is, in many instances, selective. This applies to both proteins and RNA. The mechanisms of selectivity are not fully understood, but can conceptually be divided into four components: the regulation at sites of loading into vascular conducting cells (SEs, xylem vessels/ tracheids); the immobilization within SEs, perhaps by anchoring to the plasma membrane via specific binding motifs; the presence of essential chaperones or binding partners that enable movement within the transport stream; and the selective degradation of some molecules during transport.

RNA selectivity To complete a phloem RNA signalling pathway requiring translation or sequence-specific action at its destination, selective or nonselective retrieval of RNA molecules from SE and unloading into CC are essential. One striking finding from the melon phloem transcriptome analysis by Omid et al. (2007) was that only a minority (six of 43; 14%) of phloem RNA species appeared able to transmit across a graft union. It is not clear which, if any, of the four mechanisms suggested above apply in this case.

Although pressure flow is the basis for long-distance movement in phloem, sink strength alone may be insufficient to predict patterns of macromolecule movement, especially for mRNA. In tomato plants over-expressing modified GAI genes, transcripts accumulated significantly in SAMs and young leaves, but not in fruits, despite all these tissues being classic phloem sinks (Haywood et al., 2005). Failure to detect transcript accumulation in fruit may reflect rapid turnover or lack of an unloading mechanism within the fruit phloem. Using nonprotein coding viroid RNA, Zhu et al. (2002) similarly discovered selective delivery into sink organs. Positive selectivity in RNA transport may be achieved through RNA-binding proteins that act in a size-specific, class-specific or sequence-specific manner. Some RNAs appear to travel as RNP complexes of RNA and protein and not as naked molecules (Gomez et al., 2005; Kehr & Buhtz, 2008). For example, the RNA-binding protein 50 (RPB50; Lin et al., 2009) may assist the passage of bound mRNAs though PD between CC and SE, enabling long-distance mRNA transport (Ham et al., 2009). Co-immunoprecipitation identified RPB50 binding to several RNA partners, such as PP16–1, GAI, SHOOTMERISTEMLESS, SCARECROW LIKE 14 and a MYB.

Interspecific mRNA transmission across haustoria between host plants and parasitic Cuscuta reveals further elements of selectivity (Roney et al., 2007; David-Schwartz et al., 2008). Tomato-encoded GAI, phosphofructokinase (PFK) and Rubisco small subunit RBCS rmRNAs were detected in parasite tissues, especially phloem, as were pumpkin phloem homologues of NAC [NAM, ATAF1/2 and CUC2] domain (NACP), Sucrose transporter 1 (SUT1P) and WRKY domain (WRKYD). Interestingly GAI, a pumpkin GAI homologue, was not transmitted, and neither was CmPP16, despite their well-known mobility across grafts between cucurbit species (Xoconostle-Cázares et al., 1999). Potential application in weed control has been demonstrated recently by expressing a parasite transcription factor in a tobacco host that suppresses parasite growth following RNA transmission (Alakonya et al., 2012).

Protein selectivity Selective protein delivery has been shown using an aphid stylet-based system to introduce heterologous molecular mixtures derived from pumpkin phloem sap into the phloem stream of rice (Aoki et al., 2005). Protein movement towards the SAM was passive and uncomplexed, but towards the root apex required additional molecules, such as CmPP16, eukaryotic translation initiation factor 5A (eIF5A) and translationally controlled tumor protein (TCTP), which are found in pumpkin exudate and might act as chaperones or enable PD passage. In turn, PD trafficking of CmPP16–1 itself can be enabled by the tobacco protein noncell-autonomous pathway protein 1 (NCAPP1) (Taoka et al., 2007). CmPP16 was originally detected as a weak parologue of red clover necrotic mosaic virus (RCNMV) movement protein and modifies PD SEL (Xoconostle-Cázares et al., 1999). It can also bind RNA nonspecifically and can enable RNA delivery into SE. Interaction between NCAPP1 and CmPP16–1 requires N-acetyl-glycosylation, phosphorylation and other post-translational modifications of NCAPP1. Function was elegantly demonstrated by fusing a key 36-amino-acid stretch with the normally cell autonomous glutathione S-transferase (GST), which was sufficient to confer mobility. Similarly, Lee et al. (2003) generated a mutant form of NCAPP1 lacking the transmembrane domain. Tobacco plants expressing this dominant negative NCAPP1 lost the ability to translocate PP16 across PD, but the trafficking of another noncell autonomous protein, KN1, was unimpaired. For pumpkin phloem protein, a further example of requirement for protein processing is CmPP36, which includes an N-terminal membrane targeting domain which must be cleaved for delivery into the phloem stream (Xoconostle-Cázares et al., 2000). Interestingly, the processed protein has RNA-binding properties, but does not appear to have a role in long-distance RNA transport.

One further role for mobile proteins is to act as selective binding partners to convey bioactive ligands. Lipid transfer proteins,
present in rice xylem sap and Arabidopsis phloem (Aki et al., 2008; Guellete et al., 2012), may have properties that assist the transport of hormones, such as jasmonic acid or other oxylipins (Maldonado et al., 2002; Benning et al., 2012), although specific signalling ligands have not yet been assigned to these proteins.

VI. Phloem signalling: specific examples

1. mRNA systemic signals

To date, there is limited direct evidence for the translation of mRNA delivered to distant sinks, which would necessarily occur after retrieval of transcripts from SE back into CC and beyond. Two of the clearest examples of long-distance signalling by mRNA are effects on tuberization induced by the movement of the BEL5 transcript in potato (Banerjee et al., 2006) and effects on leaf phenotype as a result of the movement of mutant forms of GAI transcripts (Haywood et al., 2005). In the latter case, post-phloem movement of mRNA into SAM tissue was demonstrated. Intriguingly, BEL5 protein acts through interaction with KNOTTED-like proteins, such as POTH1 (Hannapel, 2010), and POTH1 mRNA, at least when over-expressed from a transgene, is also graft transmissible (Mahajan et al., 2012). A further demonstration was based on a transcriptional fusion between tomato mutant LeKn2 (KNOTTED homologue) and PFP genes. The Kn2 mutation Me confers a distinctive mouse-ear leaf phenotype and alters leaf development across a graft union as a result of mRNA transport in the phloem (Kim et al., 2001). However, caution is needed because PFP, involved in carbohydrate metabolism, has its own mRNA transport properties (David-Schwartz et al., 2008), and thus KNOTTED transcripts, in effect, may hitch a ride. Indeed, no graft-transmissible phenotype resulted from nonfusion versions of KNOTTED-like transcripts (Lifschitz & Eshed, 2006).

Selective phloem movement of mRNA may depend partly on UTR sequences at both the 5′ and 3′ ends (Banerjee et al., 2006, 2009). For potato BEL5 and POTH1, specific UTR sequences enable association as RNP complexes with RNA-binding polypyrimidine tract-binding (PTB) protein and RBP domain proteins (Banerjee et al., 2009; Mahajan et al., 2012). Likewise, GAI transport is associated with a PTB interaction (Huang & Yu, 2009). The movement of FT mRNA, discussed further below, instead may depend on regions within the coding sequence (Li et al., 2009).

2. Small RNA signalling

Since the original demonstrations of long-distance spread of transgene-dependent gene silencing to most parts of the plant (Palaquí et al., 1997; Voinnet & Baulcombe, 1997), it is now known that several forms of sRNA represent graft-transmissible signals, and molecular mechanisms at source and destination have been described that cause either post-transcriptional gene silencing (PTGS) or transcriptional gene silencing (TGS). Here, the distinct systemic roles of miRNA and short-interfering RNA (siRNA) are discussed.
recent breakthroughs directly showed graft transmission of siRNA of various size classes from 21 to 24 nucleotides (Dunoyer et al., 2010; Molnar et al., 2010). A silencing donor derived from an inverted repeat of the N-terminal ‘GF’ portion of a GFP gene resulted in PTGS in receiver tissues, deduced to be a result of the accumulation of mainly GF siRNA sequences, but also some secondary siRNA (Molnar et al., 2010). Grafting of mutants shows that the transmission of 24-nucleotide siRNAs requires DCL3 and is responsible for epigenetic TGS effects on endogenous loci through DNA methylation (Bai et al., 2011; Melnyk et al., 2011). Endogenous siRNAs are encoded by inverted repeat sequences throughout the genome, many of which do not correspond to genes. However, some target sequences were detected from tryptophan biosynthesis genes and suppressive DNA methylation was seen at transposable element loci (Molnar et al., 2010).

Contrary to normal source–sink relationships, there are reports of graft transmission of silencing in Arabidopsis from nonleaf rootstocks to scions. Brosnan et al. (2007) expressed similar GFP-derived GF hairpin sequences, leading to the accumulation of GF siRNA in rootstocks which silenced GFP targets in grafted scions. However, unlike the findings from shoot-to-root transmission described earlier, no GF siRNA was detected in scions. Instead, 21-nucleotide secondary siRNAs, corresponding to the downstream ‘P’ region of the GFP gene, accumulated. Knocking out DICER-like genes (DCL2, DCL3 and DCL4), responsible for dsRNA processing into siRNAs in silencing source roots, greatly reduced the siRNA content, but did not impede the transmission of silencing (Brosnan et al., 2007). Three main conclusions can be drawn: first, the mobile silencing signal here is probably not the primary siRNA; second, secondary siRNAs are generated in the target tissue, generally from sequences lying 3′ to the original siRNA; and third, the transmission of silencing can occur from root to shoot, opposite to the normal direction of phloem flow. An explanation for this last puzzling finding comes from similar Arabidopsis grafting approaches, showing the transmission of signalling via nonvascular routes (Liang et al., 2012). A transitivity mechanism appears most likely, based on the slow spread of silencing, the requirement for the presence of the target along the transmission path and the dependence on RDR6 (Brosnan et al., 2007; Liang et al., 2012). In addition, direct exchange of cellular components, including genetic materials, may occur across the graft junction, as proposed for tobacco (Stegemann & Bock, 2009).

3. Phloem protein signals

Although phloem sap contains hundreds of proteins, to date only one, the flowering protein FT and its homologues, has been unequivocally shown to act as a long-distance signal. Current evidence is summarized here.

Systemic flowering control: breakthrough florigen discovery

Resurgent interest in long-distance developmental signals has focused particularly on the regulation of flowering time. In photoperiodic signalling, there is separation of day length perception in leaves from developmental output in the SAM, deduced from grafting experiments in which induced shoots caused flowering in uninduced graft partners. Most classic experiments were on physiologically amenable species, especially those in which flowering could readily be induced by one or a few inductive photoperiod cycles, for example, Glycine, Hyoscyamus, Nicotiana, Perilla, Pharbitis, Sinapis and Xanthium (Bernier, 1988). However, with the exception of soybean (Schmutz et al., 2010), none of these have fully sequenced genomes or extensive collections of flowering time mutants.

Genetic and molecular analysis, especially in Arabidopsis, led to the proposal of four distinct flowering time pathways: long day, vernalization, gibberellin and autonomous. Despite predictions of phloem-mobile florigen signals, the spatial dimension was not incorporated into early molecular genetic models. Combining grafting physiology with molecular biology and genetics has now provided major breakthroughs. In Arabidopsis, a CONSTANS (CO) donor rescues late flowering of grafted co mutants (An et al., 2004; Ayre & Turgeon, 2004). Because CO is central to the photoperiod pathway, it was concluded that either a direct gene product of CO, or another signal regulated by CO, was probably moving from leaf to SAM.

Several lines of evidence subsequently revealed that the protein product of FLOWERING LOCUS T (FT), a target of CO, is a mobile signal transmitted in the phloem that can act as the long-
sought florigen hormone (Fig. 3). Restricting FT expression to phloem or to the SAM was equally effective at inducing flowering (Corbesier et al., 2007), and wild-type donor shoots of pea could rescue late flowering of a grafted Pisum mutant (Hecht et al., 2011). FT proteins are present in the phloem sap of Cucurbita, Brassica and rice (Giavalisco et al., 2006; Lin et al., 2007; Aki et al., 2008), consistent with FT and some FT fusion proteins being transmitted across graft unions (Corbesier et al., 2007; Lin et al., 2007; Notaguchi et al., 2008). By contrast, immobilized versions of FT are florigen is inactive when expressed in the leaf (Jaeger & Wigge, 2007; Mathieu et al., 2007), although such fusions were still able to affect local transcription (Corbesier et al., 2007), demonstrating that FT mobility is essential for its florigenic activity.

Nevertheless, some remaining questions include the limited rescue of Arabidopsis ft mutants grafted to wild-type shoots (Turnbull & Justin, 2004; Notaguchi et al., 2008), and the complete lack of response in equivalent experiments in tomato (Lifschitz et al., 2006). Although FT-GFP and FT-myc accumulate in and around the subapical phloem endings (Corbesier et al., 2007; Jaeger & Wigge, 2007), this domain is spatially distinct from the predominant site of expression of the FT target, FD, in the SAM dome proper, leading to the transcriptional activation of early floral development genes (Abe et al., 2005; Wigge et al., 2005; Turck et al., 2008). The distance between phloem terminations and SAM presumably needs to be traversed by incoming FT molecules, yet movement out of the immediate phloem domain appears to be restricted (Corbesier et al., 2007; Jaeger & Wigge, 2007). Intriguingly, however, the antagonistic FT homologue TERMINAL FLOWER1 (TFL1) moves noncell autonomously within this domain as protein but not mRNA (Conti & Bradley, 2007). Given the very few structural changes required to interconvert FT and TFL1 protein activities (Hanzawa et al., 2005; Ahn et al., 2006), FT is likely to have similar local mobility to TFL1.

Interaction between FT and FD has recently been shown to be mediated by a 14–3–3 protein bridge in rice (Fig. 3) (Taoka et al., 2011), consistent with earlier, but previously unexplained, demonstrations of 14–3–3 binding to FT and TFL1 (Pruehl et al., 2001; Abe et al., 2005). Evidence for direct FD binding by FT and TFL1 in yeast two-hybrid screens (Pruehl et al., 2001; Wigge et al., 2005) is most probably accounted for by yeast 14–3–3 proteins being able to substitute for plant homologues in such assays.

In addition to the movement of FT protein, it remains possible that FT mRNA may be mobile and thus could convey signalling functions. Although an earlier report of the translocation of FT mRNA in Arabidopsis was retracted, more recent evidence indicates the movement of nontranslatable fusion sequences between parts of FT and a GFP gene (Li et al., 2009, 2011; Jackson & Hong, 2012), movement of fusion and mutant transcripts of FT and graft transmission of native transcripts of the TFL1 homologue ATC (Lu et al., 2012; Huang et al., 2012). Although only a small change in flowering time is seen in atc mutants, systemic transmission of ATC mRNA represents the first direct evidence of a mobile floral inhibitor. However, convincing counter-evidence, using highly sensitive nested PCR, failed to detect the movement of any transcripts of SFT, the tomato FT orthologue, across a graft union (Lifschitz et al., 2006), and similar negative results were found in Arabidopsis (Corbesier et al., 2007; Jaeger & Wigge, 2007). The role of RNA transmission in the florigen story remains controversial. A key challenge is to demonstrate whether native wild-type FT transcripts are systemically transmitted, and whether such transport leads to altered flowering time.

Nonphotoperiodic flowering pathways also involve elements of long-distance macromolecule signalling. For example, the FLC-dependent vernalization pathway in Arabidopsis operates at more than one location (Searle et al., 2006). In the meristem, FLC protein acts as a local repressive regulator, partly through binding to the promoters of both FD and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1). By contrast, in the phloem, FLC protein binds to the first intron of FT, acting to repress the systemic FT signal (Searle et al., 2006). There is also physiological evidence for systemic floral repressors (Lang et al., 1977) for which ATC, mentioned above, now represents a prime candidate (Huang et al., 2012).

In maize, INDETERMINATE 1 (ID1), a Zn finger DNA-binding protein expressed in leaves, acts systemically to regulate flowering time (Colasanti et al., 1998; Wong & Colasanti, 2007). However, the connection of ID1 to the CO/FT module is not clear: transcriptomics revealed no altered expression of CO or FT homologues in id1 mutant plants (Coneva et al., 2007). In rice, however, an ID1 homologue regulates FT in a photoperiod-independent manner, perhaps via autonomous pathway systemic signalling (Park et al., 2008). Also contrary to maize, the rice early heading date 2 (ehd2) mutant displays significantly lower expression of Hd1 (OsCO), Hd3a (OsFT) and RFT1 (FT-like) genes (Matsubara et al., 2008), possibly reflecting the photoperiodic sensitivity of rice and insensitivity of maize.

VII. Conclusions and prospects

The importance of macromolecules as long-distance signals is now very evident, with many classes of molecules participating in a wide range of biological functions. These range from full-size RNA, such as GAI mRNA, and proteins, such as FT, to siRNA, miRNA and peptides. With the accessibility of high-throughput transcriptomics and high-coverage proteomics, there is great potential to generate comparative databases for mobile protein and RNA in a wider range of model genotypes and crop species.

The increasing lists of biological examples point to the potential for the design of many other macromolecules to be delivered noncell autonomously, including via phloem long-distance transport. These might be deployed to increase resistance to pests and diseases, or to tolerate to abiotic stresses, or to modify development. Direct visualization of macromolecule movement in vivo still presents some challenges. Although genetically encoded proteins, such as GFP, are widely used, their substantial molecular mass inevitably impacts on transport properties. For RNA, although synthetic oligonucleotides can be fluorescently labelled, this is not yet possible in planta for specific genetically expressed sequences. The development of new selective tagging tools for live cell imaging will therefore enable new insights into the origins, movement pathways and destinations of macromolecule signals.
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