Advances in Plant Biology 3

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Short and Long Distance Signaling

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ISBN 978-1-4419-1531-3 e-ISBN 978-1-4419-1532-0
DOI 10.1007/978-1-4419-1532-0
Springer New York Dordrecht Heidelberg London

Library of Congress Control Number: 2011941799

Foreword

In plants, and more specifically in angiosperms, the combination of plasmodesmata and the phloem allows for the establishment of local and long-distance pathways for the delivery of nutrients (sugars, amino acids, essential minerals) and information molecules, both large and small. It has long been appreciated that plasmodesmata, the intercellular organelles that form cytoplasmic bridges between neighboring cells, play important roles in coordinating biochemical and physiological events, at the tissue and organ level. This is achieved by controlling the cell-to-cell diffusion of metabolites by adjustments in both the physical properties of these cytoplasmic channels as well as their density within specific regions of adjoining cell walls. The ability of plasmodesmata to engage in the trafficking of information macromolecules is a relatively recent discovery. However, the impact of this discovery is now working its way through all areas of research from plant development and evolution to plant pathogen interactions.

In this book, the first four chapters deal with the role of plasmodesmata in the cell-to-cell movement of proteins and RNA. The first chapter outlines the pioneering studies conducted on plant viruses. These experiments provided the first insights into the capacity of plasmodesmata to mediate in the cell-to-cell movement of virally encoded proteins that served to spread the infectious agent into neighboring cells and tissues. The discovery that plasmodesmata, like the nuclear pore complex, could engage in selective exchange of proteins and complexes of proteins and RNA/DNA led to the concept of supracellular control over physiological and developmental events. The second chapter builds on this notion by examining the role of plasmodesmata during the process of embryogenesis.

The important discovery that certain plant transcription factors function as non-cell-autonomous regulators of gene regulatory networks is covered in the third and fourth chapters. Here, the important question of how these proteins engage the cellular machinery to move beyond their cellular site of synthesis is addressed. The availability of annotated genomes for an ever expanding number of plant species, spread across the algae, non-vascular and vascular plants, is opening the door to studies that can probe the evolutionary events that led to the emergence of cell-autonomous and non-cell-autonomous transcription factors. Such insights should

afford plant biologists important tools for engineering regulatory networks that could lead to the development of agricultural crops having elite/unique morphological and/or physiological traits.

The second half of the book covers the emerging area of local and long-distance trafficking of both small RNA (short-interfering [si]- and micro [mi]-RNA) and mRNA species. This is a most exciting area of study, as it is now well known that the various forms of si/miRNA can serve to regulate an ever expanding array of developmental and physiological processes. In Chaper 5, roles for these mobile si/miRNAs are discussed in terms of local metabolic and stress response regulators, as well as in the control over such processes as gametogenesis, leaf and root development. The aspect of phloem si/miRNA delivery to distantly located tissues and organs is covered in both Chapters 5 and 6. Roles are discussed for these small RNA species in terms of epigenetic control over a broad spectrum of processes, ranging from systemic resistance to viral infection to mobile miRNA involvement in root development and nutrient stress signaling.

The discovery that phloem delivery of full length poly-adenylated mRNA into developing leaves can lead to an alteration in their overall morphology opened up a new vista in terms of our understanding of the mechanisms likely utilized by plants to coordinate developmental events at the whole-plant level. In the last chapter, the mechanisms underlying the entry and translocation of this class of long-distance-acting mRNA are discussed. The phloem translocation stream contains a significant number of RNA binding proteins and the challenge ahead will be to unravel the complex sets of codes that have evolved to allow for the assembly of specific protein-mRNA complexes that are stable enough to enter and move through the phloem to defined target cells/tissues within sink organs.

To fully understand the functions of plasmodesmata and the phloem, as critical signaling pathways, will require that we identify the proteins that comprise the supramolecular complex of the plasmodesma. Although many proteins will likely be held in common, plasmodesmal composition will likely vary depending on the location of the specific cellular interfaces within the body of the plant. As with solving any complex puzzle, identifying a few essential components often leads to rapid progress. We hope that recent progress on the isolation and characterization of a number of plasmodesmal proteins will likewise lead to rapid advances in this field of study. In any event, this book and the opportunity for pioneering discoveries in the field of cell-to-cell and long-distance signaling should certainly entice talented young scholars to join this frontier area of plant biology.

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Contents

Chapter 1
Viral Studies Point the Way: Mechanisms of Intercellular Transport

Eduardo Peña, Annette Niehl, and Manfred Heinlein

1 Introduction

Communication through PD entails the controlled cell-to-cell and systemic trafficking of a whole range of RNA and protein macromolecules, including non-cell-autonomous transcription factors, RNA-silencing signals, small RNAs, and messenger RNAs (Ishiwatari et al. 1998; Tzfira et al. 2000; Lucas et al. 2001; Haywood et al. 2002; Heinlein 2002, 2005; Wu et al. 2002; Heinlein and Epel 2004; Yoo et al. 2004; Dunoyer et al. 2005, 2010a, b; Huang et al. 2005; Kim 2005; Kurata et al. 2005; Kehr and Buhtz 2008; Lucas et al. 2009; Carlsbecker et al. 2010; Chitwood et al. 2009; Van Norman et al. 2011). Plant viruses use PD for cell-to-cell movement (Fig. 1.1) and systemic infection of their hosts and therefore represent excellent keys to the molecular mechanisms that govern these processes. Plant viruses encode a set of proteins required for their replication, intercellular movement, silencing suppression, and encapsidation. Pioneering studies in the 80s using *Tobacco mosaic virus* (TMV) led to the discovery of a dedicated virus-encoded movement protein (MP) required for cell-to-cell movement of the virus (Deom et al. 1987; Meshi et al. 1987). Further studies demonstrated that this protein accumulates in PD and increases their size exclusion limit (SEL) (Tomenius et al. 1987; Wolf et al. 1989; Atkins et al. 1991; Ding et al. 1992b; Moore et al. 1992; Oparka et al.

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F. Kragler and M. Hülskamp (eds.), *Short and Long Distance Signaling*,
Advances in Plant Biology 3, DOI 10.1007/978-1-4419-1532-0_1,

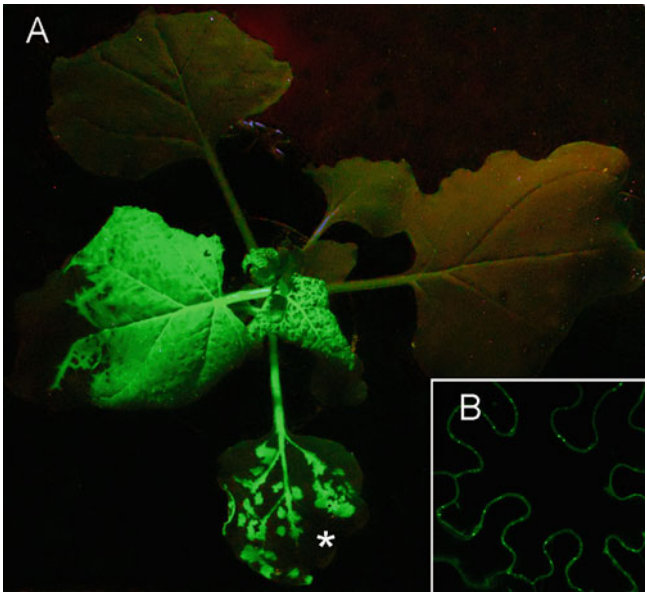

Fig. 1.1 TMV spread and the localization of MP to PD (**a**) GFP-tagged TMV infection in *Nicotiana benthamiana*. After local inoculation of one leaf (labelled with an asterisk) infection spreads cell-to-cell within the inoculated leaf and systemically into other leaves. (**b**) In infected cells, MP targets PD. The figure shows an epidermal cell in which PD are labelled with MP:GFP

1997; Heinlein et al. 1998b). Moreover, the MP was shown to bind single-stranded nucleic acids in a sequence-independent manner *in vitro* (Citovsky et al. 1990) and to form thin and elongated protein:nucleic acid complexes (Citovsky et al. 1992). These seminal findings led to a first model of virus movement proposing that the MP binds viral RNA to form a viral ribonucleoprotein (vRNP) complex that in size and structure is compatible with movement through the MP-modified PD (Citovsky et al. 1990, 1992). Subsequently, it was demonstrated that the MP is capable to mediate its own intercellular trafficking (Waigmann and Zambryski 1995; Kotlizky et al. 2001) indicating that MP itself, thus without the need for other viral proteins and infection, has the capacity to target, modify and spread through PD. Moreover, microinjected MP was capable of mediating the transport of co-injected nucleic acids (Waigmann et al. 1994) confirming that MP forms a complex with RNA *in vivo*. The formation of vRNPs was also supported by biochemical studies (Dorokhov et al. 1983, 1984; Karpova et al. 1997) as well as by elegant microinjection experiments indicating that MP functions *in vivo* as a *cis* mediator of plasmodesmal transport, requiring its physical association with the transported molecule (Waigmann and Zambryski 1995). Today, we know that most, if not all, plant viruses encode one or more MPs able to directly or indirectly modify the SEL of PD and to facilitate the spread of infection through these pores. The following paragraphs will describe the cellular targets and mechanisms by which MPs facilitate virus transport with the aim to extract general principles underlying macromolecular trafficking mechanisms through PD.

2 Mechanisms in Virus Transport

2.1 Movement as Virus Particle or vRNP Complex

The example of TMV illustrates that viruses may move cell-to-cell in the form of a vRNP. However, some viruses move through PD in virion rather than in non-virion form. Movement of virions of some specific virus species is associated with the formation of a specialized tubular transport structure assembled by MP inside the PD channel (Wellink et al. 1993; Kasteel et al. 1996). These spectacular tubules extend from the infected cell far into the cytoplasm of the non-infected cell and have been observed to be loaded with virion particles. Examples for viruses employing such tubule-guided transport of virion particles can be found among several ssRNA viruses (i.e., como-, nepo-, olea-, alfamo-, bromo-, and trichoviruses (van Lent et al. 1991; Wieczorek and Sanfacon 1993; Ritzenthaler et al. 1995; van der Wel et al. 1998; Grieco et al. 1999)), ssDNA viruses (i.e. tospoviruses; (Storms et al. 1995)), dsDNA viruses (i.e. caulimoviruses; (Kitajima et al. 1969); (Perbal et al. 1993)), and badnaviruses (Cheng et al. 1998). Nevertheless, tobamoviruses like TMV as well as viruses belonging to the diantho-, beny-, tobra-, tombus-, and hordeiviruses families move cell-to-cell in a non-virion form in a process that is independent of tubule formation. TMV is the prototype virus exemplifying this type of movement. The MP of this virus does not produce any major changes in PD structure except for fibrous material that can be labeled with anti-MP antibodies in MP-transgenic plants (Atkins et al. 1991; Ding et al. 1992b). Interestingly, MP-containing fibers have also been observed in intercellular junctions of cyanobacteria transformed with MP (Heinlein et al. 1998a; Heinlein 2006). This may suggest that the micro-PD in cyanobacteria (Giddings and Staehelin 1978) and the PD in plants share common features. However, whether the fibrous MP material has a role in increasing the SEL of PD or in active viral transport through the pore or whether this material may represent a passive fraction of MP trapped and protected within the channel cavities in continuously MP-expressing plants remains to be seen.

2.2 Requirement of Accessory Viral Proteins

Virus movement usually depends on other viral proteins in addition to MP. These include replication proteins allowing the virus to multiply in each infected cell, effector proteins to suppress host defense responses (e.g. RNA silencing), and coat proteins to protect the viral genome by encapsidation. Several RNA and DNA viruses also depend on more than one MP for virus movement. In this case, these proteins act together in forming the transport complex of the virus and targeting it to and through PD. Examples of RNA viruses encoding more than one MP are the icosahedral carmoviruses and the rod-shaped hordei- and potexviruses. These viruses encode two or three specialized MPs, referred to as double gene block

(DGB) and triple gene block (TGB) proteins, respectively. Current evidence for the role of TGB proteins in cell-to-cell movement of hordei-like viruses (hordei-, pomo-, peclu-, and benyviruses) suggests that vRNP complexes comprising TGBp1 together with genomic and subgenomic RNA (Lim et al. 2008) are transported to and through PD by the interacting integral membrane proteins TGBp2 and TGBp3, which themselves do not move between cells (Morozov and Solovyev 2003; Jackson et al. 2009; Verchot-Lubicz et al. 2010). The movement of potexviruses and, presumably, of other viruses with potex-like TGBs, depends on the CP in addition to the TGB MPs (Chapman et al. 1992; Foster et al. 1992; Sit and AbouHaidir 1993). However, it is unclear whether potexviruses move in the form of virions or rather in a non-encapsidated form. The TGBp1-containing vRNPs in potexvirus infections may contain CP (Lough et al. 1998, 2000) or may associate with CP to form virions for transport to and through PD (Santa Cruz et al. 1998). Several studies suggest that the CP may be required for efficient virus movement in cases in which the MP alone may not be sufficiently able to form stable transport complexes. This view is supported, for example, by the movement of *Cucumber mosaic bromovirus* (CMV) that can be rendered CP-independent by specific mutations in MP that increase the stability of the vRNP (Nagano et al. 2001; Andreev et al. 2004; Kim et al. 2004). Thus, strongly RNA-binding MPs like the MP of TMV could make the CP unnecessary, whereas weakly binding MPs, like the MP of CMV (Li and Palukaitis 1996) may necessitate CP, in order to encapsidate and protect the RNA (Lucas 2006). A similar view for the requirement of CP has evolved based on studies on hordei- and potexviruses. Here the requirement of CP for systemic movement appears to be inversely correlated with the size or complexity of the TGBp1 protein structure. Thus, potexviruses that encode rather small TGB1 proteins require CP for cell-to-cell and systemic movement whereas for the *Barley stripe mosaic hordeivirus* (BSMV) and *Poa semilatent hordeivirus* (PSLV), which encode larger TGB1 proteins, CP is dispensable. Consistently, certain hordei- or hordei-like viruses that encode again smaller TGB1 proteins, e.g. *Beet necrotic yellow vein virus* (BNYVV) and *Peanut clump virus* (PCV), also depend on CP for virus movement. The N-terminal domain missing in these smaller hordeivirus TGB1 proteins but present in the larger TGB1 proteins of other hordeiviruses has been proposed to be unfolded and to function as a chaperone that protects the viral RNA genome to allow phloem transport (Makarov et al. 2009). Collectively, these findings suggest a general role of CP in protecting viral RNA during movement and may be in agreement with the classical observation that long distance, systemic movement of TMV in tobacco is CP-dependent, whereas the local, intercellular movement in infected leaves is CP-independent (Holt and Beachy 1991). Thus, the CP of TMV performs specific functions during phloem entry or exit, or in the stabilization or protection of the viral RNA in the phloem. The CP of *Turnip crinkle carmovirus* (TCV) acts as the silencing suppressor of the virus and its ability to suppress silencing rather than to form virions supports viral RNA movement. However, virion formation is necessary to allow virus egress from the vasculature in systemic leaves. This illustrates that a CP can have multiple accessory roles to support movement (Cao et al. 2010).

Specific forms of movement requiring more viral proteins than a single MP are also indicated by studies using other virus families. For example, bipartite begomoviruses are DNA viruses that replicate in the nucleus and encode two MPs, one (BV1) required for shuttling the DNA genome out of the nucleus (BV1 is also referred to as nuclear shuttle protein, NSP) and the other (MP or BC1) for targeting the genome to PD (Sanderfoot et al. 1996). The movement of the bipartite viruses is independent of CP, indicating that these viruses can effectively move between cells in a non-virion form (Gardiner et al. 1988; Padidam et al. 1995). Microinjection studies established the ability of BC1 (MP) to move cell-to-cell and to mediate cell-to-cell movement of ss- and ds-DNA. However, the manner in which BV1 might transfer the viral DNA to BC1 for cell-to-cell spread is not understood (Rojas et al. 2005). The monopartitite begomoviruses lack a B-component encoding BC1 and BV1. Here, the CP and the V1 and/or C4 proteins are proposed as functional homologs of BV1 and BC1, respectively (Rojas et al. 2001). Thus, unlike bipartite viruses, the monopartite begomoviruses require CP for movement (Rigden et al. 1994; Noris et al. 1998; Rojas et al. 2001).

Another mode of cell-to-cell movement is shown by potyviruses, which represent the largest genus of plant RNA viruses. Microinjection studies performed with proteins encoded by *Lettuce mosaic virus* (LMV) and *Bean common mosaic necrosis virus* (BCMNV) established that the CP and HC-Pro (helper component-protease) proteins provide the classical MP functions for this virus, i.e. these proteins modify PD SEL, move cell-to-cell, and facilitate the movement of vRNA (Rojas et al. 1997). Mutations in the conserved core region of the *Tobacco etch virus* (TEV) CP abolished virion assembly and cell-to-cell movement, suggesting that potyviruses may move as virions (Dolja et al. 1994, 1995). The potyvirus CI protein is an RNA helicase essential for virus movement (Carrington et al. 1998) and forms conical deposits at or near PD that may function in the delivery and alignment of an HC-Pro-CP vRNA complex or of filamentous virions for transport through PD (Roberts et al. 1998). The localization of *Turnip mosaic potyvirus* (TuMV) CI to PD depends on another potyviral protein, P3N-PIPO (Wei et al. 2010).

Closteroviruses, such as *Beet yellows virus* (BYV), form exceptionally long virions and have very large RNA genomes. Movement of these viruses occurs in the form of virions and depends on four structural proteins and one MP. The latter is required for virus movement but is not an integral virion component. Three of the four structural components form a narrow virus tail essential for virion movement (Dolja et al. 2006). One of these tail components, the Hsp 70 homolog (HSP70h), localizes to PD in a myosin VIII-dependent manner and might be involved in targeting the virion to PD (Avisar et al. 2008a). In addition HSP70h might use its ATPase function to translocate the virus through the pore (Peremyslov et al. 1999).

The *Groundnut rosette umbravirus* (GRV) does not encode a CP and thus moves cell-to-cell in a non-encapsidated form. The MP of this virus interacts with PD and facilitates the transport of homologous and heterologous vRNAs through PD. However, systemic movement and transmission depends on additional mechanisms that involve the formation of specific vRNPs by the viral ORF3 protein. To form

vRNPs the ORF3 protein targets the host cell nucleolus to recruit the nucleolar protein fibrillarin. Moreover, since the virus lacks CP, virion formation and aphid-mediated inter-plant transmission require the CP of a helper luteovirus. Thus, it appears that GRV recruits a nucleolar protein and a helper virus to functionally complement the lack of a CP (Kim et al. 2007). An analogous requirement of nuclear factor for systemic movement may be represented by *Potato mop-top pomovirus* (PMTV). Similar to GRV, this virus does not require CP for long distance movement. However, the systemic spread of the virus appears to correlate with the ability of the TGB1 protein of this virus to enter the nucleolus. Thus, as in the case of GRV, also PMTV may depend on the recruitment of nuclear or nucleolar host proteins for entry into the phloem vasculature to achieve systemic plant infection (Wright et al. 2010). These examples illustrate that intercellular short-distance and systemic, phloem-mediated, long-distance communication mechanisms may depend on different interacting factors.

2.3 Viral Modification of Plasmodesmata

The basic structure of PD consists of a plasma membrane-lined pore with endoplasmic reticulum (ER) membranes running through its center (Ding et al. 1992a; Overall 1999; Maule 2008). Both the plasma membrane and the ER are continuous through the pore and thus establish membrane continuity between adjacent cells. The two leaflets of the ER are appressed within PD thus forming a central membrane structure referred to as the desmotubule. The cytoplasmic space between the desmotubule and the plasma membrane is known as the cytoplasmic annulus and may represent the major pathway for intercellular transport of water-soluble solutes and macromolecules between cells. The ER and the ER lumen represent alternative transport routes as is supported by observed intercellular movement of injected or expressed membrane probes (Cantrill et al. 1999; Martens et al. 2006; Guenoune-Gelbart et al. 2008) and the ability of plant ER membranes to transport associated macromolecules by lateral diffusion (Sparkes et al. 2009; Griffing 2010). Moreover, the desmotubule may not be a fixed structure but able to dilate in response to factors such as virus infection (Guenoune-Gelbart et al. 2008; Epel 2009; Barton et al. 2011). During development, PD undergo structural modifications leading to their transformation from simple to branched (Burch-Smith and Zambryski 2010; Ehlers and van Bel 2010; Burch-Smith et al. 2011), as observed during the sink-to-source transition (Oparka et al. 1999). PD are formed during cytokinesis (primary PD), thus when new primary cell walls are laid down between daughter cells. Additional PD may form across existing walls (secondary PD) of cells that are not clonally related, for example to connect the epidermal cell layer with the underlying cells of the leaf (Burch-Smith et al. 2011). Once established, PD regulate their SEL and thereby control communication between the cells they connect. In some cases, tissue differentiation may necessitate the degradation of established PD to permanently isolate cells from surrounding cells as, for example, in the case of stomatal guard cells (Wille and Lucas 1984).

The mechanism by which the PD SEL is regulated is intensely studied. One potential mechanism is the reversible deposition of callose, a β-1,3-glucan (Northcote et al. 1989). The role of callose in PD regulation is substantiated by the recent isolation of PD proteins associated with callose synthesis and breakdown (Levy et al. 2007; Simpson et al. 2009; Guseman et al. 2010). The SEL of PD may also be controlled by actin and myosin. Specific antibody staining demonstrated the presence of actin and myosin antigenicity in PD of algae (Blackman and Overall 1998) and higher plants (White et al. 1994; Reichelt et al. 1999; Golomb et al. 2008) and a role of the actin cytoskeleton in controlling PD permeability has been demonstrated by functional studies employing specific inhibitors. Actin-depolymerizing agents were found to increase PD aperture (White et al. 1994; Ding et al. 1996), whereas an inhibitor of actin-myosin led to constriction of PD (Radford and White 1998). Recent studies in Tradescantia (spiderwort) suggest that the SEL of PD is increased when myosin detaches from actin, as induced by BDM (2,3-butanedione monoxime), and is decreased when myosin attaches to actin, as induced by NEM (N-ethylmaleimide) (Radford and White 2011). PD are also associated with pectin methylesterase (Morvan et al. 1998) that may act to modify the SEL through changing the composition of the cell wall around PD. The SEL of PD also responds to calcium levels in the cytoplasm (Tucker 1990; Tucker and Boss 1996; Holdaway-Clarke et al. 2000), which is consistent with the presence of calcium-interacting proteins at PD, such as centrin (Blackman et al. 1999), calreticulin (Baluska et al. 1999), and a calcium-dependent kinase (Yaholom et al. 1998). Recently, proteomic studies in Arabidopsis led to the identification of several new PD protein candidates (Fernandez-Calvino et al. 2011). Further analysis of these candidates has potential to provide seminal new insights into the mechanisms that regulate the SEL of PD.

The molecular mechanism by which virus particles or viral RNP complexes are transported through the PD pore into the adjacent cell is not yet understood. However, given the small diameter of PD, structural modification of either the PD channel or the viral particles appears necessary. The formation of a tubule inside PD to facilitate cell-to-cell movement of tubule-forming viruses (van Lent et al. 1991; Storms et al. 1995) has already been mentioned. This tubule replaces the desmotubule, thus breaking the ER continuity between cells. The mechanism that drives the transport of the viral particles through the tubule is not known. Since only static images of tubules have been published it may be that the tubule acts as a container and that the whole virion-laden tubule is transported from one cell to the next. However, considering evidence that the MP of tubule forming viruses has affinity for CP and assembled capsids and that the MP domain required for virion transport is located at the inside surface of the tubule (van Lent et al. 1991; Thomas and Maule 1995; Lekkerkerker et al. 1996; Belin et al. 1999; Liu et al. 2001; Carvalho et al. 2003; Chowdhury and Savithri 2011), one may also consider a dynamic process resembling microtubule treadmilling. Thus, MP-virion complexes may co-assemble at the tubule base in the infected cell and continuous polymerization of the complex would then drive transport of the complex through the length of the tubule by treadmilling. Disassembly of the complex at the distal end of the tubule would finally release the virions into the new cell. Tubule formation seems indeed to be polar with tubule growth occurring through assembly of MP at the tubule base (Hofmann, et al., manuscript in preparation). Moreover, tubule

formation involves the interaction of MP with PDLPs, proteins of a PD-localized protein family. These proteins may act as PD-localized, cellular docking receptors for tubule assembly by MP (Amari et al. 2010).

In contrast to forming a new transport structure within the channel, the MPs of non-tubule forming viruses rather act through alignment of a transport complex near the PD channel opening (i.e. in the case of potyviruses) or by manipulating the SEL of PD. Several endogenous plant proteins are able to modify the PD SEL and to move cell-to-cell similar as, for example, TMV MP (Lucas et al. 2009; Van Norman et al. 2011). Thus, it appears likely that the MPs of non-tubule-forming viruses evolved the capacity to interact with plant endogenous mechanisms of intercellular transport. One potential mechanism by which MPs and other non-cell-autonomous factors may alter the SEL of PD may be by causing a change in local Ca^{2+} levels, which is known to affect PD SEL and is consistent with a calcium-dependent kinase and other calcium-binding proteins associated with PD (Baron-Epel et al. 1988; Tucker 1990; Lew 1994; Tucker and Boss 1996; Yaholom et al. 1998; Baluska et al. 1999; Holdaway-Clarke et al. 2000). MPs could also cause a local depletion of ATP (some MPs were shown to bind nucleotides) that is known to cause dilation of PD (Tucker 1993; Cleland et al. 1994). More recent studies indicate that the MPs of CMV and TMV have F-actin severing activity and that this activity is required to increase the PD SEL (Su et al. 2010). The authors demonstrated that the stabilization of actin by microinjection of phalloidin inhibits the ability of the MP to increase the SEL of PD, indicating that actin depolymerization is important for this activity. Moreover, in contrast to wild type, a mutant CMV MP lacking actin severing ability *in vitro* did not increase PD SEL upon microinjection into epidermal cells *in vivo*. These results provide compelling evidence for a role of the actin cytoskeleton in controlling the SEL of PD. Nevertheless, further studies are needed to demonstrate that the implied reorganization of the actin cytoskeleton leading to the SEL increase indeed occurs inside PD and is directly responsible for changing PD permeability.

MPs may dilate PD also through degradation of callose deposits at PD. Several studies showed a positive correlation of the efficiency of virus spread with the expression level of the callose-degrading enzyme β-1,3-glucanase (Iglesias and Meins 2000; Bucher et al. 2001). Moreover, the PVX TGBp2 protein interacts with host proteins that, in turn, interact with this enzyme (Fridborg et al. 2003). Recent studies indicate that the MP of TMV reduces the stress-induced deposition of PD-associated callose caused by the presence of a TMV replicon expressing only replicase (Guenoune-Gelbart et al. 2008). Reduced levels of PD-associated callose in the presence of both MP and replicase were correlated with facilitated cell-to-cell diffusion of ER-membrane-intrinsic and luminal proteins. The authors proposed that MP and replicase caused a reduction in the level of PD associated callose possibly through recruitment of β-1,3-glucanase to the pore (Epel 2009; Zavaliev et al. 2011). Another recent study revealed that the MP of TMV interacts with an ankyrin repeat-containing protein (ANK) that facilitates virus movement when overexpressed, whereas virus movement is reduced when ANK is suppressed. Importantly, expression of both MP and ANK causes reduced callose levels at PD

thus suggesting that ANK may be part of the pathway through which MP causes the degradation of callose to facilitate TMV movement (Ueki et al. 2010).

Virus movement through PD may also involve specific chaperones. A number of proteins that may function as chaperones in macromolecular transport through PD are localized to the cell-to-cell transport pathway (Aoki et al. 2002; Chen et al. 2005; Lucas 2006). A role of chaperones in cell-to-cell transport processes is indicated by a study suggesting that transport through PD may involve protein unfolding (Kragler et al. 1998) and by the observation that cells at the leading front of infection undergo a transient induction of Hsp70 expression (Havelda and Maule 2000; Whitham et al. 2003). Moreover, the closterovirus BYV expresses a Hsp70 homologue required for movement. As already mentioned, this protein is thought to facilitate virus movement by binding to a PD receptor as well as to the tail domain of the viral capsid, and to translocate the virus through PD by mechanical force (Peremyslov et al. 1999; Alzhanova et al. 2001). A requirement of structural changes in vRNPs for translocation through PD may be indicated also by a potential role of helicase activity in virus movement. Evidence is provided by a role of the helicase domain of the TMV replicase in TMV movement (Hirashima and Watanabe 2001, 2003), which may unwind the viral RNA for entering the PD pore. Helicase activity is also a feature of hordei- and potexvirus TGBp1 proteins (Kalinina et al. 2002; Makarov et al. 2009).

TMV movement as well as the intercellular movement of other non-cell-autonomous proteins (NCAPs) may depend on interaction with specific, potentially PD-localized, cellular receptor proteins. Tobacco NON-CELL-AUTONOMOUS PROTEIN PATHWAY 1 (NtNCAPP1) interacts with the MP of TMV and also with several other NCAPs, and the presence of a mutant NtNCAPP1 interfered with the capacity of TMV MP to increase the PD SEL (Lee et al. 2003; Taoka et al. 2007).

2.4 Viral Targeting of PD

Most plant RNA viruses replicate in association with membranes (Sanfacon 2005), particularly with endoplasmic reticulum (ER) membranes. Since the plant ER is highly dynamic (Sparkes et al. 2009; Griffing 2010), allows the trafficking of associated small molecules and protein complexes by lateral diffusion (Baron-Epel et al. 1988; Grabski et al. 1993; Martens et al. 2006; Runions et al. 2006; Guenoune-Gelbart et al. 2008), and is continuous between cells through the desmotubule (Ding et al. 1992a), MP and vRNA/virions could reach PD and move cell-to-cell by transport along the membrane. Consistent with this model, the MPs of many plant viruses are associated with the ER. The MP of TMV localizes to the ER shortly after synthesis (Heinlein et al. 1998b; Sambade et al. 2008) and the efficiency by which the protein is targeted to PD is reduced upon disruption of the ER-actin network (Wright et al. 2007). During viral replication, the MP localizes to distinct viral replication complexes (VRCs) formed on ER membranes (Heinlein et al. 1998b; Más and Beachy 1999). These complexes are associated with actin filaments (Liu et al. 2005;

Hofmann et al. 2009) and anchored to microtubules (Heinlein et al. 1998b). At later stages of infection, the VRCs produce CP that appears to have a role in controlling VRC size (Asurmendi et al. 2004) and is likely used for virion formation, whereas at early stages of infection the VRCs or VRC sub-complexes use their association with the ER for transport via actin/mysoin-supported lateral diffusion in the ER membrane or lumen to reach PD and to infect new cells (Kawakami et al. 2004; Guenoune-Gelbart et al. 2008; Sambade et al. 2008; Hofmann et al. 2009; Sambade and Heinlein 2009). The ER tightly interacts with actin filaments and associated motor proteins (Boevink et al. 1998; Sparkes et al. 2009; Ueda et al. 2010) and multiple evidence for a role of actin and myosins in the trafficking of various viral proteins or in the spread of infection by various viruses has been reported (Kawakami et al. 2004; Haupt et al. 2005; Prokhnevsky et al. 2005; Avisar et al. 2008a; Harries et al. 2009b, c; Hofmann et al. 2009). Consistent with a role of the ER-associated actomyosin system, TMV movement as well as motor-dependent Golgi movements can be inhibited by over-expression of actin-binding protein. This inhibition is actin-dependent, indicating that the over-expressed actin-binding protein causes obstruction of ER-embedded, motor-mediated cargo trafficking along the filament (Hofmann et al. 2009). However, TMV trafficking continues in the absence of intact actin filaments (Hofmann et al. 2009), which is consistent with the notion that the membrane itself may provide sufficient fluidity for the transport of macromolecular complexes (Sparkes et al. 2009; Griffing 2010). However, long-term (3 days and more) treatments for inhibition of actin filaments or myosins reduced the movement of several viruses tested, including TMV (Harries et al. 2009c). These findings indicate that the ER-associated acto-myosin network contributes to the efficiency or directionality of ER-mediated MP/viral RNP diffusion along the membrane.

In addition to associations with the ER/actin network, tobamoviral and also potexviral MPs have the capacity to interact with microtubules (Padgett et al. 1996; Boyko et al. 2000b; Ashby et al. 2006; Ferralli et al. 2006; Wright et al. 2010). During early stages of TMV infection, when only low levels of MP are present, microtubules appear to guide the cytoplasmic movements of mobile, MP-associated particles. These particles may represent small VRCs or VRC subcomplexes. Later on, in cells behind the leading front of infection, particle movements are inhibited and microtubules sequester overabundant MP (Boyko et al. 2007; Curin et al. 2007; Sambade et al. 2008). High-level expression and sequestration of MP by microtubules are features that are dispensable for MP function since cells behind the leading front of infection do not directly participate in movement and, indeed, much lower levels of MP are fully sufficient for the spread of infection (Arce-Johnson et al. 1995; Heinlein et al. 1998b; Szécsi et al. 1999). Interestingly, a TMV mutant exhibiting decreased accumulation of its MP along microtubules showed increased cell-to-cell movement and decreased degradation compared with the wild-type virus (Gillespie et al. 2002). Thus, accumulated MP sequestered by microtubules during late stages of infection may finally enter a degradation pathway (Padgett et al. 1996; Gillespie et al. 2002; Curin et al. 2007; Ruggenthaler et al. 2009), which is also consistent with the MP:GFP accumulation and localization pattern within infection

sites (Padgett et al. 1996; Heinlein et al. 1998b). However, although MP appears to be ubiquitinylated and degraded by the 26S proteasome in infected cells (Reichel and Beachy 2000), microtubule-associated MP is free of detectable ubiquitinylation (Ashby et al. 2006), thus indicating that the accumulation of MP on microtubules during infection and subsequent MP degradation may be unlinked processes. Consistent with the known association of protein degradation processes with the ER (Meusser et al. 2005), we recently found a MP-associated host protein belonging to a protein family involved in ER maintenance and ER-associated protein degradation (ERAD) (Niehl et al., manuscript in preparation).

The ability of MP to associate with microtubules and to form mobile particles in infection front cells is tightly correlated with functionality of MP in the spread of infection (Boyko et al. 2000b, 2007). Consistently, tobacco mutants affected in the dynamic behavior of microtubules show reduced efficiency to support TMV movement (Ouko et al. 2010). The ability of MP to interact with microtubules during infection may be important for the assembly and transport of the vRNP rather than for the targeting of MP to PD since microtubule binding-deficient MP retained the ability to target PD in infected cells (Boyko et al. 2000b, c, 2007). Transient expression experiments confirmed the formation of mobile MP particles similar to those formed during virus infection. Further studies revealed that the particles undergo stop-and-go movements along the ER, always pausing when in contact with underlying microtubules. Moreover, the particle movements were dependent on dynamic microtubule behavior (Boyko et al. 2007; Sambade et al. 2008). Using the MS2 marker system to label RNA *in vivo* (Bertrand et al. 1998), it appeared that the MP particles are associated with RNA and that the RNA colocalises with MP in PD (Sambade et al. 2008). In parallel studies, distinct mobile particles were also observed upon injection of infectious and fluorescently labeled TMV RNA into *N. benthamiana* trichome cells. The injected RNA initiated infection and associated with ER membrane in a CAP-dependent manner (Christensen et al. 2009). Together these findings suggest that early stages of TMV infection involve the association of viral RNA with the ER and the subsequent formation of mobile, ER-associated, vRNP particles that move via ER supported by the actin cytoskeleton in a manner controlled and guided by underlying microtubule contact sites through the cytoplasm and to PD. These particles may indeed represent the RNA-protein complexes that spread infection since they form when replication sites are established and TMV spreads via the ER-connected PD to non-infected neighboring cells. Moreover, as already mentioned, using conditional mutations in MP it was possible to directly correlate the formation of the particles with MP function in TMV movement (Boyko et al. 2007). A role of microtubules in these processes may also be supported by the capacity of MP to interact with GFP fused-MICROTUBULE END-BINDING PROTEIN 1 (EB1), a major integrator of microtubule dynamics and of interactions at the microtubule ends (Brandner et al. 2008).

Further studies are needed to determine the structure and composition of the MP particles. Also the mechanism by which microtubules contribute to the controlled assembly, anchorage, release, and movement of the particles deserves further studies. A major obstacle in understanding the role of microtubules in TMV movement

is the fact that the spread of TMV infection continues in the presence of microtubule inhibitors (Ashby et al. 2006). However, although this indicates that an intact microtubule cytoskeleton is not required for movement, it should be considered that components of the microtubule cytoskeleton sufficient to support the spread of virus genomes may still persist under these conditions. Indeed, microtubule inhibitors have limited effects on microtubules that are stabilized by microtubule-associated proteins (MAPs), and the MP of TMV is a particularly strongly stabilizing MAP (Ashby et al. 2006; Ferralli et al. 2006). Thus, despite the presence of inhibitors, newly infected cells at the leading front of TMV infection sites still contain numerous intact MP-labeled microtubules (Seemanpillai et al. 2006). Recent studies indicating that also the TGBp1 of *Potato mop-top pomovirus* (PMTV) interacts with microtubules (Wright et al. 2010) may underscore a widespread role of microtubules during viral infection. Although virus spread continues in the presence of microtubule inhibitors also in this case, the significance of the interaction with microtubules for the cell-to-cell movement of PMTV genomes may be revealed by further detailed studies.

As for TMV, the MPs of TGB-containing viruses such as potex- and hordeiviruses are also thought to target PD via association with the ER (Solovyev et al. 2000; Cowan et al. 2002; Gorshkova et al. 2003; Krishnamurthy et al. 2003; Ju et al. 2005; Tilsner et al. 2010). It is believed that membrane-integral TGBp3 directs the membrane protein TGBp2 and possibly soluble TGBp1-vRNA complexes to the PD pore (Morozov and Solovyev 2003; Verchot-Lubicz 2005; Verchot-Lubicz et al. 2007, 2010; Jackson et al. 2009). In contrast to the hordei-like TGBp1, which depends on TGBp2 and TGBp3 to carry it across PD, the potexvirus TGBp1 has the ability to target PD, to increase PD SEL, and to move from cell to cell when expressed on its own (Erhardt et al. 1999, 2000; Lawrence and Jackson 2001; Cowan et al. 2002; Howard et al. 2004). TGBp2 was observed to induce motile "ER-derived vesicles" (Ju et al. 2005). However, whether these vesicles play a role in the viral movement process remains to be determined. Studies on *Bamboo mosaic potexvirus* (BaMV) revealed that the TGBp2 and TGBp3 proteins of this virus form a stoichiometric protein complex localized to tubular subdomains in the cortical ER (Lee et al. 2010). In a more recent study, Wu et al. (2011) demonstrate that a hydrophobic stretch located in the cytoplasmic region of TGBp3 is required for TGBp3 oligomerization and for viral pathogenesis. The authors propose that TGBp2 forms a complex with TGBp1, CP and viral RNA and that TGBp3 targets this complex via interaction with TGBp2 to curved tubules of the cortical ER and finally to PD (Wu et al. 2011). Motile membrane-associated complexes have also been observed in association with other TGB viruses, such as pomoviruses. The PMTV TGBp2 and TGBp3 proteins occur in association with endocytic vesicles budding from the plasma membrane (Haupt et al. 2005). This observation suggests a role of endosomes in the recycling of TGB proteins upon viral RNA delivery to PD. Indeed, some MPs like the TGBp3 proteins of PMTV and PSLV contain a Tyr-based sorting motif also found in KNOLLE and other syntaxins. This motif is recognized by vesicle adapters at the plasma membrane and the ER in animals (Haupt et al. 2005) and found to be essential for PMTV movement (Tilsner et al. 2010).

The MP of TMV and also the TGB proteins are involved in the restructuring the ER network during infection. The MP of TMV associates with the ER network early in infection and induces the conversion of membrane tubules into aggregates (later proposed to represent VRCs) (Heinlein et al. 1998b). The transient formation of these aggregates in TMV-infected protoplasts correlates with the production and subsequent degradation of MP (Reichel and Beachy 1998). The ability of MP to recruit ER membranes into aggregates is maintained when the protein is ectopically expressed (Reichel and Beachy 1998). This finding is likely related to the presence of predicted transmembrane domains in MP (Brill et al. 2000; Fujiki et al. 2006) and to its behavior as an intrinsic membrane protein in cellular fractionation experiments (Reichel and Beachy 1998). The association of the MP with membranes is important but not sufficient for function in TMV movement (Fujiki et al. 2006). However, despite the importance of membrane interaction, the accumulation of MP leading to the formation of ER aggregates is dispensable (Boyko et al. 2000c) and rather solely associated with MP over-accumulation and turnover during infection. This may be different for TGB viruses for which some correlations between MP-mediated changes in ER structure and function in virus movement have been obtained. Various ER-derived structures are observed upon ectopic over-expression of TGB proteins (Solovyev et al. 2000; Cowan et al. 2002; Krishnamurthy et al. 2003; Haupt et al. 2005; Ju et al. 2005; Schepetilnikov et al. 2005; Samuels et al. 2007; Lee et al. 2010; Tilsner et al. 2010). Importantly, a specific point mutation in the PMTV TGBp3 protein (which has two trans-membrane domains) abolishes the ability of this protein to disrupt the native ER structure and also interferes with virus movement indicating that, for PMTV, membrane perturbation and virus movement are linked processes (Haupt et al. 2005; Tilsner et al. 2010). The spread of BaMV infection has been correlated with the location of TGBp3 to peripheral ER structures. However, in this case there is evidence that the TGBp3 does not shape the ER by itself. Experiments involving ectopic expression yeast cells rather indicate that the sorting signal present in this protein (see above) localizes the protein to existing ER domains associated with ER-shaping reticulon proteins Rtn1 and Yop1. This sorting signal is also required for TGBp3 localization to cortical ER structures and virus movement in plants (Lee et al. 2010; Wu et al. 2011). These observations illustrate that viral MPs may target specifically structured ER domains rather than forcing random ER membranes to which they bind into a specific structure. Whether this feature only applies to BaMV or also to other ER-interacting MPs remains to be seen. It is conceivable that in contrast to BaMV MPs, other MPs may have the ability to interact with ER reticulons (Arabidopsis has 21 reticulon homologs; Nziengui et al. 2007) to shape the ER, for example to modify the ER/desmotubule structure within PD. Indeed, the targeting to ER and the effects of MP over-expression on ER structure may reflect mechanisms by which MPs target and modify the structure of the desmotubule to allow virus movement at the leading front of infection.

Recent studies extend the range of interactions of plant viruses with membranes towards membrane rafts. Remorin, a protein associated with plasma membrane rafts and also present in PD, was shown to interact with the TGBp1 of PVX and to influence viral cell-to-cell transport. Overexpression of remorin reduced the efficiency

of virus movement whereas reduced expression enhanced the spread of the virus, suggesting that remorin binding may titrate TGBp1 available for transport (Raffaele et al. 2009). The effects of overexpressing or reducing expression of remorin may not contradict a potential role of membrane rafts in virus movement. Thus, it is conceivable that membrane raft-associated virus movement depends on a specific ratio of interaction between remorin and TGBp1. Membrane rafts could also guide PD proteins that are initially targeted to the plasma membrane by the secretory pathway to the channel. This targeting model would be consistent with the presence of GPI-anchored proteins within PD (e.g. PD callose binding protein 1, PDCB1, (Simpson et al. 2009)).

In contrast to TMV and TGB-expressing viruses, tubule-forming viruses like GFLV or CPMV do likely not use the ER to target PD since the assembly of the tubule inside the PD-pore disposes the desmotubule leading to disruption of intercellular ER membrane continuity. Instead, GFLV and CPMV appear to depend on the secretory pathway as shown by sensitivity of their PD targeting and tubule-forming capacity to the secretory pathway inhibitor Brefeldin A (Pouwels et al. 2002; Laporte et al. 2003). Recent studies demonstrate that the movement of tubule-guided viruses is promoted by a family of PD-located proteins (PDLPs) that interact with the tubule-forming MPs within PD. The PDLPs depend on the secretory pathway to reach PD thus explaining the Brefeldin A sensitivity of tubule assembly. Apparently, PDLPs act as localized MP-binding proteins that promote virus movement by catalysing tubule assembly inside PD (Thomas et al. 2008; Amari et al. 2010). Interestingly, a single transmembrane domain in PDLP is sufficient for targeting the protein to PD. Since the secretory pathway delivers proteins to the plasma membrane, it is important to determine whether the protein is delivered to PD through direct targeting of a PD-specific plasma membrane domain or via delivery to the plasma membrane and subsequent lateral diffusion in the membrane towards PD. In the latter scenario, membrane rafts could again play a role. Interestingly, when cells expressing the tubule-forming GFLV MP are treated for the disruption of the microtubule cytoskeleton, MP tubules are formed at ectopic plasma membrane sites outside PD. Since lipid rafts in plants may anchor the plasma membrane to the underlying cytoskeleton as in other systems (Bhat and Panstruga 2005; Chichili and Rodgers 2009), this finding could suggest a potential role of the plant cytoskeleton in guiding, anchoring, or restricting the movements of the lipid rafts in the membrane. Studies in protoplasts indicated that assembly of tubules by the CPMV MP is initiated at distinct punctate localizations of MP at the plasma membrane (Pouwels et al. 2004). It may be possible that these MP localizations represent membrane rafts or other sites of entrapment following MP diffusion to or within the plasma membrane. Such a membrane-based transport system seems to play a role for a number of viruses. The Brefeldin A-sensitive secretory pathway delivers viral proteins to the cellular periphery, for example *Melon necrotic spot carmovirus* (MNSV) (Genoves et al. 2010), *Potato leafroll luteovirus* (PRLV) (Vogel et al. 2007), and *Tomato spotted wilt tospovirus* (TSWV) (Ribeiro et al. 2009).

2.5 Interaction of Viral MPs with Plant Proteins

Although the above findings already indicate the role of several proteins as well as of membranes and cytoskeleton in intra-and intercellular virus movement and other macromolecular trafficking, understanding the detailed molecular mechanism of macromolecular transport will depend on the isolation and characterization of additional molecular components. Important recent advances in the isolation of PD proteins (Faulkner and Maule 2010; Fernandez-Calvino et al. 2011) will enlighten the structural composition of the channel and also have high potential for revealing proteins required for transport through the pore, as in the case for PDLP (Thomas et al. 2008; Amari et al. 2010). The isolation and characterization of MP-interacting factors is similarly promising, although these factors may be involved in functions other than PD targeting, such as viral replication and MP turnover. The list of identified MP-interacting factors is continuously growing (Table 1.1) and examples that may reflect specific viral strategies are mentioned below.

Interactions with cytokeletal proteins. The MP of TMV interacts with various host factors, including α,β-tubulin dimers (Ferralli et al. 2006), γ-tubulin (Sambade et al. 2008), assembled microtubules (Ashby et al. 2006), GFP-fused Arabidopsis EB1a (Brandner et al. 2008), and the microtubule-associated factor MPB2C (Kragler et al. 2003) that support a role of microtubules during TMV infection. As already mentioned, similar to the MP of TMV, also the pomovirus TGBp1 protein interacts with the microtubule cytoskeleton (Wright et al. 2010). There is also evidence for interaction of the putative MP (sc4) of *Sonchus yellow net rhabdovirus* (SYNV) with a microtubule-associated protein (Min et al. 2010). Moreover, interactions of viral MPs with the cytoskeleton are indicated also by the binding of the MP of TSWV to proteins resembling myosin and kinesin (van Bargen et al. 2001). Potential interactions of MP with actin remain controversial (McLean et al. 1995; Hofmann et al. 2009) and may be indirect, i.e. through association with actin-associated ER membranes. As described above the MPs of CMV and TMV have the capacity to sever actin filaments *in vitro*. Such activity exerted *in vivo* could play a role in the modification of the PD SEL (Su et al. 2010).

Interactions with secretory cargo proteins. An interaction of the MP of TMV with the secreted cell wall protein pectin-methylesterase (PME) was proposed to allow MP to target PD via the secretory pathway (Dorokhov et al. 1999; Chen et al. 2000). However, since PD targeting of the TMV MP is independent of the secretory pathway (Tagami and Watanabe 2007; Boutant et al. 2009; Amari et al. 2010) and since we could not find a colocalization of MP with PME *in vivo* (Hofmann et al. 2007), this model seems not to be correct. As mentioned above, the MPs of GFLV, and likely also the MPs of other tubule-forming viruses, find PD through targeting of PDLPs that themselves localize to PD via the secretory pathway. Since the interaction between MP and PDLP takes place at PD, the pathway that targets the MP itself to PD remains to be further investigated (Amari et al. 2010).

Table 1.1 MP-interacting proteins

Interacting protein	Virus	Virus protein	Putative function	Reference
PD and cell periphery:				
PDLP	GFLV	2B	PD docking	(Amari et al. 2010)
TIP	PVX	TGBp2	Interact with beta-glucanase, thought to modulate PD SEL	(Fridborg et al. 2003)
PME	TMV, TVCV, CaMV	MP	PD delivery, PD modification?	(Dorokhov et al. 1999; Chen et al. 2000)
Calreticulin	TMV	MP	vRNP movement	(Chen et al. 2005)
PAPK (casein kinase I)	TMV, BDMV	MP	Regulation of movement through PD by MP phosphorylation	(Lee et al. 2005)
AtP8	TCV	MP	Unknown	(Lin and Heaton 2001)
SIUPTG1 (reversibly glycosylated peptide)	TLCV	V1	Cell wall polysaccharide biosynthesis	(Selth et al. 2006)
Cytoskeleton				
Tubulin, microtubules	TMV	MP	vRNA movement	(Heinlein et al. 1995; McLean et al. 1995; Heinlein et al. 1998b; Boyko et al. 2000a, b, c, 2002, 2007; Ashby et al. 2006; Ferralli et al. 2006; Seemanpillai et al. 2006; Sambade et al. 2008)
Microtubules	PMTV	TGBp1	unknown	(Wright et al. 2010)
γ-tubulin	TMV	MP	MT nucleation	(Sambade et al. 2008)
EB1a	TMV	MP	vRNP movement	(Brandner et al. 2008)
MPB2C	TMV	MP	Regulator of MP function, sequestration of MP	(Kragler et al. 2003)
Actin	CMV, TMV	MP	Increase PD SEL, actin severing	(Su et al. 2010)
Actin	TMV	MP	vRNA movement along ER	(McLean et al. 1995)
Vesicle trafficking/ intracellular translocation:				
KNOLLE	GFLV	2B	t-SNARE, vesicle mediated targeting to cell plate	(Laporte et al. 2003)

Synaptotagmin	TMV, SqLCV, CaLCuV	MP (BC1)	Ca^{2+}-sensor, endocytic recycling	(Lewis and Lazarowitz 2010)
MP17	CaMV	MP	Vesicle-associated membrane protein	(Huang et al. 2001)
NbNACa1	BMV	3aMP	Fidelity of translocation of polypeptides, regulation of MP localisation to PD	(Kaido et al. 2007)
Chaperones:				
NtMPIP1	TMV	MP	DNAJ-like chaperone	(Shimizu et al. 2009)
NtCPIP (DNAJ-like)	PVY	CP	HSF70 recruitment	(Hofius et al. 2007)
DNAJ-like	TSWV	MP	Chaperone, regulation of HSP70	(Soellick et al. 2000)
DNAJ-like	TSWV	MP	Chaperone	(van Bargen et al. 2001)
cpHSC70-1	AbMV	MP	Chaperone	(Krenz et al. 2010)
Nucleus:				
Fibrillarin	GRV	ORF3 protein	Formation of viral transport-competent RNP particles	(Kim et al. 2007)
KELP	ToMV	MP	Transcriptional co-activator of PR-protein	(Matsushita et al. 2001)
MBF1	ToMV	MP	Transcriptional co-activator	(Matsushita et al. 2002)
NIG	CaLCuV, TGMV, TCrLYV	NSP	GTPase	(Carvalho et al. 2008)
HFi22	TBSV	P22	Leucine zipper homeodomain protein	(Desvoyes et al. 2002)
MP modification:				
CK2	PVX, ToMV	MP	MP phosphorylation	(Matsushita et al. 2003; Modena et al. 2008)
RIO kinase	ToMV, CMV	MP	Maturation of rRNAs (yeast), MP phosphorylation	(Yoshioka et al. 2004)

(continued)

Table 1.1 (continued)

Interacting protein	Virus	Virus protein	Putative function	Reference
LeNIK, GmNIK	TGMV, TCrLYV	NSP	LRR-RLK	(Santos et al. 2010; Mariano et al. 2004)
PERK (Proline-rich extensin-like receptor protein kinase)	CaLCuV	NSP	Regulation of NSP function, NSP transport	(Florentino et al. 2006)
Other:				
eIF4E	Potyviruses	VPg	Eucaryotic translation initiation factor, virus movement	(Wittmann et al. 1997; Leonard et al. 2000, 2004; Schaad et al. 2000; Robaglia and Caranta 2006)
PVIP	PSbMV	VPg	Required for virus movement, PHD finger domain	(Dunoyer et al. 2004)
2bip	CMV	2b	unknown	(Ham et al. 1999)
IP-L (Interacting protein L)	ToMV	CP	Systemic movement, similar to tobacco elicitor response protein	(Li et al. 2005)
AtNSI	CaLCuV	NSP	Acetyltransferase, facilitates transport of viral genome	(McGarry et al. 2003; Carvalho et al. 2006)
PSI-K	PPV	CI	Photosystem I component, inhibits infection	(Jimenez et al. 2006)

AbMV abutilon mosaic begomovirus, *BDMV* bean dwarf mosaic begomovirus, *BMV* brome mosaic bromovirus, *CaLCuV* cabbage leaf curl begomovirus, *CaMV* cauliflower mosaic caulimovirus, *CMV* cucumber mosaic cucumovirus, *GFLV* grapevine fanleaf nepovirus, *GRV* groundnut rosette umbravirus, *PPV* plum pox potyvirus, *PSbMV* pea seed-borne mosaic potyvirus, *PVX* potato potexvirus X, *PVY* potato potyvirus Y, *SqLCV* squash leaf curl begomovirus, *TBSV* tomato bushy stunt tombusvirus, *TCrLYV* tomato crinkle leaf yellows begomovirus, *TCV* turnip crinkle carmovirus, *TGMV* tomato golden mosaic begomovirus, *TLCV* tomato leaf curl begomovirus, *TMV* tobacco mosaic tobamovirus, *ToMV* tomato mosaic tobamovirus, *TSWV* tomato spotted wilt tospovirus, *TVCV* turnip vein clearing tobamovirus

Interactions with membrane proteins. The MPs of the begomoviruses *Cabagge leaf curl virus* (CaLCuV) and *Squash leaf curl virus* (SqLCV), and also the MP of TMV, interact with an Arabidopsis synaptotagmin (SYTA) (Lewis and Lazarowitz 2010), a calcium sensor that regulates endo-and exocytotic processes. In plant mutants deficient for SYTA, systemic virus infection is delayed and the intercellular spread of MP is inhibited, thus hinting to an important role of endo- and exocytic trafficking in virus movement (Lewis and Lazarowitz 2010). However, whether the targeting of MP to PD occurs via vesicles or whether SYTA regulates intercellular trafficking through other, more indirect and potentially calcium-dependent, mechanisms remains to be shown. Potential interactions of MPs with the vesicle trafficking machinery are also indicated by interactions of the GFLV MP with the vesicle syntaxin KNOLLE (Laporte et al. 2003) and of the CaMV MP with a Rab acceptor homolog (Huang et al. 2001). Moreover, PVX and PMTV TGBp2 proteins associate with vesicle-like structures (Haupt et al. 2005; Verchot-Lubicz et al. 2007, 2010), and the PMTV TGBp2 interacts with an RME8 homolog, a J-domain protein involved in endocytosis (Haupt et al. 2005). Recent studies indicate that the movement-related *Turnip mosaic potyvirus*-encoded CI protein interacts with the recently discovered potyviral protein P3N-PIPO and that both proteins are delivered to PD by virtue of exocytic vesicles (Wei et al. 2010).

Interactions with other NCAPs. Viruses may also interact with host NCAPs to support their own intercellular spread. The P22 MP of *Tomato bushy stunt tombusvirus* (TBSV) was shown to interact with a homeodomain protein (Desvoyes et al. 2002). Since certain homeodomain proteins act non-cell autonomously and move between cells in plants (Lucas et al. 1995; Perbal et al. 1996; Ruiz-Medrano et al. 2004; Van Norman et al. 2011), P22 may represent an example for MPs which may achieve intercellular transport through interaction with other proteins that by themselves move cell-to-cell. Another example might be the TSWV MP, which interacts with a host At-4/1 protein that moves cell-to-cell (Paape et al. 2006).

Possible interactions with extracellular matrix proteins. Protein p8, one of the two MPs required for the spread of TCV, interacted *in vitro* and in a yeast two-hybrid system with an Arabidopsis protein containing 'RGD' sequences. RGD sequences are usually carried by extracellular matrix proteins and mediate cellular adhesion through interaction with cellular integrins. Integrin-like proteins and plasma membrane RGD binding sites have been described in plants (Canut et al. 1998; Faik et al. 1998; Laval et al. 1999; Nagpal and Quatrano 1999; Sun et al. 2000; Senchou et al. 2004).

Interactions with chaperones. Virus movement through the narrow pore of PD may depend on chaperones that assist in the unfolding and refolding of proteins or in the disassembly and reassembly of macromolecular complexes. As already mentioned, a role of chaperones in cell-to-cell transport processes is indicated by a study suggesting that transport through PD may indeed involve protein unfolding (Kragler et al. 1998) and by the observation that cells at the leading front of infection undergo a transient induction of Hsp70 expression (Havelda and Maule 2000; Whitham et al. 2003). Moreover, as also already mentioned, BYV movement involves a virus-encoded Hsp 70 homolog (HSP70h) that localizes to PD in a myosin VIII dependent

manner and might be involved in targeting the virion to PD (Avisar et al. 2008a). The HSP70h protein has also been proposed to use its ATPase function to translocate the virus through the pore (Peremyslov et al. 1999). Unlike for closteroviruses, movement of other viruses may depend on interaction of their MP with cellular chaperones. Consistently, there is evidence for interactions of a number of MPs with HSF-like chaperones and J-protein co-factors (Soellick et al. 2000; van Bargen et al. 2001; Hofius et al. 2007; Shimizu et al. 2009; Krenz et al. 2010).

Interaction with nuclear factors. Plant viruses may interact with nuclear factors to facilitate movement. The case of GRV that recruits a nucleolar protein and a helper virus to functionally complement the lack of a CP in virus systemic movement (Kim et al. 2007) has already been mentioned. In several additional cases a role of the interaction of a viral MP with the nucleus or nuclear factors has been described. Thus, systemic movement of PMTV correlates with the ability of its TGB1 protein to enter the nucleolus (Wright et al. 2010). Some viral MPs interact with transcriptional activators (Matsushita et al. 2001; Matsushita et al. 2002). This suggests that MPs may facilitate the spread of infection through manipulating the transcriptional program of the host cells.

2.6 Regulation of Virus Movement

Although certain virus:host interactions can have adverse effects to plant development leading to disease symptoms and crop losses, a virus should evolve means to protect its host against damage caused by infection. Since PD gating by virus-encoded MP affects intercellular communication, the activity of viral MPs is controlled at several levels. For example, the expression of the MP of TMV is restricted to early stages of infection (Watanabe et al. 1984; Blum et al. 1989) and, when the protein has performed its function and allowed viral RNA to move into non-infected cells at the infection front, the protein is degraded by the 26S proteasome (Reichel and Beachy 2000). Cessation of MP expression and degradation of remaining MP in cells behind the leading edge of TMV infection sites leads to a ring-shaped fluorescence pattern if MP is fused to fluorescent proteins (Heinlein et al. 1995, 1998b; Padgett et al. 1996; Szécsi et al. 1999; Hofmann et al. 2009). Moreover, the ability of MP to gate PD in TMV infection is restricted to the leading edge even though the PD of cells in the centers of infection sites remain labeled with MP:GFP (Oparka et al. 1997). Thus, MP appears to be rendered non-functional for PD gating after the virus has spread. It shall be reminded here that also the ability of MP to form mobile particles is restricted to cells at the leading front. Behind the leading front, MP may still be associated with particles; however, these are rendered immobile (Boyko et al. 2007). MP activity may be controlled at the level of post-translational modification since the protein is phosphorylated at multiple sites *in vivo* (Watanabe et al. 1992; Citovsky et al. 1993; Haley et al. 1995; Kawakami et al. 1999; Waigmann et al. 2000). Cell-wall enriched protein fractions appear to contain a number of calcium-dependent and calcium-independent protein kinases able to phosphorylate

MP (Citovsky et al. 1993; Karpova et al. 1997, 1999; Yaholom et al. 1998; Waigmann et al. 2000; Lee et al. 2005). A cell wall-associated kinase (Citovsky et al. 1993) was shown to reduce the ability of MP to dilate PD (Waigmann et al. 2000; Trutnyeva et al. 2005) suggesting that the cell-wall-associated kinase may be localized at PD. A 34 kDa protein belonging to the casein kinase I family was indeed shown to colocalize with MP in PD and to phosphorylate C-terminal residues in MP previously implemented in contributing to the control of the PD gating activity (Lee et al. 2005). However, the role of a PD localized kinase requires further investigation since another study indicates that the majority of MP molecules undergo C-terminal phosphorylation on ER membranes before reaching PD (Tyulkina et al. 2010). MP activity is also controlled at the level of the vRNP. Thus, MP appears to repress the translation of vRNA in *in vitro* MP–vRNA complexes (Karpova et al. 1997) when in non-phosphorylated form. However, when MP is phosphorylated, the vRNA is translatable (Karpova et al. 1999). Therefore, by inactivating the PD gating capacity of MP and by rendering vRNA translatable, phosphorylation of MP during passage of the vRNP complex through PD may serve as a crucial molecular switch from vRNA transport to vRNA translation/replication (Rhee et al. 2000). In addition to the MP of TMV, several other viral MPs have been shown to be phosphorylated during the infection process or *in vitro* (Sokolova et al. 1997; Kawakami et al. 1999; Matsushita et al. 2000; Yoshioka et al. 2004; Florentino et al. 2006; Akamatsu et al. 2007; Modena et al. 2008; Kleinow et al. 2009).

Other mechanisms of controlling MP activity may include sequestration by binding proteins. Overexpression of the MP-interacting factors calreticulin and MPB2C inhibited virus movement (Kragler et al. 2003; Chen et al. 2005). This inhibition was correlated with increased accumulation of MP on microtubules, which may reflect removal of MP from other sites. Alternatively, overexpression of calreticulin or MPB2C may have created a downstream bottleneck in the pathway that targets vRNPs to PD, thus leading to overaccumulation of MP at upstream sites. The same sites, for example microtubules, accumulate MP when the MP itself is highly expressed (Heinlein et al. 1998b).

MP activity and virus movement are also controlled by the specific expression levels of MP proteins and host factors that must interact with each other during this process. For example, hordei-, potex-, and potyvirus movement and PD-targeting depend on the expression levels of different movement-related proteins relative to each other (Lim et al. 2008; Tilsner et al. 2010; Wei et al. 2010). The efficiency of virus movement might be restricted by limiting host factors essential for the movement process. Thus, virus movement may be facilitated or limited depending on the balance between movement-associated viral and/or cellular proteins.

Virus movement is, of course, also controlled by plant defense mechanisms. As mentioned, one mechanism is the occlusion of PD by callose deposition to interfere with virus trafficking (Iglesias and Meins 2000; Bucher et al. 2001; Guenoune-Gelbart et al. 2008; Epel 2009; Ueki et al. 2010; Lee and Lu 2011). Several PD-associated host proteins, including PD-callose binding proteins (PDCBs) (Simpson et al. 2009) and reversibly glycosylated proteins (RGPs; (Sagi et al. 2005)) may be involved in recruiting and stabilizing callose depositions at PD.

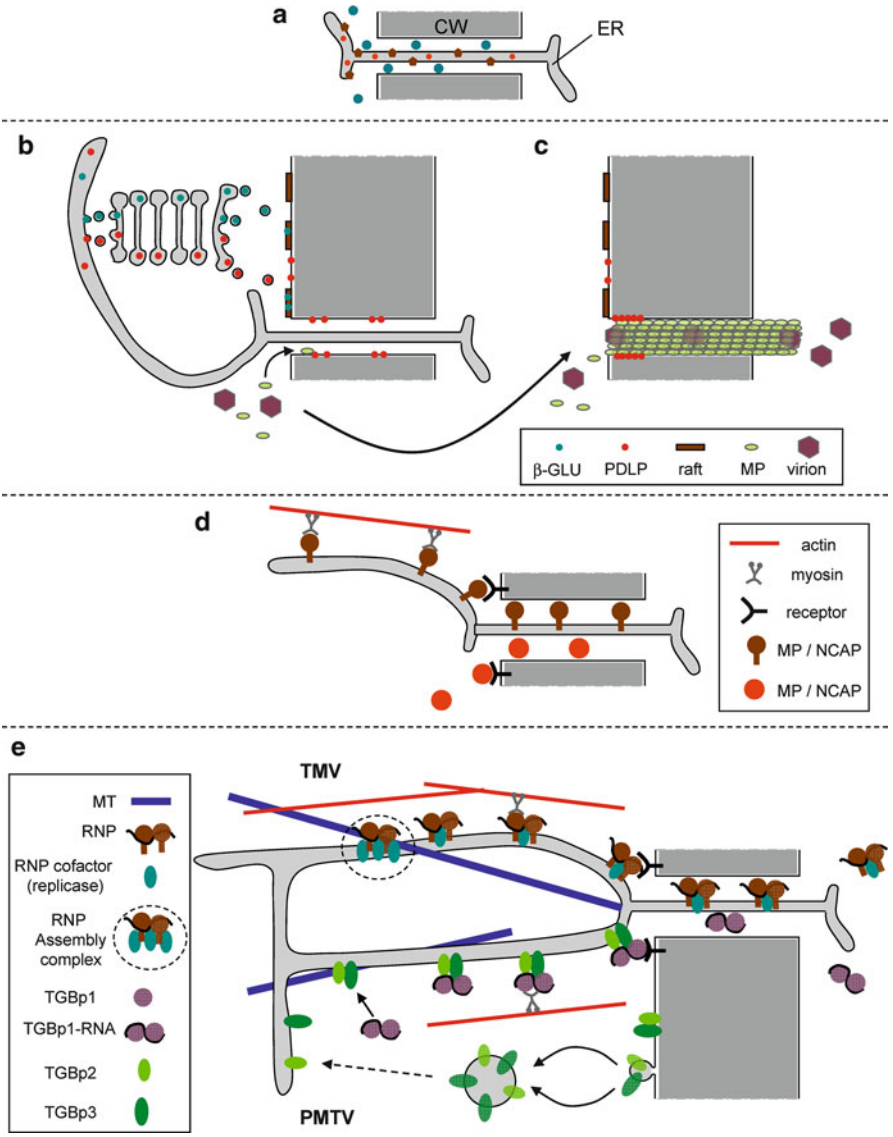

Fig. 1.2 Models of macromolecular targeting and movement through PD. (**a**) Macromolecular movement through the PD channel can occur by diffusion or facilitated transport (evidence provided by the presence of actin and myosin in PD) via the cytoplasmic annulus (cytoplasmic proteins, blue dots), the ER membrane (ER-associated proteins, brown dots), or the ER lumen (ER luminal proteins, orange dots). In the case of non-targeted movement, the ability of the macromolecules to move between cells is restricted by the given PD SEL. (**b**) Macromolecules may be secreted to the plasma membrane via the secretory pathway and then transported to PD along the membrane. β-1,3-glucanase (β-GLU, blue dots) is a GPI-anchored protein that once at the plasma membrane may be targeted to PD by lipid rafts. Another example is PDLP (red dots), which targets PD by virtue of a specific trans-membrane domain (TMD). This protein may diffuse

Virus movement is also controlled by antiviral RNA silencing (Bayne et al. 2005; Ding and Voinnet 2007). To sustain their reproduction and spreading efficiency viruses have evolved mechanisms to suppress the systemic RNA-silencing system (Wu et al. 2010; see also Chap. 5, Bernie Carrol). Viruses also induce the expression and/or recruit enzymes to degrade callose and thus allow their movement through PD (Fridborg et al. 2003; Epel 2009; Ueki et al. 2010; Zavaliev et al. 2011).

3 General Principles of Macromolecular Transport

Fluorescent recovery after photobleaching (FRAP) analysis established that molecules as large as 500 kDa can diffuse relatively freely within the cytoplasm (Seksek et al. 1997; Luby-Phelps 2000). Thus, non-targeted proteins with no specific intracellular location such as GFP may find PD by chance and move cell-to-cell by diffusion if the SEL of PD is sufficient to allow this movement, as for example during embrogenesis (Kim et al. 2005a) or in sink tissues of developing leaves (Oparka et al. 1999). Using transient transfection by bombardment, it has been shown that native GFP can move several cells away from its source. Non-targeted diffusion of proteins through PD may occur via the cytoplasmic annulus, the ER membrane or the ER lumen (Guenoune-Gelbart et al. 2008; Barton et al. 2011) (Fig. 1.2a) and

Fig. 1.2 (continued) in the membrane to target a specific plasma membrane domain at or inside PD. PDLP acts as a receptor for tubule-forming viral MPs (arrow). These MPs assemble into tubules inside PD (replacing the desmotubule) to create highly modified channels for virion movement (**c**). (**d**) PD-targeted proteins (viral MPs, NCAPs) interact with a component of the intercellular trafficking pathway (for example, a PD-localized receptor complex, as shown). Interaction leads to an increase in PD SEL (e.g. through activation of β-glucanase activity to degrade PD-associated callose). MP/NCAPs may target the receptor in association with the ER (potentially facilitated by actin and myosin) for movement along the desmotubule (brown dots) or in the cytoplasm for movement along the cytoplasmic annulus (orange dots). (**e**) Targeted RNA transport requires additional mechanisms. A model based on TMV movement is shown in the upper part of the figure. Here, RNA particles are assembled at microtubule (MT)-associated ER sites before they are released for transport along the ER with support by ER-associated actin and myosin. The role of actin and myosin may be direct (RNP as myosin cargo, as shown) or indirect (RNP may be transported as a consequence of myosin-driven macromolecular bulk flow in the membrane). At PD, the RNP-associated MP/NCAP interacts with the PD-localized mechanism (potentially receptor-mediated, as shown) to increase SEL and for continued movement along the desmotubule. A model based on pomo/hordeivirus movement is shown at the lower part of the figure. In contrast to the TMV-based model, the RNP (TGBp1-RNA) binds to a mobile carrier complex (TGBp2/TGBp3) in the ER for delivery to PD. Whereas the RNP interacts with the mechanism to modify PD SEL and continues its movement along the PD channel into the adjacent cell, the carrier complex remains in the donor cell and is recycled via endosomes back to the ER. Pomovirus TGBp1 binds to microtubules (MT), but the role of microtubule binding is not yet known. In analogy to the TMV model, one may speculate that MTs anchor the ER-located carrier complex for association with the RNP and thus allow maturation of the mobile RNA particle. ER-mediated movement of the RNP may again be directly or indirectly facilitated by ER-associated actin and myosin

largely depends on the probability of attaining PD which, for example, may be higher with ER-associated proteins. Factors allowing or limiting diffusion via PD could be found also in the electrochemical gradient between cells, parameters that restrict diffusion inside the channel (e.g. hydrophobicity, charge, and Stoke's radius of the transported molecule, size of cytoplasmic annulus/ER lumen, length of the channel, and potential surface interactions between the transported molecules and channel components) (Heinlein and Epel 2004). Targeted movement as in the case of viral MPs involves interaction of the molecules with pathways and mechanisms that increase the probability of attaining PD and lead to the formation of a dedicated transport structure (for example, tubule-forming viruses; Fig. 1.2c) or to an increase in PD SEL (Fig. 1.2d), thus allowing movement even if the native SEL of an infected cell would normally be limiting. Several non-cell-autonomous proteins have the ability to interact with PD and modify PD SEL similar to viral MPs. A prominent example for such targeted movement is the non-cell-autonomous movement of the maize homeobox meristem identity transcription factor KNOTTED 1 (KN1) (Lucas et al. 1995; Kim et al. 2002) (see also Chap. 4). In contrast to targeted movement of viral MPs, KN1, or other NCAPs such as the trichome fate protein CAPRICE (Kurata et al. 2005), non-targeted movement of GFP or of the Arabidopsis flower meristem identity transcription factor LEAFY (Wu et al. 2003) is not affected by deletion mutations in the protein and movement is strictly restricted by the given PD SEL of the cells. However, both targeted and non-targeted movements are governed by associations of the proteins with other factors and cellular components (Crawford and Zambryski 2000, 2001). Thus, proteins anchored to a cellular site (e.g. cytoskeleton) or host factor, or trapped in a cellular compartment such as the nucleus, are *a priori* immobile. The proteins will also not move if their PD modification domains are inhibited (e.g. by phosphorylation, as in the case of TMV MP) or, in the case of non-targeted movement, if the proteins form homo-or heteromers causing them to exceed the size compatible for movement through the PD of the given cells. For example, the cell-to-cell movement of the SHORTROOT protein in the developing Arabidopsis root is inhibited and trapped in the endodermal cell layer upon interaction with SCARECROW and the subsequent formation of a complex in the endodermal cell nucleus (Cui et al. 2007).

Although MPs and other molecules may find PD by diffusion and specific receptor-mediated docking at PD (Fig 1.2b, d), their association with various intracellular components and the presence of transport-mediating motifs suggests additional mechanisms. Also, associations and mechanisms required for PD targeting of proteins may not be sufficient for the PD targeting of RNPs. Indeed, much larger RNP complexes likely require specific compartmentation and transport mechanisms to ensure coordinated assembly and subsequent delivery to the PD pore (Fig. 1.2e). Specific requirements for the PD targeting and the spread of RNP complexes were demonstrated, for example, in studies using TMV variants carrying conditional mutations in MP. Although at non-permissive conditions the vRNP failed to spread cell-to-cell, the MP still accumulated in PD. Thus, MP targeting to PD is required but insufficient for the transport of viral RNA (Boyko et al. 2000b, 2007).

Based on the studies on viruses the following principles can be derived for targeted movement of proteins and nucleic acids through PD:

1. *Proteins that move cell-to-cell by targeted movement may interact with common components in the intercellular transport pathway.* The targeting of other viral or host proteins to PD may involve specific recruitment by a PD-localized binding protein. The MP of GFLV interacts with PDLP at PD, a reaction that appears to be common to tubule-forming viruses (Amari et al. 2010) (Fig. 1.2b). Moreover, a peptide reflecting a motif located within the N-terminus of the KN1 protein was shown to act as a powerful antagonist against the ability of the KN1 protein, the CMV MP, and other NCAPs, to potentiate an increase in PD SEL. This suggests the existence of a component in the intercellular transport pathway through PD that is common to many endogenous and viral proteins (Kragler et al. 2000). Moreover, NON-CELL AUTONOMOUS PATHWAY PROTEIN 1 (NCAPP1) was reported to bind to both viral MPs and plants NCAPs and thus may represent a key component in PD transport (Lee et al. 2003; Ruiz-Medrano et al. 2004). These examples indicate the existence of receptor complexes that mediate responses leading to an increase in PD SEL (Fig. 1.2d).

   It has been proposed that PD have features in common with nuclear pore complexes and that PD-mediated trafficking may involve specific protein motifs that function in analogy to a nuclear-localization signal (NLS) (Lee et al. 2000). Here, it appears interesting that the homeodomain of KN1 shown to be necessary and sufficient for intercellular trafficking contains an NLS (Kim et al. 2005b). Nevertheless, a general PD-localizing motif common to NCAPs has not been identified.
2. *Proteins may use different mechanisms to find the pore.* The GFLV MP may exemplify a targeted mechanism in which after diffusion in the cytoplasm an entrapment occurs at PD by interaction with a PD-localized binding partner (Amari et al. 2010). By locally concentrating the MP at PD, this interaction may function to catalyse MP tubule formation (Fig. 1.2b, c). Finding PD could also be facilitated by association with the ER (Fig. 1.2a, d). The MP of TMV contains hydrophobic domains that allow it to interact with ER membranes and likely be transported by membrane flow with support by ER-associated actin and myosin (Fig. 1.2d). A role of ER membranes in the targeting of PD is also exemplified by the pomo-, hordei- and potexvirus TGB2/3 proteins (Fig. 1.2e). Proteins may also find PD via the secretory pathway (Fig. 1.2b). Examples of proteins taking this route are PDLPs (Thomas et al. 2008; Amari et al. 2010), RGPs (Sagi et al. 2005), and β-1,3-glucanase (Levy et al. 2007). It is unknown how a secreted protein finally accumulates in PD. However, the presence of remorin in PD and the interaction of remorin with a potexvirus TGBp1 suggest a role of lipid rafts in PD targeting (Raffaele et al. 2009). Such a role of lipid rafts is supported by the notion that several of the recently identified PD proteins are glycosylphosphatidylinositol (GPI)-anchored (Faulkner and Maule 2010; Fernandez-Calvino et al. 2011), and GPI-anchored proteins represent a major class of raft-resident proteins (Brown and Rose 1992; Rao et al. 2011). Thus, in addition to PD targeting

via ER or by diffusion in the cytoplasm, proteins may find PD by delivery to the plasma membrane via the secretory pathway followed by lateral transport via lipid rafts (Fig. 1.2b). Proteins may target PD also indirectly by 'hitching-a-ride' in association with other proteins transported along these pathways. The MP of TMV may serve as an example since this protein was reported to interact with the secreted cell wall protein pectin-methylesterase (Dorokhov et al. 1999; Chen et al. 2000; Chen and Citovsky 2003). Since several MPs were reported to interact with endocytic vesicles or vesicular markers, endocytic pathways appear to contribute to the targeting and recycling of PD proteins (Fig. 1.2e).

Transport to PD likely involves direct or indirect support by the acto-myosin system (Fig. 1.2d, e). This is shown by numerous reports indicating the involvement of actin and myosin in the trafficking of viral proteins (Haupt et al. 2005; Liu et al. 2005; Prokhnevsky et al. 2005; Vogel et al. 2007; Wright et al. 2007; Avisar et al. 2008a; Harries et al. 2009a, b), viral protein-associated vesicles (Ju et al. 2005; Cotton et al. 2009), in the spread of infection (Kawakami et al. 2004; Harries et al. 2009c; Hofmann et al. 2009), in cytoplasmic streaming (Shimmen and Yokota 2004), and the movements of ER and ER-resident proteins (Runions et al. 2006; Ueda et al. 2010), and other organelles (Avisar et al. 2008b; Prokhnevsky et al. 2008). Although interactions of viral MPs with microtubules have been reported (Heinlein et al. 1995; Boyko et al. 2000b; Wright et al. 2010), microtubules contribute to the maturation and transport of larger RNPs rather than of proteins (Fig. 1.2e, see below).

3. *RNP transport involves additional mechanisms.* Whereas the above membrane and actin-supported mechanisms allow proteins to target PD, additional mechanisms are required for the transport of RNPs (Fig. 1.2e). For example, whereas peptide antagonists of the PD targeting pathway used by KN1 caused full inhibition of KN1-mediated transport of KN1-RNA complexes, the movement of the KN1 protein itself was only partially blocked (Kragler et al. 2000). Moreover, mutations in the TMV MP that interfere with its ability to transport viral RNA do not necessarily interfere with the PD targeting and intercellular transport of MP (Boyko et al. 2000b, c, 2007; Kahn et al. 1998; Vogler et al. 2008). Localization of MP particles at microtubule/ER junctions in cells at the spreading front of viral infection suggest a role of microtubules in the anchorage or positioning of replication sites and in the assembly of movement-competent vRNPs (Sambade et al. 2008; Sambade and Heinlein 2009) (Fig. 1.2e). This model is in agreement with other evidence suggesting that cortical microtubules may have a general role in the anchorage and maturation of large trafficking assemblies. For example, cortical microtubules tether specific compartments for the positioned delivery of cellulase synthase complexes to the plasma membrane (Gutierrez et al. 2009). RNA transport may also involve specific RNA folding or unfolding, as is suggested by the potential roles for RNA helicases in RNA virus movement (Carrington et al. 1998; Hirashima and Watanabe 2001, 2003; Kalinina et al. 2002; Makarov et al. 2009).
4. *NCAP transport through the PD channel may involve different mechanisms.* Similar to proteins that move-cell-to-cell via non-targeted movement, NCAPs

may pass through the PD channel by diffusion through the cytoplasmic annulus or via the desmotubule, either by lateral diffusion in the desmotubular ER membrane or through the desmotubular lumen. However, unlike for proteins using the non-targeted mechanism for movement, targeted NCAP movement involves a modification of PD SEL. Recent models depicting PD structure show the desmotubule wrapped by a helical actin filament structure (Overall and Blackman 1996; Maule 2008; Faulkner and Maule 2010). In addition to EM data, this model consists with observations indicating a role of the actin cytoskeleton in restricting the SEL of PD (Ding et al. 1996; Su et al. 2010; Radford and White 2011). Thus, to move cell-to-cell, NCAPs may be able to cause a modification in actin function within the channel. This model gains support by recent evidence indicating that the MPs of CMV and TMV sever actin filaments and that this activity is required to increase the SEL of PD (Su et al. 2010). To spread between cells, NCAPs may also cause changes in Ca^{2+} or ATP levels since both are known to influence the SEL of PD (Tucker 1990, 1993; Cleland et al. 1994; Lew 1994; Tucker and Boss 1996; Holdaway-Clarke et al. 2000). NCAPs may also be able to directly or indirectly recruit or enhance the activity of β-glucanase and thus cause the degradation of callose in the neck regions of PD, as has been proposed, for example, for the MPs of TMV (Epel 2009) and PVX (Fridborg et al. 2003). Experimental evidence suggests that NCAP trafficking through PD may involve a degree of protein unfolding (Kragler et al. 1998). This observation and the capacity of specific HSP70 chaperones to increase PD SEL (Aoki et al. 2002) suggests that the interaction of NCAPs with chaperones facilitates movement.

5. *NCAP trafficking is regulated.* Regulation of NCAP trafficking may occur by specific expression in donor cells and inhibition of further movement by entrapment in recipient cells, as has been demonstrated for SHORTROOT movement (Cui et al. 2007). As already discussed, NCAP movement may also be controlled by endocytic mechanisms that remove proteins from PD. Post-translational mechanisms may play important roles in controlling the ability of the proteins to interact with other components of the intercellular communication pathway. A role of post-translational modification in the regulation of NCAP trafficking is suggested, for example, by the role of phosphorylation in influencing the ability of the TMV MP to increase PD SEL (Waigmann and Zambryski 2000; Trutnyeva et al. 2005). Moreover, phosphorylation and glycosylation of specific amino acids were shown to affect the interaction of the *Cucurbita maxima* mobile phloem protein CmPP16 with NCAPP1 and its ability to move cell-to-cell (Taoka et al. 2007) (see also Chap. 7).

4 Conclusions and Perspectives

Studying plant virus movement has provided important knowledge about the potential cellular pathways by which macromolecules and macromolecular complexes are targeted to PD and transported through the channel. However, we are still far

away from understanding the mechanisms of macromolecular trafficking at the molecular level. Studying viruses will continue to provide important new insights, for example, through the further identification and characterization of MP-interacting proteins and their respective interaction network. Since significant progress has been made in the proteomic analysis of PD components (Faulkner and Maule 2010; Fernandez-Calvino et al. 2011) viruses may be utilized to further extend this type of analysis to the isolation and analysis of viral and non-viral complexes that target proteins and nucleic acids to the channel. However, it is clear that many more studies will be needed to decipher the molecular interactions and functional significance of identified components. Nevertheless, genetic testing of identified components and applying recently developed high-resolution fluorescent imaging techniques (Fitzgibbon et al. 2010; Bell and Oparka 2011) to investigate their *in vivo* localization and activities in association with other components, PD proteins, and cellular markers, will likely lead to major advances in this field. Moreover, in using viruses, it will be interesting to distinguish complexes and pathways involved in macromolecular movement from those that might be provoked by host defense responses. Taken together, these studies will also allow us to identify components and pathways used to transport proteins and RNAs from cell to cell that are of general importance for plant development.

Acknowledgment A.N. and E.P were supported through research grants provided by the HFSPO (RGP2006/22) and the ANR (ANR-08-BLAN-0244).

References

Akamatsu N, Takeda A, Kishimoto M, Kaido M, Okuno T, Mise K (2007) Phosphorylation and interaction of the movement and coat proteins of *Brome mosaic virus* in infected barley protoplasts. Arch Virol 152:2087–2093

Alzhanova DV, Napuli AJ, Creamer R, Dolja VV (2001) Cell-to-cell movement and assembly of a plant closterovirus: roles for the capsid proteins and Hsp70 homolog. EMBO J 20:6997–7007

Amari K, Boutant E, Hofmann C, Schmitt-Keichinger C, Fernandez-Calvino L, Didier P, Lerich A, Mutterer J, Thomas CL, Heinlein M, Mely Y, Maule AJ, Ritzenthaler C (2010) A family of plasmodesmal proteins with receptor-like properties for plant viral movement proteins. PLoS Pathog 6:e1001119

Andreev IA, Hyon Kim S, Kalinina NO, Rakitina DV, Fitzgerald AG, Palukaitis P, Taliansky ME (2004) Molecular interactions between a plant virus movement protein and RNA: force spectroscopy investigation. J Mol Biol 339:1041–1047

Aoki K, Kragler F, Xoconostle-Cazares B, Lucas WJ (2002) A subclass of plant heat shock cognate 70 chaperones carries a motif that facilitates trafficking through plasmodesmata. Proc Natl Acad Sci USA 99:16342–16347

Arce-Johnson P, Kahn TW, Reimann-Philipp U, Rivera-Bustamente R, Beachy RN (1995) The amount of movement protein produced in transgenic plants influences the establishment, local movement, and systemic spread of infection by movement protein-deficient *Tobacco mosaic virus*. Mol Plant Microbe Interact 3:415–423

Ashby J, Boutant E, Seemanpillai M, Groner A, Sambade A, Ritzenthaler C, Heinlein M (2006) *Tobacco mosaic virus* movement protein functions as a structural microtubule-associated protein. J Virol 80:8329–8344

Asurmendi S, Berg RH, Koo JC, Beachy RN (2004) Coat protein regulates formation of replication complexes during *Tobacco mosaic virus* infection. Proc Natl Acad Sci USA 101:1415–1420

Atkins D, Hull R, Wells B, Roberts K, Moore P, Beachy RN (1991) The *Tobacco mosaic virus* 30 K movement protein in transgenic tobacco plants is localized to plasmodesmata. J Gen Virol 72:209–211

Avisar D, Prokhnevsky AI, Dolja VV (2008a) Class VIII myosins are required for plasmodesmatal localization of a closterovirus Hsp70 homolog. J Virol 82:2836–2843

Avisar D, Prokhnevsky AI, Makarova KS, Koonin EV, Dolja VV (2008b) Myosin XI-K is required for rapid trafficking of golgi stacks, peroxisomes, and mitochondria in leaf cells of *Nicotiana benthamiana*. Plant Physiol 146:1098–1108

Baluska F, Samaj J, Napier R, Volkmann D (1999) Maize calreticulin localizes preferentially to plasmodesmata in root apex. Plant J 19:481–488

Baron-Epel O, Hernandez D, Jiang LW, Meiners S, Schindler M (1988) Dynamic continuity of cytoplasmic and membrane compartments between plant cells. J Cell Biol 106:715–721

Barton DA, Cole L, Collings DA, Liu DY, Smith PM, Day DA, Overall RL (2011) Cell-to-cell transport via the lumen of the endoplasmic reticulum. Plant J 66:806–817

Bayne EH, Rakitina DV, Morozov SY, Baulcombe DC (2005) Cell-to-cell movement of *Potato potexvirus X* is dependent on suppression of RNA silencing. Plant J 44:471–482

Belin C, Schmitt C, Gaire F, Walter B, Demangeat G, Pinck L (1999) The nine C-terminal residues of the *Grapevine fanleaf nepovirus* movement protein are critical for systemic virus spread. J Gen Virol 80:1347–1356

Bell K, Oparka K (2011) Imaging plasmodesmata. Protoplasma 248:9–25

Bertrand E, Chartrand P, Schaefer M, Shenoy SM, Singer RH, Long RM (1998) Localization of *ASH1* mRNA particles in living yeast. Mol Cell 2:437–445

Bhat RA, Panstruga R (2005) Lipid rafts in plants. Planta 223:5–19

Blackman LM, Overall RL (1998) Immunolocalization of the cytoskeleton to plasmodesmata of *Chara corallina*. Plant J 14:733–741

Blackman LM, Harper JDI, Overall RL (1999) Localization of a centrin-like protein to higher plant plasmodesmata. Eur J Cell Biol 78:297–304

Blum H, Gross HJ, Beier H (1989) The expression of the TMV-specific 30-kDa protein in tobacco protoplasts is strongly and selectively enhanced by actinomycin. Virology 169:51–61

Boevink P, Oparka K, Santa Cruz S, Martin B, Betteridge A, Hawes C (1998) Stacks on tracks: the plant Golgi apparatus traffics on an actin/ER network. Plant J 15:441–447

Boutant E, Fitterer C, Ritzenthaler C, Heinlein M (2009) Interaction of the *Tobacco mosaic virus* movement protein with microtubules during the cell cycle in tobacco BY-2 cells. Protoplasma 237:3–12

Boyko V, Ferralli J, Heinlein M (2000a) Cell-to-cell movement of TMV RNA is temperature-dependent and corresponds to the association of movement protein with microtubules. Plant J 22:315–325

Boyko V, Ferralli J, Ashby J, Schellenbaum P, Heinlein M (2000b) Function of microtubules in intercellular transport of plant virus RNA. Nat Cell Biol 2:826–832

Boyko V, van der Laak J, Ferralli J, Suslova E, Kwon M-O, Heinlein M (2000c) Cellular targets of functional and dysfunctional mutants of *Tobacco mosaic virus* movement protein fused to GFP. J Virol 74:11339–11346

Boyko V, Ashby JA, Suslova E, Ferralli J, Sterthaus O, Deom CM, Heinlein M (2002) Intramolecular complementing mutations in *Tobacco mosaic virus* movement protein confirm a role for microtubule association in viral RNA transport. J Virol 76:3974–3980

Boyko V, Hu Q, Seemanpillai M, Ashby J, Heinlein M (2007) Validation of microtubule-associated *Tobacco mosaic virus* RNA movement and involvement of microtubule-aligned particle trafficking. Plant J 51:589–603

Brandner K, Sambade A, Boutant E, Didier P, Mely Y, Ritzenthaler C, Heinlein M (2008) TMV movement protein interacts with GFP-tagged microtubule Endbinding Protein 1 (EB1). Plant Physiol 147:611–623

Brill LM, Nunn RS, Kahn TW, Yeager M, Beachy RN (2000) Recombinant *Tobacco mosaic virus* movement protein is an RNA-binding, α-helical membrane protein. Proc Natl Acad Sci USA 97:7112–7117

Brown DA, Rose JK (1992) Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell 68:533–544

Bucher GL, Tarina C, Heinlein M, Di Serio F, Meins F Jr, Iglesias VA (2001) Local expression of enzymatically active class 1 beta-1,3-glucanase enhances symptoms of TMV infection in tobacco. Plant J 28:361–369

Burch-Smith TM, Zambryski PC (2010) Loss of increased size exclusion limit (ISE)1 or ISE2 increases the formation of secondary plasmodesmata. Curr Biol 20:989–993

Burch-Smith TM, Stonebloom S, Xu M, Zambryski PC (2011) Plasmodesmata during development: re-examination of the importance of primary, secondary, and branched plasmodesmata structure versus function. Protoplasma 248:61–74

Cantrill LC, Overall RL, Goodwin PB (1999) Cell-to-cell communication via plant endomembranes. Cell Biol Int 23:653–661

Canut H, Carrasco A, Galaud JP, Cassan C, Bouyssou H, Vita N, Ferrara P, Pont-Lezica R (1998) High affinity RGD-binding sites at the plasma membrane of *Arabidopsis thaliana* links the cell wall. Plant J 16:63–71

Cao M, Ye X, Willie K, Lin J, Zhang X, Redinbaugh MG, Simon AE, Morris TJ, Qu F (2010) The capsid protein of *Turnip crinkle virus* overcomes two separate defense barriers to facilitate systemic movement of the virus in Arabidopsis. J Virol 84:7793–7802

Carlsbecker A, Lee JY, Roberts CJ, Dettmer J, Lehesranta S, Zhou J, Lindgren O, Moreno-Risueno MA, Vaten A, Thitamadee S, Campilho A, Sebastian J, Bowman JL, Helariutta Y, Benfey PN (2010) Cell signalling by microRNA165/6 directs gene dose-dependent root cell fate. Nature 465:316–321

Carrington JC, Jensen PE, Schaad MC (1998) Genetic evidence for an essential role for potyvirus CI protein in cell-to-cell movement. Plant J 14:393–400

Carvalho CM, Wellink J, Ribeiro SG, Goldbach RW, Van Lent JW (2003) The C-terminal region of the movement protein of *Cowpea mosaic virus* is involved in binding to the large but not to the small coat protein. J Gen Virol 84:2271–2277

Carvalho MF, Turgeon R, Lazarowitz SG (2006) The geminivirus nuclear shuttle protein NSP inhibits the activity of AtNSI, a vascular-expressed Arabidopsis acetyltransferase regulated with the sink-to-source transition. Plant Physiol 140:1317–1330

Carvalho CM, Fontenelle MR, Florentino LH, Santos AA, Zerbini FM, Fontes EP (2008) A novel nucleocytoplasmic traffic GTPase identified as a functional target of the bipartite geminivirus nuclear shuttle protein. Plant J 55:869–880

Chapman SN, Hills G, Watts J, Baulcombe DC (1992) Mutational analysis of the coat protein gene of *Potato virus X*: effects on virion morphology and viral pathogenicity. Virology 191: 223–230

Chen M-H, Citovsky V (2003) Systemic movement of a tobamovirus requires host cell pectin methylesterase. Plant J 35:386–392

Chen M-H, Sheng J, Hind G, Handa AK, Citovsky V (2000) Interaction between the *Tobacco mosaic virus* movement protein and host cell pectin methylesterases is required for viral cell-to-cell movement. EMBO J 19:913–920

Chen MH, Tian GW, Gafni Y, Citovsky V (2005) Effects of calreticulin on viral cell-to-cell movement. Plant Physiol 138:1866–1876

Cheng CP, Tzafrir I, Lockhart BE, Olszewski NE (1998) Tubules containing virions are present in plant tissues infected with *Commelina yellow mottle badnavirus*. J Gen Virol 79:925–929

Chichili GR, Rodgers W (2009) Cytoskeleton-membrane interactions in membrane raft structure. Cell Mol Life Sci 66:2319–2328

Chitwood DH, Nogueira FT, Howell MD, Montgomery TA, Carrington JC, Timmermans MC (2009) Pattern formation via small RNA mobility. Genes Dev 23:549–554

Chowdhury SR, Savithri HS (2011) Interaction of *Sesbania mosaic virus* movement protein with the coat protein – implications for viral spread. FEBS J 278:257–272

Christensen N, Tilsner J, Bell K, Hammann P, Parton R, Lacomme C, Oparka K (2009) The 5′cap of *Tobacco Mosaic Virus* (TMV) is required for virion attachment to the actin/ER network during early infection. Traffic 10:536–551

Citovsky V, Knorr D, Schuster G, Zambryski P (1990) The P30 movement protein of *Tobacco mosaic virus* is a single-stranded nucleic acid binding protein. Cell 60:637–647

Citovsky V, Wong ML, Shaw AL, Venkataram Prasad BV, Zambryski P (1992) Visualization and characterization of *Tobacco mosaic virus* movement protein binding to single-stranded nucleic acids. Plant Cell 4:397–411

Citovsky V, McLean BG, Zupan JR, Zambryski P (1993) Phosphorylation of *Tobacco mosaic virus* cell-to-cell movement protein by a developmentally regulated plant cell wall-associated protein kinase. Genes Dev 7:904–910

Cleland RE, Fujiwara T, Lucas WJ (1994) Plasmodesmal-mediated cell-to-cell transport in wheat roots is modulated by anaerobic stress. Protoplasma 178:81–85

Cotton S, Grangeon R, Thivierge K, Mathieu I, Ide C, Wei T, Wang A, Laliberte JF (2009) *Turnip mosaic virus* RNA replication complex vesicles are mobile, align with microfilaments, and are each derived from a single viral genome. J Virol 83:10460–10471

Cowan GH, Lioliopoulou F, Ziegler A, Torrance L (2002) Subcellular localization, protein interactions, and RNA binding activity of *Potato mop-top virus* triple gene block proteins. Virology 298:106–115

Crawford KM, Zambryski PC (2000) Subcellular localization determines the availability of non-targeted proteins to plasmodesmatal transport. Curr Biol 10:1032–1040

Crawford KM, Zambryski PC (2001) Non-targeted and targeted protein movement through plasmodesmata in leaves in different developmental and physiological states. Plant Physiol 125: 1802–1812

Cui H, Levesque MP, Vernoux T, Jung JW, Paquette AJ, Gallagher KL, Wang JY, Blilou I, Scheres B, Benfey PN (2007) An evolutionarily conserved mechanism delimiting SHR movement defines a single layer of endodermis in plants. Science 316:421–425

Curin M, Ojangu EL, Trutnyeva K, Ilau B, Truve E, Waigmann E (2007) MPB2C, a microtubule-associated plant factor, is required for microtubular accumulation of *Tobacco mosaic virus* movement protein in plants. Plant Physiol 143:801–811

Deom CM, Oliver MJ, Beachy RN (1987) The 30-kilodalton gene product of *Tobacco mosaic virus* potentiates virus movement. Science 237:384–389

Desvoyes B, Faure-Rabasse S, Chen MH, Park JW, Scholthof HB (2002) A novel plant homeodomain protein interacts in a functionally relevant manner with a virus movement protein. Plant Physiol 129:1521–1532

Ding SW, Voinnet O (2007) Antiviral immunity directed by small RNAs. Cell 130:413–426

Ding B, Turgeon R, Parthasarathy MV (1992a) Substructure of freeze-substituted plasmodesmata. Protoplasma 169:28–41

Ding B, Haudenshield JS, Hull RJ, Wolf S, Beachy RN, Lucas WJ (1992b) Secondary plasmodesmata are specific sites of localization of the *Tobacco mosaic virus* movement protein in transgenic tobacco plants. Plant Cell 4:915–928

Ding B, Kwon M-O, Warnberg L (1996) Evidence that actin filaments are involved in controlling the permeability of plasmodesmata in tobacco mesophyll. Plant J 10:157–164

Dolja VV, Haldeman R, Robertson NL, Dougherty WG, Carrington JC (1994) Distinct functions of capsid protein in assembly and movement of *Tobacco etch virus*. EMBO J 13:1482–1491

Dolja VV, Haldeman-Cahill R, Montgomery AE, Vandenbosch KA, Carrington JC (1995) Capsid protein determinants involved in cell-to-cell and long distance movement of *Tobacco etch potyvirus*. Virology 206:1007–1016

Dolja VV, Kreuze JF, Valkonen JPT (2006) Comparative and functional genomics of closteroviruses. Virus Res 117:38–51

Dorokhov YL, Alexandrov NM, Miroshnichenko NA, Atabekov JG (1983) Isolation and analysis of virus-specific ribonucleoprotein of *Tobacco mosaic virus*-infected tobacco. Virology 127: 237–252

Dorokhov YL, Alexandrova NM, Miroshnichenko NA, Atabekov JG (1984) The informosome-like virus-specific ribonucleoprotein (vRNP) may be involved in the transport of *Tobacco mosaic virus* infection. Virology 137:127–134

Dorokhov YL, Mäkinen K, Yu O, Merits A, Saarinen J, Kalkkinen N, Atabekov JG, Saarma M (1999) A novel function for a ubiquitous plant enzyme pectin methylesterase: the host-cell receptor for the *Tobacco mosaic virus* movement protein. FEBS Lett 461:223–228

Dunoyer P, Thomas C, Harrison S, Revers F, Maule A (2004) A cysteine-rich plant protein potentiates potyvirus movement through an interaction with the virus genome-linked protein VPg. J Virol 78:2301–2309

Dunoyer P, Himber C, Voinnet O (2005) DICER-LIKE 4 is required for RNA interference and produces the 21-nucleotide small interfering RNA component of the plant cell-to-cell silencing signal. Nat Genet 37:1356–1360

Dunoyer P, Schott G, Himber C, Meyer D, Takeda A, Carrington JC, Voinnet O (2010a) Small RNA duplexes function as mobile silencing signals between plant cells. Science 328:912–916

Dunoyer P, Brosnan CA, Schott G, Wang Y, Jay F, Alioua A, Himber C, Voinnet O (2010b) An endogenous, systemic RNAi pathway in plants. EMBO J 29:1699–1712

Ehlers K, van Bel AJ (2010) Dynamics of plasmodesmal connectivity in successive interfaces of the cambial zone. Planta 231:371–385

Epel BL (2009) Plant viruses spread by diffusion on ER-associated movement-protein-rafts through plasmodesmata gated by viral induced host beta-1,3-glucanases. Semin Cell Dev Biol 20:1074–1081

Erhardt M, Stussi-Garaud C, Guilley H, Richards KE, Jonard G, Bouzoubaa S (1999) The first triple gene block protein of *Peanut clump virus* localizes to the plasmodesmata during virus infection. Virology 264:220–229

Erhardt M, Morant M, Ritzenthaler C, Stussi-Garaud C, Guilley H, Richards K, Jonard G, Bouzoubaa S, Gilmer D (2000) P42 movement protein of *Beet necrotic yellow vein virus* is targeted by the movement proteins P13 and P15 to punctate bodies associated with plasmodesmata. Mol Plant Microbe Interact 13:520–528

Faik A, Laboure AM, Gulino D, Mandaron P, Falconet D (1998) A plant surface protein sharing structural properties with animal integrins. Eur J Biochem 253:552–559

Faulkner C, Maule A (2010) Opportunities and successes in the search for plasmodesmal proteins. Protoplasma 248:27–38

Fernandez-Calvino L, Faulkner C, Walshaw J, Saalbach G, Bayer E, Benitez-Alfonso Y, Maule A (2011) Arabidopsis plasmodesmal proteome. PLoS One 6:e18880

Ferralli J, Ashby J, Fasler M, Boyko V, Heinlein M (2006) Disruption of microtubule organization and centrosome function by expression of *Tobacco mosaic virus* movement protein. J Virol 80:5807–5821

Fitzgibbon J, Bell K, King E, Oparka K (2010) Super-resolution imaging of plasmodesmata using three-dimensional structured illumination microscopy. Plant Physiol 153:1453–1463

Florentino LH, Santos AA, Fontenelle MR, Pinheiro GL, Zerbini FM, Baracat-Pereira MC, Fontes EP (2006) A PERK-like receptor kinase interacts with the geminivirus nuclear shuttle protein and potentiates viral infection. J Virol 80:6648–6656

Foster RLS, Beck DL, Guilford PJ, Voot DM, Van Dolleweerd CJ, Andersen MT (1992) The coat protein of *White clover mosaic potexvirus* has a role in facilitating cell-to-cell transport in plants. Virology 191:480–484

Fridborg I, Grainger J, Page A, Coleman M, Findlay K, Angell S (2003) TIP, a novel host factor linking callose degradation with the cell-to-cell movement of *Potato virus X*. Mol Plant Microbe Interact 16:132–140

Fujiki M, Kawakami S, Kim RW, Beachy RN (2006) Domains of *Tobacco mosaic virus* movement protein essential for its membrane association. J Gen Virol 87:2699–2707

Gardiner WE, Sunter G, Brand L, Elmer JS, Rogers SG, Bisaro DM (1988) Genetic analysis of *Tomato golden mosaic virus*: the coat protein is not required for systemic spread or symptom development. EMBO J 7:899–904

Genoves A, Navarro JA, Pallas V (2010) The Intra- and intercellular movement of *Melon necrotic spot virus* (MNSV) depends on an active secretory pathway. Mol Plant Microbe Interact 23: 263–272

Giddings TH, Staehelin LA (1978) Plasma membrane architecture of *Anabaena cylindrica*: occurrence of microplasmodesmata and changes associated with heterocyst development and the cell cycle. Eur J Cell Biol 16:235–249

Gillespie T, Boevink P, Haupt S, Roberts AG, Toth R, Vantine T, Chapman S, Oparka KJ (2002) Functional analysis of a DNA shuffled movement protein reveals that microtubules are dispensable for the cell-to-cell movement of *Tobacco mosaic virus*. Plant Cell 14:1207–1222

Golomb L, Abu-Abied M, Belausov E, Sadot E (2008) Different subcellular localizations and functions of Arabidopsis myosin VIII. BMC Plant Biol 8:3

Gorshkova EN, Erokhina TN, Stroganova TA, Yelina NE, Zamyatin AA, Kalinina NO, Schiemann J, Solovyev AG, Morozov SY (2003) Immunodetection and fluorescence microscopy of transgenically expressed hordeivirus TGBp3 movement protein reveals its association with endoplasmic reticulum elements in close proximity to plasmodesmata. J Gen Virol 84:985–994

Grabski S, de Feijter AW, Schindler M (1993) Endoplasmic reticulum forms a dynamic continuum for lipid diffusion between contiguous soybean root cells. Plant Cell 5:25–38

Grieco F, Castellano MA, Di Sansebastiano GP, Maggipinto G, Neuhaus JM, Martelli GP (1999) Subcellular localization and *in vivo* identification of the putative movement protein of *Olive latent virus 2*. J Gen Virol 80(Pt 5):1103–1109

Griffing LR (2010) Networking in the endoplasmic reticulum. Biochem Soc Trans 38:747–753

Guenoune-Gelbart D, Elbaum M, Sagi G, Levy A, Epel BL (2008) *Tobacco mosaic virus* (TMV) replicase and movement protein function synergistically in facilitating TMV spread by lateral diffusion in the plasmodesmal desmotubule of *Nicotiana benthamiana*. Mol Plant Microbe Interact 21:335–345

Guseman JM, Lee JS, Bogenschutz NL, Peterson KM, Virata RE, Xie B, Kanaoka MM, Hong Z, Torii KU (2010) Dysregulation of cell-to-cell connectivity and stomatal patterning by loss-of-function mutation in Arabidopsis *chorus* (glucan synthase-like 8). Development 137:1731–1741

Gutierrez R, Lindeboom JJ, Paredez AR, Emons AM, Ehrhardt DW (2009) Arabidopsis cortical microtubules position cellulose synthase delivery to the plasma membrane and interact with cellulose synthase trafficking compartments. Nat Cell Biol 11:797–806

Haley A, Hunter T, Kiberstis P, Zimmern D (1995) Multiple serine phosphorylation sites on the 30 kDa TMV cell-to-cell movement protein synthesized in tobacco protoplasts. Plant J 8: 715–724

Ham B-K, Lee T-H, You JS, Nam Y-W, Kim J-K, Paek K-H (1999) Isolation of a putative tobacco host factor interacting with *Cucumber mosaic virus* 2b protein by yeast two-hybrid screening. Mol Cells 9:548–555

Harries PA, Schoelz JE, Nelson RS (2009a) Covering common ground: F-actin-dependent transport of plant viral protein inclusions reveals a novel mechanism for movement utilized by unrelated viral proteins. Plant Signal Behav 4:454–456

Harries PA, Palanichelvam K, Yu W, Schoelz JE, Nelson RS (2009b) The *Cauliflower mosaic virus* protein P6 forms motile inclusions that traffic along actin microfilaments and stabilize microtubules. Plant Physiol 149:1005–1016

Harries PA, Park JW, Sasaki N, Ballard KD, Maule AJ, Nelson RS (2009c) Differing requirements for actin and myosin by plant viruses for sustained intercellular movement. Proc Natl Acad Sci USA 106:17594–17599

Haupt S, Cowan GH, Ziegler A, Roberts AG, Oparka KJ, Torrance L (2005) Two plant-viral movement proteins traffic in the endocytic recycling pathway. Plant Cell 17:164–181

Havelda Z, Maule AJ (2000) Complex spatial responses to *Cucumber mosaic virus* infection in susceptible *Cucurbita pepo* cotyledons. Plant Cell 12:1975–1985

Haywood V, Kragler F, Lucas WJ (2002) Plasmodesmata: pathways for protein and ribonucleoprotein signaling. Plant Cell 14(Supplement):S303–S325

Heinlein M (2002) Plasmodesmata: dynamic regulation and role in macromolecular cell-to-cell signalling. Curr Opin Plant Biol 5:543–552
Heinlein M (2005) Systemic RNA silencing. In: Oparka K (ed) Plasmodesmata. Oxford, Blackwell, pp 212–240
Heinlein M (2006) TMV movement protein targets cell-cell channels in plants and prokaryotes: possible roles of tubulin- and FtsZ-based cytoskeletons. In: Baluska F, Volkmann D, Barlow PW (eds) Cell-cell channels. Landes Bioscience, Austin, pp 176–182
Heinlein M, Epel BL (2004) Macromolecular transport and signaling through plasmodesmata. Int Rev Cytol 235:93–164
Heinlein M, Epel BL, Padgett HS, Beachy RN (1995) Interaction of tobamovirus movement proteins with the plant cytoskeleton. Science 270:1983–1985
Heinlein M, Wood MR, Thiel T, Beachy RN (1998a) Targeting and modification of prokaryotic cell-cell junctions by *Tobacco mosaic virus* cell-to-cell movement protein. Plant J 14:345–351
Heinlein M, Padgett HS, Gens JS, Pickard BG, Casper SJ, Epel BL, Beachy RN (1998b) Changing patterns of localization of the *Tobacco mosaic virus* movement protein and replicase to the endoplasmic reticulum and microtubules during infection. Plant Cell 10:1107–1120
Hirashima K, Watanabe Y (2001) Tobamovirus replicase coding region is involved in cell-to-cell movement. J Virol 75:8831–8836
Hirashima K, Watanabe Y (2003) RNA helicase domain of tobamovirus replicase executes cell-to-cell movement possibly through collaboration with its nonconserved region. J Virol 77: 12357–12362
Hofius D, Maier AT, Dietrich C, Jungkunz I, Bornke F, Maiss E, Sonnewald U (2007) Capsid protein-mediated recruitment of host DnaJ-like proteins is required for *Potato virus Y* infection in tobacco plants. J Virol 81:11870–11880
Hofmann C, Sambade A, Heinlein M (2007) Plasmodesmata and intercellular transport of viral RNA. Biochem Soc Trans 35:142–145
Hofmann C, Niehl A, Sambade A, Steinmetz A, Heinlein M (2009) Inhibition of *Tobacco mosaic virus* movement by expression of an actin-binding protein. Plant Physiol 149:1810–1823
Holdaway-Clarke TL, Walker NA, Hepler PK, Overall RL (2000) Physiological elevations in cytoplasmic free calcium by cold or ion injection result in transient closure of higher plant plasmodesmata. Planta 210:329–335
Holt CA, Beachy RN (1991) *In vivo* complementation of infectious transcripts from mutant *Tobacco mosaic virus* cDNAs in transgenic plants. Virology 181:109–117
Howard AR, Heppler ML, Ju HJ, Krishnamurthy K, Payton ME, Verchot-Lubicz J (2004) *Potato virus X* TGBp1 induces plasmodesmata gating and moves between cells in several host species whereas CP moves only in *N. benthamiana* leaves. Virology 328:185–197
Huang Z, Andianov VM, Han Y, Howell SH (2001) Identification of Arabidopsis proteins that interact with the *Cauliflower mosaic virus* (CaMV) movement protein. Plant Mol Biol 47: 663–675
Huang T, Bohlenius H, Eriksson S, Parcy F, Nilsson O (2005) The mRNA of the Arabidopsis gene FT moves from leaf to shoot apex and induces flowering. Science 309:1694–1696
Iglesias VA, Meins F Jr (2000) Movement of plant viruses is delayed in a β-1,3-glucanase-deficient mutant showing a reduced plasmodesmatal size exclusion limit and enhanced callose deposition. Plant J 21:157–166
Ishiwatari Y, Fujiwara T, McFarland KC, Nemoto K, Hayashi H, Chino M, Lucas WJ (1998) Rice phloem thioredoxin h has the capacity to mediate its own cell-to-cell transport through plasmodesmata. Planta 205:12–22
Jackson AO, Lim HS, Bragg J, Ganesan U, Lee MY (2009) Hordeivirus replication, movement, and pathogenesis. Annu Rev Phytopathol 47:385–422
Jimenez I, Lopez L, Alamillo JM, Valli A, Garcia JA (2006) Identification of a *Plum pox virus* CI-interacting protein from chloroplast that has a negative effect in virus infection. Mol Plant Microbe Interact 19:350–358

Ju HJ, Samuels TD, Wang YS, Blancaflor E, Payton M, Mitra R, Krishnamurthy K, Nelson RS, Verchot-Lubicz J (2005) *The Potato virus X* TGBp2 movement protein associates with endoplasmic reticulum-derived vesicles during virus infection. Plant Physiol 138:1877–1895

Kahn TW, Lapidot M, Heinlein M, Reichel C, Cooper B, Gafny R, Beachy RN (1998) Domains of the TMV movement protein involved in subcellular localization. Plant J 15:15–25

Kaido M, Inoue Y, Takeda Y, Sugiyama K, Takeda A, Mori M, Tamai A, Meshi T, Okuno T, Mise K (2007) Downregulation of the NbNACa1 gene encoding a movement-protein-interacting protein reduces cell-to-cell movement of *Brome mosaic virus* in *Nicotiana benthamiana*. Mol Plant Microbe Interact 20:671–681

Kalinina NO, Rakitina DA, Solovyev AG, Schiemann J, Morozov SY (2002) RNA helicase activity of the plant virus movement proteins encoded by the first gene of the triple gene block. Virology 296:321–329

Karpova OV, Ivanov KI, Rodionova P, Dorokhov YL, Atabekov JG (1997) Nontranslatability and dissimilar behavior in plants and protoplasts of viral RNA and movement protein complexes formed *in vitro*. Virology 230:11–21

Karpova OV, Rodionova NP, Ivanov KI, Kozlovsky SV, Dorokhov YL, Atabekov JG (1999) Phosphorylation of *Tobacco mosaic virus* movement protein abolishes its translation repressing ability. Virology 261:20–24

Kasteel DTJ, Perbal M-C, Boyer J-C, Wellink J, Goldbach RW, Maule AJ, van Lent JWM (1996) The movement proteins of *Cowpea mosaic virus* and *Cauliflower mosaic virus* induce tubular structures in plant and insect cells. J Gen Virol 77:2857–2864

Kawakami S, Padgett HS, Hosokawa D, Okada Y, Beachy RN, Watanabe Y (1999) Phosphorylation and/or presence of serine 37 in the movement protein of *Tomato mosaic tobamovirus* is essential for intracellular localization and stability *in vivo*. J Virol 73:6831–6840

Kawakami S, Watanabe Y, Beachy RN (2004) *Tobacco mosaic virus* infection spreads cell to cell as intact replication complexes. Proc Natl Acad Sci USA 101:6291–6296

Kehr J, Buhtz A (2008) Long distance transport and movement of RNA through the phloem. J Exp Bot 59:85–92

Kim JY (2005) Regulation of short-distance transport of RNA and protein. Curr Opin Plant Biol 8:45–52

Kim JY, Yan Z, Cilia M, Khalfan-Jagani Z, Jackson D (2002) Intercellular trafficking of a *KNOTTED1* green fluorescent protein fusion in the leaf and shoot meristem of *Arabidopsis*. Proc Natl Acad Sci USA 99:4103–4108

Kim SH, Kalinina NO, Andreev I, Ryabov EV, Fitzgerald AG, Taliansky ME, Palukaitis P (2004) The C-terminal 33 amino acids of the *Cucumber mosaic virus* 3a protein affect virus movement, RNA binding and inhibition of infection and translation. J Gen Virol 85:221–230

Kim I, Cho E, Crawford K, Hempel FD, Zambryski PC (2005a) Cell-to-cell movement of GFP during embryogenesis and early seedling development in Arabidopsis. Proc Natl Acad Sci USA 102:2227–2231

Kim JY, Rim Y, Wang J, Jackson D (2005b) A novel cell-to-cell trafficking assay indicates that the KNOX homeodomain is necessary and sufficient for intercellular protein and mRNA trafficking. Genes Dev 19:788–793

Kim SH, Macfarlane S, Kalinina NO, Rakitina DV, Ryabov EV, Gillespie T, Haupt S, Brown JW, Taliansky M (2007) Interaction of a plant virus-encoded protein with the major nucleolar protein fibrillarin is required for systemic virus infection. Proc Natl Acad Sci USA 104: 11115–11120

Kitajima EW, Lauritis JA, Swift H (1969) Fine structure of zinnial leaf tissues infected with *Dahlia mosaic virus*. Virology 39:240–249

Kleinow T, Nischang M, Beck A, Kratzer U, Tanwir F, Preiss W, Kepp G, Jeske H (2009) Three C-terminal phosphorylation sites in the *Abutilon mosaic virus* movement protein affect symptom development and viral DNA accumulation. Virology 390:89–101

Kotlizky G, Katz A, van der Laak J, Boyko V, Lapidot M, Beachy RN, Heinlein M, Epel BL (2001) A dysfunctional movement protein of *Tobacco mosaic virus* interferes with targeting of wild type movement protein to microtubules. Mol Plant Microbe Interact 7:895–904

Kragler F, Monzer J, Shash K, Xoconoctle-Cazares B, Lucas WJ (1998) Cell-to-cell transport of proteins: requirement for unfolding and characterization of binding to a putative plasmodesmal receptor. Plant J 15:367–381

Kragler F, Monzer J, Xoconostle-Cazares B, Lucas WJ (2000) Peptide antagonists of the plasmodesmal macromolecular trafficking pathway. EMBO J 19:2856–2868

Kragler F, Curin M, Trutnyeva K, Gansch A, Waigmann E (2003) MPB2C, a microtubule-associated plant protein binds to and interferes with cell-to-cell transport of *Tobacco mosaic virus* movement protein. Plant Physiol 132:1870–1883

Krenz B, Windeisen V, Wege C, Jeske H, Kleinow T (2010) A plastid-targeted heat shock cognate 70 kDa protein interacts with the *Abutilon mosaic virus* movement protein. Virology 401:6–17

Krishnamurthy K, Heppler M, Mitra R, Blancaflor E, Payton M, Nelson RS, Verchot-Lubicz J (2003) *The Potato virus X* TGBp3 protein associates with the ER network for virus cell-to-cell movement. Virology 309:135–151

Kurata T, Ishida T, Kawabata-Awai C, Noguchi M, Hattori S, Sano R, Nagasaka R, Tominaga R, Koshino-Kimura Y, Kato T, Sato S, Tabata S, Okada K, Wada T (2005) Cell-to-cell movement of the CAPRICE protein in Arabidopsis root epidermal cell differentiation. Development 132:5387–5398

Laporte C, Vetter G, Loudes AM, Robinson DG, Hillmer S, Stussi-Garaud C, Ritzenthaler C (2003) Involvement of the secretory pathway and the cytoskeleton in intracellular targeting and tubule assembly of *Grapevine fanleaf virus* movement protein in tobacco BY-2 cells. Plant Cell 15:2058–2075

Laval V, Chabannes M, Carriere M, Canut H, Barre A, Rouge P, Pont-Lezica R, Galaud J (1999) A family of Arabidopsis plasma membrane receptors presenting animal beta-integrin domains. Biochim Biophys Acta 1435:61–70

Lawrence DM, Jackson AO (2001) Interactions of the TGB1 protein during cell-to-cell movement of *Barley stripe mosaic virus*. J Virol 75:8712–8723

Lee JY, Lu H (2011) Plasmodesmata: the battleground against intruders. Trends Plant Sci 16: 201–210

Lee J-Y, Yoo B-C, Lucas WJ (2000) Parallels between nuclear-pore and plasmodesmal trafficking of informational molecules. Planta 210:177–187

Lee J-Y, Yoo B-C, Rojas MR, Gomez-Ospina N, Staehelin LA, Lucas WJ (2003) Selective trafficking of non-cell-autonomous proteins mediated by NtNCAPP1. Science 299:392–396

Lee JY, Taoka K, Yoo BC, Ben-Nissan G, Kim DJ, Lucas WJ (2005) Plasmodesmal-associated protein kinase in tobacco and Arabidopsis recognizes a subset of non-cell-autonomous proteins. Plant Cell 17:2817–2831

Lee SC, Wu CH, Wang CW (2010) Traffic of a viral movement protein complex to the highly curved tubules of the cortical endoplasmic reticulum. Traffic 11:912–930

Lekkerkerker A, Wellink J, Yuan P, van Lent J, Goldbach R, van Kammen AB (1996) Distinct functional domains in the *Cowpea mosaic virus* movement protein. J Virol 70:5658–5661

Leonard S, Plante D, Wittmann S, Daigneault N, Fortin MG, Laliberte JF (2000) Complex formation between potyvirus VPg and translation eukaryotic initiation factor 4E correlates with virus infectivity. J Virol 74:7730–7737

Leonard S, Viel C, Beauchemin C, Daigneault N, Fortin MG, Laliberte JF (2004) Interaction of VPg-Pro of *Turnip mosaic virus* with the translation initiation factor 4E and the poly(A)-binding protein in planta. J Gen Virol 85:1055–1063

Levy A, Erlanger M, Rosenthal M, Epel BL (2007) A plasmodesmata-associated beta-1, 3-glucanase in Arabidopsis. Plant J 49:669–682

Lew RR (1994) Regulation of electrical coupling between Arabidopsis root hairs. Planta 193: 67–73

Lewis JD, Lazarowitz SG (2010) Arabidopsis synaptotagmin SYTA regulates endocytosis and virus movement protein cell-to-cell transport. Proc Natl Acad Sci USA 107:2491–2496

Li Q, Palukaitis P (1996) Comparison of the nucleic acid- and NTP-binding properties of the movement protein of *Cucumber mosaic cucumovirus* and *Tobacco mosaic tobamovirus*. Virology 216:71–79

Li Y, Wu MY, Song HH, Hu X, Qiu BS (2005) Identification of a tobacco protein interacting with *Tomato mosaic virus* coat protein and facilitating long-distance movement of virus. Arch Virol 150:1993–2008

Lim HS, Bragg JN, Ganesan U, Lawrence DM, Yu J, Isogai M, Hammond J, Jackson AO (2008) Triple gene block protein interactions involved in movement of *Barley stripe mosaic virus*. J Virol 82:4991–5006

Lin B, Heaton LA (2001) An *Arabidopsis thaliana* protein interacts with a movement protein of *Turnip crinkle virus* in yeast cells and *in vitro*. J Gen Virol 82:1245–1251

Liu H, Boulton MI, Oparka KJ, Davies JW (2001) Interaction of the movement and coat proteins of *Maize streak virus*: implications for the transport of viral DNA. J Gen Virol 82:35–44

Liu J-Z, Blancaflor EB, Nelson RS (2005) The *Tobacco mosaic virus* 126-kilodalton protein, a constituent of the virus replication complex, alone or within the complex aligns with and traffics along microfilaments. Plant Physiol 138:1877–1895

Lough TJ, Shash K, Xoconostle-Cazares B, Hofstra KR, Beck DL, Balmori E, Forster RL, Lucas WJ (1998) Molecular dissection of the mechanism by which potexvirus triple gene block proteins mediate cell-to-cell transport of infectious RNA. Mol Plant Microbe Interact 11:801–814

Lough TJ, Netzler NE, Emerson SJ, Sutherland P, Carr F, Beck DL, Lucas WJ, Forster RL (2000) Cell-to-cell movement of potexviruses: evidence for a ribonucleoprotein complex involving the coat protein and first triple gene block protein. Mol Plant Microbe Interact 13:962–974

Luby-Phelps K (2000) Cytoarchitecture and physical properties of cytoplasm: volume, viscosity, diffusion, intracellular surface area. Int Rev Cytol 192:189–221

Lucas WJ (2006) Plant viral movement proteins: agents for cell-to-cell trafficking of viral genomes. Virology 344:169–184

Lucas WJ, Bouche-Pillon S, Jackson DP, Nguyen L, Baker L, Ding B, Hake S (1995) Selective trafficking of KNOTTED1 homeodomain protein and its RNA through plasmodesmata. Science 270:1980–1983

Lucas WJ, Yoo B-C, Kragler F (2001) RNA as a long-distance information macromolecule in plants. Nat Rev Mol Cell Biol 2:849–857

Lucas WJ, Ham BK, Kim JY (2009) Plasmodesmata – bridging the gap between neighboring plant cells. Trends Cell Biol 19:495–503

Makarov VV, Rybakova EN, Efimov AV, Dobrov EN, Serebryakova MV, Solovyev AG, Yaminsky IV, Taliansky ME, Morozov SY, Kalinina NO (2009) Domain organization of the N-terminal portion of hordeivirus movement protein TGBp1. J Gen Virol 90:3022–3032

Mariano AC, Andrade MO, Santos AA, Carolino SM, Oliveira ML, Baracat-Pereira MC, Brommonshenkel SH, Fontes EP (2004) Identification of a novel receptor-like protein kinase that interacts with a geminivirus nuclear shuttle protein. Virology 318:24–31

Martens HJ, Roberts AG, Oparka KJ, Schulz A (2006) Quantification of plasmodesmatal endoplasmic reticulum coupling between sieve elements and companion cells using fluorescence redistribution after photobleaching. Plant Physiol 142:471–480

Más P, Beachy RN (1999) Replication of *Tobacco mosaic virus* on endoplasmic reticulum and role of the cytoskeleton and virus movement in intracellular distribution of viral RNA. J Cell Biol 147:945–958

Matsushita Y, Hanazawa K, Yoshioka K, Oguchi T, Kawakami S, Watanabe Y, Nishiguchi M, Nyunoya H (2000) *In vitro* phosphorylation of the movement protein of *Tomato mosaic tobamovirus* by a cellular kinase. J Gen Virol 81:2095–2102

Matsushita Y, Deguchi M, Youda M, Nishiguchi M, Nyunoya H (2001) The *Tomato mosaic tobamovirus* movement protein interacts with a putative transcriptional coactivator KELP. Mol Cells 12:57–66

Matsushita M, Miyakawa O, Deguchi M, Nishiguchi M, Nyunoya H (2002) Cloning of a tobacco cDNA coding for a putative transcriptional coactivator MBF1 that interacts with the *Tomato mosaic virus* movement protein. J Exp Bot 53:1531–1532

Matsushita Y, Ohshima M, Yoshioka K, Nishiguchi M, Nyunoya H (2003) The catalytic subunit of protein kinase CK2 phosphorylates *in vitro* the movement protein of T*omato mosaic virus*. J Gen Virol 84:497–505

Maule AJ (2008) Plasmodesmata: structure, function and biogenesis. Curr Opin Plant Biol 11: 680–686

McGarry RC, Barron YD, Carvalho MF, Hill JE, Gold D, Cheung E, kraus WL, Lazarowitz SG (2003) A novel Arabidopsis acetyltransferase interacts with the geminivirus movement protein NSP. Plant Cell 15:1605–1618

McLean BG, Zupan J, Zambryski PC (1995) *Tobacco mosaic virus* movement protein associates with the cytoskeleton in tobacco plants. Plant Cell 7:2101–2114

Meshi T, Watanabe Y, Saito T, Sugimoto A, Maeda T, Okada Y (1987) Function of the 30 kd protein of *Tobacco mosaic virus*: involvement in cell-to-cell movement and dispensability for replication. EMBO J 6:2557–2563

Meusser B, Hirsch C, Jarosch E, Sommer T (2005) ERAD: the long road to destruction. Nat Cell Biol 7:766–772

Min BE, Martin K, Wang RY, Tafelmeyer P, Bridges M, Goodin M (2010) A host-factor interaction and localization map for a plant-adapted rhabdovirus implicates cytoplasm-tethered transcription activators in cell-to-cell movement. Mol Plant Microbe Interact 23:1420–1432

Modena NA, Zelada AM, Conte F, Mentaberry A (2008) Phosphorylation of the TGBp1 movement protein of *Potato virus X* by a *Nicotiana tabacum* CK2-like activity. Virus Res 137:16–23

Moore P, Fenczik CA, Deom CM, Beachy RN (1992) Developmental changes in plasmodesmata in transgenic tobacco expressing the movement protein of *Tobacco mosaic virus*. Protoplasma 170:115–127

Morozov SY, Solovyev AG (2003) Triple gene block: modular design of a multifunctional machine for plant virus movement. J Gen Virol 84:1351–1366

Morvan O, Quentin M, Jauneau A, Mareck A, Morvan C (1998) Immunogold localization of pectin methylesterases in the cortical tissues of flax hypocotyl. Protoplasma 202:175–184

Nagano H, Mise K, Furusawa I, Okuno T (2001) Conversion in the requirement of coat protein in cell-to-cell movement mediated by the *Cucumber mosaic virus* movement protein. J Virol 75:8045–8053

Nagpal P, Quatrano RS (1999) Isolation and characterization of a cDNA clone from *Arabidopsis thaliana* with partial sequence similarity to integrins. Gene 230:33–40

Noris E, Vaira AM, Caciagli P, Masenga V, Gronenborn B, Accotto GP (1998) Amino acids in the capsid protein of *Tomato yellow leaf curl virus* that are crucial for systemic infection, particle formation, and insect transmission. J Virol 72:10050–10057

Northcote DH, Davey R, Lay J (1989) Use of antisera to localize callose, xylan and arabinogalactan in the cell-plate, primary and secondary walls of plant-cells. Planta 178:353–366

Nziengui H, Bouhidel K, Pillon D, Der C, Marty F, Schoefs B (2007) Reticulon-like proteins in *Arabidopsis thaliana*: structural organization and ER localization. FEBS Lett 581:3356–3362

Oparka KJ, Prior DAM, Santa Cruz S, Padgett HS, Beachy RN (1997) Gating of epidermal plasmodesmata is restricted to the leading edge of expanding infection sites of *Tobacco mosaic virus*. Plant J 12:781–789

Oparka KJ, Roberts AG, Boevink P, Santa Cruz S, Roberts I, Pradel KS, Imlau A, Kotlizky G, Sauer N, Epel B (1999) Simple, but not branched, plasmodesmata allow the nonspecific trafficking of proteins in developing tobacco leaves. Cell 97:743–754

Ouko MO, Sambade A, Brandner K, Niehl A, Peña E, Ahad A, Heinlein M, Nick P (2010) Tobacco mutants with reduced microtubule dynamics are less susceptible to TMV. Plant J 62:829–839

Overall RL (1999) Structure of plasmodesmata. In: van Bel AJE, van Kesteren WJP (eds) Plasmodesmata, structure, function, role in cell communication. Springer, Berlin/Heidelberg/ New York, pp 129–148

Overall RL, Blackman LM (1996) A model of the macromolecular structure of plasmodesmata. Trends Plant Sci 9:307–311

Paape M, Solovyev AG, Erokhina TN, Minina EA, Schepetilnikov MV, Lesemann DE, Schiemann J, Morozov SY, Kellmann JW (2006) At-4/1, an interactor of the *Tomato spotted wilt virus*

movement protein, belongs to a new family of plant proteins capable of directed intra- and intercellular trafficking. Mol Plant Microbe Interact 19:874–883
Padgett HS, Epel BL, Kahn TW, Heinlein M, Watanabe Y, Beachy RN (1996) Distribution of tobamovirus movement protein in infected cells and implications for cell-to-cell spread of infection. Plant J 10:1079–1088
Padidam M, Beachy RN, Fauquet CM (1995) *Tomato leaf curl geminivirus* from India has a bipartite genome and coat protein is not essential for infectivity. J Gen Virol 76:25–35
Perbal M-C, Thomas CL, Maule AJ (1993) C*auliflower mosaic virus* gene I product (P1) forms tubular structures which extend from the surface of infected protoplasts. Virology 195: 281–285
Perbal M-C, Haughn G, Saedler H, Schwarz-Sommer Z (1996) Non-autonomous function of *Antirrhinum* floral homeotic proteins DEFICIENS and GLOBOSA is exerted by their polar cell-to-cell trafficking. Development 122:3433–3441
Peremyslov VV, Hagiwara Y, Dolya VV (1999) HSP70 homolog functions in cell-to-cell movement of a plant virus. Proc Natl Acad Sci USA 96:14771–14776
Pouwels J, Van Der Krogt GN, Van Lent J, Bisseling T, Wellink J (2002) The cytoskeleton and the secretory pathway are not involved in targeting the *Cowpea mosaic virus* movement protein to the cell periphery. Virology 297:48–56
Pouwels J, van der Velden T, Willemse J, Borst JW, van Lent J, Bisseling T, Wellink J (2004) Studies on the origin and structure of tubules made by the movement protein of *Cowpea mosaic virus*. J Gen Virol 85:3787–3796
Prokhnevsky AI, Peremyslov VV, Dolja VV (2005) Actin cytoskeleton is involved in targeting of a viral Hsp70 homolog to the cell periphery. J Virol 79:14421–14428
Prokhnevsky AI, Peremyslov VV, Dolja VV (2008) Overlapping functions of the four class XI myosins in Arabidopsis growth, root hair elongation, and organelle motility. Proc Natl Acad Sci USA 105:19744–19749
Radford JE, White RG (1998) Localization of a myosin-like protein to plasmodesmata. Plant J 14:743–750
Radford JE, White RG (2011) Inhibitors of myosin, but not actin, alter transport through *Tradescantia* plasmodesmata. Protoplasma 248:205–216
Raffaele S, Bayer E, Lafarge D, Cluzet S, German Retana S, Boubekeur T, Leborgne-Castel N, Carde JP, Lherminier J, Noirot E, Satiat-Jeunemaitre B, Laroche-Traineau J, Moreau P, Ott T, Maule AJ, Reymond P, Simon-Plas F, Farmer EE, Bessoule JJ, Mongrand S (2009) Remorin, a solanaceae protein resident in membrane rafts and plasmodesmata, impairs *Potato virus X* movement. Plant Cell 21:1541–1555
Rao W, Isaac RE, Keen JN (2011) An analysis of the *Caenorhabditis elegans* lipid raft proteome using geLC-MS/MS. J Proteomics 74:242–253
Reichel C, Beachy RN (1998) *Tobacco mosaic virus* infection induces severe morphological changes of the endoplasmatic reticulum. Proc Natl Acad Sci USA 95:11169–11174
Reichel C, Beachy RN (2000) Degradation of the *Tobacco mosaic virus* movement protein by the 26S proteasome. J Virol 74:3330–3337
Reichelt S, Knight AE, Hodge TP, Baluska F, Samaj J, Volkmann D, Kendrick-Jones J (1999) Characterization of the unconventional myosin VIII in plant cells and its localization at the post-cytokinetic cell wall. Plant J 19:555–569
Rhee Y, Tzfira T, Chen MH, Waigmann E, Citovsky V (2000) Cell-to-cell movement of *Tobacco mosaic virus*: enigmas and explanations. Mol Plant Pathol 1:33–39
Ribeiro D, Goldbach R, Kormelink R (2009) Requirements for ER-arrest and sequential exit to the golgi of *Tomato spotted wilt virus* glycoproteins. Traffic 10:664–672
Rigden JE, Krake LR, Rezaian MA, Dry IB (1994) ORF C4 of *Tomato leaf curl geminivirus* is a determinant of symptom severity. Virology 204:847–850
Ritzenthaler C, Schmidt A-C, Michler P, Stussi-Garaud C, Pinck L (1995) *Grapevine fanleaf nepovirus* putative movement protein is involved in tubule formation *in vivo*. Mol Plant Microbe Interact 8:379–387

Robaglia C, Caranta C (2006) Translation initiation factors: a weak link in plant RNA virus infection. Trends Plant Sci 11:40–45

Roberts IM, Wang D, Findlay K, Maule AJ (1998) Ultrastructural and temporal observations of the potyvirus cylindrical inclusions (CIs) show that the CI protein acts transiently in aiding virus movement. Virology 245:173–181

Rojas MR, Zerbini M, Allison RF, Gilbertson RL, Lucas WJ (1997) Capsid protein and helper component-proteinase function as potyvirus cell-to-cell movement proteins. Virology 237: 283–295

Rojas MR, Jiang H, Salati R, Xoconostle-Cazares B, Sudarshana MR, Lucas WJ, Gilbertson RL (2001) Functional analysis of proteins involved in movement of the monopartite begomovirus, *Tomato yellow leaf curl virus*. Virology 291:110–125

Rojas MR, Hagen C, Lucas WJ, Gilbertson RL (2005) Exploiting chinks in the plant's armor: evolution and emergence of geminiviruses. Annu Rev Phytopathol 43:361–394

Ruggenthaler P, Fichtenbauer D, Krasensky J, Jonak C, Waigmann E (2009) Microtubule-associated protein AtMPB2C plays a role in organization of cortical microtubules, stomata patterning, and tobamovirus infectivity. Plant Physiol 149:1354–1365

Ruiz-Medrano R, Xoconostle-Cazares B, Kragler F (2004) The plasmodesmatal transport pathway for homeotic proteins, silencing signals and viruses. Curr Opin Plant Biol 7:641–650

Runions J, Brach T, Kuhner S, Hawes C (2006) Photoactivation of GFP reveals protein dynamics within the endoplasmic reticulum membrane. J Exp Bot 57:43–50

Sagi G, Katz A, Guenoune-Gelbart D, Epel BL (2005) Class 1 reversibly glycosylated polypeptides are plasmodesmal-associated proteins delivered to plasmodesmata via the golgi apparatus. Plant Cell 17:1788–1800

Sambade A, Heinlein M (2009) Approaching the cellular mechanism that supports the intercellular spread of *Tobacco mosaic virus*. Plant Signal Behav 4:35–38

Sambade A, Brandner K, Hofmann C, Seemanpillai M, Mutterer J, Heinlein M (2008) Transport of TMV movement protein particles associated with the targeting of RNA to plasmodesmata. Traffic 9:2073–2088

Samuels TD, Ju HJ, Ye CM, Motes CM, Blancaflor EB, Verchot-Lubicz J (2007) Subcellular targeting and interactions among the *Potato virus X* TGB proteins. Virology 367(2):375–389

Sanderfoot AA, Ingham DJ, Lazarowitz SG (1996) A viral movement protein as a nuclear shuttle. The geminivirus BR1 movement protein contains domains essential for interaction with BL1 and nuclear localization. Plant Physiol 110:23–33

Sanfacon H (2005) Replication of positive-strand RNA viruses in plants: contact points between plant and virus components. Can J Bot 83:1529–1549

Santa Cruz S, Roberts AG, Prior DAM, Chapman S, Oparka KJ (1998) Cell-to-cell and phloem-mediated transport of *Potato virus X*: the role of virions. Plant Cell 10:495–510

Santos AA, Lopes KV, Apfata JA, Fontes EP (2010) NSP-interacting kinase, NIK: a transducer of plant defence signalling. J Exp Bot 61(14):3839–3845

Schaad MC, Anderberg RJ, Carrington JC (2000) Strain-specific interaction of the *Tobacco etch virus* NIa protein with the translation initiation factor eIF4E in the yeast two-hybrid system. Virology 273:300–306

Schepetilnikov MV, Manske U, Solovyev AG, Zamyatnin AA Jr, Schiemann J, Morozov SY (2005) The hydrophobic segment of *Potato virus X* TGBp3 is a major determinant of the protein intracellular trafficking. J Gen Virol 86:2379–2391

Seemanpillai M, Elamawi R, Ritzenthaler C, Heinlein M (2006) Challenging the role of microtubules in *Tobacco mosaic virus* movement by drug treatments is disputable. J Virol 80: 6712–6715

Seksek O, Biwersi J, Verkman AS (1997) Translational diffusion of macromolecule-sized solutes in cytoplasm and nucleus. J Cell Biol 138:131–142

Selth LA, Dogra SC, Rasheed MS, Randles JW, Rezaian MA (2006) Identification and characterization of a host reversibly glycosylated peptide that interacts with the *Tomato leaf curl virus* V1 protein. Plant Mol Biol 61:297–310

Senchou V, Weide R, Carrasco A, Bouyssou H, Pont-Lezica R, Govers F, Canut H (2004) High affinity recognition of a *Phytophthora* protein by Arabidopsis via an RGD motif. Cell Mol Life Sci 61:502–509

Shimizu T, Yoshii A, Sakurai K, Hamada K, Yamaji Y, Suzuki M, Namba S, Hibi T (2009) Identification of a novel tobacco DnaJ-like protein that interacts with the movement protein of *Tobacco mosaic virus*. Arch Virol 154:959–967

Shimmen T, Yokota E (2004) Cytoplasmic streaming in plants. Curr Opin Cell Biol 16:68–72

Simpson C, Thomas C, Findlay K, Bayer E, Maule AJ (2009) An Arabidopsis GPI-anchor plasmodesmal neck protein with callose binding activity and potential to regulate cell-to-cell trafficking. Plant Cell 21:581–594

Sit TL, AbouHaidir MG (1993) Infectious RNA transcripts derived from cloned cDNA of *Papaya mosaic virus*: effect of mutations to the capsid and polymerase proteins. J Gen Virol 74: 1133–1140

Soellick TR, Uhrig JF, Bucher GL, Kellmann JW, Schreier PH (2000) The movement protein NSm of *Tomato spotted wilt tospovirus*: RNA binding, interaction with TSWV N protein, and identification of interacting plant proteins. Proc Natl Acad Sci USA 97:2373–2378

Sokolova M, Prufer D, Tacke E, Rohde W (1997) The *Potato leafroll virus* 17 k movement protein is phosphorylated by a membrane-associated protein kinase from potato with biochemical features of protein kinase C. FEBS Lett 400:201–205

Solovyev AG, Stroganova TA, Jr Zamyatin AA, Fedorkin ON, Schiemann J, Morozov SY (2000) Subcellular sorting of small membrane-associated triple gene block proteins: TGBp3-assisted targeting of TGBp2. Virology 269:113–127

Sparkes I, Runions J, Hawes C, Griffing L (2009) Movement and remodeling of the endoplasmic reticulum in nondividing cells of tobacco leaves. Plant Cell 21:3937–3949

Storms MMH, Kormelink R, Peters D, van Lent JWM, Goldbach RW (1995) The nonstructural NSm protein of *Tomato spotted wilt virus* induces tubular structures in plant and insect cells. Virology 214:485–493

Su S, Liu Z, Chen C, Zhang Y, Wang X, Zhu L, Miao L, Wang XC, Yuan M (2010) *Cucumber mosaic virus* movement protein severs actin filaments to increase the plasmodesmal size exclusion limit in tobacco. Plant Cell 22:1373–1387

Sun Y, Qian H, Xu XD, Han Y, Yen LF, Sun DY (2000) Integrin-like proteins in the pollen tube: detection, localization and function. Plant Cell Physiol 41:1136–1142

Szécsi J, Ding XS, Lim CO, Bendahmane M, Cho MJ, Nelson RS, Beachy RN (1999) Development of *Tobacco mosaic virus* infection sites in *Nicothiana benthamiana*. Mol Plant Microbe Interact 2:143–152

Tagami Y, Watanabe Y (2007) Effects of brefeldin A on the localization of tobamovirus movement protein and cell-to-cell movement of the virus. Virology 361:133–140

Taoka K, Ham BK, Xoconostle-Cazares B, Rojas MR, Lucas WJ (2007) Reciprocal phosphorylation and glycosylation recognition motifs control NCAPP1 interaction with pumpkin phloem proteins and their cell-to-cell movement. Plant Cell 19:1866–1884

Thomas CL, Maule AJ (1995) Identification of structural domains within the *Cauliflower mosaic virus* movement protein by scanning deletion mutagenesis and epitope tagging. Plant Cell 7:561–572

Thomas CL, Bayer EM, Ritzenthaler C, Fernandez-Calvino L, Maule AJ (2008) Specific targeting of a plasmodesmal protein affecting cell-to-cell communication. PLoS Biol 6:e7

Tilsner J, Cowan GH, Roberts AG, Chapman SN, Ziegler A, Savenkov E, Torrance L (2010) Plasmodesmal targeting and intercellular movement of *Potato mop-top pomovirus* is mediated by a membrane anchored tyrosine-based motif on the lumenal side of the endoplasmic reticulum and the C-terminal transmembrane domain in the TGB3 movement protein. Virology 402:41–51

Tomenius K, Clapham D, Meshi T (1987) Localization by immunogold cytochemistry of the virus-coded 30 K protein in plasmodesmata of leaves infected with *Tobacco mosaic virus*. Virology 160:363–371

Trutnyeva K, Bachmaier R, Waigmann E (2005) Mimicking carboxyterminal phosphorylation differentially effects subcellular distribution and cell-to-cell movement of *Tobacco mosaic virus* movement protein. Virology 332:563–577

Tucker EB (1990) Calcium-loaded 1,2-bis(2-aminophenoxy)ethane-N, N, N′, N′- tetraacetic acid blocks cell-to-cell diffusion of carboxyfluorescein in staminal hairs of *Setcreasea purpurea*. Planta 182:34–38

Tucker EB (1993) Azide treatment enhances cell-to-cell diffusion in staminal hairs of *Setcreasea purpurea*. Protoplasma 174:45–49

Tucker EB, Boss WF (1996) Mastoparan induced intracellular Ca^{2+} fluxes may regulate cell-to-cell communication in plants. Plant Physiol 111:459–467

Tyulkina LG, Karger EM, Sheveleva AA, Atabekov JG (2010) Binding of monoclonal antibodies to the movement protein (MP) of *Tobacco mosaic virus*: influence of subcellular MP localization and phosphorylation. J Gen Virol 91:1621–1628

Tzfira T, Rhee Y, Chen M-H, Kunik T, Citovsky V (2000) Nucleic acid transport in plant-microbe interactions: the molecules that walk through the walls. Annu Rev Microbiol 54:187–219

Ueda H, Yokota E, Kutsuna N, Shimada T, Tamura K, Shimmen T, Hasezawa S, Dolja VV, Hara-Nishimura I (2010) Myosin-dependent endoplasmic reticulum motility and F-actin organization in plant cells. Proc Natl Acad Sci USA 107:6894–6899

Ueki S, Spektor R, Natale DM, Citovsky V (2010) ANK, a host cytoplasmic receptor for the *Tobacco mosaic virus* cell-to-cell movement protein, facilitates intercellular transport through plasmodesmata. PLoS Pathog 6:e1001201

van Bargen S, Salchert K, Paape M, Piechulla B, Kellmann J-W (2001) Interactions between *Tomato spotted wilt virus* movement protein and plant proteins showing homologies to myosin, kinesin, and DNAJ-like chaperones. Plant Physiol Biochem 39:1083–1093

van der Wel NN, Goldbach R, van Lent J (1998) The movement protein and coat protein of *Alfalfa mosaic virus* accumulate in structurally modified plasmodesmata. Virology 244:322–329

van Lent J, Storms M, van der Meer F, Wellink J, Goldbach R (1991) Tubular structures involved in movement of *Cowpea mosaic virus* are also formed in infected cowpea protoplasts. J Gen Virol 72:2615–2623

Van Norman JM, Breakfield NW, Benfey PN (2011) Intercellular communication during plant development. Plant Cell 23:855–864

Verchot-Lubicz J (2005) A new cell-to-cell transport model for potexviruses. Mol Plant Microbe Interact 18:283–290

Verchot-Lubicz J, Ye CM, Bamunusinghe D (2007) Molecular biology of potexviruses: recent advances. J Gen Virol 88:1643–1655

Verchot-Lubicz J, Torrance L, Solovyev AG, Morozov SY, Jackson AO, Gilmer D (2010) Varied movement strategies employed by triple gene block-encoding viruses. Mol Plant Microbe Interact 23:1231–1247

Vogel F, Hofius D, Sonnewald U (2007) Intracellular trafficking of *Potato leafroll virus* movement protein in transgenic Arabidopsis. Traffic 8:1205–1214

Vogler H, Kwon MO, Dang V, Sambade A, Fasler M, Ashby J, Heinlein M (2008) *Tobacco mosaic virus* movement protein enhances the spread of RNA silencing. PLoS Pathog 4:e1000038

Waigmann E, Zambryski P (1995) *Tobacco mosaic virus* movement protein-mediated protein transport between trichome cells. Plant Cell 7:2069–2079

Waigmann E, Zambryski P (2000) Trichome plasmodesmata: a model system for cell-to-cell movement. Adv Bot Res 31:261–283

Waigmann E, Lucas W, Citovsky V, Zambryski P (1994) Direct functional assay for *Tobacco mosaic virus* cell-to-cell movement protein and identification of a domain involved in increasing plasmodesmal permeability. Proc Natl Acad Sci USA 91:1433–1437

Waigmann E, Chen M-H, Bachmeier R, Ghoshroy S, Citovsky V (2000) Regulation of plasmodesmal transport by phosphorylation of *Tobacco mosaic virus* cell-to-cell movement protein. EMBO J 19:4875–4884

Watanabe Y, Emori Y, Ooshika I, Meshi T, Ohno T, Okada Y (1984) Synthesis of TMV-specific RNAs and proteins at the early stage of infection in tobacco protoplasts: transient expression of 30 k protein and its mRNA. Virology 133:18–24

Watanabe Y, Meshi T, Okada Y (1992) *In vivo* phosphorylation of the 30-kDa protein of *Tobacco mosaic virus*. FEBS Lett 313:181–184

Wei T, Zhang C, Hong J, Xiong R, Kasschau KD, Zhou X, Carrington JC, Wang A (2010) Formation of complexes at plasmodesmata for potyvirus intercellular movement is mediated by the viral protein P3N-PIPO. PLoS Pathog 6:e1000962

Wellink J, van Lent JWM, Verver J, Sijen T, Goldbach RW, van Kammen A (1993) The *Cowpea mosaic virus* M RNA-encoded 48-kilodalton protein is responsible for induction of tubular structures in protoplasts. J Virol 67:3660–3664

White RG, Badelt K, Overall RL, Vesk M (1994) Actin associated with plasmodesmata. Protoplasma 180:169–184

Whitham SA, Quan S, Chang HS, Cooper B, Estes B, Zhu T, Wang X, Hou YM (2003) Diverse RNA viruses elicit the expression of common sets of genes in susceptible Arabidopsis plants. Plant J 33:271–283

Wieczorek A, Sanfacon H (1993) Characterization and subcellular location of *Tomato ringspot nepovirus* putative movement protein. Virology 194:734–742

Wille A, Lucas WJ (1984) Ultrastructural and histochemical changes on guard cells. Planta 160: 129–142

Wittmann S, Chatel H, Fortin MG, Laliberte JF (1997) Interaction of the viral protein genome linked of *Turnip mosaic potyvirus* with the translational eukaryotic initiation factor (iso) 4E of *Arabidopsis thaliana* using the yeast two-hybrid system. Virology 234:84–92

Wolf S, Deom CM, Beachy RN, Lucas WJ (1989) Movement protein of *Tobacco mosaic virus* modifies plasmodesmatal size exclusion limit. Science 246:377–379

Wright KM, Wood NT, Roberts AG, Chapman S, Boevink P, Mackenzie KM, Oparka KJ (2007) Targeting of TMV movement protein to plasmodesmata requires the Actin/ER network; evidence from FRAP. Traffic 8:21–31

Wright KM, Cowan GH, Lukhovitskaya NI, Tilsner J, Roberts AG, Savenkov EI, Torrance L (2010) The N-terminal domain of PMTV TGB1 movement protein is required for nucleolar localization, microtubule association, and long-distance movement. Mol Plant Microbe Interact 23:1486–1497

Wu X, Weigel D, Wigge PA (2002) Signaling in plants by intercellular RNA and protein movement. Genes Dev 16:151–158

Wu X, Dinneny JR, Crawford KM, Rhee Y, Citovsky V, Zambryski PC, Weigel D (2003) Modes of intercellular transcription factor movement in the Arabidopsis apex. Development 130: 3735–3745

Wu Q, Wang X, Ding SW (2010) Viral suppressors of RNA-based viral immunity: host targets. Cell Host Microbe 8:12–15

Wu CH, Lee SC, Wang CW (2011) Viral protein targeting to the cortical endoplasmic reticulum is required for cell-cell spreading in plants. J Cell Biol 193:521–535

Yaholom A, Lando R, Katz A, Epel BL (1998) A calcium-dependent protein kinase is associated with maize mesocotyl plasmodesmata. J Plant Physiol 153:354–362

Yoo BC, Kragler F, Varkonyi-Gasic E, Haywood V, Archer-Evans S, Lee YM, Lough TJ, Lucas WJ (2004) A systemic small RNA signaling system in plants. Plant Cell 16:1979–2000

Yoshioka K, Matsushita Y, Kasahara M, Konagaya K, Nyunoya H (2004) Interaction of *Tomato mosaic virus* movement protein with tobacco RIO kinase. Mol Cells 17:223–229

Zavaliev R, Ueki S, Epel BL, Citovsky V (2011) Biology of callose (beta-1,3-glucan) turnover at plasmodesmata. Protoplasma 248:117–130

Chapter 2
Embryogenesis As a Model System to Dissect the Genetic and Developmental Regulation of Cell-to-Cell Transport Via Plasmodesmata

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1 Introduction

1.1 Embryogenesis

Arabidopsis offers a remarkably accessible model system to study plant embryogenesis largely because its seedpod, called a silique, carries 2 rows of 20 embryos at exactly the same stage of development along its length. This arrangement is a general feature of the Brassicaceae family. Also notably, each flowering *Arabidopsis* plant has up to 50 siliques along its inflorescence axis, and each silique will carry embryos from a different stage of development, from older (at the base) to younger (at the tip). This extremely ordered array of embryos allowed detailed studies to map the regular pattern of cell divisions in specific groups of cells in the early embryo that develop into specific cells and tissues of more mature embryos and seedlings (Jurgens and Mayer 1994). The mature embryo shows an apical-basal organization along the main axis that is composed of structures such as shoot apical meristem (SAM), cotyledons, hypocotyl and root, and clonal analyses predict the contribution of each embryonic cell to this body plan (Laux et al. 2004) (Fig. 2.1). Generally, positional information determines the overall body pattern, and lineage-dependent cell fate specifies local patterning (Mansfield and Briarty 1991; Scheres et al. 1994; Poethig et al. 1986; Laux et al. 2004). Auxin signaling in conjunction with differential gene expression then facilitate specific morphogenesis (reviewed in Saulsberry et al. 2002; Capron et al. 2009).

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F. Kragler and M. Hülskamp (eds.), *Short and Long Distance Signaling*, Advances in Plant Biology 3, DOI 10.1007/978-1-4419-1532-0_2,

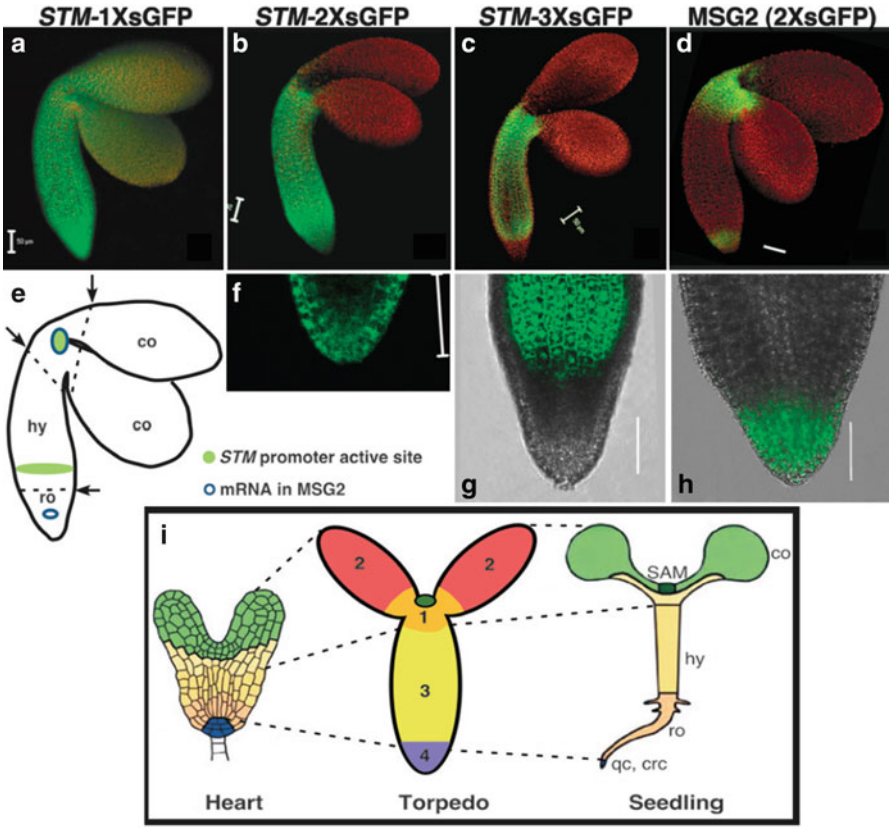

Fig. 2.1 *sGFP movement in Arabidopsis midtorpedo embryos*.This figure is reprinted in its entirety from Kim and Zambryski 2005, with permission (from Elsevier, and includes data previously published in Kim et al. 2005a, b). 1XsGFP expressed by the *STM* promoter in the SAM and the base of hypocotyls (hy) (see *green* areas in (**e**)) freely moves throughout the whole embryo (**a**). 2XsGFP fails to move into cotyledons (co) (**b**) but moves to the root tip (**f**). 3XsGFP fails to move to the root (ro) as well as cotyledons (**c**, **g**). These results indicate the formation of at least two symplastic sub-domains, the cotyledon and root. 2XsGFP expressed in the SAM and RAM in MSG2 line (see *dark blue* circles for where expression occurs in (**e**)) stays within sub-domains of the shoot apex and the root, respectively (**d**, **h**). These results, together with (**b**), reveal the boundary between the shoot apex and hypocotyl sub-domains. Root sub-domains from embryos in (**c**) and (**d**) are shown in larger magnification views in (**g**) and (**h**) under each whole mid torpedo image, and include quiescent center (qc), part of the RAM, and central root caps (crc). (**i**) Four symplastic sub-domains, shoot apex including SAM (*1*), cotyledons (*2*), hypocotyl (*3*), and root (*4*) are extrapolated to the body parts in heart embryos and seedlings shown to the *left* and *right*. Same colors in heart embryo and seedling represent regions of development with common clonal origins. Sub-domains of the torpedo embryo, as determined by their cell-to-cell transport via PD, also correspond to the apical-basal body pattern of the heart embryo (and seedling) by their positions; these regions are diagrammed with different colors to indicate they were defined by a different assay. Scale bars, 50 μm

Plasmodesmata (PD) directly connect the cytoplasm of adjacent cells, and convey positional information during axial patterning in embryogenesis by determining whether or not specific cells are symplastically coupled. The cytoplasm between adjacent plant cells is continuous due to PD connections; such adjacent cells share cytoplasm and thus are symplastically coupled. Embryos develop from a single cell, and cell division results in symplastic coupling between cells. Oriented patterns of cell division then result in morphogenesis. Early heart stage embryos, comprised of about 250 cells, are interconnected by open PD and constitute a single symplast of continuous cytoplasm bounded by the plasma membranes of connected cells (McLean et al. 1997). However, as embryonic development proceeds to the later torpedo stages, groups of cells become symplastically isolated from neighboring cells to form symplastic sub-domains (described below).

Arabidopsis embryos provide a very tractable system to study the genetic and developmental regulation of PD. Importantly, maternal transport of macromolecules ceases by the early heart stage. Thus, one can monitor cell-to-cell transport in isolated embryos independent of maternal effects by observing fluorescent tracers that are either added transiently or stably expressed in transgenic lines. Also, embryos are easily released from developing seed coats by mild pressure.

We review the establishment of symplastic domains in *Arabidopsis* embryos, genetic identification of mutations that alter PD function during embryogenesis, PD ultrastructure during embryogenesis, and discuss novel findings that have emerged from studies of PD transport during embryogenesis.

2 Symplastic Domain Formation During Arabidopsis Embryogenesis

We first studied cell-to-cell transport during embryogenesis by transiently and exogenously adding fluorescent (F) tracers. How are F-tracers introduced into embryos? We developed a simple strategy (Kim et al. 2002) that relies on the fact that certain cells of the embryo are more likely to break during extrusion of embryos from their seed coats. First, developing seeds are removed from the silique by running a needle horizontally along its length. Then the seeds are placed in plant liquid media under a cover slip on a slide. Gentle pressure to the cover slip then releases the embryos from their seed coats. This pressure often causes breaks at the junction between cotyledons and the meristem as the cotyledons are bent at a severe angle in the small space of the seed coat. Similarly the tip of the embryonic root often sustains small breaks in its terminal cells during extrusion. Following extrusion embryos are incubated with F-tracers and then washed extensively to remove unincorporated tracer. The broken cells provide entry points for the tracer, and then depending on the SEL of their connecting PD, the tracer either moves into the tissue or not.

The small F-tracer 8-hyroxypyrene-1,3,6-trisulfonic acid (HPTS, 0.5 kDa) moves through all cells of embryos during all stages of embryonic development examined from early heart to mid and late torpedo. However, the use of higher molecular weight tracers, such as 10-kDa F-dextran, reveals that PD aperture is down regulated, for such larger macromolecules, as development proceeds. 10-kDa F-dextrans are transported cell-to-cell in 50% of heart, 20% of early torpedo, and 0% of mid torpedo embryos (Kim et al. 2002). This developmental transition was used as a screen to select for mutants with altered cell-to-cell transport via PD (discussed in Sect. 3 below).

We then used transgenic approaches to express fluorescent proteins such as green fluorescent protein (GFP). In fact, GFP is a boon for PD research. GFP is an exogenous protein with no particular cellular address so that its expression results in nonspecific cytoplasmic and nuclear localization, and depending on the aperture of PD, it can move between cells by diffusion (Crawford and Zambryski 2000). Since GFP is not sequestered in cellular compartments, its movement between cells gives an indication of the PD-mediated connectivity between groups of cells. Observations of changes in PD aperture during embryonic development especially illustrate the usefulness of GFP. Note, PD aperture is also described as PD size exclusion limit (SEL), reflecting the molecular weight of the largest molecules that can be transported via PD connecting particular sets of cells. We use both terms in this review, but prefer the term "aperture" as it describes PD themselves versus the size of molecules they transport.

We used two different promoters to drive GFP expression in meristematic regions. The *SHOOT MERISTEMLESS (STM)* promoter was used to express 1X, 2X, and 3XGFP in the shoot apical meristem (SAM) and a subset of cells in the hypocotyl just above the root meristem (Kim et al. 2005b). In addition, the cell-type-specific enhancer of the Haseloff enhancer trap line J2341 (Cho and Zambryski 2011) induced expression of 2XGFP in the SAM and the root apical meristem (RAM) in the MSG2 line (Fig. 2.1e). The subsequent movement of these various sized tracers from their site of synthesis was monitored at three stages of embryogenesis to reveal several major findings (Kim et al. 2005a, b). Remarkably, 2×GFP (54 kDa) moves throughout the entire early heart embryo demonstrating that PD apertures in early embryos are quite dilated (Kim et al. 2005b). Importantly, these data reinforce the observations made with exogenously applied HPTS and F-dextran tracers, again revealing that the entire embryo is a single symplast, where all cells can freely transport GFP cell-to-cell via PD. Significantly, different regions of the embryo have distinct PD apertures/SELs defining specific symplastic sub-domains by the mid torpedo stage.

Figure 2.1 a–d illustrate the movement of 1X to 3XGFP at the late-torpedo stage. Mid-torpedo cotyledons do not allow transport of probes larger than 1XGFP (27 kDa). Mid-torpedo roots do not allow transport of probes larger than 2XGFP (54 kDa). Finally, the movement of 3XGFP (81 kDa) is more restricted to the regions immediately surrounding its site of synthesis at the SAM and at the base of the hypocotyl. Strikingly, these sub-domains of cytoplasmic continuity correspond to

the basic layout of the organs of the adult body along the apical-basal body axis: the shoot apex, cotyledons, hypocotyls and root (Fig. 2.1i and legend). These sub-domains can be extrapolated to morphogenetic regions of the early embryo (and seedling) defined by gene expression profiles and clonal analyses (Fig. 2.1i) (Poethig et al. 1986; Mansfield and Briarty 1991; Scheres et al. 1994; Laux et al. 2004).

An independent study of GFP transport following expression mediated by the cell-type-specific enhancer of the Haseloff reporter line J2341 (Kim et al. 2005a) provides salient additional evidence for a boundary with distinct PD aperture between the root tip and the hypocotyl. Figure 2.1f, g shows that 2XGFP and 3XGFP expressed from the STM promoter can, or cannot, move down into the root tip, respectively. Figure 2.1h shows that 2XGFP expressed at the root tip under the control of the J2341 enhancer cannot move up into the hypocotyl. Thus, PD at the root-hypocotyl boundary allow directional movement of 2XGFP downward, but not upward.

In summary, there are boundaries between each of four symplastic sub-domains, SAM, cotyledons, hypocotyl, and root, that control embryo-specific intercellular transport (Fig. 2.1i). Each boundary likely has a distinct PD aperture/SEL. The boundary between the shoot apex and the cotyledons has a SEL between 27 and 54 kDa, as 1XGFP but not 2XGFP moves from the SAM to the cotyledons (Fig. 2.1a, b, and e). The boundary between the hypocotyl and the root has a SEL between 54 kDa and 81 kDa, as 2XGFP but not 3XGFP moves from the hypocotyl to the root (Fig. 2.1f, g). The boundary between the hypocotyl and shoot apex domains has a SEL less than 54 kDa as evidenced in the MSG2 line where 2XGFP moves from its site of synthesis at the SAM to surrounding cells but it fails to move downward into the hypocotyl (Fig. 2.1d, e). Movement within the hypocotyl is only observed when GFP expression occurs within the hypocotyl itself (just above the root) under the control of the *STM* promoter; in this case, 2X and 3XGFP move upward from their site of synthesis (Fig. 2.1b, c, and e).

Thus, groups of cells with similar developmental fates carry PD with similar apertures. Differential PD connectivity likely is critical during organ formation, providing a means whereby groups of cells can exchange factors essential for common developmental programming. PD connectivity also varies in adult plants, being highest in young organs and more restricted in mature tissues (reviewed in Burch-Smith et al. 2011).

2.1 Further Refinement of Localized Embryonic Symplastic Sub-domains

Additional embryonic symplastic sub-domains were defined by promoter-specific expression of 1XGFP in the protodermis or stele of the embryo (Stadler et al. 2005). The *GLABRA 2* promoter drives expression in the protodermis, and when 1XGFP was expressed in the outermost protodermal layer of the heart-stage embryonic hypocotyl, it moved inward to internal ground tissues and to neighboring protodermal

cells in cotyledons. Such movement was reduced at the torpedo stage (Stadler et al. 2005). When 1XGFP was expressed under the *SUCROSE TRANSPORTER3 (AtSUC3)* promoter it moves freely between cells of the hypocotyl in earlier stages (Stadler et al. 2005), but becomes restricted to the stele in the mid-torpedo stage (Fig. 2.3b). Thus, cell-to-cell transport inward from the protodermis and outward from the stele is regulated during the torpedo stage of embryogenesis.

Significantly, the above data underscore that the extent of cell-to-cell movement is dramatically affected by the location of the initial site of GFP synthesis. 1XGFP freely moves to every cell in embryos following expression in the SAM, (Kim et al. 2005a, b), but its movement is limited when expressed from epidermal cells (Stadler et al. 2005). Potentially, PD in and around the SAM are more active, or have larger apertures, than those in the epidermis as meristems are likely the source of morphological signals to enable patterning during embryogenesis.

The above studies provoke many questions. What factors regulate the establishment of embryonic symplastic domains? Do such factors bind directly to PD to regulate their aperture? Do different factors differentially regulate PD at boundaries between symplastic domains? Are PD in boundary cells more tightly regulated than PD within symplastic domains?

2.2 Maternal Influences on PD Function Just Before and During Embryogenesis

While this review focuses on PD function and regulation during embryogenesis, we also alert the reader to studies of PD function during the developmental period immediately prior to embryogenesis. A recent study with stunning images documents PD and phloem transport during ovule development, and provides evidence for two switches in transport capacity (Werner et al. 2011). Ovules initiate their development in stage 11 flowers during which time they are symplastically connected to the maternal phloem. However, when the female gametophyte is produced during stage 12, phloem unloading is switched off. Phloem unloading is resumed in developing embryos of stage 13 flowers when fertilization occurs, though the resumption of symplastic unloading is not fertilization-dependent.

2.3 Embryo Suspensor Cell-to-Cell Communication

After fertilization the zygote undergoes polar cell division. The upper apical cell will become the embryo and lower basal cell forms the suspensor. We previously reviewed (Kim and Zambryski 2005) GFP transport from the embryo to the suspensor and *vice versa*, but it is important to highlight here too. The suspensor is a terminally differentiated linear file of embryonic cells that connects the embryo

to maternal tissues and provides a direct route for nutrient transport from material tissues to the early embryo. In globular embryos, GFP can move in both directions, implying that the embryo and suspensor together form a single symplast at the globular stage. However, as the embryo makes the transition from radial to bilateral symmetry at the heart stage, PD mediated movement from the suspensor to the embryo restricts maternal input.

2.4 Embryo-Seed Coat Cell-to-Cell Transport

Finally, GFP moves differentially in the five cell layers of the *Arabidopsis* seed coat consisting of the innermost endothelial layer, followed by two cell layers each of inner and outer integuments. GFP expressed in the outer integument cannot move to the inner integument layers and *vice versa*. Thus, the inner and outer integuments are separate symplastic domains. Even small tracers such as HPTS (0.5 kDa) are not transported across the boundary between the outer and the inner integuments (Stadler et al. 2005).

The above studies highlight both dynamic and highly regulated plant intercellular transport during ovule development, early embryo development, stage specific embryogenesis, and seed coat development.

3 Isolation of Mutants with Altered PD Transport During Embryogenesis

Genetics is an obvious classical approach to identify PD-specific components. However, alterations in components that affect PD structure or function are likely to have severe effects on plant growth and development. Indeed, PD defects are expected to manifest at the first stages of plant growth, during embryogenesis, and thereby exhibit a defective or even lethal phenotype. However, embryos make an especially excellent system for genetic screens because embryo-lethal/defective mutants can be propagated as heterozygote plants, and segregating defective embryos can be detected in seedpods. This is especially true for Arabidopsis because their seedpods, siliques, contain approximately 40 embryos in a linear file that are easily observed by simple light microscopy. Furthermore, because embryogenesis is a vitally important phase in plant development where basic tissues and anatomy of a plant are established, one might expect to see regulation of PD function during this time frame.

As mentioned above, we used exogenously added 10-kDa F-tracers to determine that PD aperture is reduced significantly during the mid-torpedo stage of embryogenesis. We then used this time frame to screen 5,000 embryo-lethal mutants for alterations in cell-to-cell transport of fluorescent tracers (Kim et al. 2002); see Sect. 2.1

above for description of the F-dextran loading assay. Fifteen mutants that continued to traffic 10 kDa F-dextran at the torpedo stage were identified and called *increased size exclusion limit (ise)*. In a separate study we screened for the opposite phenotype, reduced transport of the low molecular weight tracer HTPS, and identified a single mutant called *decreased size exclusion limit* (*dse*) (XM, unpublished).

To date we have cloned and characterized three mutants, *ise1*, *ise2*, and *dse*. All three mutations were induced by ethane methane sulfonate (EMS) that causes alterations in single nucleotides. This less severe mutagenesis strategy was in fact critical to our successful identification of mutants with altered PD function. After each mutant gene was identified, we could then obtain the relevant T-DNA insertion alleles. In all cases, T-DNA-induced null alleles of the identified genes are severely retarded; *ise1* null mutants form only a large mass of cells, and *ise2* and *dse* null mutants do not develop beyond the torpedo stage.

Contrary to our expectations, none of the encoded products of *ISE1*, *ISE2*, or *DSE*, localize to PD. The *ISE1* gene encodes a DEAD-box RNA helicase that localizes to mitochondria (Stonebloom et al. 2009). *ISE2* encodes a DEVH-box RNA helicase first shown to localize to the cytoplasm and chloroplasts (Kobayashi et al. 2007). Recent higher resolution microscopy reveals that ISE2 localizes entirely within chloroplasts Burch-Smith et al. 2011.

3.1 Mutants with Altered PD Function Increase De Novo Formation of PD

Significantly, *ise1* and *ise2* increase PD formation (Burch-Smith and Zambryski 2010), as would be expected from their effects on increasing PD transport. PD can have several different morphologies: simple linear channels, twinned PD (simple PD within 100 nm of each other) (Faulkner et al. 2008), moderately branched, or highly branched. The latter highly branched structures are found in mature tissues such as source leaves. PD that arise during cell division are called primary PD, and those that arise post division are called secondary PD. As the overall morphology of *ise1*, *ise2*, or *dse* torpedo embryos are similar to wild type torpedo embryos, these mutations do not affect cell division patterns and therefore are unlikely to effect primary PD formation. Instead we predicted that mutations in *ise1, ise2*, or *dse1* might affect the *de novo* formation of secondary PD.

Figure 2.2a illustrates the frequencies of twinned and branched PD in hypocotyls of late-staged torpedo embryos in three mutant lines for which published data is available. Wild type embryos contain ~9% twinned and branched PD and *ise1* (26%) and *ise2* (14%) mutant embryos have significantly increased frequencies of twinned and branched PD. Twinned PD are likely the result of duplication of a simple PD (Faulkner et al. 2008); by definition then twinned PD are secondary. Branched PD may arise from both primary and secondary simple PD, so it is impossible to know the origin of branched PD from their structure.

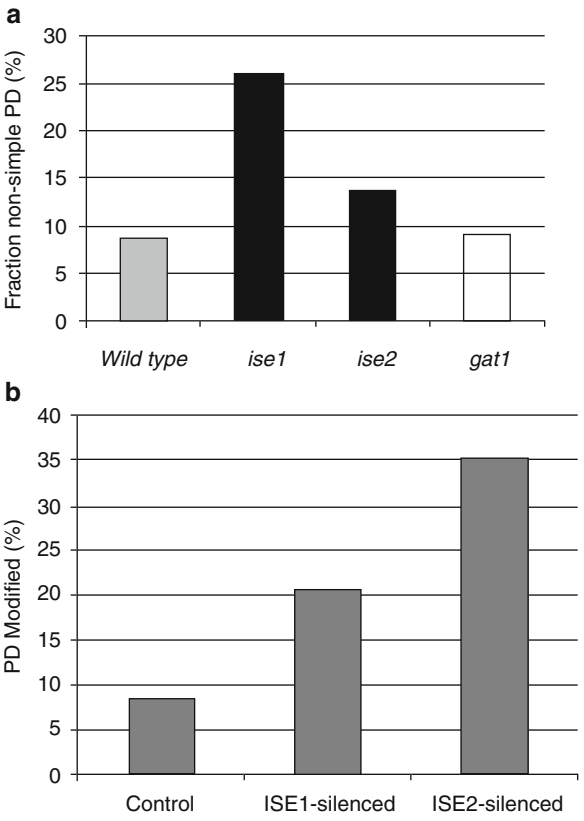

Fig. 2.2 *Fraction of total PD that are not simple*. (**a**) The fraction of non-simple PD is plotted as a percentage of the total PD. Data for wild type (*grey bar*), *ise1* and *ise2* embryos (*black bars*) are from Burch-Smith and Zambryski 2010. Data for *gat1* seedlings are from Benitez-Alfonso et al. 2009. (**b**) Compares the frequency of de novo secondary PD formation in non-silenced leaves compared to *ISE1* or *ISE2* silenced leaves. Secondary PD were defined as PD in the cell walls between the epidermal and mesophyll layer as in Fig. 2.3b

However, secondary twinned or branched PD can be unambiguously identified if they occur in cell walls that are explicitly non-division walls. Non-division walls are easily identified in longitudinal cell files, such as those of the expansion zone of the root (Scheres et al. 2002). In addition, leaf epidermis-mesophyll cell interfaces do not result from cell division, and provide a ready supply of tissue for specific analyses of secondary PD (Burch-Smith and Zambryski 2010). Thus, we determined if the increased or decreased numbers of branched PD we observed in mutant embryos reflect increased or decreased branching of secondary PD by an independent approach that specifically examined unambiguous secondary PD in the cell walls at epidermal-mesophyll interfaces of the leaf. To induce the loss of ISE1, or ISE2 in leaves, we performed virus induced gene silencing (VIGS) (Burch-Smith and Zambryski 2010) of *ISE1*, and *ISE2*. As VIGS is easy and efficient in *Nicotiana benthamiana* (Burch-Smith et al. 2004) we first identified homologs of *ISE1*, and *ISE2* in *N. benthamiana*. Following VIGS of *ISE1* and *ISE2*, we then performed ultrastructural analyses to monitor the frequency of *de novo* secondary PD formation at the cell wall interfaces between epidermal and mesophyll cells of young leaves.

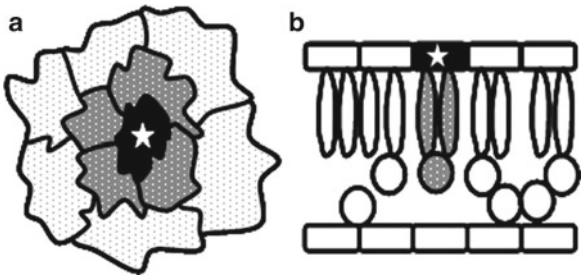

Fig. 2.3 *Movement assay in leaf cells*. Agrobacterium carrying constructs to express GFP fused to TMV MP were infiltrated into leaf epidermal cells at low dilution to ensure transfection of single cells. An initially transfected cell is indicated as a black cell with a white star. (**a**) Depicts movement to two rings of cells away from the transfected cell as *grey* and *white* cells respectively. (**b**) Depicts movement from a single epidermal cell down into the mesophyll layer (*grey cells*). Palisade mesophyll cells are elongated and spongy mesophyll cells are shown as *circles*

Our results were dramatic. Control non-silenced wild-type leaves contained 8% twinned and branched PD in the cell walls between the leaf epidermis and the underlying mesophyll cells that specifically contain secondary PD. Silencing of *ISE1* or *ISE2* lead to 20% and 35% twinned and branched PD respectively (Fig. 2.2b). These results lead us to extrapolate that the increased twinned and branched PD observed in embryos are also likely to be secondary.

3.2 Gene Silencing of ISE1 or ISE2 in Leaves Increases PD Cell-to-Cell Transport

In addition, we used our gene silencing strategy to demonstrate that silencing of *ISE1* or *ISE2* increases cell-to-cell transport in leaves, reiterating the effects of *ise1*, and *ise2* mutants during embryogenesis. Leaves silenced for *ISE1* or *ISE2* were assayed for the movement of tobacco mosaic virus (TMV) movement protein (MP) P30 fused to GFP. To more sensitively detect potential increased movement in *ISE1* or *ISE2* silenced leaves we monitored 2XGFP fused to P30 (P30-2XGFP). P30-2XGFP-fusion proteins were detected following *Agrobacterium*-mediated transient expression. The results obtained were exactly as predicted, and demonstrated that loss of *ISE1* or *ISE2* also impacts intercellular movement via PD in mature leaf cells. Figure 2.3 diagrams the movement assay. Movement was quantified first as the number of rings of cells to which the P30-2XGFP moved away from the initially transfected single cell (Fig. 2.3a). Thus, leaves silenced for *ISE1* or *ISE2* resulted in more rings of P30-2XGFP movement than control non-silenced leaves (Burch-Smith and Zambryski 2010). Besides horizontal movement (which includes primary and secondary PD-mediated transport) we also specifically assayed for movement via secondary PD, assessed as movement from the epidermal cell

layer into the mesophyll layer (Fig. 2.3b). Movement via secondary PD was increased in *ISE1* and *ISE2*-silenced leaves (Burch-Smith and Zambryski 2010). Importantly, in the above studies we infiltrated leaves with Agrobacterium (carrying the P30-2XGFP construct) at very low dilutions to ensure that foci observed and analyzed represent single transfection events.

3.3 Additional Mutants with Altered PD Transport

In an independent study Benitez-Alfonso et al. (Benitez-Alfonso et al. 2009) mutagenized transgenic seedlings expressing GFP in phloem-specific cells that leads to GFP movement cell-to-cell into surrounding tissues. They screened for mutants, called *GFP arrested trafficking* (*gat*) that exhibit reduced PD function by failing to unload GFP from the phloem in seedling roots. *GAT1* encodes a plastid thioredoxin and *gat1* mutants increase callose production (Benitez-Alfonso et al. 2009) (discussed further below). Benitez-Alfonso et al. (2009) also analyzed PD frequency and structure in *gat1* seedling roots. They found no increase in PD frequency but suggested there was an increase in branched PD. Figure 2.2 shows that the frequency of PD branching (9%) observed in *gat1* seedling roots is identical to the frequency of twinned and branched PD observed in wild type late torpedo hypocotyls. We also demonstrated that early and mid-torpedo hyocotyls contain ~9% twinned and branched PD (Burch-Smith and Zambryski 2010), but here we specifically illustrate our results for the late torpedo stage hypocotyls as they are most comparable to early seedling roots analyzed by Benitez-Alfonso et al. (2009).

Finally, we continue to analyse mutants that lead to increased cell-to-cell movement at the mid-torpedo stage of embryogenesis identified in our original screen (Kim et al. 2002), and are currently mapping the mutant loci to identify their affected genes. We are most far along with the characterization of the *dse* mutant line; in terms of the ultrastructual and functional assays mentioned above for *ise1* and *ise2*.

4 Surprising Novel Results from Studies of PD Transport in Embryos

4.1 Twinned and Branched PD Occur in Wild Type Embryos and Young Tissues

We (Kobayashi et al. 2007), and others (Benitez-Alfonso et al. 2009), previously assumed that embryos or seedling root meristems only contain simple unbranched PD; both reports mention the presence of branched PD specifically as a feature of the

mutant (*ise2* or *gat1*) phenotype. However, quantitative and statistically significant measurements of PD structures during embryogenesis now reveal that wild type embryonic hypocotyls contain 9% twinned and branched PD between early and late-torpedo stages of development (Burch-Smith and Zambryski 2010). Thus, the 9% branched PD reported in seedling roots (Benitez-Alfonso et al. 2009) is not significantly different from the frequency observed in wild type embryos; a quantitative study of simple, versus twinned and branched PD remains to be performed in wild type root seedlings. While Kobayashi et al. (Kobayashi et al. 2007) noted 15% branched PD in their mutant, they incorrectly reported no branched PD occur in wild type tissues because insufficient numbers of PD were analyzed in wild type tissues. Thus, to accurately determine the frequencies of different PD structures in particular cell types it is essential to analyze a sufficiently large number of PD in both experimental **and** wild type tissues. Thus, we missed the existence of twinned and branched PD in our earlier report as we counted too few PD in wild type tissues.

A recent review re-evaluates the evidence for different types of PD in diverse tissues, emphasizing that, contrary to previous assumptions, young tissues do not contain "only" simple unmodified PD, but also contain twinned and branched PD, and proposing a re-evaluation of some of the older data with regard to the nomenclature of the different PD forms (Burch-Smith et al. 2011).

4.2 Plant Viral Movement Proteins as Probes to Study PD During Embryogenesis

The movement protein (MP) of TMV is a veritable workhorse and remarkably useful tool for studying intercellular transport via PD. Besides its ability to move extensively from cell to cell, it targets to PD so that GFP fusions to TMV MP mark PD as puncta in the cell perimeter. Besides moving via PD TMV MP also "gates" PD aperture allowing large F-tracers to move cell-to-cell in trans (Wolf et al. 1989). Proteins that potentially localize to PD are assayed by their co-localization with TMV MP. Finally a transgenic line constitutively expressing TMV MP has lead to important insights in distinguishing PD transport in sink and source leaves. However, these data lead to the idea that TMV-MP "only" localizes to complex branched PD with central cavities, called "secondary" PD by the authors, in mature tissues (Ding et al. 1992). Many reports subsequently asserted that viral MPs preferentially localize to "secondary" PD in mature tissues (e.g. Epel 1994; Hofius et al. 2001) and TMV MP was used to distinguish "secondary" PD from primary PD (Roberts et al. 2001).

During our studies to characterize the movement of different sized GFP tracers (1X, 2X, and 3XGFP) during embryogenesis (described above) we also performed studies using the same *STM* promoter to drive expression of 1X and 2XGFP fused to TMV MP (Kim et al. 2005b). We embarked on these studies to determine if TMV MP could also "gate" PD aperture in embryos to allow more extensive cell-to-cell transport than similarly sized soluble GFP. Comparison of the movement of 2XGFP (54 kDa) versus MP-GFP (57 kDa) revealed that MP-GFP moved more extensively

down into the root tip and within the hypocotyl than 2xGFP (Kim et al. 2005b). Thus, MP can gate PD in embryos to facilitate its own transport.

Importantly, MP-GFP targets to PD in embryos and forms the classic punctate pattern previously seen for its PD localization in more mature tissues. As more than 90% of embryonic PD are simple (Burch-Smith and Zambryski 2010) these data unambiguously demonstrate that MP also localizes to simple PD. We further confirmed that MP-GFP localizes to primary PD in the differentiation zone just above the root meristem (Burch-Smith et al. 2011).

In summary, studies to monitor cell-to-cell transport in embryos have revealed new properties of TMV MP, to target to and gate simple PD, and thereby increase the utility of TMV MP to now investigate PD structure and function in developing tissues.

4.3 Reactive Oxygen Species Production During Embryogenesis Alters PD Transport

As mentioned, embryo-defective *ise1* mutants increase PD transport and exhibit delayed development (Kim et al. 2002). *ISE1* encodes a nuclear-encoded mitochondria localized RNA helicase that is likely essential for mitochondrial mRNA processing, and *ise1* mutants are defective in the formation of mitochondrial electron transport gradients necessary for ATP production (Stonebloom et al. 2009). In addition, *ise1* mutants produce increased levels of reactive oxygen species (ROS). The *gat1* mutant produces increased levels of ROS as well (Benitez-Alfonso et al. 2009). *GAT1* encodes a plastid thioredoxin and *gat1* mutants increase callose production, leading to PD closure (Benitez-Alfonso et al. 2009). ROS are thought to act as critical signalling molecules mediating cross-talk between mitochondria, plastids and the nucleus. However, there is no "one size fits all" for the role of ROS in PD transport as *ise1* and *gat1* mutants have the opposite effect on intercellular trafficking (Benitez-Alfonso et al. 2009; Stonebloom et al. 2009). Thus, we proposed (Stonebloom et al. 2009) that the site of ROS production, mitochondria versus plastids, differentially regulates cellular processes and associated specific targets that in turn affect PD function. We recently performed a systematic study to chemically induce ROS in mitochondria versus chloroplasts and the results support our hypothesis (Stonebloom et al. 2011). Thus, oxidized mitochondria lead to increased cell-to-cell transport and oxidized plastids lead to reduced cell-to-cell transport. In the same study, we assayed the redox state of the mitochondria versus plastids following gene silencing of *ISE1* or *ISE2*. Loss of ISE1 leads to mitochondrial oxidation as expected. However, loss of ISE2 leads to reduction of plastid redox state, which supports that loss of ISE2 has the opposite effect on PD transport compared to *gat1* (which is expected to have oxidized plastids). In addition, we proposed (Stonebloom et al. 2009) that relative amount of ROS may differentially affect PD function, as the levels of ROS produced in *gat1* likely are higher than those produced in *ise1*. Indeed, a recent study showed that treatment of *Arabidopsis* roots with low concentrations of H_2O_2 drastically increased PD permeability while high concentrations induced PD occlusion (Rutschow et al. 2011).

5 New Concept: Cellular Homeostasis Affects PD Formation and PD Function

The data revealing *de novo* formation of secondary PD in *ise1* and *ise2* mutants and following gene silencing of *ISE1* or *ISE2* are the only demonstration to date of genetic pathways that regulates PD biogenesis. When we embarked on our mutant screen for PD transport alterations, we expected to uncover genes whose products localized to PD. In hindsight this was a naïve expectation. However, our findings are not disappointing. Instead they reveal that cellular homeostasis and cross talk between important cellular organelles (mitochondria for ISE1 and chloroplasts for ISE2) are vital to establishing the degree of intercellular transport via PD and the *de novo* formation of PD. Studies with the *gat1* mutant reinforce this idea and reveal that interfering with plastid homeostasis by altering redox state via thioredoxin and ROS levels negatively regulates PD function.

We are currently evaluating the transcriptomes of *ise1* and *ise2* embryos compared to wild type embryos. Since both mutants have similar overall morphology and similar effects on PD (increased PD transport and increased secondary PD formation) yet their gene products reside in different organelles, mitochondria and chloroplasts, we expect to uncover genes that are commonly effected in both mutants that will allow us to dissect their respective roles in PD formation/function. It is important to note that ISE1 and ISE2 are both RNA helicases. RNA helicases are essential in all stages of mRNA production and turnover. Thus, defects in ISE1 and ISE2 are expected to affect mRNA levels in either mitochondria or chloroplasts. Thus, acute defects resulting from loss of mitochondrial or chloroplast function should allow systematic investigation of their downstream effects on PD.

Our analyses of gene expression in *ise1* and *ise2* embryos found that the most significantly affected class of transcripts in both mutants encodes products targeting to and enabling plastid function (Burch-Smith et al. 2011). Indeed, ultrastructural analyses show that plastid development is defective in both mutants (Burch-Smith et al. 2011). While defective plastids were expected following loss of plastid-localized ISE2, the defective plastids formed following loss of mitochondrial ISE1 were surprising. These results reinforce the importance of plastid-mitochondria-nucleus crosstalk, and PD as critical players in the plant cell communication network. Thus, they illuminate a new signaling pathway we dub organelle-nucleus-plasmodesmata signaling (ONPS).

6 Conclusions and Perspectives

Arabidopsis embryogenesis occurs relatively rapidly. Torpedo embryos are formed within 4 days post fertilization, and different stages of embryogenesis can be easily visualized in independent siliques along the inflorescence axis. *Arabidopsis* embryogenesis is an especially attractive system to study genes critical for development as severely defective mutants can nevertheless be propagated in heterozygote plants

that carry both wild type and mutant embryos segregating in the same silique for direct comparison.

Surprisingly, while mutants such as *ise1* and *ise2* cause significant alterations in PD formation and function, their gene products do not localize to PD. Similarly, a seedling defective mutant with altered PD transport, *gat1*, also does not localize to PD. These data underscore how little we know about the intricacies of PD synthesis and modes of action, but nevertheless open the door to investigate more global cellular processes to uncover their impact on PD synthesis and regulation. These studies are extremely important given the remarkable ability of PD to transport a range of developmentally critical factors, from small growth regulators and nutrients, to macromolecules, such as transcription factors, gene silencing RNAs and microRNAs.

Acknowledgments SS was supported by an NSF Predoctoral Fellowship. TBS acknowledges the UC Berkeley Miller Institute for Basic Research in Science postdoctoral fellowship. PD work in the Zambryski lab is supported by National Institutes of Health Grant GM45244.

References

Benitez-Alfonso Y, Cilia M, San Roman A, Thomas C, Maule A, Hearn S, Jackson D (2009) Control of Arabidopsis meristem development by thioredoxin-dependent regulation of intercellular transport. Proc Natl Acad Sci USA 106:3615–3620

Burch-Smith TM, Zambryski PC (2010) Loss of increased size exclusion limit (ISE)1 or ISE2 increases the formation of secondary plasmodesmata. Curr Biol 20:989–993

Burch-Smith TM, Anderson JC, Martin GB, Dinesh-Kumar SP (2004) Applications and advantages of virus-induced gene silencing for gene function studies in plants. Plant J 39:734–746

Burch-Smith TM, Stonebloom S, Xu M, Zambryski PC (2011) Plasmodesmata during development: re-examination of the importance of primary, secondary, and branched plasmodesmata structure versus function. Protoplasma 248:61–74

Burch-Smith TM, Brunkard J, Choi YG, ZAmbryski PC (2011) Organelle-nucleus crosstalk regulates plant intercellular communication via plasmodesmata. In press

Capron A, Chatfield S, Provart N, Berleth T (2009) Embryogenesis: pattern from a single cell. The Arabidopsis Book 7:e0126. doi:10.1199/tab.0126

Cho E, Zambryski PC (2011) Organ boundary1 defines a gene expressed at the junction between the shoot apical meristem and lateral organs. Proc Natl Acad Sci USA 108:2154–2159

Crawford KM, Zambryski PC (2000) Subcellular localization determines the availability of non-targeted proteins to plasmodesmatal transport. Curr Biol 10:1032–1040

Ding B, Haudenshield JS, Hull RJ, Wolf S, Beachy RN, Lucas WJ (1992) Secondary plasmodesmata are specific sites of localization of the tobacco mosaic virus movement protein in transgenic tobacco plants. Plant Cell 4:915–928

Epel BL (1994) Plasmodesmata: composition, structure and trafficking. Plant Mol Biol 26: 1343–1356

Faulkner C, Akman OE, Bell K, Jeffree C, Oparka K (2008) Peeking into pit fields: a multiple twinning model of secondary plasmodesmata formation in tobacco. Plant Cell 20:1504–1518

Hofius D, Herbers K, Melzer M, Omid A, Tacke E, Wolf S, Sonnewald U (2001) Evidence for expression level-dependent modulation of carbohydrate status and viral resistance by the potato leafroll virus movement protein in transgenic tobacco plants. Plant J 28:529–543

Jurgens G, Mayer U (1994) A colour atlas of developing embryos. (Harcourt Health Sciences)

Kim I, Zambryski PC (2005) Cell-to-cell communication via plasmodesmata during Arabidopsis embryogenesis. Curr Opin Plant Biol 8:593–599

Kim I, Hempel FD, Sha K, Pfluger J, Zambryski PC (2002) Identification of a developmental transition in plasmodesmatal function during embryogenesis in Arabidopsis thaliana. Development 129:1261–1272

Kim I, Cho E, Crawford K, Hempel FD, Zambryski PC (2005a) Cell-to-cell movement of GFP during embryogenesis and early seedling development in Arabidopsis. Proc Natl Acad Sci USA 102:2227–2231

Kim I, Kobayashi K, Cho E, Zambryski PC (2005b) Subdomains for transport via plasmodesmata corresponding to the apical-basal axis are established during Arabidopsis embryogenesis. Proc Natl Acad Sci USA 102:11945–11950

Kobayashi K, Otegui MS, Krishnakumar S, Mindrinos M, Zambryski P (2007) Increased size exclusion limit 2 encodes a putative DEVH box RNA helicase involved in plasmodesmata function during Arabidopsis embryogenesis. Plant Cell 19:1885–1897

Laux T, Wurschum T, Breuninger H (2004) Genetic regulation of embryonic pattern formation. Plant Cell 16(Suppl):S190–202

Mansfield SG, Briarty LG (1991) Early embryogenesis in *Arabidopsis thaliana*: II. The developing embryo. Can J Bot 69:461–476

McLean BG, Hempel FD, Zambryski PC (1997) Plant intercellular communication via plasmodesmata. Plant Cell 9:1043–1054

Poethig SR, Coe EH, Johri MM (1986) Cell lineage patterns in maize embryogenesis: a clonal analysis. Dev Biol 117:392–404

Roberts IM, Boevink P, Roberts AG, Sauer N, Reichel C, Oparka KJ (2001) Dynamic changes in the frequency and architecture of plasmodesmata during the sink-source transition in tobacco leaves. Protoplasma 218:31–44

Rutschow HL, Baskin TI, Kramer EM (2011) Regulation of solute flux through plasmodesmata in the root meristem. Plant Physiol doi:10.1104/pp. 110.168187

Saulsberry A, Martin PR, O'Brien T, Sieburth LE, Pickett FB (2002) The induced sector Arabidopsis apical embryonic fate map. Development 129:3403–3410

Scheres B, Wolkenfelt H, Willemsen V, Terlouw M, Lawson E, Dean C, Weisbeek P (1994) Embryonic origin of the *Arabidopsis* primary root and root meristem initials. Development 120:2475–2487

Scheres B, Benfey P, Dolan L (2002) Root development. The Arabidopsis Book 1:e0101. doi:10.1199/tab.0101

Stadler R, Lauterbach C, Sauer N (2005) Cell-to-cell movement of green fluorescent protein reveals post-phloem transport in the outer integument and identifies symplastic domains in Arabidopsis seeds and embryos. Plant Physiol 139:701–712

Stonebloom S, Burch-Smith T, Kim I, Meinke D, Mindrinos M, Zambryski P (2009) Loss of the plant DEAD-box protein ISE1 leads to defective mitochondria and increased cell-to-cell transport via plasmodesmata. Proc Natl Acad Sci USA 106:17229–17234

Stonebloom S, Brunkard JO, Cheung, AC, Juang K, Feldman L, Zambryski PC (2011) Redox states of plastids and mitochondria differentially regulate intercellular transport via plasmodesmata. In press

Werner D, Gerlitz N, Stadler R (2011) A dual switch in phloem unloading during ovule development in Arabidopsis. Protoplasma 248:225–235

Wolf S, Deom CM, Beachy RN, Lucas WJ (1989) Movement protein of tobacco mosaic virus modifies plasmodesmatal size exclusion limit. Science 246:377–379

Chapter 3
Function and Identification of Mobile Transcription Factors

Ji-Young Lee and Jing Zhou

1 Introduction

Coordinated growth and development of multi-cellular organisms require mechanisms that allow for extensive cell-to-cell communication. Sometimes these communications are mediated by signaling molecules that are directly transported from one cell to the other. Since cells in plants, in contrast to animal cells, cannot migrate, the direct transport of mobile molecules seems to play a more significant role in such communications than in animal system. Sessile nature of plant growth also necessitated the evolution of mechanisms that rapidly transmit signaling molecules in response to environmental changes or pathogen attacks. These mobile signals in plants have been found in forms of proteins, RNAs, and small molecules. They are transmitted through the vascular system in a long distance (between organs), or through plasmodesmata in a short distance (between cell types).

As mobile signals, transcription factors have been found to play crucial roles in a growing number of studies. For example, KNOTTED1 in maize is the first transcription factor that has been identified to move between cells. It regulates shoot apical meristem (SAM) activities by moving from the second cell layer (L2) of the meristem to the first cell layer (L1). SHORT ROOT (SHR) in Arabidopsis roots moves from the vascular cylinder to endodermis/cortex initials and then drives asymmetric cell division that generates endodermis and cortex. Recently cell-to-cell movement of SHR was also shown to be required for cell type patterning in the vascular tissues. SHR does this in the endodermis by activating the expression of mobile microRNAs, which subsequently move out of the endodermis and affect their targets in the vascular stem cells. Cell-to-cell movement of these and several other transcription factors,

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F. Kragler and M. Hülskamp (eds.), *Short and Long Distance Signaling*, Advances in Plant Biology 3, DOI 10.1007/978-1-4419-1532-0_3,

which will be reviewed in this chapter, plays an indispensable role for providing positional information during specification and patterning of cell types in plants.

Transcription factors move in short distance mainly through plasmodesmata. However, only few mobile transcription factor proteins are smaller than the size exclusion limit of plasmodesmata, a maximum size of molecules that is allowed for free diffusion through openings of plasmodesmata. This suggests that many of transcription factors, which are mobile as proteins, might be actively recruited by components that increase the size exclusion limit and facilitate cell-to-cell movement. A lot of effort is invested to find the cellular components facilitating intercellular transport of proteins and RNAs (see also Chap. 1, Manfred Heinlein; Chap. 2, P. Zambrisky; Chap. 4, D. Jackson, and Chap. 7, Ruiz-Medrano).

Transcription factors move not only between cell layers but also between organs. Movement of transcription factors in a long distance has been found to happen through phloem sieve cells in a form of RNA. For example, BEL5 mRNAs in potatoes move from shoot apices to stolons, which will develop into potato tubers. Messenger RNAs move in the form of protein-RNA complexes by binding to RNA binding proteins, which protect and facilitate the mRNA movement through phloem.

So far there are only a small number of transcription factors that have been found to be mobile and influence plant development. Recent progress in technology in genomics and proteomics and other biotechnologies will enable the discovery of more mobile transcription factors in coming years.

In this chapter, we will comprehensively review mobile transcription factors: factors that have been discovered, mechanisms of their mobility, new tools that will lead to the discovery of mobile transcription factors, and putative mobile transcription factors inferred from cell-type specific RNA profiling data.

2 Mobile Transcription Factors in Plants

2.1 Cell-To-Cell Movement of Transcription Factor Proteins

2.1.1 KNOTTED1, the First Mobile Transcription Factor Discovered

KNOTTED1 (KN1), a homeobox family transcription factor, is the first transcription factor that has been discovered to move between cells (Lucas et al. 1995). Its gene was identified from a dominant mutant in maize, which develops knots on developing leaves (Vollbrecht et al. 1991). Expression analysis of KN1 showed the distinctive distribution between its RNAs and proteins' (Jackson et al. 1994). KN1 RNAs were detected in the cells below the L1 layer of SAM, however its proteins were found throughout the meristem. Such a difference in expression domains provoked the idea that KN1 proteins might move from inner cells to cells in the L1 layer of SAM. The localization of KN1 proteins was monitored after injecting the KN1 tagged with fluorescent molecules into mesophyll cells of tobacco and maize leaves.

This experiment showed that KN1 proteins move from one cell to the other (Lucas et al. 1995). Further domain analyses in the same study suggested that the homeodomain is critical for cell-cell trafficking of KN1. Interestingly, movement of KN1 proteins also facilitated its own mRNA movement. More detailed movement mechanisms of KN1 are described in Chap. 4 (Dave Jackson).

As an important developmental regulator, KN1 shows the conserved cell-to-cell movement in flowering plants (Kim et al. 2002, 2003). In *Arabidopsis* homeobox family, KNOTTED 1-like homeobox protein 1/BREVIPEDICELLUS (KNAT1/BP) and SHOOTMERISTEMLESS (STM) are most closely related to KN1. STM and KN1 have very similar function in initiating and maintaining the SAM (Long et al. 1996). KNAT1/BP is involved in the regulation of inflorescence architecture (Venglat et al. 2002). Kim et al. demonstrated that both KNAT1 and STM proteins are mobile from L1 to inner layers in the shoot apical meristem when they were expressed under a L1 specific promoter (Kim et al. 2003). In rice, three KN1-like homeobox class 1 transcription factors, Oskn1~3, were tested for their cell-to-cell trafficking by transiently expressing them in onion epidermis. Among these, Oskn1 (OSH1) showed extensive mobility between cells (Kuijt et al. 2004).

2.1.2 Mobile LAX PANICLE1 in the Axillary Meristem Formation

In grasses, formation of axillary meristems affects the overall plant architecture. Among several mutants that affect axillary meristem formation, *lax panicle1* (*lax1*) phenotype (Komatsu et al. 2001) was found to be caused by the mutation in a bHLH transcription factor. *LAX1* mRNA is expressed in an adaxial boundary between the axillary meristem and shoot apical meristem, however LAX1 function is required inside the axillary meristem. This indicated that LAX1 might function in a non-cell autonomous manner. Such non-cell autonomous behavior is explained by the mobility of LAX1 proteins. LAX1 proteins fused to a GFP monomer were distributed inside the axillary meristem as well as in the adaxial junction whereas its RNA was only restricted to the junction (Oikawa and Kyozuka 2009). Since GFP:LAX1 fusion proteins only move toward the axillary meristem, the movement of LAX1 seems to be directional. When the size of LAX1 fusion proteins was increased by attaching 3xGFP, the mobility of 3xGFP:LAX1 decreased significantly, which might suggest that LAX1 does not influence the size exclusion limit of plasmodesmata.

2.1.3 Bidirectional Communication of Mobile Transcription Factors in the Hair Cell Development

Epidermis in the root and leaf is a cell layer in direct contact with outer environments. Root hairs formed in the root epidermal layer are key cells that absorb water and minerals from soil. Above ground similar epidermis-derived cellular extensions are formed and named leaf trichomes. They function in protecting leaves from predators

and pathogens, and preventing the overheating of leaf surface. Both, root and leaf hair cells, appear in a regular pattern, which is established by a similar set of transcription factors that act as signaling molecules between hair and neighboring non-hair cells. Detailed gene regulatory programs in epidermal cell patterning are reviewed elsewhere (Ishida et al. 2008; Schiefelbein et al. 2009). Transcription factors that belong to R3 Myb and bHLH families contribute to the patterning of root hairs and trichomes by moving between cells.

CAPRICE (CPC), a small R3 Myb transcription factor, promotes root hair formation but inhibits trichome development (Wada et al. 1997, 2002; Kurata et al. 2005). CPC proteins, generated in the atrichoblasts (precursors of hairless cells), travel to the trichoblasts (precursors of hair cells) where CPC promotes the formation of root hairs. Kurata et al. (2005) demonstrated that two discrete regions in the N-terminus and Myb domain are required for CPC movement. A single amino acid substitution in W76 and M78 in the Myb domain of CPC drastically reduced its mobility. During its movement, CPC seems to increase the size exclusion limit of plasmodesmata. When *CPC* fused to multimerized GFP was expressed under *CPC* promoter, these proteins moved between cells whereas the same multimerized GFP without CPC did not. The inter-cellular movement of CPC also seems to be regulated in a tissue-specific manner. *CPC* was expressed under promoters that drive gene expression in the root stele (vascular tissues and pericycle) and trichoblasts, respectively. CPC proteins moved from trichoblasts to atrichoblasts, however, they did not move from the stele to an outer cell layer.

The mobility of two other R3 MYB family proteins, ENHANCER OF TRY AND CPC 3 (ETC3) and TRIPTYCHON (TRY), has been observed in the leaf epidermis (Digiuni et al. 2008; Wester et al. 2009). Wester et al. reported ETC3 transport between epidermal cells as well as between the epidermis and underlying cells in the leaves (Wester et al. 2009). In the *etc3* null mutant, trichome density increased suggesting a negative role of ETC3 in the trichome identity.

CPC, ETC3, and TRY interact with GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3), bHLH transcription factors. In the root, these two transcription factors move from trichoblasts to atrichoblasts to define the non-hair cell fate (Bernhardt et al. 2005). Studies indicated that *GL3* and *EGL3* are preferentially upregulated by CPC in the trichoblasts, while they repress their own expression after traveling to atrichoblasts. Therefore, CPC and GL3/EGL3 movement forms a bidirectional regulatory circuit that mediates the communication between trichoblasts and atrichoblasts. Furthermore, an elevated level of GL3 proteins in the over-expression lines significantly reduced mobility of CPC, suggesting the protein-protein interaction interferes with the cell-to-cell movement of CPC (Wester et al. 2009).

2.1.4 SHORT ROOT Movement in the Root

Cell type patterning and growth in the root are governed by root apical meristem. Plant roots are composed of radial tissues that are centripetally organized: epidermis, cortex, endodermis, and pericycle. Inside the pericycle, vascular tissues are organized in multi-symmetry. Development of these root tissues are driven by

activities of cell division and cell type specification in the stem cell niche, which is composed of undifferentiated stem cells and the quiescent center, a stem cell organizer (Bennett and Scheres 2010; Sablowski 2011).

Cell-to-cell movement of SHORT ROOT (SHR), a member of plant specific GRAS family transcription factors, plays a critical role in the patterning of root tissues (Helariutta et al. 2000). SHR protein is produced in the stele, and then moves into adjacent cell layers including quiescent center, cortex/endodermal initials, and endodermis. In these cell types, SHR directly activates the expression of *SCARECROW* (*SCR*), which encodes another GRAS family transcription factor (Helariutta et al. 2000; Nakajima et al. 2001). The presence of SHR and SCR in the endodermis/cortex initials subsequently trigger the asymmetric cell division that forms endodermis and cortex. Recently, SHR and SCR in the endodermis were also shown to be required for the vascular cell type patterning by activating the expression of microRNA 165/6 as a retrograde signal (Carlsbecker et al. 2010; Miyashima et al. 2011). Mobile microRNA 165/6 establishes the graded distribution of the mRNAs that encode class III Homeodomain Leucine-Zipper family (HD-ZIP III) transcription factors via post-transcriptional regulation, which is low in the stele periphery and high in the center.

Cell-to-cell movement of SHR is regulated in a tissue specific manner. When *SHR* was expressed in the cell types where it is not normally expressed, SHR proteins did not move between cells (Sena et al. 2004). Studies by Gallagher et al. proposed a model that a balance between cytoplasmic and nuclear localization is required for SHR movement (Gallagher and Benfey 2009). Similar to CPC, more than one region were found to be important for both mobility and function of SHR. However, a deletion of LNELDV in the second leucine heptad repeat region disrupted only mobility but not functionality. It was also shown that signals for intercellular movement are likely to be conserved within GRAS domain since a modified SCR protein without nuclear localization signal (NLS) moved from cell to cell like SHR.

For proper patterning of endodermis and cortex, SHR has to be confined to the endodermis as ectopic SHR expression in the cortex causes supernumerary cell layers. Interestingly, a study demonstrated that SCR proteins, produced by transcriptional activation of SHR, interact with SHR and thereby restrict SHR proteins to the nucleus and inhibit their further movement (Cui et al. 2007). This is consistent with the finding that SHR needs to be in the cytoplasm to move to a neighboring cell layer (Gallagher et al. 2004).

2.1.5 Mobile bHLH Transcription Factors in the Root Initiation and Growth

Three mobile transcription factors in the bHLH family have been found to play various roles in the root initiation and growth. One of them is *TARGET OF MP 7* (*TMO7*), a direct target of auxin-dependent transcription factor MONOPTEROS (MP) (Schlereth et al. 2010). MP, also known as *AUXIN RESPONSE FACTOR 5* (*ARF5*), regulates gene expression in response to auxin (Hardtke and Berleth 1998).

MP activity has been shown to be important for controlling hypophysis specification and embryonic root initiation. Schlereth et al. (2010) showed that MP directly activates the expression of *TMO7* in the embryo cells adjacent to hypophysis. TMO7 protein was then found to move into the hypophysis precursor cells. Such directional transport allows TMO7 to act as intercellular signal that mediates MP regulation on the initiation of root stem cells. Unlike CPC, the cell-to-cell movement of TMO7 seems to be dependent on the size. At the same time, TMO7 movement seems to be directional. The addition of triple-GFP (84 kD) to the 11 kD TMO7 protein blocked movement beyond the expression domain, and led to a mostly cytoplasmic localization. This TMO7:GFP fusion protein was detected in the hypophysis, but not in the apical half of the embryo.

Another mobile bHLH transcription factor recently identified is UPBEAT1 (UPB1). UPB1 controls the transition from cell proliferation to differentiation in the root (Tsukagoshi et al. 2010). UPB1 proteins seem to move from the lateral root cap (LRC) to vascular tissues. In vascular tissues, UPB1 positively regulates expression of peroxidases which promote the production of reactive oxygen species (ROS). ROS subsequently promote the transition of cell status from meristem to differentiation. Correlation between LRC and ROS activation zone indicates that the LRC emits the positional information which is provided by the UPB1 moving horizontally and triggering the production of ROS in the right place. The movement of UPB1 was significantly reduced when it was fused to 3xYFP but not completely abolished.

Another bHLH transcription factor, named POPEYE, is very interesting because it seems to regulate root development and protein movement in response to abiotic stresses. In plants exposed to iron deficiency, expression of *POPEYE* is induced specifically in the pericycle and root cap (Long et al. 2010). From there POPEYE protein moves to the neighboring root meristem and stele. Though not been emphasized in the paper by Long et al. (2010), POPEYE proteins seem to change their sub-cellular localization in response to iron deficiency. Exposed to iron rich conditions, POPEYE localizes in the cytoplasms and cell boundaries. However, under iron deficiency, POPEYE is found mainly in the nuclei suggesting that nuclear localization might be required for the cell-to-cell mobility (Gallagher and Benfey 2009).

2.1.6 Cell-To-Cell Movement of Transcription Factors in the Flower Development

There are several transcription factors documented to be mobile which are involved in flower development. Comprehensive reviews for floral meristem formation and development are available elsewhere (Goto et al. 2001; Jack 2004; Causier et al. 2009). LFY, an important floral meristem regulator, has been shown to move in a non-directional manner, which is different from the mode of cell-to-cell movement of most of aforementioned transcription factors (Sessions et al. 2000). When *LFY* was expressed controlled by a promoter that drives expression in the L1 layer of

developing flowers, the *lfy* mutant phenotype was fully rescued. In such transgenic plants, LFY proteins, but not LFY RNAs, were detected in all layers indicating that LFY proteins move from the L1 into inner the layers. The mobility of LFY proteins into the inner cell layers of floral meristems was gradual and dependent on the size of protein, suggesting that LFY movement could be based on diffusion. Such type of intercellular trafficking might be related to the lack of particular domains required for targeted cell-to-cell movement, as indicated by a domain analysis of LFY (Wu et al. 2003).

Two MADS-box family transcription factors, DEFICIENS (DEF) and GLOBOSA (GLO), function in a non-cell-autonomous manner to control petal and stamen organ identity in *Antirrhinum* (Perbal et al. 1996). In *DEF* or *GLO* mutants, organ primordia in the second and third whirls in a flower, which form petals and stamens in wild-type plants, turn into sepals and carpels, which represent organs of the first and fourth whorls (Carpenter and Coen 1990). When *DEF* expression was specifically established in the L2 and L3 layers of floral meristem in the *def* mutant, petals developed in the second whirl and deposited pigments in the L1 layer. Such full recovery of petals seems to be achieved by DEF proteins that move from the L2 and L3 layers into the L1 layer. However, the respective *Arabidopsis* orthologous proteins APETALA3 (AP3) and PISTILLATA (PI) do not seem to have similar activities (Jenik and Irish 2001). Another MADS-box family transcription factor, AGAMOUS (AG) has been shown to be able to move between cells in the L1 layer as well as into the inner cells from the L1 layer in the floral meristem. AG cell-to-cell movement might suppress *WUS* expression in the underlying cells, thereby driving determinate formation of floral organs (Urbanus et al. 2010).

2.2 Transcription Factors Moving Long Distance

2.2.1 Mobile *LeT6* Affects Leaf Architecture

In wild-type tomato, leaves develop as unipinnate compound leaves with acutely pointed tips. Leaves in a dominant mutant *Mouse ears* (*Me*) develop round-shaped leaves with higher orders of compoundness. The *Me* mutant phenotype is caused by a fusion between *LeT6*, a gene encoding a KNOTTED-1–like homeobox (KNOX) transcription factor, and *PFP*, a gene coding for PYROPHOSPHATE-DEPENDENT PHOSPHOFRUCTOKINASE, glycolytic pathway enzyme (Chen et al. 1997). This gene fusion, which has 10 kb of native *PFP* upstream sequence and a part of the *PFP* coding region, enhances *LeT6* expression in *Me* mutant, thereby increasing the complexity of leaf structure. Grafting studies conducted by Kim et al. (2001) suggested that *LeT6* RNAs moves through the phloem (Kim et al. 2001). When wild-type tomato plants were grafted onto *Me* stocks, newly developing leaves on the wild-type scion turned into leaves similar to *Me* mutants. Further more *in situ* RT-PCR experiments suggested the presence of *PFP-LeT6* RNA in the shoot apices of wild-type scions supported by mutant stock plants.

2.2.2 Movement of *CmNACP* Through Phloem

Phloem sieve elements are thought to contribute to the transport of signaling molecules (see also Chap. 7, Ruiz-Medrano et. al.). To find whether those molecules include mRNA and to reveal their identity, mRNAs were isolated from the phloem sap of pumpkin (*Cucurbita maxima*) and a cDNA library was produced and sequenced. By this means RNA species were identified that encode several transcription factors highly similar to NAM (CmNACP), RING (CmRINGP), GAI (CmGAIP), WRKY (CmWRKYP), and STM (CmSTMP) (Ruiz-Medrano et al. 1999). The mobility of these mRNAs was further confirmed by hetero-grafting experiments using a pumpkin as stock and a cucumber as scion. In these experiments, all of these transcription factor RNAs were detected in the phloem sap of cucumber stem when grafted onto pumpkin stock. Further analyses using *in situ* RT-PCR suggested, that, despite RNA movement into sieve tubes in the stem happens widely, movement of RNA into the meristem is more selective. Among five mobile transcription factors that showed mobility along the phloem system, only *CmNACP* and *CmGAIP* mRNAs were detected in the apical region of the cucumber scion after the hetero-grafting.

2.2.3 *St BEL5* Regulates the Development of Potato Tubers

Tuber formation in potatoes is a complex developmental process that requires the interaction of environmental, biochemical, and genetic factors. In potato genotypes such as *S. tuberosum* ssp *andigena*, short-day (SD) photoperiods are strictly required for tuber formation, whereas long-day (LD) conditions inhibit tuberization. *St BEL5* is ubiquitously expressed in potato plants but accumulation of its RNA is enhanced in leaves and stolons by SD conditions (Chen et al. 2003). *St BEL5* RNA levels accumulate in an increasing concentration gradient from the shoot tip (low levels) through the stem to the stolon tip in response to SD conditions. Under LD conditions, the highest levels of RNA are observed in shoot tips and stolons with the lowest levels in the lower portion of the stem. Over-expression of *St BEL5* induced tuber formation even in non-inductive condition.

Grafting experiments suggested that *St BEL5* RNAs are mobile moving through the phloem. Transport is induced by short day photoperiod and regulated by the 3′ untranslated region of *St BEL5*, indicating that the 3′ untranslated region may contain a motif which is recognized by RNA-binding proteins (Banerjee et al. 2006, 2009; Hannapel 2010).

2.2.4 Conserved Mobility of *GIBBERELLIC ACID-INSENSITIVE*

Arabidopsis gene *GIBBERELLIC ACID-INSENSITIVE* (*GAI*) encodes a protein that belongs to the GRAS family, and functions as a negative regulator of gibberellic acid (GA) responses. Mutated GAI carrying deletions in the DELLA domain inhibits some components of the GA signaling pathway and causes dark green

dwarf phenotype in a semi-dominant manner. The mobility of *GAI* RNA along sieve cells in the stem was initially found in the pumpkin (Ruiz-Medrano et al. 1999) and further characterized in detail in pumpkin, tomato, and Arabidopsis (Haywood et al. 2005). Hetero-grafting experiments showed that RNAs of *GAI* from *Cucurbita maxima* (*CmGAIP*) are mobile moving from a pumpkin stock to a cucumber scion. The same phenomenon was observed when the wild-type scions of Arabidopsis and tomato were grafted onto stocks ectopically expressing *CmGAIP* or Arabidopsis Δ*DELLA-gai*. Also in tomato Δ*DELLA-gai* moved from a stock to a scion affecting the colors of leaves forming on scions. This suggests that mobile Δ*DELLA-gai* RNAs were translated to functional proteins. The mobility of *GAI* RNA was also observed in apples (Xu et al. 2010). However, unlike in the studies of Arabidopsis, pumpkin, and tomato, which showed directional *GAI* RNAs transport from the stock to the scion, *GAI* RNAs were mobile in to both directions.

This evolutionarily conserved RNA mobility of *GAI* transcripts seems to be mediated by an secondary RNA structure (Huang and Yu 2009). Other closely related paralogues of *GAI* did not show the RNA mobility suggesting that mobility has specifically evolved in *GAI*. Domain analyses of *GAI* RNA suggested that a part of the coding region of RNA which starts ~1 kb after the translational start site and the 3′ untranslated region are required for the RNA mobility. A random mutagenesis analysis of this region indicated that the secondary RNA structure involving a stem-and-loop structure might play a more important role than a specific nucleotide sequence.

3 Cellular Contexts That Influence Cell-To-Cell Movement of Transcription Factors

As described in the previous section, a growing number of transcription factors have been found to move between cells in forms of proteins or RNAs. For many of them, their mobility plays an important role in plant functions. However, it remains an open question how many transcription factors move between cells. In a recent study, a comparison was made between cell type specific root expression data and expression patterns of transcriptional and translational GFPs driven by promoters of 24 cell type enriched transcription factors in the Arabidopsis root (Lee et al. 2006). Comparison of three data sets, which include transcriptional GFPs driven by endogenous promoters of individual transcription factors, translational GFPs which are fused to the coding regions of transcription factors and driven under promoters of corresponding transcription factors, and cell-type specific root expression data, indicated that nearly 25% of transcription factors (6 out of 24) might move between cells as proteins in the Arabidopsis root. This frequency is much higher than anticipated based on the number of mobile transcription factors identified so far (Table 3.1).

Now it is well established that proteins and RNAs move between cells through plasmodesmata. Cell-to-cell movement of transcription factors as proteins involves

Table 3.1 A list of transcription factors that move between cells as RNAs or proteins

Transcription factor	Gene family	Molecular weight[a]	What is moving?	Mechanism	Origin	Target	Organism	Literature
CAPRICE	R3-type MYB	11 kD	Protein	Targeted	Atrichoblast	Trichoblast	Arabidopsis	(Wada et al. 2002)
KNAT1/BREVIPEDICELLUS	KNOX I Homeobox	46 kD	Protein	Targeted	L1 of shoot meristem	L2/3 of shoot meristem	Arabidopsis	(Kim et al. 2003)
KNOTTED1	KNOX I Homeobox	40 kD	RNA/ protein	Targeted	Below L1 of SAM	L1 of SAM	Maize	(Lucas et al. 1995)
STM	KNOX I Homeobox	43 kD	Protein	Targeted	L1 of shoot meristem	L2/3 of shoot meristem	Arabidopsis	(Kim et al. 2003)
LEAFY	LFY	47 kD	Protein	Non-targeted	L1 of shoot meristem	L2/3 of shoot meristem	Arabidopsis	(Sessions et al. 2000)
LAX PANICLE1 (LAX1)	bHLH	23 kD	Protein	Targeted	Adaxial boundary between axillary meristem and shoot apical meristem	Rest of axillary meristem	Rice	(Oikawa and Kyozuka 2009)
SHORT ROOT (SHR)	GRAS	59 kD	Protein	Targeted	Stele in the root	Endodermis and QC	Arabidopsis	(Nakajima et al. 2001)
TARGET OF MP 7 (TMO7)	bHLH	11 kD	Protein	Non-targeted	Outside the hypophysis	Hypophysis and vascular tissues	Arabidopsis	(Schlereth et al. 2010)
AT4G00940	C2C2-Dof	34 kD	Protein		Pericycle in the root	Endodermis	Arabidopsis	(Lee et al. 2006)
AT4G27410/RD26	NAC	33 kD	Protein		Pericycle and endodermis in the root	Cortex and epidermis	Arabidopsis	(Lee et al. 2006)
AT3G61850/DAG1	C2C2-Dof	33 kD	Protein		Stele in the root	Endodermis	Arabidopsis	(Lee et al. 2006)
AT2G22850	bZIP	26 kD	Protein		Phloem pole pericycle	Endodermis and stele	Arabidopsis	(Lee et al. 2006)
AT4G37940/AGL21	MADS	26 kD	Protein		Procambium and columella root cap	Stele, endodermis, columella, and epidermis	Arabidopsis	(Lee et al. 2006)

ETC3	R3-type MYB	9 kD	Protein	Trichome	Neighboring epidermal cells around a trichome	Arabidopsis	(Wester et al. 2009)
TRIPTYCHON (TRY)	MYB	13 kD	Protein			Arabidopsis	(Digiuni et al. 2008)
GL3	bHLH	70.5 kD	Protein	Hair cell	Non-hair cell	Arabidopsis	(Bernhardt et al. 2005)
UPBEAT1	bHLH	12 kD	Protein	Lateral root cap	Root elongation zone	Arabidopsis	(Tsukagoshi et al. 2010)
DEFICIENS	MADS	26 kD	Protein	L2 or L3 of floral organ primordia	L1	Antirrhinum	(Perbal et al. 1996)
GLOBOSA	MADS	25 kD	Protein	L2 or L3 of floral organ primordia	L1	Antirrhinum	(Perbal et al. 1996)
AGAMOUS	MADS	29 kD	Protein	L1 of floral meristem	L2 and L3	Arabidopsis	(Urbanus et al. 2010)
POPEYE	bHLH	27 kD	Protein	Root cap	Root meristem	Arabidopsis	(Long et al. 2010)
St BEL5	homeobox		RNA	Shoot	Stolon	Potato	(Banerjee et al. 2006)
GAI	GRAS		RNA	Stock	Scion	Pumpkin, tomato, Arabidopsis	(Haywood et al. 2005; Xu et al. 2010)
LeT6	KNOX I Homeobox		RNA	Stock	Scion	Tomato	(Kim et al. 2001)
CmNACP	NAC		RNA	Stock	Scion	Pumpkin	(Ruiz-Medrano et al. 1999)
CmRINGP	RING		RNA	Stock	Scion	Pumpkin	(Ruiz-Medrano et al. 1999)
CmWRKYP	WRKY		RNA	Stock	Scion	Pumpkin	(Ruiz-Medrano et al. 1999)
CmSTMP	Homeobox		RNA	Stock	Scion	Pumpkin	(Ruiz-Medrano et al. 1999)

[a]The molecular weights of mobile proteins and not of mobile RNAs are shown

the alteration of size exclusion limit of plasmodesmata, however, the detailed mechanism of protein and RNA movement through plasmodesmata is described in other chapters of the book (Chap. 1, Manfred Heinlein; Chap. 2, P. Zambrysky; and Chap. 7, Ruiz-Medrano et al.). In recent studies, size exclusion limits have been estimated using free GFP (Crawford and Zambryski 2000; Kim et al. 2005a, b). Monitoring the movement of GFP proteins of various sizes suggested that plasmodesmatal size exclusion limits are regulated dynamically. In *Nicotiana* sp.leaf epidermis, 27 kD free GFPs were observed to move between cells at a relatively high frequency (23%). When the size of GFP was doubled (54 kD), GFP movement decreased dramatically (Crawford and Zambryski 2000). By contrast, a similar size of GFP fusion proteins that include P30 of the *Tobbaco mosaic virus* (57 kD) moved between cells at a frequency 30 times higher than 2xGFP. In addition to the mobility, free GFP and P30-GFP showed a difference in the sub-cellular localization. Whereas free GFP proteins were localized throughout the cell including the nucleus in a diffusive manner, P30-GFP proteins were located to punctae on the cell walls, which co-localize to plasmodesmata. Based on the distinctive nature of these proteins (GFP versus P30-GFP), Crawford and Zambryski categorized cell-to-cell protein movement into two types: targeted and non-targeted.

Non-targeted movement is more like diffusion, which is affected by the concentration and size of proteins. By contrast, targeted movement involves the interaction between mobile proteins and plasmodesmata components and thereby affects the size exclusion limit. Consistent with the idea that targeted movement involves the active regulation of size exclusion limit of plasmodesmata, targeted movement seems not to be strongly affected much by changes in environments and developmental stages (Crawford and Zambryski 2001). However, non-targeted movement was dramatically reduced by these factors.

As shown in Table 3.1, protein sizes of mobile transcription factors are distributed at a broad range. Some seem to be sufficient small to move to neighboring cells by diffusion. However, studies indicate that the protein size does not predict the mode of cell-cell movement. For example, CPC is a small 11 kD protein, however it moves between cells in a targeted manner (Kurata et al. 2005). When the protein size of CPC was dramatically increased by fusing it to 3xYFP, CPC:3xYFP the fusion still moved between cells. Furthermore, CPC moves only between epidermal cells and not in to the inner cell layers, suggesting that specific factors in the epidermis might direct cell-type specific movement. In contrast to CPC, a 47 kD large LFY fused to GFP seems to move from the L1 layer to inner layers by diffusion (Wu et al. 2003). This suggests that the size of transcription factors proteins is not a good indicator for predicting the mode of cell-to-cell trafficking.

Although the mode of protein movement between cells was categorized into two, complex behaviors of mobile proteins, additional data suggest that there might be multiple regulatory mechanisms governing protein mobility. One important factor that affects protein movement is protein-protein interaction. A number of studies indicate that protein-protein interaction(s) can interfere with the cell-to-cell mobility. SHR proteins do not move any further once they reach the endodermis where they can interact with SCR. These SHR-SCR protein complexes are formed in the nuclei,

and thereby seem to restrict SHR movement to the next cell layer (Cui et al. 2007). In the absence of SCR, SHR proteins are present in both cytoplasms and nuclei, and in the next cell layer (Sena et al. 2004; Cui et al. 2007). Similarly, the cell-to-cell movement of CPC was proposed to be restricted by the interaction with GL3 (Wester et al. 2009).

The second factor that was shown to affect protein movement is the sub-cellular localization. For SHR to move, it seems be present in two cellular compartments the nucleius and cytoplasm (Gallagher et al. 2004; Gallagher and Benfey 2009). When SHR was fused to the GFP with a nuclear localization signal (NLS), SHR movement was significantly reduced suggesting that cytoplasmic localization of SHR is necessary for cell-to-cell trafficking. However, when domains that are crucial for the SHR movement are deleted, SHR proteins were localized to the cytoplasm. Interestingly, as modified non-mobile SHR proteins were retargeted to the nuclei by fusing to NLS-GFP, these proteins were able to move again between cells. These studies suggest the presence of an unknown mechanism that requires the proper sub-cellular localization to facilitate cell-to-cell trafficking.

The third factor that affects protein mobility can be found in cell type specific mechanisms affecting cell-to-cell trafficking in an either positive or negative manner. Unlike free GFP and P30-GFP that move radially from the foci of their expression, movement of many mobile transcription factors seems to be directional. SHR expressed in phloem sieve cells or in the epidermis did not move (Sena et al. 2004). CPC expressed in the stele did not move either (Kurata et al. 2005). These observations suggest the presence of cell type specific factors that specifically facilitate or repress the movement of SHR and CPC. Although diffusive behaviors of LAX1 and TMO7 proteins suggest their transport might be non-targeted, LAX1 and TMO7 move in a directional manner (Oikawa and Kyozuka 2009; Schlereth et al. 2010). LAX1 proteins fused to a GFP monomer are distributed inside the axillary meristem as well as in the adaxial junction, but not to the opposite direction. TMO7 preferably moves in the shoot-ward direction. In the future it will be interesting to find whether these preferences in the direction of cell-to-cell trafficking are caused by a cell-type specific size exclusion limit of plasmodesmata or by other cell factors that actively regulate directional mobility.

4 Genome-Wide Approaches to Identifying Mobile Transcription Factors

Recent advances in technologies for profiling proteins and RNA at a global scale, isolating cell types and imaging are speeding up the discovery of transcription factors mobile as proteins and RNAs. Here, several existing approaches that can enable the efficient discovery of mobile transcription factors at a global level are introduced. Furthermore, though not listed in published manuscripts, transcription factors that might move as RNA in the phloem sieve cells are predicted from the cell type specific expression data.

4.1 Trichome-Assay Based Approach

Using trichome assay system (Kim et al. 2005c), domains required for cell-to-cell movement in KN1 were identified (Fig. 3.1a) (see also Chap. 4, Dave Jackson). In this approach, GLABRA1 (GL1), a transcription factor that promotes trichome formation on leaf epidermis, was expressed under *rbcS* promoter, which drives gene expression in the mesophyll cell, in *agl1* mutant background. In *agl1* mutant, trichomes do not develop on the leaf epidermis. Since GL1 acts in a cell-autonomous manner, GL1 expressed in the mesophyll cells cannot complement *gl1* mutant. However, when KN1 was translationally fused to GL1 expressed in *gl1* lines, trichomes developed because KN1:GL1 proteins moved from the mesophyll cells to the epidermal cells. This system can be applied in a cost-efficient manner to identify cellular factors altering the capacity of transcription factors to move between cells (Winter et al. 2007) or to identify novel transcription factors that actively move between cells.

4.2 Analysis of Transcription Factor Movement Using Cell Type Specific Promoters

The mobility of transcription factors can be assessed by expressing transcription factors, which are translationally fused to the visual markers, under a cell type specific promoter. This approach was used in the studies on the cell-to-cell movement of SHR in the root (Sena et al. 2004). SHR proteins, generated in the stele, move to the endodermis layer in the Arabidopsis root. SHR was expressed in the phloem companion cells in the root or in the epidermis. In either cell types, SHR proteins did not move to other cells. However, when SHR was expressed in the epidermis in the *scr* mutant background, SHR proteins moved into the cortex layer suggesting that components regulated by SCR are involved in controlling the cell-to-cell movement of SHR.

A similar approach was used to investigate the movement of KN1 (Kim et al. 2002, 2005c). GFP fused to KN1 was expressed controlled by a WUSCHEL promoter, which drives gene expression specifically in the L3 layer of shoot apical meristem, and under SCR, which drives gene expression in the L1 layer of shoot apical meristem. Under both promoters, expansion of proteins was observed beyond where they were produced, suggesting that KN1 can move in either direction in the shoot apical meristem.

Again, L1 specific promoter of *ATML1* was used to assess the movement of LFY and AG (Wu et al. 2003; Urbanus et al. 2010). Both transcription factors moved to inner cells of floral meristems and partially complemented their mutant phenotypes.

There are rich sources of cell type specific promoters in Arabidopsis that can be used for systematic screening of potentially mobile transcription factors. However, the assessment of transcription factor mobility should be made carefully because studies show the cell-to-cell movement is significantly dependent upon cellular contexts. For example, a random analysis of selected 23 transcription factors by expressing

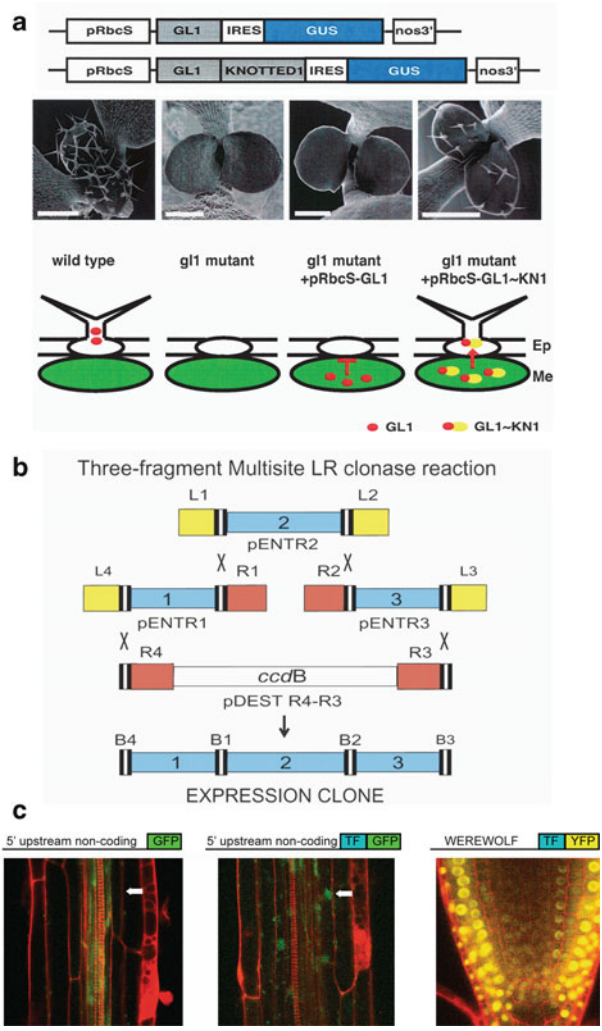

Fig. 3.1 Genetic and molecular tool kits for identifying mobile transcription factors. (**a**) Functional protein trafficking assay using trichome rescue. *Upper panel*; GL1 fusion constructs used for trichome rescue assay. Constructs include the mesophyll-specific RbcS promoter (pRbcS), GL1 or GL1~KN1 fusions, an internal ribosome entry sequence (IRES) upstream of the GUS coding sequence, and the nopaline synthase (nos) 3′ terminator. *Middle and bottom panels* show images of leaf surfaces in different genetic backgrounds and cartoons that explain the phenotypes. Trichome formation is promoted by GL1~KN1 that moves from the mesophyll cells into the epidermis. Image modified from Fig. 3.1 by Kim et al. (2005c) with copyright permission from Cold Spring Harbor Press. (**b**) Versatile cloning of multiple components using multisite Gateway recombination system (Invitrogen). Image modified from Fig. 3.1 by Karimi et al. (2007) with copyright permission from American Society of Plant Biologists. (**c**) Identification of a mobile transcription factor (DAG1) in the Arabidopsis roots with transgenes generated with the multisite Gateway recombination and the laser scanning confocal microscopy (Lee et al. 2006). *Left panel*; transcriptional GFP (endoplasmic-reticulum localized) expressed under the DAG1 promoter. *Middle panel*; translational free GFP fused to the coding region of DAG1 expressed under its own promoter. *Right panel*; YFP fused to DAG1 expressed by the *WEREWOLF* promoter. *WEREWOLF* promoter drives expression in the lateral root cap and epidermis. Arrows indicate the endodermis layer whether DAG1 proteins move from the stele

their GFP fusion under *WEREWOLF* promoter, which drives gene expression in the lateral root cap and atrichoblasts, more than 40% (ten transcription factors) expanded their GFP domains (Lee, unpublished results; an example is shown in Fig. 3.1c). This is a much higher frequency than when they are expressed in their endogenous expression domains.

4.3 Analysis of Transcription Factor Movement Using a Versatile Cloning Technology

Technologies for isolating gene promoters and coding regions became very efficient with the development of DNA polymerase with a high proof reading function and recombination based cloning technology (Fig. 3.1b). Several binary vectors that can integrate multiple DNA fragments in frame using gateway cloning system have been developed (see the reviews (Chung et al. 2005; Karimi et al. 2005, 2007)). ~1,600 transcription factors in Arabidopsis have been cloned into a gateway vector (Ou et al. 2011). Using this highly efficient cloning system, individual transcription factors translationally fused to GFP were expressed under their own promoters in transgenic Arabidopsis. GFP expression patterns of these lines (translational fusion) were compared with the ER localized GFP expressed under the same promoters (transcriptional fusion). In this experiment (Lee et al. 2006), transcription factors that are expressed in a cell type enriched manner were selected from the genome-wide expression data generated from several root cell type specific GFP expression lines. Distinctive expression domains of transcriptional GFPs made it feasible to identify translational GFPs that had different expression patterns. Among 61 transcription factors whose GFP fusion lines generated, 24 yielded lines with GFP patterns that clearly recapitulated cell type specific root expression data. Six of them (25%) were found to have translational fusion GFP with expression domains broader than the corresponding transcriptional GFP, suggesting that their proteins might move between cells (Fig. 3.1c).

4.4 Profiling RNAs Transported by RNA Binding Phloem Proteins

RNA-binding proteins (RBPs) form ribonucleoprotein (RNP) complexes and thereby play crucial roles in RNA stability, processing, targeted delivery and novel modes of mRNA protection (Shyu et al. 2008). Recent studies have established that in plants some RNP complexes can act non-cell-autonomously (Gómez et al. 2005; Ham et al. 2009). These RNP complexes help a unique population of mRNA species to move through the phloem sieve cells. Profiling RNA species bound to RBPs can help to find new RNA species that are transported through the phloem.

Recently, 50-kD pumpkin phloem RNA-binding protein (RBP50), a polypyrimidine tract binding protein, has been characterized as a non-cell-autonomous RNA-binding protein (Ham et al. 2009). This phloem mobile protein constitutes RBP50-based RNP complexes with multiple phloem proteins (Lin et al. 2009). To identify RNA that binds to RBP50, RBP50 RNP complexes were co-immunoprecipitated, from which RNAs were extracted, reversed transcribed and then amplified. From these, mRNAs of *PP16-1*, *GIBBERELLIC ACID-INSENSITIVE PHLOEM PROTEIN*, *SHOOT MERISTEMLESS*, *SCARECROW-LIKE 14* and *MYELOBLASTOSIS* family transcription factor were identified. To further investigate the degree to which RBP50 exhibits specificity in its binding to these identified phloem transcripts, co-immunoprecipitation experiments were performed using PP2, a phloem protein previously shown to bind RNA in a non-sequence-specific manner (Gómez et al. 2005). RNAs isolated from these PP2 complexes were broader than those from RBP50; in contrast with the six mRNA species identified from the RBP50 RNP complexes, 75 different transcripts were cloned from the PP2 complexes. Some of these transcripts encode transcription factors. The heterogeneous nature and high number of the transcripts identified from the PP2 co-immunoprecipitation contrasted markedly from the six transcripts identified from the RBP50 experiments. This suggests that RBP50 binds to a specific set of phloem-mobile mRNA species. All the six transcripts bound by RBP50 contain canonical polypyrimidine tract binding motifs. Further tests of RBP50 binding to these sequences confirmed that polypyrimidine tract binding motifs are required for the interaction.

4.5 Identification of Potential Mobile Transcription Factor RNAs from Cell Type Specific RNA Profiling

Most of mobile transcription factors as RNAs or proteins so far have been identified in studies focusing on individual transcription factors. Thanks to the enhanced efficiency of molecular cloning and *in vivo* imaging, the number of newly identified mobile transcription factors is growing fast. Recent advances of cell type specific profiling of RNA, however, will further facilitate finding more mobile transcription factors. Here, recent data generated in the phloem sieve cells and companion cells using various techniques are described and potential mobile transcription factor RNAs are introduced.

4.5.1 Phloem Expression Profiling

To find mRNAs that are mobile through phloem, Deeken et al. generated a combination of datasets using various approaches (Deeken et al. 2008). The first data set was based on microarray experiments on mRNAs found in the whole phloem tissue harvested by Laser Microdissection Pressure Catapulting (LMPC) from the

Arabidopsis inflorescence stems. The second microarray data were generated from mRNA extracted from the stem exudates. The last set of data was 444 ESTs generated from RNAs, which were isolated from companion cells (Ivashikina et al. 2003). Having confirmed the quality of the microarray data, only genes with hybridization signals that met the significance criterion of a *P*-value ≤0.01 on all three replicate microarray data were referred to as present in the phloem sap or LMPC-derived phloem tissue. According to these criteria, 2,417 transcripts were detected by microarrays in the phloem sap and 1,291 in LMPC-derived phloem tissue of Arabidopsis. From these lists, a total of 828 genes were found in both phloem sap and phloem tissue and 13 of them were transcription factors (Table 3.2). Among 444 ESTs from companion cells, 144 ESTs were also found in both, exudates transcripts and LMPC-based phloem transcripts, suggesting that these mRNAs from companion cells might be unloaded into the phloem sieve cells.

Table 3.2 Potential mobile transcription factor RNAs inferred from cell-type specific RNA profiling

TAIR number	Transcription factor gene family	Reference
AT3G60490	AP2-EREBP	(Mustroph et al. 2009)[a]
AT4G11140	AP2-EREBP	(Brady et al. 2007)[b]
AT1G17880	Basic transcription factor	(Deeken et al. 2008)[c]
AT4G14410	bHLH	(Deeken et al. 2008)
AT1G59640	bHLH	(Brady et al. 2007)
AT1G61660	bHLH	(Mustroph et al. 2009)
AT3G23210	bHLH	(Mustroph et al. 2009)
AT5G04150	bHLH	(Mustroph et al. 2009)
AT5G67110	bHLH	(Mustroph et al. 2009)
AT1G75390	bZIP	(Mustroph et al. 2009)
AT1G66140	C2H2	(Mustroph et al. 2009)
AT2G36930	C2H2	(Deeken et al. 2008)
AT5G16470	C2H2	(Deeken et al. 2008)
AT1G07640	Dof-type zinc finger	(Brady et al. 2007; Mustroph et al. 2009)
AT2G28510	Dof-type zinc finger	(Mustroph et al. 2009)
AT2G28810	Dof-type zinc finger	(Mustroph et al. 2009)
AT2G37590	Dof-type zinc finger	(Brady et al. 2007)
AT3G45610	Dof-type zinc finger	(Brady et al. 2007)
AT3G55370	Dof-type zinc finger	(Mustroph et al. 2009)
AT3G61850	Dof-type zinc finger	(Mustroph et al. 2009)
AT1G49560	G2-like	(Mustroph et al. 2009)
AT1G69580	G2-like	(Mustroph et al. 2009)
AT1G79430	G2-like	(Brady et al. 2007; Mustroph et al. 2009)
AT3G04030	G2-like	(Mustroph et al. 2009)

(continued)

Table 3.2 (continued)

TAIR number	Transcription factor gene family	Reference
AT4G18020	G2-like	(Mustroph et al. 2009)
AT4G37180	G2-like	(Mustroph et al. 2009)
AT5G18240	G2-like	(Mustroph et al. 2009)
AT5G29000	G2-like	(Mustroph et al. 2009)
AT2G18380	GATA	(Brady et al. 2007)
AT4G36620	GATA	(Brady et al. 2007)
AT2G04890	GRAS	(Brady et al. 2007)
AT5G52510	GRAS	(Mustroph et al. 2009)
AT1G04880	HMG (high mobility group)	(Brady et al. 2007)
AT1G69580	Homeodomain	(Brady et al. 2007)
AT2G03500	Homeodomain	(Brady et al. 2007; Mustroph et al. 2009)
AT3G04030	Homeodomain	(Brady et al. 2007)
AT3G12730	Homeodomain	(Brady et al. 2007; Mustroph et al. 2009)
AT1G19000	Homeodomain	(Deeken et al. 2008)
AT2G33310	IAA13	(Deeken et al. 2008)
AT1G72450	JASMONATE-ZIM-DOMAIN PROTEIN 6 (JAZ6)	(Deeken et al. 2008)
AT2G22540	MADS	(Deeken et al. 2008)
AT1G71692	MADS	(Mustroph et al. 2009)
AT2G42830	MADS	(Brady et al. 2007)
AT5G05790	MYB	(Mustroph et al. 2009)
AT1G54330	NAC	(Brady et al. 2007)
AT1G60350	NAC	(Brady et al. 2007)
AT1G65910	NAC	(Brady et al. 2007)
AT2G27300	NAC	(Brady et al. 2007)
AT3G03200	NAC	(Brady et al. 2007)
AT5G17260	NAC	(Brady et al. 2007)
AT1G73230	NAC	(Deeken et al. 2008)
AT4G29080	Phytochrome-associated protein 2 (PAP2)	(Deeken et al. 2008)
AT1G43000	PLATZ transcription factor family	(Brady et al. 2007)
AT1G20823	RING/U-box superfamily protein	(Deeken et al. 2008)
AT1G33480	RING/U-box superfamily protein	(Brady et al. 2007)
AT1G06040	Salt tolerance protein (STO)	(Deeken et al. 2008)
AT2G46020	SWI/SNF	(Deeken et al. 2008)
AT1G28520	VOZ-9	(Mustroph et al. 2009)
AT2G04880	WRKY	(Mustroph et al. 2009)
AT4G26640	WRKY	(Mustroph et al. 2009)

[a] Enriched in the companion cells. Selection criteria for significantly enriched RNAs for each pairwise comparison: Signal-log-ratio >1; False discovery rate <0.01

[b] RNAs enriched in both, S32 and APL, expression domains (>1.2-fold and False discovery rate <0.001)

[c] Expression was considered 'present' if the significance call *P* value equals or is below 0.01

4.5.2 Cell-Type Specific Expression Map in the Arabidopsis Root

The root expression map generated by cell sorting/microarray technology can serve as a great resource for identifying putative transcription factors that are mobile in form of RNA (Birnbaum et al. 2003; Nawy et al. 2005; Lee et al. 2006; Levesque et al. 2006; Brady et al. 2007). Cell sorting combined with microarray technology uses transgenic plants that express fluorescent markers in cells of interest. Fluorescent cells among protoplasts are selectively isolated through the Fluorescence Activated Cell Sorter and their RNAs are purified and processed for expression profiling using microarray or next-generation sequencing technology (Birnbaum et al. 2005).

A recently combined root expression map includes expression profiles from 19 cell type specific lines, which cover 14 non-overlapping cell types in the root (Brady et al. 2007). In this expression map, data generated using three phloem lines are available, S32, APL, and SUC2. S32, APL, and SUC2 data respectively cover mRNA profiles from protophloem sieve cells starting from initials, companion cells starting from the region of root meristem, and companion cells starting from root maturation zone (Fig. 3.2). Using these and expression data from other cell types, phloem-enriched mRNA species that are mobile from companion cells into sieve cells can be predicted. For example, in the study by Brady et al. (2007), genes that are highly enriched in S32, APL, and SUC2 were predicted from the pair-wise comparison of gene expression values in each of phloem data against those in non-phloem cell types. 544 and 317 transcripts are respectively enriched in cell types labeled by S32 and APL. A surprisingly large number of genes (266) are expressed in both cell types in a highly enriched manner. Among these, 23 genes encode transcription factors (Table 3.2). GFP expression domains in S32 and APL lines slightly overlap in the early stage of phloem. Therefore, some of these overlapping genes could be from the shared expression domain. In contrast, GFP expressed by the SUC2 promoter is expressed specifically in companion cells. Expression of 224 genes is predicted to be highly enriched in the SUC2 domains. Among these, 97 genes are also enriched in the S32 domain. Taken together, comparison of enriched genes in phloem cell types suggests that a high proportion but not all of mRNA species expressed in companion cells might be loaded into sieve tubes. Such conclusion is also consistent with what was found by Deekens et al. (2008) (see above).

4.5.3 Cell-Type Specific Translatome Data

Cell-type specific root expression map generated using the cell-sorting/microarray technique provides a list of transcription factor transcripts that might be worthwhile to be tested for their capacity to allocate into phloem sieve tube cells. A major concern in this approach, however, is the contamination of protoplasts by neighboring cells, which could potentially result in false positives.

Recently, mRNAs associated with ribosomes from specific cell populations were used for profiling on microarrays to find cell type specific changes of translatomes under hypoxia (Mustroph et al. 2009). In this study, FLAG-tagged ribosomal protein

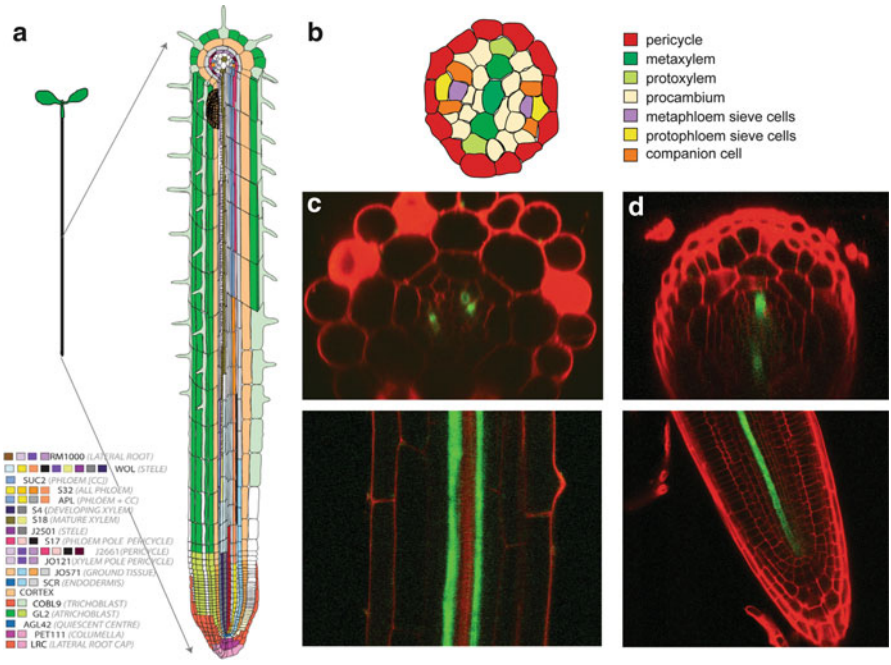

Fig. 3.2 Cell-type specific mRNA profiling in the Arabidopsis root for predicting potentially mobile transcription factor RNAs through phloem. (**a**) Microarray expression profiles of 19 fluorescently sorted GFP-marked lines were analyzed by Brady et al. (2007). The colors associated with each marker line reflect the cell types sampled. Image modified from Fig. 3.1 by Brady et al. (2007) with copyright permission from AAAS. (**b**) Diagram of cell types inside the Arabidopsis stele. (**c**) Cross (*upper panel*) and longitudinal (*lower panel*) sections of Arabidopsis roots that show GFP expression driven by *APL* promoter. Images were taken with a laser scanning confocal microscope. (**d**) Cross (*upper panel*) and longitudinal (*lower panel*) GFP expression driven by *S32* (AT2G18380) promoter in the Arabidopsis root

L18 (RPL18), a component of ribosome complexes, was expressed under the cell type specific promoters in Arabidopsis, and then mRNA-ribosome complexes were purified by immunoprecipitation using FLAG antibodies.

These translatomes obtained from phloem companion cell populations can help predict mobile transcription factor RNAs. In a study by Mustroph et al. (2009), two promoters were used to profile translatome in the companion cells. *pSUC2:GFP-RPL18* was expressed in companion cells of the entire root whereas *pSULTR2;2:GFP-RPL18* was limited to companion cells present in the root elongation and maturation region. Consistent with the regional distinctions in expression of these promoters, the *pSUC2* and *pSULTR2;2* root translatomes were highly overlapping but not identical. The 270 co-enriched transcripts included a number of phloem companion cell markers such as *SUC2*, Sucrose-H^+ symporter (At1g22710); *AHA3*, plasma membrane H^+ ATPase (At5g57350); *APL*, G2-type transcription factor associated with phloem development (At1g79430); two phloem-specific lectins (At4g19840 and At2g02230),

supporting the conclusion that mRNAs were effectively isolated from the targeted cell type. The comparison of the shoot and root *pSUC2* translatomes with mRNAs obtained from *pSUC2:GFP* protoplasts of seedling roots (Brady et al. 2007) identified 214 enriched mRNAs present in all three samples. Furthermore, 78 of the reported phloem sap mRNAs were markedly enriched in the shoot *pSUC2* translatome (Deeken et al. 2008). In Table 3.2, transcription factors that are enriched in all the companion cell data are listed. Some of these overlap with enriched transcription factors from a cell-sorting based approach. Considering that these lists were generated using different data set and different search criteria, it is very encouraging to find several overlapping transcription factors that might be mobile.

4.6 Bioinformatics Approach to Predict Genes That Are Expressed in the Vascular Tissues

Studies using various approaches suggest that a large number of RNAs and proteins that move through the phloem sieve elements might be unloaded from the neighboring companion cells. Therefore, genes that are highly expressed in the cells associated with phloem sieve cells are good candidates as mobile proteins or mRNAs. Gene expression patterns can be predicted if a small set of transcription factors drive the gene expression in particular cell types. A recent study suggests that it might be the case for phloem-enriched genes (Ruiz-Medrano et al. 2011).

Previously EST sequence database was generated for more than 1,200 transcripts from pumpkin phloem sap. This dataset was compared against Arabidopsis genes to pinpoint the putative orthologous genes. In this analysis, 150 Arabidopsis genes encoding putative transcription factors, protein kinases, protein phosphatases, cell cycle regulators, and hormone response factors were identified. Enriched motifs in 150 genes were searched using several motif search algorithms. In this search, motifs with CT and GA rich repeats were identified. Promoter analysis of a subset of 150 genes showed that genes that are expressed specifically in vascular tissues have these repeats at higher rates than those that are expressed in vascular tissues as well as other tissues. Furthermore, each of CT and GA motifs was able to drive vascular expression when they were attached to the 35S minimal promoter, confirming the importance of transcription factors that bind to these motifs in driving gene expression in vascular tissues.

5 Conclusions and Perspectives

The sessile nature of plants and different functions of plant tissues require a versatile communication system between cells and organs in response to changes inside and outside the plant body. Studies that uncovered and investigated mobile transcription factors clearly indicate the functional significance of their cell-to-cell movement activity. An unbiased localization study of cell-type specific transcription factors suggests that nearly a quarter of transcription factors might move between cells.

The underlying mechanisms of cell-to-cell movement of transcription factors have started to emerge, however, it is still an open question what cellular factors determine the cell-to-cell movement activity of proteins and mRNAs.

Recent advances in technologies enabling us to profile RNA and proteins at a high resolution will propel this research field at an unprecedented level. Simple comparison of cell type specific RNA profiling data already suggests that a high proportion of RNA species generated in the phloem companion cells might be unloaded into the sieve cells. With more systematic data analyses and validation experiments, a large number of transcription factors as mobile signals will be unveiled.

In addition to the discovery of mobile transcription factors, unraveling a biological significance behind their mobility remains as one of the big challenges. Furthermore, so far only few transcription factors have been reported to move between cells in evolutionarily divergent species. Also very little progress has been made in understanding the meaning of cell-to-cell movement of transcription factors in the context of plant evolution. However, deep-sequencing and automatized mass-screening techniques combined with the complete genome information and molecular toolkits that have become available in various plant lineages, a deeper understanding of the intercellular communication system based on mobile transcription factors might soon emerge.

Acknowledgments This work was funded by National Science Foundation (IOS-0818071) and Boyce Thompson Institute to J-Y Lee.

References

Banerjee AK, Chatterjee M, Yu Y, Suh S-G, Miller WA, Hannapel DJ (2006) Dynamics of a mobile RNA of potato involved in a long-distance signaling pathway. Plant Cell 18:3443–3457

Banerjee AK, Lin T, Hannapel DJ (2009) Untranslated regions of a mobile transcript mediate RNA metabolism. Plant Physiol 151:1831–1843

Bennett T, Scheres B (2010) Root development-two meristems for the price of one? Curr Top Dev Biol (Academic Press) 91:67–102

Bernhardt C, Zhao M, Gonzalez A, Lloyd A, Schiefelbein J (2005) The bHLH genes GL3 and EGL3 participate in an intercellular regulatory circuit that controls cell patterning in the Arabidopsis root epidermis. Development 132:291–298

Birnbaum K, Shasha DE, Wang JY, Jung JW, Lambert GM, Galbraith DW, Benfey PN (2003) A gene expression map of the Arabidopsis root. Science 302:1956–1960

Birnbaum K, Jung JW, Wang JY, Lambert GM, Hirst JA, Galbraith DW, Benfey PN (2005) Cell type-specific expression profiling in plants via cell sorting of protoplasts from fluorescent reporter lines. Nat Methods 2:615–619

Brady SM, Orlando DA, Lee J-Y, Wang JY, Koch J, Dinneny JR, Mace D, Ohler U, Benfey PN (2007) A high-resolution root spatiotemporal map reveals dominant expression patterns. Science 318:801–806

Carlsbecker A, Lee J-Y, Roberts CJ, Dettmer J, Lehesranta S, Zhou J, Lindgren O, Moreno-Risueno MA, Vatén A, Thitamadee S, Campilho A, Sebastian J, Bowman JL, Helariutta Y, Benfey PN (2010) Cell signalling by microRNA165/6 directs gene dose-dependent root cell fate. Nature 465:316–321

Carpenter R, Coen ES (1990) Floral homeotic mutations produced by transposon-mutagenesis in Antirrhinum majus. Genes Dev 4:1483–1493

Causier B, Schwarz-Sommer Z, Davies B (2009) Floral organ identity: 20 years of ABCs. Semin Cell Dev Biol 21:73–79

Chen JJ, Janssen BJ, Williams A, Sinha N (1997) A gene fusion at a homeobox locus: alterations in leaf shape and implications for morphological evolution. Plant Cell 9:1289–1304

Chen H, Rosin FM, Prat S, Hannapel DJ (2003) Interacting transcription factors from the three-amino acid loop extension superclass regulate tuber formation. Plant Physiol 132:1391–1404

Chung S-M, Frankman EL, Tzfira T (2005) A versatile vector system for multiple gene expression in plants. Trends Plant Sci 10:357–361

Crawford KM, Zambryski PC (2000) Subcellular localization determines the availability of non-targeted proteins to plasmodesmatal transport. Curr Biol 10:1032–1040

Crawford KM, Zambryski PC (2001) Non-targeted and targeted protein movement through plasmodesmata in leaves in different developmental and physiological states. Plant Physiol 125: 1802–1812

Cui H, Levesque MP, Vernoux T, Jung JW, Paquette AJ, Gallagher KL, Wang JY, Blilou I, Scheres B, Benfey PN (2007) An evolutionarily conserved mechanism delimiting SHR movement defines a single layer of endodermis in plants. Science 316:421–425

Deeken R, Ache P, Kajahn I, Klinkenberg J, Bringmann G, Hedrich R (2008) Identification of Arabidopsis thaliana phloem RNAs provides a search criterion for phloem-based transcripts hidden in complex datasets of microarray experiments. Plant J 55:746–759

Digiuni S, Schellmann S, Geier F, Greese B, Pesch M, Wester K, Dartan B, Mach V, Srinivas BP, Timmer J, Fleck C, Hulskamp M (2008) A competitive complex formation mechanism underlies trichome patterning on Arabidopsis leaves. Mol Syst Biol 4:217

Gallagher KL, Benfey PN (2009) Both the conserved GRAS domain and nuclear localization are required for SHORT-ROOT movement. Plant J 57:785–797

Gallagher KL, Paquette AJ, Nakajima K, Benfey PN (2004) Mechanisms regulating SHORT-ROOT intercellular movement. Curr Biol 14:1847–1851

Gómez G, Torres H, Pallás V (2005) Identification of translocatable RNA-binding phloem proteins from melon, potential components of the long-distance RNA transport system. Plant J 41:107–116

Goto K, Kyozuka J, Bowman JL (2001) Turning floral organs into leaves, leaves into floral organs. Curr Opin Genet Dev 11:449–456

Ham B-K, Brandom JL, Xoconostle-Cázares B, Ringgold V, Lough TJ, Lucas WJ (2009) A polypyrimidine tract binding protein, pumpkin RBP50, forms the basis of a phloem-mobile ribonucleoprotein complex. Plant Cell 21:197–215

Hannapel DJ (2010) A model system of development regulated by the long-distance transport of mRNA. J Integr Plant Biol 52:40–52

Hardtke CS, Berleth T (1998) The Arabidopsis gene MONOPTEROS encodes a transcription factor mediating embryo axis formation and vascular development. EMBO J 17:1405–1411

Haywood V, Yu T-S, Huang N-C, Lucas WJ (2005) Phloem long-distance trafficking of GIBBERELLIC ACID-INSENSITIVE RNA regulates leaf development. Plant J 42:49–68

Helariutta Y, Fukaki H, Wysocka-Diller J, Nakajima K, Jung J, Sena G, Hauser M-T, Benfey PN (2000) The SHORT-ROOT gene controls radial patterning of the Arabidopsis root through radial signaling. Cell 101:555–567

Huang N-C, Yu T-S (2009) The sequences of Arabidopsis GA-INSENSITIVE RNA constitute the motifs that are necessary and sufficient for RNA long-distance trafficking. Plant J 59:921–929

Ishida T, Kurata T, Okada K, Wada T (2008) A genetic regulatory network in the development of trichomes and root hairs. Annu Rev Plant Biol 59:365–386

Ivashikina N, Deeken R, Ache P, Kranz E, Pommerrenig B, Sauer N, Hedrich R (2003) Isolation of AtSUC2 promoter-GFP-marked companion cells for patch-clamp studies and expression profiling. Plant J 36:931–945

Jack T (2004) Molecular and genetic mechanisms of floral control. Plant Cell 16:S1–S17

Jackson D, Veit B, Hake S (1994) Expression of maize KNOTTED1 related homeobox genes in the shoot apical meristem predicts patterns of morphogenesis in the vegetative shoot. Development 120:405–413

Jenik PD, Irish VF (2001) The Arabidopsis floral homeotic gene APETALA3 differentially regulates intercellular signaling required for petal and stamen development. Development 128:13–23
Karimi M, De Meyer B, Hilson P (2005) Modular cloning in plant cells. Trends Plant Sci 10:103–105
Karimi M, Depicker A, Hilson P (2007) Recombinational cloning with plant gateway vectors. Plant Physiol 145:1144–1154
Kim M, Canio W, Kessler S, Sinha N (2001) Developmental changes due to long-distance movement of a homeobox fusion transcript in tomato. Science 293:287–289
Kim JY, Yuan Z, Cilia M, Khalfan-Jagani Z, Jackson D (2002) Intercellular trafficking of a KNOTTED1 green fluorescent protein fusion in the leaf and shoot meristem of Arabidopsis. Proc Natl Acad Sci USA 99:4103–4108
Kim J-Y, Yuan Z, Jackson D (2003) Developmental regulation and significance of KNOX protein trafficking in Arabidopsis. Development 130:4351–4362
Kim I, Kobayashi K, Cho E, Zambryski PC (2005a) Subdomains for transport via plasmodesmata corresponding to the apical-basal axis are established during Arabidopsis embryogenesis. Proc Natl Acad Sci USA 102:11945–11950
Kim I, Cho E, Crawford K, Hempel FD, Zambryski PC (2005b) Cell-to-cell movement of GFP during embryogenesis and early seedling development in Arabidopsis. Proc Natl Acad Sci USA 102:2227–2231
Kim J-Y, Rim Y, Wang J, Jackson D (2005c) A novel cell-to-cell trafficking assay indicates that the KNOX homeodomain is necessary and sufficient for intercellular protein and mRNA trafficking. Genes Dev 19:788–793
Komatsu M, Maekawa M, Shimamoto K, Kyozuka J (2001) The LAX1 and FRIZZY PANICLE 2 genes determine the inflorescence architecture of rice by controlling rachis-branch and spikelet development. Dev Biol 231:364–373
Kuijt SJH, Lamers GEM, Rueb S, Scarpella E, Ouwerkerk PBF, Spaink HP, Meijer AH (2004) Different subcellular localization and trafficking properties of KNOX class 1 homeodomain proteins from rice. Plant Mol Biol 55:781–796
Kurata T, Ishida T, Kawabata-Awai C, Noguchi M, Hattori S, Sano R, Nagasaka R, Tominaga R, Koshino-Kimura Y, Kato T, Sato S, Tabata S, Okada K, Wada T (2005) Cell-to-cell movement of the CAPRICE protein in Arabidopsis root epidermal cell differentiation. Development 132:5387–5398
Lee J-Y, Colinas J, Wang JY, Mace D, Ohler U, Benfey PN (2006) Transcriptional and posttranscriptional regulation of transcription factor expression in Arabidopsis roots. Proc Natl Acad Sci USA 103:6055–6060
Levesque MP, Vernoux T, Busch W, Cui H, Wang JY, Blilou I, Hassan H, Nakajima K, Matsumoto N, Lohmann JU, Scheres B, Benfey PN (2006) Whole-genome analysis of the SHORT-ROOT developmental pathway in Arabidopsis. PLoS Biol 4:e143
Lin M-K, Lee Y-J, Lough TJ, Phinney BS, Lucas WJ (2009) Analysis of the pumpkin phloem proteome provides insights into angiosperm sieve tube function. Mol Cell Proteomics 8:343–356
Long J, Moan E, Medford J, Barton M (1996) A member of the KNOTTED class of homeodomain proteins encoded by the STM gene of Arabidopsis. Nature 379:66–69
Long TA, Tsukagoshi H, Busch W, Lahner B, Salt DE, Benfey PN (2010) The bHLH transcription factor POPEYE regulates response to iron deficiency in arabidopsis roots. Plant Cell 22: 2219–2236
Lucas WJ, Bouché-Pillon S, Jackson DP, Nguyen L, Baker L, Ding B, Hake S (1995) Selective trafficking of KNOTTED1 homeodomain protein and its mRNA through plasmodesmata. Science 270:1980–1983
Miyashima S, Koi S, Hashimoto T, Nakajima K (2011) Non-cell-autonomous microRNA165 acts in a dose-dependent manner to regulate multiple differentiation status in the Arabidopsis root. Development 138:2303–2313
Mustroph A, Zanetti ME, Jang CJH, Holtan HE, Repetti PP, Galbraith DW, Girke T, Bailey-Serres J (2009) Profiling translatomes of discrete cell populations resolves altered cellular priorities during hypoxia in Arabidopsis. Proc Natl Acad Sci USA 106:18843–18848

Nakajima K, Sena G, Nawy T, Benfey PN (2001) Intercellular movement of the putative transcription factor SHR in root patterning. Nature 413:307–311
Nawy T, Lee J-Y, Colinas J, Wang JY, Thongrod SC, Malamy JE, Birnbaum K, Benfey PN (2005) Transcriptional profile of the Arabidopsis root quiescent center. Plant Cell 17:1908–1925
Oikawa T, Kyozuka J (2009) Two-step regulation of LAX PANICLE1 protein accumulation in Axillary meristem formation in rice. Plant Cell 21:1095–1108
Ou B, Yin K-Q, Liu S-N, Yang Y, Gu T, Wing Hui JM, Zhang L, Miao J, Kondou Y, Matsui M, Gu H-Y, Qu L-J (2011) A high-throughput screening system for arabidopsis transcription factors and its application to Med25-dependent transcriptional regulation. Mol Plant 4:546–555
Perbal MC, Haughn G, Saedler H, Schwarz-Sommer Z (1996) Non-cell-autonomous function of the Antirrhinum floral homeotic proteins DEFICIENS and GLOBOSA is exerted by their polar cell-to-cell trafficking. Development 122:3433–3441
Ruiz-Medrano R, Xoconostle-Cazares B, Lucas WJ (1999) Phloem long-distance transport of CmNACP mRNA: implications for supracellular regulation in plants. Development 126: 4405–4419
Ruiz-Medrano R, Xoconostle-Cázares B, Ham B-K, Li G, Lucas WJ (2011) Vascular expression in Arabidopsis is predicted by the frequency of CT/GA-rich repeats in gene promoters. Plant J 67:130–144
Sablowski R (2011) Plant stem cell niches: from signalling to execution. Curr Opin Plant Biol 14:4–9
Schiefelbein J, Kwak S-H, Wieckowski Y, Barron C, Bruex A (2009) The gene regulatory network for root epidermal cell-type pattern formation in Arabidopsis. J Exp Bot 60(5):1515–1521, ern339
Schlereth A, Moller B, Liu W, Kientz M, Flipse J, Rademacher EH, Schmid M, Jurgens G, Weijers D (2010) MONOPTEROS controls embryonic root initiation by regulating a mobile transcription factor. Nature 464:913–916
Sena G, Jung JW, Benfey PN (2004) A broad competence to respond to SHORT ROOT revealed by tissue-specific ectopic expression. Development 131:2817–2826
Sessions A, Yanofsky MF, Weigel D (2000) Cell-cell signaling and movement by the floral transcription factors LEAFY and APETALA1. Science 289:779–781
Shyu A-B, Wilkinson MF, van Hoof A (2008) Messenger RNA regulation: to translate or to degrade. EMBO J 27:471–481
Tsukagoshi H, Busch W, Benfey PN (2010) Transcriptional regulation of ROS controls transition from proliferation to differentiation in the root. Cell 143:606–616
Urbanus SL, Martinelli AP, Dinh QD, Aizza LCB, Dornelas MC, Angenent GC, Immink RGH (2010) Intercellular transport of epidermis-expressed MADS domain transcription factors and their effect on plant morphology and floral transition. Plant J 63:60–72
Venglat SP, Dumonceaux T, Rozwadowski K, Parnell L, Babic V, Keller W, Martienssen R, Selvaraj G, Datla R (2002) The homeobox gene BREVIPEDICELLUS is a key regulator of inflorescence architecture in Arabidopsis. Proc Natl Acad Sci USA 99:4730–4735
Vollbrecht E, Veit B, Sinha N, Hake S (1991) The developmental gene Knotted-1 is a member of a maize homeobox gene family. Nature 350:241–243
Wada T, Tachibana T, Shimura Y, Okada K (1997) Epidermal cell differentiation in Arabidopsis determined by a Myb homolog, CPC. Science 277:1113–1116
Wada T, Kurata T, Tominaga R, Koshino-Kimura Y, Tachibana T, Goto K, Marks MD, Shimura Y, Okada K (2002) Role of a positive regulator of root hair development, CAPRICE, in Arabidopsis root epidermal cell differentiation. Development 129:5409–5419
Wester K, Digiuni S, Geier F, Timmer J, Fleck C, Hülskamp M (2009) Functional diversity of R3 single-repeat genes in trichome development. Development 136:1487–1496
Winter N, Kollwig G, Zhang S, Kragler F (2007) MPB2C, a microtubule-associated protein, regulates non-cell-autonomy of the homeodomain protein KNOTTED1. Plant Cell 19:3001–3018
Wu XL, Dinneny JR, Crawford KM, Rhee Y, Citovsky V, Zambryski PC, Weigel D (2003) Modes of intercellular transcription factor movement in the Arabidopsis apex. Development 130: 3735–3745
Xu H, Zhang W, Li M, Harada T, Han Z, Li T (2010) Gibberellic acid insensitive mRNA transport in both directions between stock and scion in Malus. Tree Genet Genom 6:1013–1019

Chapter 4
Cell-to-Cell Movement of Homeodomain Transcription Factors: Yesterday, Today and Tomorrow

Xianfeng Morgan Xu and Dave Jackson

1 Discovery and History of KN1 Cell-to-Cell Movement

Maize *knotted1 (kn1)* was the first plant homeobox gene to be functionally characterized, and the founding member of the *knotted1*-like homeobox (KNOX) gene family. Members of this family of homeodomain proteins function to maintain the shoot apical meristem (SAM) in an indeterminate, stem cell state, in part by regulating different classes of plant hormones (Hake et al. 2004). However, the *KN1* gene was first characterized by dominant gain-of-function *Kn1* mutations that cause ectopic growth ("knots") on the leaf blade (Hake and Freeling 1986). These knots result from extra cell divisions in all cell layers of the leaf, which suggest that *Kn1* plays a role in cell proliferation and/or fate determination (Fig. 4.1a, b).

1.1 Genetic and Histological Hints

Mosaic analysis showed that the dominant *Kn1* allele act non-cell autonomously, because the presence of the *Kn1* allele only in the inner cell layers (mesophyll and vasculature) can induce the epidermal cells to divide and form knots (Hake and Freeling 1986). At the time, though, it was not clear if the non-cell autonomy was mediated directly by the *Kn1* gene product, or by a signal acting downstream of *Kn1*, such as a hormone or other mobile signal.

Upon isolation of the *KN1* gene, and investigation of its mRNA and protein localization patterns, clues started to emerge as to what the mobile "knot inducing" signal might be. *KN1* was found to encode the first plant homeodomain transcription factor with a genetically defined function (Vollbrecht et al. 1991). Surprisingly,

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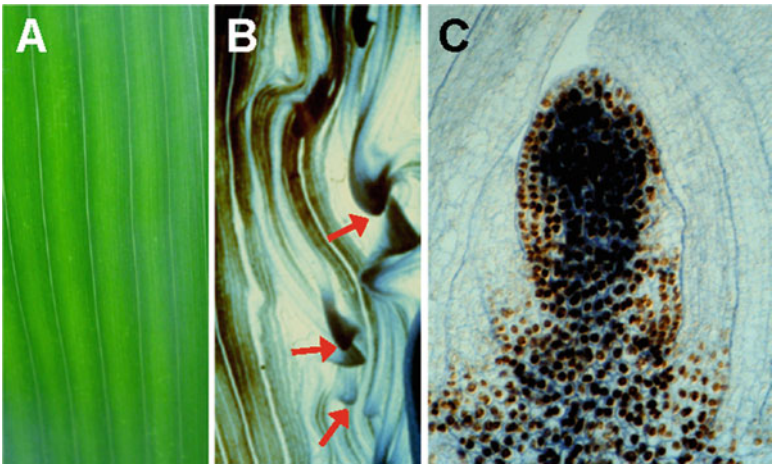

Fig. 4.1 Compared to normal maize leaves (**a**), *Kn1* mutant leaves develop knots (*arrows* in (**b**)). Double labeling of KN1 protein (*brown*) and mRNA (*black*) in the maize SAM reveals their differential distribution ((**c**), Adapted from (Jackson 2002))

it was found that KN1 is normally not expressed in the maize leaf, but the dominant mutations cause ectopic expression of KN1 in leaves, leading to the proliferative cell divisions and knot formation. In *Kn1* mutant leaves, KN1 mRNA expression was limited to the vascular cells, whereas the KN1 protein was present in all cell layers of the leaf, including the epidermis (Smith et al. 1992; Jackson et al. 1994). This discrepancy between the KN1 mRNA and protein distribution patterns was also seen in the maize shoot apical meristem (SAM), where KN1 is normally expressed. KN1 mRNA is expressed throughout the inner L2 layer of the shoot meristem; KN1 protein, however, is abundant in all shoot meristem cells, including the L1 (Fig. 4.1c) (Jackson et al. 1994; Lucas et al. 1995). These observations led to the hypothesis that KN1 protein might serve as the mobile signal between cells.

1.2 Direct Demonstration by Microinjection

The movement of KN1 protein between plant cells was first demonstrated by microinjection of fluorescently labeled recombinant KN1 protein into either maize or tobacco leaves (Lucas et al. 1995). When KN1 was microinjected into mesophyll cells, it spread into surrounding cells within seconds. This movement of KN1 resembled the spread of KN1 in the dominant *Kn1* maize mutant leaves. KN1 also showed the classic PD "gating" capacity, similar to that of viral movement proteins (MPs), in that it could modify the size exclusion limit (SEL) of plasmodesmata (PDs) and allow the cell-to-cell trafficking of otherwise cell-autonomous dextran reporters. This gating effect strongly suggested that KN1 moves through PD in an active and selective manner. Interestingly, like viral MPs, KN1 was also able to bind to and traffic its mRNA. The significance of this finding still remains somewhat obscure, since it is contradictory to the results of the KN1 localization experiments.

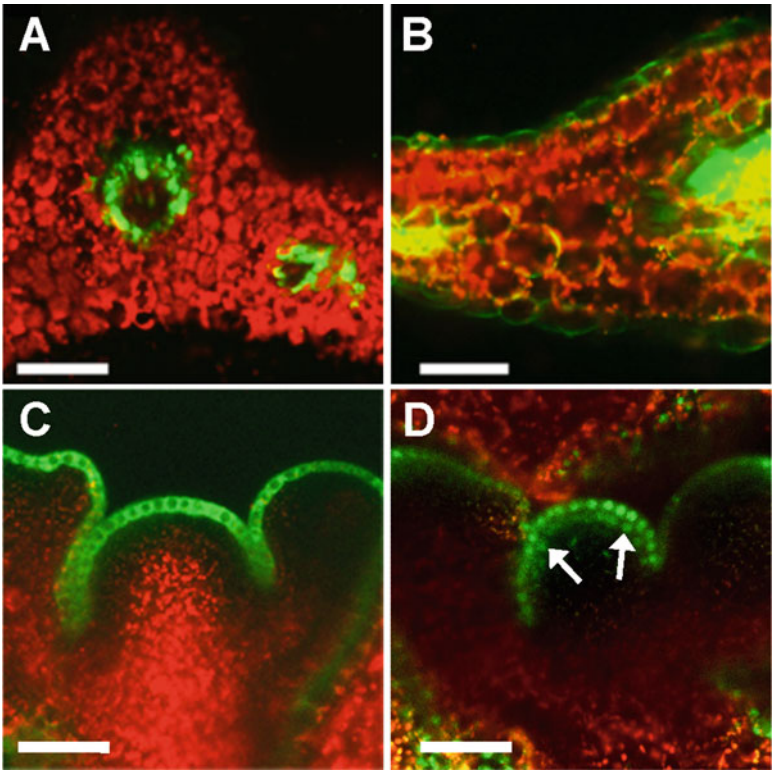

Fig. 4.2 The enhancer trap line J2111 drives expression of the cell autonomous GFP^{ER} in the leaf vasculature (**a**). GFP~KN1, when under the control of the J2111 driver, moves out of the vasculature and reaches the epidermis (**b**). The *pAtML1* promoter enables L1 specific expression of GFP^{ER} (**c**), while *pAtML1::GFP~STM* plants show trafficking of GFP~STM into the inner layer (*arrows* in (**d**)). *Arabidopsis* leaf cross sections (**a**, **b**) and SAM (**c**, **d**) are shown. In all panels, chlorophyll fluorescence is in red

1.3 In Vivo Evidence from Tissue-Specific Expression

The microinjection assay bears a limitation that the injected proteins are at high concentrations, which may not be physiologically relevant, and the cells that can be injected are developmentally very different than those where KN1 normally is thought to move. To confirm that these were not significant issues, and to independently demonstrate KN1 movement, KN1 tagged with green fluorescent protein (GFP) was expressed in *Arabidopsis* or onion epidermal cells using micro-projectile bombardment (Kim et al. 2002). Trafficking of GFP~KN1 to the surrounding cells provided independent and direct visualization of protein movement *in vivo*. More importantly, tissue-specific expression of GFP~KN1, using the GAL4 enhancer trap system led to the first direct demonstration of KN1 trafficking across different tissue layers (Fig. 4.2a, b). In addition, the cell-to-cell trafficking property appeared to be conserved in some closely related class I KNOX proteins from different species (Fig. 4.2c, d), suggesting trafficking may be associated with their normal functions (Kim et al. 2003, 2005).

2 Mechanism and Regulation of KN1 Trafficking

2.1 Connection Between Cell-to-Cell Trafficking and Nuclear Transport

Given that KN1 trafficking appears to be an active process, attempts have been made to understand what motifs or protein domains of KN1 contribute to its trafficking. Microinjection of various Alanine-scanning substitution mutants of KN1 revealed that residues within the nuclear localization signal (NLS) are essential for KN1 trafficking, which raises an interesting link between cell-to-cell trafficking and nucleocytoplasmic trafficking (Lucas et al. 1995). In general, protein subcellular localization appears to affect diffusion-based trafficking through PDs, due to competition between delivery to different destinations (Crawford and Zambryski 2000). However, the nuclear localization of KN1 appears to be a prerequisite, rather than competiting for its ability to move between cells. Studies with another non-cell autonomous transcription factor, SHORTROOT (SHR), also support this puzzling link. SHR, a GRAS family transcription factor, is transcribed in the stele and the protein moves into the ground tissue layer to promote endodermal cell fate specification (Nakajima et al. 2001). SHR protein is both nuclear and cytoplasmic in the stele. One *shr* allele (shr^{T289I}) renders the SHR protein exclusively cytoplasmic, and also leads to loss of its mobility (Gallagher et al. 2004). Restoring the nuclear localization by attaching an NLS to the SHR^{T289I} mutant protein rescues its mobility, further strengthening the correlation between nuclear accumulation or passage, and the ability to move between cells (Gallagher and Benfey 2009).

2.2 The KN1 Homeodomain Is Necessary and Sufficient for Trafficking

To further define whether KN1 contains specific trafficking domains, a novel gain-of-function trafficking assay was developed to mimic the dominant *Kn1* maize mutant (Kim et al. 2005). This assay depends on the rescue of trichomes in *Arabidopsis glabrous1* (*gl1*) mutants. Trichomes are leaf hairs, and their development is dependent on GL1, a Myb-domain transcription factor that acts cell autonomously in the leaf epidermis to specify trichome cell fate. When GL1 alone is expressed in the mesophyll cell layers under the control of the *Rubisco Small Subunit 2b* promoter, *gl1* mutants fail to make trichomes (Fig. 4.3a). However, when GL1 is fused to KN1, and the fusion protein is again expressed specifically in the mesophyll layers, the GL1 ~ KN1 fusion traffics to the epidermal layer and rescues trichome development (Fig. 4.3b). Thus a simple, tractable phenotype (the presence or absence of trichomes) serves as a simple reporter system for KN1 cell-to-cell trafficking *in vivo*. Using this system, the homeodomain of KN1, including the

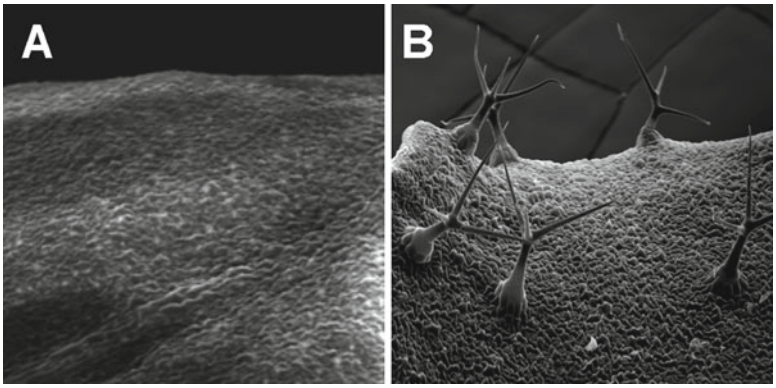

Fig. 4.3 The trichome rescue system reports KN1 trafficking in an easy visual manner, suitable for genetic screening. In (**a**), mesophyll specific expression of GL1 fails to rescue trichome production in a *gl1* mutant plant. In contrast, mesophyll specific expression of a GL1 ~ KN1 fusion can complement trichome formation in *gl1* mutants (**b**)

NLS, was found to be both necessary, and sufficient, for KN1 trafficking in leaves (Kim et al. 2005). In the SAM, the homeodomain also appears necessary and sufficient for movement (Kim et al. 2005).

The KN1 homeodomain consists of about 60 amino acids, and could be considered large to be defined as a trafficking motif. However, attempts to further delineate the minimal trafficking motif were fruitless, suggesting the whole homeodomain is indeed needed for movement. Similar results were observed when the trafficking domain of SHR was defined (Gallagher and Benfey 2009). It appears the whole GRAS domain, not any particular short segments or motifs, enable SHR movement. Trafficking domain-mapping studies of other non-autonomous proteins, such as CAPRICE (CPC) and the 16-kD C. maxima phloem protein 1 (CmPP16), have all defined significant portions of the protein as being essential for movement (Kurata et al. 2005; Taoka et al. 2007). However, sequence comparisons between all these minimal trafficking domains failed to come up with any consensus. This is consistent with the earlier notion that structural properties, rather than primary sequences of trafficking domains, are important for their movement (Haywood et al. 2002).

2.3 Co-trafficking of KN1 Protein and mRNA

Interestingly, in addition to its own movement, KN1 also has the ability to transport RNA between cells. Moreover, KN1 is selective in terms of the RNA that it can traffic, as opposed to viral MPs that bind and traffic RNA non-specifically. It was first demonstrated by microinjection that KN1 can potentiate the trafficking of its own mRNA (Lucas et al. 1995). Later, *in vivo* evidence from the trichome rescue system confirmed this result (Kim et al. 2005). Both the microinjection and

trichome rescue assays indicated that KN1 mRNA movement depends on the KN1 protein. However, the KN1 protein moves into the L1 layer of the maize SAM, without any detectable accumulation of its mRNA in the L1. This contradicts the mRNA-protein co-trafficking model, however, it could mean that stringent controls regulate KN1 mRNA trafficking.

The significance of binding and co-trafficking of the KN1 protein and mRNA remains unclear. Ideas may be drawn from the specific homeodomain-mRNA interactions observed in *Drosophila*, where BICOID homeodomain protein binds to the *Caudal* mRNA to regulate its translation in the embryo (Dubnau and Struhl 1996). Another idea is that the co-trafficked mRNA may provide a mechanism to amplify the signal that would be obtained by protein trafficking alone (Jackson and Hake 1997).

2.4 Comparing and Contrasting with Viral MPs

Viral MPs have been the pioneer agents for us to understand macromolecular trafficking through PDs. It therefore pays to compare and contrast KN1 trafficking with that of MPs. There are some notable differences and yet interesting links between the trafficking of these two different types of proteins.

2.4.1 Distinctions

KNOX protein trafficking in the meristem is relatively restricted, compared to the viral MPs or free diffusion of small proteins, like GFP. This suggests KNOX protein trafficking is more regulated, for short range signaling. It is noteworthy, though, that *KNOX* genes might also mediate long-distance signaling through the trafficking of their mRNA, rather than protein (Kim et al. 2001).

In contrast to MPs that enrich at the PDs near the viral infection front, KN1 shows no strong accumulation at the PDs in the SAM. This may be attributable to the types and state of those PDs in the SAM (primary/simple versus secondary/modified), or the much higher expression levels of viral MPs. On the other hand, it may also reflect the different functional significance of trafficking, with MPs modifying the PD SEL (gating) to enable viral spread, and KN1 moving into the target cells to determine cell fate.

2.4.2 Molecular Connections

An interesting link between the trafficking of KN1 and MPs came from studies with the microtubule-associated protein, Movement Protein Binding protein 2C (MPB2C). As its name suggests, MPB2C was identified as a binding protein for the MP of *Tobacco Mosaic Virus* (*TMV*). MPB2C mediates the accumulation of TMV MP at microtubules, and interferes with its trafficking (Kragler et al. 2003).

Interestingly, MPB2C also interacts with KN1 and STM via their homeodomains, which are the trafficking domains. In both transient assays and the trichome rescue system, expression of MPB2C negatively regulates KN1 association with PDs, and prevents KN1 cell-to-cell movement (Winter et al. 2007). Therefore it appears that MPB2C functions in a common mechanism, possibly involving sequestration, to regulate the trafficking both of KNOX proteins and of viral MPs.

Another unexpected finding showing the complex interplay between KNOX proteins and MPs is the identification of a class II KNOX protein from tobacco, NTH201, which facilitates MP accumulation and the spreading of TMV (Yoshii et al. 2008). KNOX proteins are divided into class I and II, based on sequence conservation and expression patterns, and these classes also differ in movement ability (Kerstetter et al. 1994; Kim et al. 2005). Similar to other *Arabidopsis* class II KNOX proteins, NTH201 has no ability to traffic itself (Kim et al. 2005; Yoshii et al. 2008). However, this KNOX protein co-localizes with the TMV MP at the PD when co-expressed. How NTH201 modulates MP trafficking is still unknown, but the fact that these two proteins form a ternary complex with a DnaJ-like protein suggests an involvement of regulated chaperone activities (Shimizu et al. 2009).

2.5 Movement of Homeodomain Proteins Between Mammalian Cells

Notwithstanding all the suggestive evidence that KN1 moves through PD, such as its association with cell wall associated speckles resembling PD in leaf cells, and its PD gating effect in microinjection assays, direct observation of KN1 transport through PD is still missing. A surprising finding is that homeodomain proteins have the capacity to traffic between mammalian cells, which obviously lack PD. Therefore we cannot exclude the possibility that KN1 may also move following routes other than PD. It is currently believed that unconventional secretion and internalization mechanisms promote homeodomain trafficking between mammalian cells (Maizel et al. 2002). In fact, the KN1 homeodomain also moves cell-to-cell when expressed in mammalian cells (Maizel et al. 2002). Since plants and animals have independently evolved multi-cellularity, homeodomain trafficking might have been co-opted separately for cell-to-cell communication in these two lineages, and might therefore represent convergent evolution.

Further dissection of the mobile mammalian homeodomain proteins has revealed specific signals responsible for distinct steps during trafficking. A short region in the chicken ENGRAILED homeodomain is necessary for its secretion (Maizel et al. 1999). A different region, the third helix, of the *Drosophila* ANTENNAPEDIA homeodomain, serves as a signal for cellular internalization, and has now been explored for therapeutic delivery purposes (Derossi et al. 1994; Joliot and Prochiantz 2004). Analogous efforts in unraveling similar type of signals in the KN1 homeodomain have not been successful (Kim et al. 2005). This may reflect differential requirements for entering the unconventional secretion/internalization pathway versus the PD trafficking pathway.

3 Functional Significance of KNOX Trafficking

3.1 KNOX Trafficking Appears Functionally Significant

Since the first discovery of KN1 cell-to-cell movement, its functional significance has been a major question. Several lines of evidence have suggested that KNOX trafficking is functionally significant. First, L1-specific expression of KN1, but not of a non-trafficking version of KN1, can partially complement null mutants of *SHOOTMERISTEMLESS* (*STM)*, a close *Arabidopsis* homolog of KN1 with similar loss-of-function phenotypes (Long et al. 1996; Kim et al. 2003). This suggests that KN1 retains its function in SAM maintenance after trafficking into target cells. In addition, through the trichome rescue system, the homeodomains of other *Arabidopsis* or tomato class I KNOX proteins have also been shown to have trafficking ability (Kim et al. 2005). This functional conservation lends support from an evolutionary perspective for KN1 trafficking being biologically relevant.

Another line of evidence supporting the idea that KN1 trafficking is significant came from observations that it is under specific developmental regulation (Kim et al. 2003). KN1 was found to traffic from the inner layers of the leaf to the epidermis, but not in the opposite direction, from epidermis to mesophyll. In contrast, KN1 is able to traffic inward from the epidermal (L1) layer in the SAM. Thus KN1 movement out of the L1 is developmentally regulated. Similar uni-directional movement between L1 and L2 layers has also been observed in flowers with the floral organ identity protein *DEFICIENS* (*DEF*) (Perbal et al. 1996). Additionally, SHR movement also appears uni-directional, from the stele to the endodermis, and the movement is limited to only one cell layer. In this case, SHR, when it is transported into the endodermis, stays there due to its interaction with other proteins, and its nuclear sequestration (Gallagher et al. 2004; Cui et al. 2007; Gallagher and Benfey 2009). Hence, directional trafficking may be a common theme in protein movement regulation.

3.2 Proposed Developmental Significance of KNOX Protein Trafficking

Despite all the evidence suggesting KN1 trafficking being functionally relevant, its exact molecular and developmental significance remains unclear. Here we summarize several ideas that have been proposed and that await further testing.

First, given that KN1 protein moves from the inner L2 layer to the epidermal L1 layer in the wild-type maize meristem, it may serve as a signal to coordinate the development of the L1 in response to that of the L2. However, this hypothesis, if correct, can only account for part of the significance. In light of the fact that *STM*, which is also mobile, has no reported difference in its mRNA and protein distribution between SAM layers, the inter-layer coordination hypothesis may not hold true

in all plants. Double labeling of the KN1 mRNA and protein also revealed more subtle distribution differences and some novel sites for KN1 protein trafficking in the maize shoot apical region (Fig. 4.1c) (Jackson 2002). The double labeling analysis also led to the observation of a concentration gradient of the KN1 protein between the SAM and the incipient leaf primordium. This KN1 protein gradient may form as a result of protein trafficking, and leads to a second hypothesis that KN1 cell-to-cell trafficking is used for establishing concentration gradients. Although the biological function of the KN1 gradient is unknown, studies with *Drosophila* embryos may shed some light. In *Drosophila*, gradients of homeodomain proteins are used to activate different target genes at different protein concentrations (Dubnau and Struhl 1996). Hence it is possible that the KN1 gradient provides positional information to specify SAM boundary identity.

Other hypotheses have also been proposed. Similar to the role proposed for the trafficking of the floral regulator LEAFY (Sessions et al. 2000), cell-to-cell trafficking of KN1/STM within its mRNA expression domain may provide a fail-safe mechanism to ensure all cells maintain a SAM fate. Alternatively, KN1 and STM may receive post-translational modifications, and gain novel molecular functions, during cell-to-cell movement. KN1 appears to be partially unfolded for passing through PDs, thus potentially gets exposed to various modifications (Kragler et al. 1998). Consistent with this, phosphorylation and glycosylation of other trafficking proteins have been demonstrated (Taoka et al. 2007). The observation that ectopic expression of a cell-autonomous version of KN1 fails to cause the same phenotypic effects as the wild type, non-autonomous KN1, lends some support to the protein modification during trafficking model (Kim et al. 2003).

4 Summary Remarks and Some Remaining Challenges

A specific group of plant homeodomain transcription factors, with KN1 and STM as the best understood, move between cells, presumably through PDs. The homeodomain, including the NLS, is both necessary and sufficient for movement, and the movement process is under tight temporal and spatial control during plant development. Despite our limited understanding about its functional significance, KNOX protein trafficking appears to establish a novel signaling pathway in plants for coordinated meristem development.

Many intriguing questions remain unanswered and deserve some thoughts. First, it is unclear how dynamic the KN1 trafficking process is. By understanding this aspect, we may get a grasp on the temporal resolution of molecular responses that follow KN1 trafficking. In microinjection experiments, KN1 appears to move into neighboring cells within seconds. However, it remains to be seen whether this reflects the trafficking dynamics of endogenously expressed proteins. The rate of trafficking might also be regulated in different tissues and at different developmental stages. To address this issue, fluorescence recovery after photobleaching (FRAP), which has taught us kinetics of viral MP trafficking (Wright et al. 2007), may hold the key.

As to the interaction and co-trafficking of KN1 protein and mRNA, it remains to be tested if KN1 protein can bind and enable the trafficking of specific sequences in its mRNA. If specific RNA motifs are involved, can we predict, through bioinformatics, any other RNAs that may harbor those motifs? Alternatively, RNA immunoprecipitation coupled with high-throughput sequencing could be used to systematically identify other RNAs that KN1 might bind and traffic (Selth et al. 2009). These motifs could also be mutated, to test the functional significance of KN1 mRNA trafficking.

The most interesting and challenging question in KNOX trafficking is to identify the molecular players that mediate the process. So far, only MPB2C has been implicated in KNOX protein movement, though its precise role in KNOX trafficking remains to be addressed. Some ongoing efforts, such as using the trichome rescue system for genetic screens, and proteomic studies of KNOX interacting proteins, may be key to bring us mechanistic understanding on how KNOX proteins traffic. Indeed, a study published while this review was in proof (Xu et al. 2011), showed that a specific class of chaperones, called chaperonins, are necessary facilitators of KN1 trafficking. Furthermore, studies of chaperonin mutant interactions demonstrated a clear function for KNOX protein trafficking in maintenance of stem cells in the shoot meristem. These studies set the stage for the use of the trichome rescue screen, and other innovative approaches, to enrich our understanding of this fascinating developmental process.

Acknowledgements Thanks to Kate Creasey for critical reading. X. M. X. is a LSRF Fellow of the Life Sciences Research Foundation and a Chapman Fellow of the Chapman Foundation. Relevant research in the authors' laboratory is supported by the National Science Foundation (award 1027003).

References

Crawford KM, Zambryski PC (2000) Subcellular localization determines the availability of non-targeted proteins to plasmodesmatal transport. Curr Biol 10:1032–1040

Cui H, Levesque MP, Vernoux T, Jung JW, Paquette AJ, Gallagher KL, Wang JY, Blilou I, Scheres B, Benfey PN (2007) An evolutionarily conserved mechanism delimiting SHR movement defines a single layer of endodermis in plants. Science 316:421–425

Derossi D, Joliot AH, Chassaing G, Prochiantz A (1994) The third helix of the Antennapedia homeodomain translocates through biological membranes. J Biol Chem 269:10444–10450

Dubnau J, Struhl G (1996) RNA recognition and translational regulation by a homeodomain protein. Nature 379:694–699

Gallagher KL, Benfey PN (2009) Both the conserved GRAS domain and nuclear localization are required for SHORT-ROOT movement. Plant J 57:785–797

Gallagher KL, Paquette AJ, Nakajima K, Benfey PN (2004) Mechanisms regulating short-root intercellular movement. Curr Biol 14:1847–1851

Hake S, Freeling M (1986) Analysis of genetic mosaics shows that the extra epidermal cell divisions in *Knotted* mutant maize plants are induced by adjacent mesophyll cells. Nature (Lond) 320:621–623

Hake S, Smith HM, Holtan H, Magnani E, Mele G, Ramirez J (2004) The role of knox genes in plant development. Annu Rev Cell Dev Biol 20:125–151

Haywood V, Kragler F, Lucas WJ (2002) Plasmodesmata: pathways for protein and ribonucleoprotein signaling. Plant Cell 14:303–325

Jackson D (2002) Double labeling of KNOTTED1 mRNA and protein reveals multiple potential sites of protein trafficking in the shoot apex. Plant Physiol 129:1423–1429

Jackson D, Hake S (1997) Morphogenesis on the move: intercellular trafficking of regulatory proteins in plants. Curr Opin Genet Dev 7:495–500

Jackson D, Veit B, Hake S (1994) Expression of maize *KNOTTED1* related homeobox genes in the shoot apical meristem predicts patterns of morphogenesis in the vegetative shoot. Development 120:405–413

Joliot A, Prochiantz A (2004) Transduction peptides: from technology to physiology. Nat Cell Biol 6:189–196

Kerstetter R, Vollbrecht E, Lowe B, Veit B, Yamaguchi J, Hake S, Department of Plant Biology, U.o.C.B (1994) Sequence analysis and expression patterns divide the maize knotted1-like homeobox genes into two classes. Plant Cell 6(12):1877–1887

Kim JY, Yuan Z, Jackson D (2003) Developmental regulation and significance of KNOX protein trafficking in Arabidopsis. Development 130:4351–4362

Kim JY, Rim Y, Wang J, Jackson D (2005) A novel cell-to-cell trafficking assay indicates that the KNOX homeodomain is necessary and sufficient for intercellular protein and mRNA trafficking. Genes Dev 19:788–793

Kim JY, Yuan Z, Cilia M, Khalfan-Jagani Z, Jackson D (2002) Intercellular trafficking of a KNOTTED1 green fluorescent protein fusion in the leaf and shoot meristem of Arabidopsis. Proc Natl Acad Sci USA 99:4103–4108

Kim M, Canio W, Kessler S, Sinha N (2001) Developmental changes due to long-distance movement of a homeobox fusion transcript in tomato. Science 293:287–289

Kragler F, Monzer J, Shash K, Xoconostle-Cazares B, Lucas WJ (1998) Cell-to-cell transport of proteins: requirement for unfolding and characterization of binding to a putative plasmodesmal receptor. Plant J 15:367–381

Kragler F, Curin M, Trutnyeva K, Gansch A, Waigmann E (2003) MPB2C, a microtubule-associated plant protein binds to and interferes with cell-to-cell transport of tobacco mosaic virus movement protein. Plant Physiol 132:1870–1883

Kurata T, Ishida T, Kawabata-Awai C, Noguchi M, Hattori S, Sano R, Nagasaka R, Tominaga R, Koshino-Kimura Y, Kato T, Sato S, Tabata S, Okada K, Wada T (2005) Cell-to-cell movement of the CAPRICE protein in Arabidopsis root epidermal cell differentiation. Development 132:5387–5398

Long JA, Moan EI, Medford JI, Barton MK (1996) A member of the KNOTTED class of homeodomain proteins encoded by the SHOOTMERISTEMLESS gene of Arabidopsis. Nature 379:66–69

Lucas WJ, Bouche-Pillon S, Jackson DP, Nguyen L, Baker L, Ding B, Hake S (1995) Selective trafficking of KNOTTED1 homeodomain protein and its mRNA through plasmodesmata. Science (Washington, DC) 270:1980–1983

Maizel A, Bensaude O, Prochiantz A, Joliot A (1999) A short region of its homeodomain is necessary for engrailed nuclear export and secretion. Development 126(14):3183–3190

Maizel A, Tassetto M, Filhol O, Cochet C, Prochiantz A, Joliot A (2002) Engrailed homeoprotein secretion is a regulated process. Development 129:3545–3553

Nakajima K, Sena G, Nawy T, Benfey PN (2001) Intercellular movement of the putative transcription factor SHR in root patterning. Nature 413:307–311

Perbal M-C, Haughn G, Saedler H, Schwarz-Sommer Z (1996) Non-cell-autonomous function of the Antirrhinum floral homeotic proteins DEFICIENS and GLOBOSA is exerted by their polar cell-to-cell trafficking. Development 122:3433–3441

Selth LA, Gilbert C, Svejstrup JQ (2009) RNA immunoprecipitation to determine RNA-protein associations *in vivo*. Cold Spring Harb Protoc **2009,** pdb prot5234

Sessions A, Yanofsky MF, Weigel D (2000) Cell-cell signaling and movement by the floral transcription factors LEAFY and APETALA1. Science 289:779–782

Shimizu T, Yoshii A, Sakurai K, Hamada K, Yamaji Y, Suzuki M, Namba S, Hibi T (2009) Identification of a novel tobacco DnaJ-like protein that interacts with the movement protein of tobacco mosaic virus. Arch Virol 154:959–967

Smith LG, Greene B, Veit B, Hake S (1992) A dominant mutation in the maize homeobox gene, *Knotted-1*, causes its ectopic expression in leaf cells with altered fates. Development 116: 21–30

Taoka K, Ham BK, Xoconostle-Cázares B, Rojas MR, Lucas WJ (2007) Reciprocal phosphorylation and glycosylation recognition motifs control NCAPP1 interaction with pumpkin phloem proteins and their cell-to-cell movement. Plant Cell 19:1866–1884

Vollbrecht E, Veit B, Sinha N, Hake S (1991) The developmental gene Knotted-1 is a member of a maize homeobox gene family. Nature 350:241–243

Winter N, Kollwig G, Zhang S, Kragler F (2007) MPB2C, a microtubule-associated protein, regulates non-cell-autonomy of the homeodomain protein KNOTTED1. Plant Cell 19:3001–3018

Wright KM, Wood NT, Roberts AG, Chapman S, Boevink P, Mackenzie KM, Oparka KJ (2007) Targeting of TMV movement protein to plasmodesmata requires the actin/ER network: evidence from FRAP. Traffic 8:21–31

Yoshii A, Shimizu T, Yoshida A, Hamada K, Sakurai K, Yamaji Y, Suzuki M, Namba S, Hibi T (2008) NTH201, a novel class II KNOTTED1-like protein, facilitates the cell-to-cell movement of Tobacco mosaic virus in tobacco. Mol Plant Microbe Interact 21:586–596

Xu MX, Wang J, Xuan Z, Goldshmidt A, Borrill P.G.M, Harihanan N, Kim JY and Jackson D, (2011) Chaperonins facilitate KNOTTED1 cell-to-cell trafficking and stem cell function. Science 333:1141–1144

Chapter 5
Mechanism of Small RNA Movement

Nial R. Gursanscky and Bernard J. Carroll

1 Introduction

1.1 Small Regulatory RNAs (sRNAs)

Although sRNAs are well known regulators of gene expression, it is worthwhile to review current knowledge of their biogenesis and action before we discuss cell-to-cell and systemic sRNA movement. Small RNAs are potent sequence-specific, negative regulators of gene expression (Boutet et al. 2003; Vaucheret 2008). They play a crucial role in many fundamental processes in plants including developmental regulation of gene expression (Chitwood et al. 2007, 2009; Nogueira et al. 2007, 2009; Schwab et al. 2009; Carlsbecker et al. 2010), regulation of chromatin structure during mitosis and meiosis (Matzke and Birchler 2005), and defence against viral (Havelda et al. 2003; Himber et al. 2003; Dunoyer et al. 2007), bacterial (Dunoyer et al. 2006; Navarro et al. 2006; Agorio and Vera 2007) and fungal (Ellendorff et al. 2009) pathogens. It is becoming clear that the movement of sRNAs from cell-to-cell is essential for some of these activities.

There are several types of sRNAs and their biogenesis is similar but distinct. The differences in biogenesis could contribute to whether sRNAs act as cell-to-cell or systemic signals rather than in restricted cell autonomous functions. The key similarity that all types of sRNA share is their origin from double-stranded RNA (dsRNA) (Vaucheret 2008; Xie and Qi 2008). In plants, sRNAs are invariably derived from dsRNA that is processed by one of four members of the Dicer-Like family of ribonucleases (DCL1 to DCL4) (Xie and Qi 2008). The source of the dsRNA substrate and the DCL enzyme that carries out the cleavage are the first

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F. Kragler and M. Hülskamp (eds.), *Short and Long Distance Signaling*,
Advances in Plant Biology 3, DOI 10.1007/978-1-4419-1532-0_5,

defining characteristics of the transcriptional and post-transcriptional gene silencing pathways in plants.

Each DCL has a different, albeit sometimes redundant, role in gene silencing. This is partly reflected by the length of the sRNA produced by each DCL. DCL1 predominantly produces microRNAs (miRNAs), usually 21 nucleotides (nt) in length, which can direct mRNA cleavage or translational inhibition (Kurihara and Watanabe 2004; Gandikota et al. 2007). DCL2 produces 22 nt small interfering RNAs (siRNAs), which are primarily required to direct cleavage of virus RNA. DCL4 produces 21 nt siRNAs from both endogenous (Gasciolli et al. 2005; Xie et al. 2005) and exogenous (i.e. viral or transgene) (Deleris et al. 2006) substrates to play the key role in various forms of post-transcriptional gene silencing (PTGS). Finally, DCL3 produces 24 nt sRNA products are responsible for directing epigenetic modifications such as DNA methylation and chromatin modification, i.e. transcriptional gene silencing (TGS) (Daxinger et al. 2009). In particular, 24 nt siRNAs are responsible for maintaining epigenetic marks necessary to prevent transposon activity and maintain heterochromatin structure (Henderson et al. 2006). This 24 nt size class of sRNAs represent the largest proportion of sRNA in *Arabidopsis* (Kasschau et al. 2007; Zhang et al. 2007; Mosher et al. 2009; Cuperus et al. 2010), but that may not be true for all cell types and all plant species (Wong et al. 2011).

Once miRNAs and siRNAs are formed, they associate with Argonaute (AGO) proteins to guide silencing of complementary RNA and DNA (Vaucheret 2008). In *Arabidopsis*, there are ten AGO proteins and like the DCL family, each AGO has specialized roles (Vaucheret 2008).

1.2 MicroRNAs (miRNAs) and Trans-acting Small Interfering RNAs (ta-siRNAs)

The first miRNA lin-4 was identified in the worm *Caenorhabditis elegans,* and was demonstrated to down-regulate an endogenous gene called *lin-14*. miRNA lin-4 binds to complimentary sites in the 3′-UTR of the *lin-14 mRNA* and prevents translation (Lee et al. 1993; Wightman et al. 1993). The second miRNA described (let-7) was also found in *C. elegans*, and it also down-regulates gene expression through translational inhibition (Reinhart et al. 2000).

The defining features of miRNAs are: (1) they are typically between 20 and 24 nt in length, (2) they are processed from a single-stranded RNA that folds into a double-stranded RNA precursor, which is also usually a non-protein coding transcript, and (3) they usually have a complimentary site within the transcript of other protein-coding genes (Reinhart et al. 2002; Ambros et al. 2003).

More recently, functional miRNA have been identified in many eukaryotic species ranging from single-celled algae (Molnar et al. 2007; Zhao et al. 2007b) to higher plants (Reinhart et al. 2002; Palatnik et al. 2003; Zhang et al. 2006a) and humans (Pasquinelli et al. 2000; Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001). The biogenesis of miRNAs has been the subject of intense study and is relatively well understood.

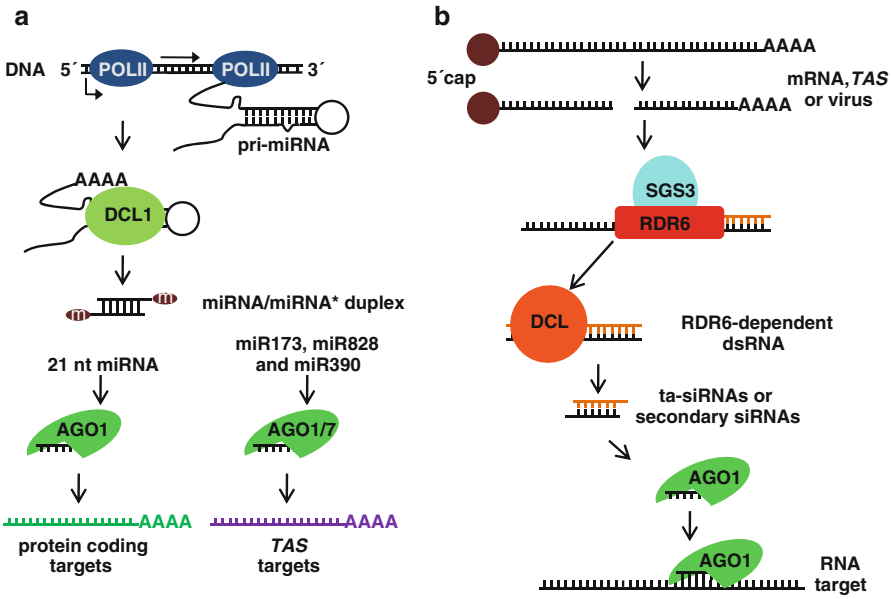

Fig. 5.1 Biogenesis of small RNAs and post-transcriptional gene silencing (PTGS): (**a**) Biogenesis and action of miRNAs. pri-miRNAs are produced by RNA polymerase II (POL II) and form a double-stranded RNA hairpin structure. The pri-miRNA is recognised by a group of proteins, most notably DICER-like 1 (DCL1). The mature miRNA/miRNA* duplex produced by DCL1 is methylated (m) on the 3′ nucleotides by HEN1, and incorporated into an AGO protein. Most miRNAs are bound by AGO1 and direct cleavage or translational repression of protein coding target transcripts. A few miRNAs in *Arabidopsis* (miR173, miR828 and miR390) in association with AGO1 or AGO7 direct cleavage of noncoding *TAS* transcripts that results in generation of *trans*-acting siRNAs (ta-siRNAs). (**b**) Biogenesis of ta-siRNAs and secondary siRNAs. ta-siRNAs and secondary siRNAs are produced from RNA lacking either a 5′ cap or a 3′ poly(A) tail. These RNA include cleaved *TAS* transcripts (in the case of ta-siRNAs), mRNAs or viral RNAs. De-capped and deadenylated RNA become substrates for RNA-Dependent RNA polymerase 6 (RDR6), resulting in production of double-stranded RNA. The activity of RDR6 is facilitated by SGS3. A DCL protein then cuts the double-stranded RNA into produce siRNAs that guide post-transcriptional gene silencing (PTGS). For further details, see text

Here, we will focus on the biogenesis of miRNAs in plants, and in particular, on *Arabidopsis*. Plant miRNAs are derived from transcription of endogenous genes, which are usually non-protein coding but still have their own promoters and 3′ terminators (Reinhart et al. 2002; Ambros et al. 2003). Like mRNAs, the primary miRNA transcripts (pri-miRNAs) are transcribed by RNA polymerase II (Pol II) and require the transcriptional enhancer MEDIATOR (Kim et al. 2011). However, unlike protein coding mRNAs, pri-miRNAs have a high level of secondary structure. This secondary structure forms a double-stranded RNA (dsRNA) known as a hairpin, which normally contains some bulges and mismatches (Ambros et al. 2003) (Fig. 5.1). The intracellular location of all the steps involved in processing pri-miRNAs into mature miRNAs in plant cells is not clear, and there are possibly several sequential processing sites. The first steps in pri-miRNAs processing occur within sub-nuclear compartments (Fang and Spector 2007).

The accurate cleavage of pri-miRNA by DCL1 depends on interactions between DCL1 and Double-stranded RNA Binding protein 1 (DRB1; also called Hyponastic Leaves 1 or HYL1) (Kurihara et al. 2006), as well as the zinc finger protein SERRATE (Yang et al. 2006a; Dong et al. 2008), the FHA domain protein DAWDLE (DDL) (Yu et al. 2008) and the secondary structure of the pri-miRNA (Parizotto et al. 2004; Kurihara et al. 2006; Dong et al. 2008; Eamens et al. 2009). The first DCL1 dependent cut in the pri-miRNA acts as a position marker for the second DCL1 dependent cut that produces a miRNA/miRNA* duplex (miRNA* refers to the strand of the duplex that is complementary to the mature miRNA strand) (Dong et al. 2008; Schwab and Voinnet 2009). In plants, almost all miRNAs are 21 nt in length. However, in some cases, a bulge or mismatch in the dsRNA between the first and second cleavage sites results in a 22 or 23 nt miRNA (Chen et al. 2010; Cuperus et al. 2010).

The miRNA/miRNA* duplex has 2 nt overhangs on each 3′ end, and the 3′ nt on each strand is methylated by HEN1 (Yu et al. 2005; Yang et al. 2006b). This methylation protects the miRNA from degradation (Li et al. 2005; Yu et al. 2005; Yang et al. 2006b). The correct orientation of the miRNA and miRNA* strands upon loading into AGO1 (or sometimes AGO7) depends on DRB1 (HYL1), and also differences in thermostability between the two ends of the miRNA/miRNA* duplex (Eamens et al. 2009). After loading into AGO1 the miRNA* strand is then cut and degraded, allowing the miRNA to then guide AGO1 specifically to complementary sequences in the respective target transcripts (Eamens et al. 2009).

In plants, miRNAs most commonly regulate gene expression by guiding sequence-specific cleavage of mRNAs (Vaucheret et al. 2004), but they can also block translation (Aukerman and Sakai 2003; Chen 2004; Schwab et al. 2005; Zhang et al. 2006b; Brodersen et al. 2008). After mRNA cleavage by AGO1, the cleaved mRNA is typically degraded by exonucleases from the XRN family (Gregory et al. 2008). More recently, it has been shown that when mRNAs are targeted by 22 nt miRNAs (e.g. miR173 and miR828), rather than be degraded, they become substrates for RNA-dependent RNA polymerase 6 (RDR6) in *Arabidopsis*. This results in amplification of dsRNA and secondary siRNA biogenesis along the length of the cleaved transcript (Chen et al. 2010; Cuperus et al. 2010). In particular, some *trans*-acting small interfering RNAs are produced in this way.

Trans-acting small interfering RNAs (ta-siRNAs) are similar to siRNAs, but are distinguished by two points. Firstly, they are derived from non-coding *TAS* transcripts rather than protein coding transcripts. Secondly, like miRNAs, ta-siRNAs act in *trans* to down-regulate expression of endogenous protein coding transcripts (Peragine et al. 2004; Allen et al. 2005; Xie et al. 2005; Axtell et al. 2006; Rajagopalan et al. 2006). There are eight known ta-siRNA generating loci in *Arabidopsis*, grouped into four families, *TAS1-4*. These non-coding transcripts are processed by one of two possible ways (Peragine et al. 2004; Allen et al. 2005; Xie et al. 2005; Axtell et al. 2006; Rajagopalan et al. 2006). *TAS 1, 2* and *4* transcripts are cleaved by AGO1 and the 3′ transcript fragment becomes a template for RDR6 (Montgomery et al. 2008b). The resulting dsRNA is then processed into 21 nt ta-siRNAs by DCL4 (Peragine et al. 2004; Allen et al. 2005; Xie et al. 2005; Axtell

et al. 2006; Rajagopalan et al. 2006) (Fig. 5.1). In the case of *TAS1, 2* (targeted by miR173) and *TAS4* (targeted by miR828), the size of the miRNA directs the cleavage product into the RDR6 secondary siRNA biogenesis pathway. As indicated earlier, both miR173 and miR828 are 22 nt long rather than the more common miRNA length of 21 nt (Chen et al. 2010; Cuperus et al. 2010).

Artificial miR173 and miR828 precursors that produce 21 nt versions of miR173 and miR828 do not lead to secondary siRNA production after the non-coding *TAS* transcript is cleaved (Chen et al. 2010; Cuperus et al. 2010). Why 22 nt but not 21 nt miRNAs induce dsRNA production by RDR6 is not clear, but one possibility is that the different size miRNA induces a conformational change in AGO1, which could enhance recruitment of RDR6 through an unknown mechanism (Chen et al. 2010; Cuperus et al. 2010).

TAS3 transcripts are processed a slightly different way, and these represent the second known class of ta-siRNAs. To begin with, they specifically require miR390 in association with AGO7 (rather than AGO1) to bind to two miRNA recognition site at opposite ends of the *TAS3* transcript (Fig. 5.1) (Axtell et al. 2006; Montgomery et al. 2008a). One recognition site remains uncut, while the other is cleaved. The cleaved *TAS3* transcript then becomes a substrate for RDR6, and as for the first class of ta-siRNAs, the resulting dsRNA that is then cut by DCL4 into 21 nt ta-siRNAs with the phase set by the original AGO7-mediated cleavage (Allen et al. 2005; Axtell et al. 2006; Montgomery et al. 2008a). As will be discussed later in Sect. 2, *TAS3* ta-siRNAs have important non-cell-autonomous roles in leaf polarity and root development. Interestingly, other ta-siRNAs appear to act only in a cell-autonomous manner, implying a form of molecular discrimination that prevents random intercellular spreading of ta-siRNAs.

ta-siRNA duplexes generated from *TAS* transcripts are sorted into AGOs by their binding preference for the 5′ nucleotide of the ta-siRNA. Like miRNAs, ta-siRNAs with a 5′ U are usually loaded into AGO1 where they guide sequence-specific cleavage of target mRNAs (Mi et al. 2008; Allen and Howell 2010).

1.3 Small Interfering RNAs (siRNAs) and Post-Transcriptional Gene Silencing

Post-Transcriptional Gene Silencing (PTGS) describes a range of related phenomena dependent upon siRNAs produced usually by DCL4 and DCL2 in *Arabidopsis*. Targeted RNAs can be endogenous and transgene mRNAs, transposons and RNA viruses.

DCL4 plays the predominant role in PTGS but it has a degree of functional redundancy with DCL2 and DCL3 (Henderson et al. 2006). Single mutants in any of these genes have relatively limited consequences, although *dcl4* mutants lack ta-siRNAs and have elongated, epinastic leaves (Dunoyer et al. 2005; Gasciolli et al. 2005; Xie et al. 2005). Due to the redundancy between DCL genes, it was initially

not easy to show which DCL contributes to a particular aspect of PTGS. Indeed, under conditions of high transgene expression all DCLs are able to produce siRNAs that can be loaded into AGO1 regardless of their size, to effect PTGS (Dunoyer et al. 2007). DCL4-dependent 21 nt siRNAs are most important for viral defence and transgene silencing (Dunoyer et al. 2005; Ding and Voinnet 2007; Garcia-Ruiz et al. 2010). However, DCL4-dependent 21 nt siRNAs cannot initiate transitive production of secondary siRNAs from transgenes (Mlotshwa et al. 2008) or from endogenous genes (Cuperus et al. 2010). On the other hand, DCL2-dependent 22 nt siRNAs can also be loaded into AGO1 to guide sequence-specific PTGS, but additionally, they can trigger RDR6-dependent biogenesis of secondary siRNAs from transgenes (Mlotshwa et al. 2008).

DCL3 plays the major role in RNA-mediated epigenetic modification including DNA methylation and histone modification (Xie et al. 2004). However, RNAs from viruses, endogenous overlapping complimentary transcripts, and transcribed endogenous and transgenic inverted repeats form a double-stranded substrate for DCL2, DCL3 and DCL4 (Borsani et al. 2005; Blevins et al. 2006; Henderson et al. 2006). But when all are present, it is DCL4 that produces most siRNAs that act in the PTGS pathway (Dunoyer et al. 2005; Henderson et al. 2006).

It has been suggested that the breakdown of hierarchical DCL processing may be related to viral defence. When plants are infected with virus, extremely high levels of dsRNA may occur and provide substrate for DCL2, DCL3 and DCL4. Furthermore, viruses have evolved suppressor proteins that interfere with the production, action and transport of DCL4-dependent siRNAs (Mallory et al. 2001; Dunoyer et al. 2004; Blevins et al. 2006; Deleris et al. 2006). Given that DCL2 and DCL3 produce different sized siRNAs to DCL4, their siRNAs may not be recognised by the same viral silencing suppressors. In this way, DCL2 and DCL3 are able to reinforce viral defence provided by DCL4 (Dunoyer et al. 2007).

Endogenous and transgenic single-stranded RNAs (ssRNAs) require processing by RDRs into dsRNA before they become substrates for DCLs and siRNA biogenesis. PTGS often occurs in transgenic plants that have engineered to over-express endogenous genes. This type of silencing is called sense-induced PTGS, co-suppression or spontaneous silencing (Napoli et al. 1990; van der Krol et al. 1990). The exact mechanism that induces co-suppression is still unclear, although it is generally accepted that high level transgene expression leads to a proportion of the transcripts being aberrant RNAs. Aberrant RNAs are most likely transcripts lacking a 5′ cap or a 3′ poly(A) tail, as these are very effective templates for RDR6-dependent dsRNA production (Gazzani et al. 2004; Luo and Chen 2007).

DCL2, DCL3 and DCL4 mediated cleavage of dsRNA produces siRNA duplexes with 2 nt 3′ overhangs that are subsequently processed like miRNA/miRNA* duplexes (see Sect. 1.2). As with miRNAs, siRNAs are incorporated into an AGO according to preferential binding of different AGOs to specific 5′ nucleotides of siRNAs and thermodynamic stability of the ends of the siRNA duplex (Mi et al. 2008; Takeda et al. 2008). These AGO-bound siRNAs can guide either cleavage or translational repression of mRNAs (mainly by AGO1), or chromatin modification (mostly mediated by AGO4 and AGO6) (Zilberman et al. 2003; Baumberger and Baulcombe 2005; Zheng et al. 2007).

The first phase of siRNA production and AGO-mediated RNA cleavage provides an additional opportunity for further amplification of secondary siRNAs. As for the production of some ta-siRNAs by 22 nt miRNAs, RNA cleavage that is guided by 22 nt siRNAs also produces an effective substrate for RDR6 (Moissiard et al. 2007). In this case, 22 nt siRNAs from DCL2 guide AGO-mediated cleavage of the transcript or virus (Moissiard et al. 2007; Mlotshwa et al. 2008; Chen et al. 2010; Cuperus et al. 2010). DCL2 therefore plays an important role in secondary siRNA biogenesis associated with transgene silencing and resistance to RNA viruses.

RDR6 and secondary siRNA biogenesis are also required for systemic transgene silencing (Schwach et al. 2005; Brosnan et al. 2007). It has been proposed that this form of systemic transgene silencing is simply a reiteration of cell-to-cell movement, with amplification occurring in each recipient cell to allow silencing to spread through the entire plant (Himber et al. 2003). While it has been shown that both endogenous genes and transgenes are able to give rise to siRNAs that move systemically without cell-to-cell amplification of secondary siRNAs (Dunoyer et al. 2010a; Molnar et al. 2010), expression of an abundant target mRNA or an RNA virus throughout the plant would enhance the chances of secondary siRNA biogenesis and systemic spreading of PTGS via this mechanism.

1.4 Small Interfering RNAs and Transcriptional Gene Silencing (TGS)

Epigenetic modifications directed by DCL3-dependent 24 nt siRNAs lead to transcriptional silencing through alteration of chromatin structure and DNA methylation. However, epigenetic regulation also occurs in a broad range of siRNA-independent contexts (Lister et al. 2008). Epigenetic regulation at the level of chromatin is itself a highly complex set of processes, and analysis of the factors involved is beyond the scope of this chapter (for recent review see Law and Jacobsen 2010). We will briefly summarise the biogenesis and action of the DCL3-dependent 24 nt siRNAs, and briefly define the boundary between siRNA-dependent and siRNA-independent epigenetic maintenance.

The main source of dsRNA substrate for DCL3 is from loci transcribed by RNA Polymerase IV (Pol IV), which are converted to dsRNA by the activity of RNA-Dependent RNA Polymerase 2 (RDR2) (Daxinger et al. 2009). DCL3 cleavage then yields 24 nt siRNAs. As mentioned earlier, DCL3 can also act upon additional sources of dsRNA, including endogenous inverted repeat transcripts, overlapping complementary transcripts, dsRNA from viruses and dsRNA produced by RDR6 from transgenes (Henderson et al. 2006).

DCL3-derived 24 nt siRNAs are bound predominantly by AGO4, although AGO6 and AGO9 also bind 24 nt siRNAs in some tissues (Havecker et al. 2010; Olmedo-Monfil et al. 2010). AGO4 is guided by the bound 24 nt siRNA to methylate homologous DNA sequence in a process known as RNA-directed DNA Methylation (RdDM) (Cao et al. 2003; Zilberman et al. 2004; Wierzbicki et al. 2009). In addition to Pol IV, Pol V is required for RdDM of some target loci.

Initially, the presence of complementary siRNAs results in methylation of cytosines in all sequence contexts (CG, CNG and CNN) by DRM2, a homologue of mammalian DNA methytransferase 3 (DNMT3) (Cao et al. 2003). Maintenance of CG and CNG methylation in the absence of siRNAs is then carried out by DNA METHYLTRANSFERASE 1 (MET1) and CHROMOMETHYLASE 3 (CMT3), respectively (Cao et al. 2003). In parallel with DNA methylation, siRNA-directed histone modifications lead to heterochromatin formation and silencing of the target locus (Numa et al. 2010). RNA-directed DNA methylation and histone deacetylation result in DNA that is less accessible to Pol II, resulting in lower transcription and a corresponding decrease in expression (Taunton et al. 1996; Tian and Chen 2001; Aufsatz et al. 2007; Wierzbicki et al. 2008).

Recent reports have demonstrated that all size classes of siRNAs are graft-transmissible. However, 24 nt siRNAs are particularly graft-transmissible and can direct methylation of DNA in recipient cells (Dunoyer et al. 2010a; Molnar et al. 2010). Furthermore, the non-cell autonomous movement of 24 nt siRNAs is crucial to ovule development in *Arabidopsis*. These discoveries will be discussed in more detail later.

2 Small Regulatory RNAs as Intercellular Signals in Plants

Mobile sRNAs are essential signals for a range of processes in plants. We can group known mobile sRNAs by their function into categories of metabolic and stress response regulators, developmental regulators, and pathogen defence factors.

2.1 Small RNAs as Metabolic and Stress Response Regulators

Expression of some miRNAs is induced by environmental stresses such as hypoxia (Moldovan et al. 2010), drought (Zhao et al. 2007a; Kantar et al. 2010; Zhou et al. 2010), nutrient deficiency (Chiou 2007) and salinity (Borsani et al. 2005; Jagadeeswaran et al. 2009; Zhao et al. 2009). In the case of phosphate and sulphate deficiency, these miRNAs are capable of moving long distances via the phloem (Buhtz et al. 2008; Buhtz et al. 2010). The mechanisms of phloem-mediated long-distance transport of miRNAs are discussed in detail in the next chapter (see Chap. 6, Kehr), and will not be covered further here.

2.2 Small RNAs as Intercellular Regulators of Development

Mobile sRNAs are also essential regulators for many aspects of development, including gametogenesis, leaf and root development.

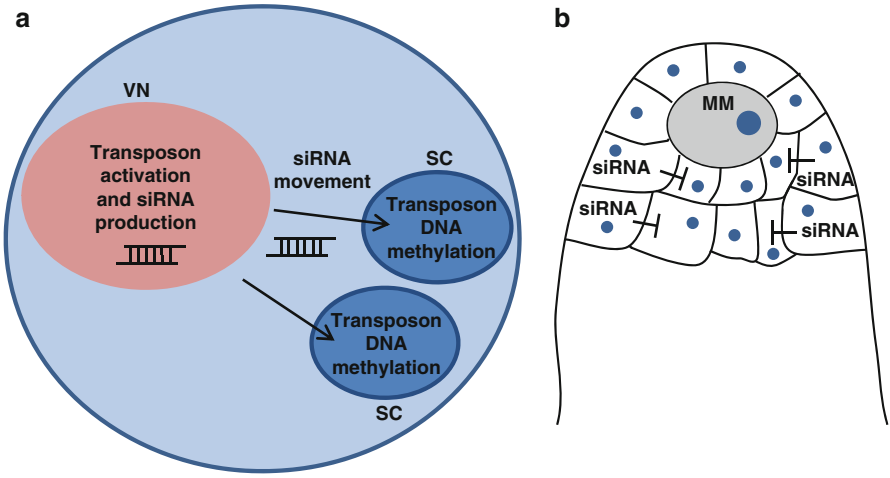

Fig. 5.2 Cell-to-cell small RNA movement in male and female gametophyte development: (**a**) During pollen development in *Arabidopsis*, transposons are reactivated and processed into 21 nt siRNAs in the vegetative nucleus (VN). These 21 nt siRNAs move from the VN to the sperm cells (SC), and reinforce DNA methylation and transcriptional silencing of transposons in the germline. This ensures maintenance of genome stability in the next generation. (**b**) The female *Arabidopsis* gamete develops from the megaspore mother cell (MM), also known as a gametophyte precursor cell. The MM differentiates from a single cell in the L2 layer of cells, under the epidermis of the pre-meiotic ovule. The epidermal cells of the ovule produce siRNAs dependent on RDR2, POLIV and POLV, DCL3, and RDR6. These siRNAs act non-cell autonomously, in an AGO9-dependent manner to restrict differentiation of cells in the L2 so that only a single L2 cell per ovule differentiates into a MM (gametophytic precursor cell)

2.2.1 Gametogenesis

Gametogenesis in plants is a complex developmental process. Unlike organisms that produce simple haploid gametes from meiosis, flowering plants undergo meiosis followed by additional rounds of mitotic cell division to produce the gametes.

Male gametogenesis produces pollen grains (microgametophytes), which contain two sperm cells (SCs) enclosed within a vegetative cell (Walbot and Evans 2003) (Fig. 5.2). Following meiosis, there are two rounds of mitosis to produce the mature pollen. Firstly, the haploid cell formed by meiosis divides to produce the genetically identical vegetative and generative cells. The generative cell then divides to produce two SCs. At fertilization, these two SCs fertilize the egg cell and the central cell within the female megagametophyte, in a double fertilization (Walbot and Evans 2003). The vegetative nucleus (VN) of the pollen does not contribute DNA to the embryo, but is required to control delivery of the SCs to the female megagametophyte (Walbot and Evans 2003).

During this process of pollen formation, a gene called *Decreased DNA Methylation 1 (DDM1)* is required to maintain epigenetic silencing of transposons and heterochromatin. However, *DDM1* is specifically down-regulated in the VN,

while its expression is maintained in the SCs (Slotkin et al. 2009). Corresponding with down-regulation of *DDM1* in the VN, there is a dramatic increase in the level of transposon activation and transposition in the VN (Slotkin et al. 2009). This transposon activity is only present in the VN and is accompanied by high levels of 21 nt siRNA production from transposon transcripts (Fig. 5.2). These 21 nt siRNAs derived from the reactivated transposons are transported from the VN to the SCs where they accumulate (Slotkin et al. 2009). Remarkably, the expression of transposons in the VN and subsequent siRNA intercellular movement and accumulation in the SCs reinforces silencing of transposons in the germline SCs (Slotkin et al. 2009).

As with all known intercellular movement of sRNAs, it is not known whether this movement of siRNAs from the vegetative cell to the SCs is an active process or passive diffusion. However, HASTY, the *Arabidopsis* orthologue of the human Exportin 5, is involved in export of miRNAs from the nucleus (Park et al. 2005). HASTY would also be a good candidate for facilitating export of siRNAs out of the VN of pollen, but how the siRNA move from the cytoplasm of the vegetative cells into the SCs awaits discovery (see also Chap. 7, Wolf et al.).

On the female side of plant reproduction, it has been also shown that during ovule development to produce the female gametophyte, the action of non-cell-autonomous siRNAs is necessary to restrict the number of gametophyte precursor cells to one per ovule. Mutations in *AGO9* produce ovules with multiple gametophyte precursor cells (Olmedo-Monfil et al. 2010). AGO9 binds to 24 nt siRNAs derived from transposons. Thus, AGO9 is required to silence transposons in the gametophyte and the associated cells of the ovule (Olmedo-Monfil et al. 2010) (Fig. 5.2). The biogenesis of these AGO9-associated 24 nt siRNAs is dependent on Pol IV and Pol V, RDR2 and DCL3, which are known components of the 24 nt siRNA biogenesis or chromatin silencing pathway (Sect. 1.4). Surprisingly, however, their biogenesis also appears to be dependent on RDR6, a key component of the PTGS pathway (Sect. 1.3). Single or double mutants in these genes produced a phenocopy of the *ago9* gametogenesis phenotype (Olmedo-Monfil et al. 2010). In summary, these results indicate that mobile 24 nt siRNAs derived from transposons play a key role in cell identity within the ovule.

In addition to these findings involving AGO9, it has been reported that there is exclusively maternal expression of Pol IV-dependent 24 nt siRNAs in the developing endosperm, indicating an additional process that may direct gametophyte identity through a non-cell-autonomous pathway involving 24 nt siRNAs (Mosher et al. 2009).

2.2.2 Leaf Development

Both leaf and root development requires cell-to-cell signalling to define the symmetry and polarity of the growing organs. As a leaf primordium forms, intercellular signals define the upper (adaxial) and lower (abaxial) surfaces of the developing primordium (Sussex 1954; Kidner and Martienssen 2004; Garcia et al. 2006; Chitwood et al. 2007; Nogueira et al. 2007; Chitwood et al. 2009; Nogueira et al. 2009).

In *Arabidopsis*, *TAS3* expression is limited to the two most adaxial cell layers of the leaf primordium (Schwab et al. 2009). miR390-guided cleavage of *TAS3* transcripts leads to DCL4-dependent production of ta-siRNAs (also called tasiR-ARFs), which target the *AUXIN RESPONSE FACTOR 3 and 4* (*ARF3, ARF4*) genes (Allen et al. 2005; Williams et al. 2005; Garcia et al. 2006). Expression of *ARF3* and *ARF4* is required for abaxial cell identity (Pekker et al. 2005; Fahlgren et al. 2006; Garcia et al. 2006). The tasiR-ARFs move out of the adaxial cells and create a gradient of low adaxial to high abaxial expression of *ARF3* and *ARF4* down through the leaf primordium. This results in *ARF3* and *ARF4* accumulation only in the abaxial cells (Chitwood et al. 2007, 2009; Nogueira et al. 2007, 2009; Schwab et al. 2009).

As mentioned earlier, DCL4 is required for biogenesis of ta-siRNAs and *dcl4* mutants have an elongated, epinastic leaf phenotype. Further evidence for the mobility of the ta-siRNAs is that a *DCL4* transgene expressed from the phloem-specific *SUC2* promoter, is able to suppress the epinastic leaf phenotype in a *dcl4* mutant background (Dunoyer et al. 2010b). Presumably, the *DCL4* transgene is able to produce sufficient ta-siRNAs in the meristem region and vasculature to allow ta-siRNA movement into the leaf primordial to confer normal leaf development.

The primary determinants of adaxial identity in leaves are the HD-ZIP III transcription factors PHABULOSA (PHB), PHAVOLUTA (PHV) and REVOLUTA (REV) (McConnell et al. 2001). PHB, PHV and REV are all targets for miR165/166-dependent mRNA cleavage (Floyd and Bowman 2004). miR165/166 are expressed only in the abaxial cell layers, and prevent PHB, PHV and REV from being expressed in the abaxial side of the developing leaf (Floyd and Bowman 2004; Kidner and Martienssen 2004). It has not been demonstrated yet that intercellular movement of miR165/166 is required for its role in leaf development. However, considering the role of miR165/166 in root development (discussed below), mobility of these miRNAs may be central to their function in leaf development as well.

2.2.3 Root Development

Non-cell-autonomous sRNAs are also required for normal root development in *Arabidopsis*. During root development, strict cell layer differentiation is required to produce the correct radial patterning of root tissues (Fig. 5.3). The patterning of these cell layers is regulated in part by the transcription factors SHORT ROOT (SHR) and SCARECROW (SCR). These transcription factors act together to induce expression of miR165/166 in the endodermal cell layer (Carlsbecker et al. 2010). Upon induction of miR165/166 expression in the endodermis, mature miR165/166 moves from the endodermis into the central vascular cylinder and targets the HD-ZIP III genes, in particular, PHABULOSA (PHB). This creates a radial gradient of PHB expression that drives dosage dependent differentiation of the protoxylem and metaxylem in the central vascular tissues (Carlsbecker et al. 2010) (Fig. 5.3).

In addition to the role of miR165/166 in determining the radial pattern of developing roots, miR390 and the tasiR-ARFs are also required for directing initiation and formation of lateral roots (Marin et al. 2010). Lateral root formation is initiated

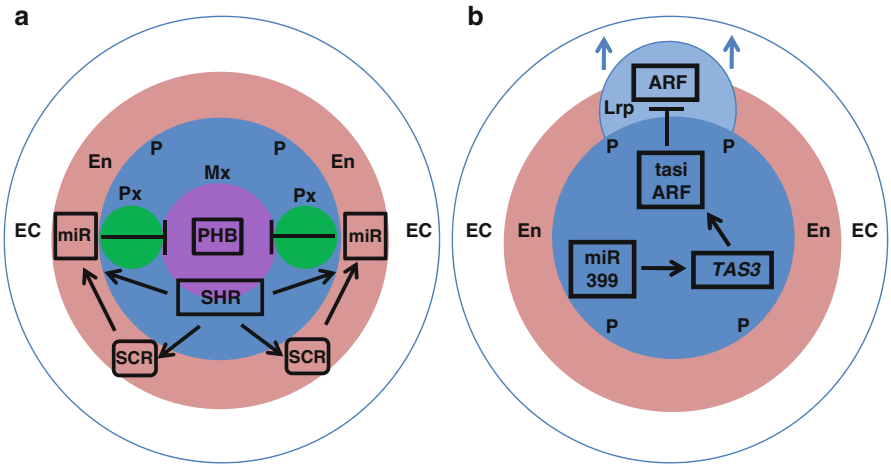

Fig. 5.3 Intercellular movement of small RNA in root development: (**a**) Radial model of the root for intercellular movement of miR165/166 in differentiation of xylem cell types in the central vascular cylinder. The signalling pathway starts with expression of the transcription factor SHORT ROOT (SHR) in the developing central vascular cylinder (*blue*). SHR moves outwards into the endodermis and induces expression of the transcription factor SCARECROW (SCR). SHR and SCR act synergistically to induce transcription of miR165 (miR) in the endodermal cell layer (En). miR165 moves into the central vascular cylinder and represses *PHABULOSA* (PHB) in the outer portion of the vascular cylinder, thereby directing the cell identity of metaxylem (Mx) versus protoxylem (Px). (**b**) Radial model for intercellular movement of tasiR-ARFs (tasi ARF) in formation of lateral root primordia (Lrp; *light blue*). Lateral root formation is initiated from division of a small number of cells the pericycle (P), the outer cell layer of the central vascular cylinder (*blue*). tasiR-ARFs are produced where miR390 overlaps with TAS3 expression in the central vascular cylinder of the main root. Cleavage of TAS3 transcripts results in generation of tasiR-ARFs (tasi ARF). From there, tasiR-ARFs (tasi ARF) move outwards to repress ARF2, ARF3 and ARF4 (ARF) in developing lateral root primordium (Lrp). Light blue arrows indicate direction of outgrowth of the lateral root. *EC* epidermis and cortex, *En* endodermis, *P* pericycle, *Px* protoxylem, *Mx* metaxylem

at local maxima of auxin. tasiR-ARFs are produced where miR390 overlaps with *TAS3* expression in the central vascular cylinder of the main root (Fig. 5.3). From there, both miR390 and tasiR-ARFs move outwards to repress *ARF2, ARF3* and *ARF4* in developing lateral root primordium. Both miR390 and tasiR-ARFs are detected in the developing root primordium several cell layers away from where miR390 and *TAS3* are expressed in the central vascular tissue of the main root (Marin et al. 2010) (Fig. 5.3).

As discussed in the previous section, miR165/166, miR390 and tasiR-ARFs are also required for normal abaxial/adaxial patterning of new leaves. The fact that these sRNA families have been identified as mobile signals and that they both function in root and leaf development, may indicate that there is a unique feature they share to enable their mobile, intercellular signalling function. However, such a feature has not yet been identified.

2.3 Small RNAs and Defence Against Pathogens

Viruses rely on active cell-to-cell and/or phloem-mediated transport of their genomes to achieve systemic infection (see Chap. 1; Heinlein and Chap. 7, Ruiz-Medrano et al.). Various strategies are employed by the host plant to limit virus movement, replication and survival. At the same time, viruses have developed many strategies to evade plant defences (Ding and Voinnet 2007). Plants recognise viral dsRNA and produce siRNAs that direct silencing of viral RNA and thereby prevent virus establishment (Ding and Voinnet 2007). Cell-to-cell virus movement is dependent upon a variety of viral-encoded movement proteins, which differ between virus species, but are generally required to interact with host proteins for cell-to-cell movement through plasmodesmata (reviewed in Scholthof (2005)) (see also Chap. 1, Heinlein).

Facilitating viral defence is a key role for cell-to-cell and systemic movement of siRNAs that target viruses (Havelda et al. 2003; Himber et al. 2003; Dunoyer et al. 2007). As discussed previously (Sect. 1.3), the PTGS pathway and in particular DCL4, DCL2 and AGO1, are key components of the siRNA immune response directed against RNA viruses.

There are several lines of evidence that imply a requirement of long-distance movement of siRNAs for plant antiviral immunity. There are numerous examples of viral encoded silencing suppressor proteins, which inhibit movement of siRNA-mediated silencing (Voinnet 2005). The 2b protein encoded by the *Cucumber Mosaic Virus* (CMV) is unable to prevent intracellular PTGS, but prevents the transmission of a defensive viral silencing signal (sRNA) to distal tissues (Guo and Ding 2002). HcPro and P19 both bind to 21 nt siRNAs and inhibit transmission of silencing by sequestering siRNA, while P38 produces an outcome in favour of the virus by binding to 21 and 22 nt siRNAs produced by DCL4 and DCL2, respectively (Lakatos et al. 2006; Ding and Voinnet 2007).

Another indication that long distance movement of siRNA-mediated silencing is a key antiviral defence mechanism in plants is that RDR6 activity is required to exclude Potato Virus X (PVX) from the apical meristems of tobacco plants (Schwach et al. 2005). It is highly probable that viruses and homologous siRNAs travel systemically at the same speed, and that siRNA-guided cleavage of the viral RNA after it enters a new cell, facilitates rapid production of dsRNA by RDR6. Subsequent biogenesis of additional antiviral siRNAs would provide a rapid defence and systemic response against RNA virus infection (Schwach et al. 2005).

DCL3-generated, 24 nt siRNA-mediated methylation of gemniviruses (DNA viruses) has also been reported as a systemic defence mechanism against these viruses. In support of this concept, DCL3-dependent, AGO4-associated 24 nt siRNAs appear to be required for host recovery of resistance to some gemniviral infections (Raja et al. 2008, 2010). Plants are often susceptible when first inoculated with a virus, but new and younger leaves on the plant can recover and become resistant to the virus (Wingard 1928). Geminivirus DNA recovered from secondary infected but resistant tissue showed hypermethylation, indicating that the 24 nt siRNAs may be able to move from the primary infected tissue into the secondary infected tissue to confer resistance to the virus (Raja et al. 2008).

Although viral-derived siRNAs differ from other siRNAs because of their origins and targets, their biogenesis is similar. Furthermore, other non-viral siRNAs are systemically mobile (Dunoyer et al. 2010a; Molnar et al. 2010) and therefore, a viral origin is probably not required for siRNA mobility. However, further research is required to fully address this question.

3 Possible Mechanisms Affecting Intercellular Movement of sRNAs in Plants

Higher plants are complex organisms with highly regulated mechanisms for local and systemic movement of nutrients, water and developmental signals. Direct cell-to-cell movement of molecules can occur through plasmodesmata, which are pore structures joining neighbouring cells (Fig. 5.4). It is generally thought that passage though plasmodesmata requires active transport for larger molecules, while small molecules may move by passive diffusion (reviewed in Lucas and Lee (2004)). Transport through plasmodesmata is regulated by altering the size exclusion limit (SEL). Altering the SEL requires movement of ligands to bind to channel proteins and subsequent alteration of the actin cytoskeleton to increase or decrease the pore size (Kragler et al. 2000; Oparka 2004; Ruiz-Medrano et al. 2004). One such ligand is the *Nicotiana tobacum* NON CELL AUTONOMOUS PATHWAY PROTEIN 1 (NtNCAPP1), which facilitates transport of selected proteins through plasmodesmata. It appears to be localised in the endoplasmic reticulum at the cell periphery and is essential for correct development (Lee et al. 2003), but its role in sRNA movement remains to be demonstrated.

Systemic signal movement in higher plants is thought to occur through the vascular system, which consists of the phloem and xylem (reviewed in Turgeon and Wolf (2009)) (see also Chap. 7; Ruiz-Medrano et al.). The xylem is a system of enucleated cells forming hollow tubes that transport mostly water and soil derived nutrients upward from the roots to the leaves and flowers. The phloem consists of the sieve elements, which carry the phloem sap, and companion cells, which regulate loading and unloading of the molecules between the phloem and the mesophyll cells. The phloem transports the energy derived from photosynthesis in the form of sugar, but also other signals that contribute to developmental processes.

The direction of movement of solutes in the phloem over a whole plant is a very complex issue. Generally, the source tissues (where phloem flows from) are older leaves that produce more energy than they use, whereas the sink tissues (where phloem flows to) are the young leaves and the roots that require more energy than they can produce through photosynthesis (Turgeon and Wolf 2009) (Fig. 5.4). Signal molecules along with photo-assimilates, are thought to be transported from the source tissue via cell-to-cell movement through plasmodesmata across the leaf, and into the companion cells of the phloem. The companion cells then facilitate some aspects of molecule transfer into the sieve elements by specific transporter proteins,

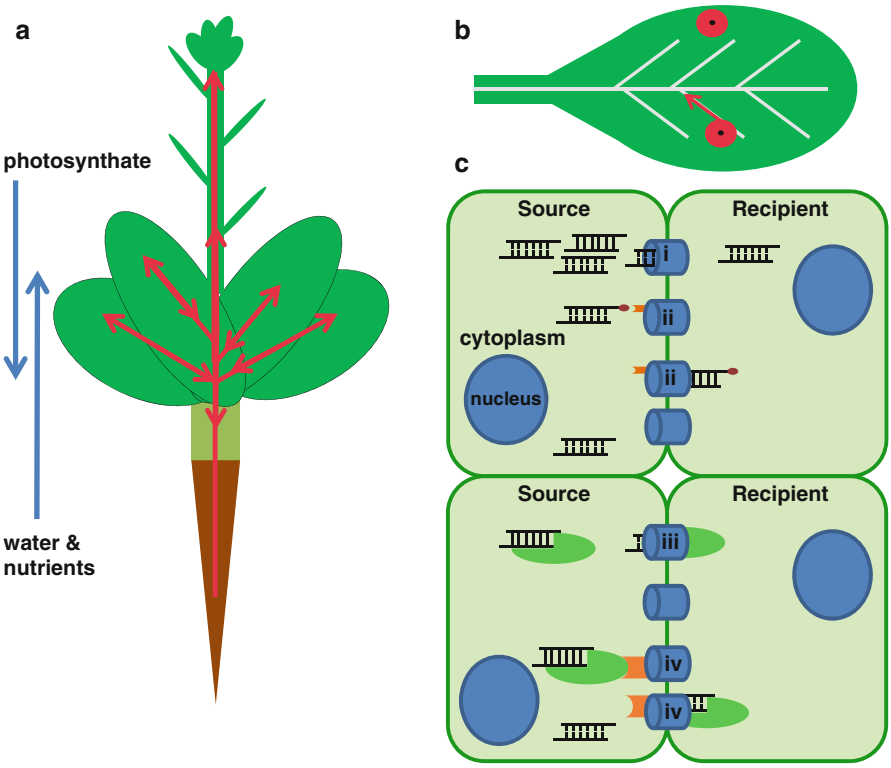

Fig 5.4 Channels for intercellular movement of molecules in higher plants: (**a**) A simple model of the vascular system of *Arabidopsis*. The xylem transports water and nutrients from the soil to the above ground parts of the plant. The phloem transports photo-assimilates (energy in the form of sugars) from mature leaves to the sink tissues (developing or non-photosynthetic tissues including young leaves, flowers, fruits, as well as roots). (**b**) Model for spreading of transgene silencing from a mature leaf. siRNA biogenesis induces transgene silencing in a single cell (*black dot*). Double-stranded and/or single-stranded siRNAs can spread cell-to-cell over a distance of 10–15 cells (*red circle*), and also possibly enter the phloem (*grey veins*) and travel systemically to induce transgene silencing. (**c**) Models for movement of small regulatory RNAs (sRNAs) from cell-to-cell. The possible ways that double-stranded and/or single-stranded siRNAs move between cells via the plasmodesmata include: (1) simple diffusion due to a concentration gradient, (2) facilitation by receptors in the plasmodesmata that increase the pore size to allow movement, (3) facilitation by an active transport factor, or (4) facilitation by a transfer factor that acts as a bridging ligand between the siRNA duplex and a plasmodesmata receptor

but there is also undirected diffusion of some molecules, as the SEL of companion cell plasmodesmata is reported to be as high as 67 kDa (Stadler et al. 2005). However, the SEL varies considerably with the cell type. It has been shown that only fluorescent labelled molecules less than 8 kDa are able to move between pumpkin leaf cells via plasmodesmata (Balachandran et al. 1997), and the SEL of plasmodesmata in mature tobacco leaves has been reported to be as low as 1 kDa (Wolf et al. 1989).

The presence of small RNA in pumpkin phloem sap and the possible function of the small RNA binding protein *Cucurbita maxima* PHLOEM SMALL RNA BINDING PROTEIN 1 (CmPSRP1) in triggering intercellular transport, indicates an active sRNA transport mechanism is present within the sieve tube system (Yoo et al. 2004). The molecular weight of greater than 7 and 14 kDa for single-stranded and double-stranded siRNA, respectively, also suggests that sRNAs may require active transport through plasmodesmata (Fig. 5.4). On the other hand, it has also been reported that the 27 kDa Green Fluorescent Protein (GFP) is able to move from the phloem companion cells into the sieve elements, and then unload into the sink tissues and move cell-to-cell without any additional proteins required (Imlau et al. 1999). The simplest conclusion that can be drawn from this collection of data is that transport of some molecules but not others is regulated through plasmodesmata (see also Chap. 2; Zambryski et al.). However, at this point, it has still not been unequivocally demonstrated whether transport of sRNAs through plasmodesmata is an active process or not.

We have presented evidence that sRNAs are able to move between cells, and that this movement is required for normal development and resistance against viruses. However, the question remains, why some sRNAs move and others do not. As we discussed previously, it is possible that differences in the biogenesis of some sRNAs predisposes them to intercellular motility, while others are prevented from leaving the cells in which they are formed. However, differences in biogenesis alone would appear insufficient to explain the different mobility of particular sRNA species.

miRNA production is usually tightly regulated at the transcriptional level. This results in different members of the same miRNA family being expressed specifically in different organs, or in one cell layer but not in the next (Baker et al. 2005; Aung et al. 2006; Nikovics et al. 2006; Sieber et al. 2007; Raman et al. 2008). This tight regulation and the clear evidence of cell-specific miRNA function (Chen 2004; Kidner and Martienssen 2004; Parizotto et al. 2004), in spite of the demonstrated ability of some sRNAs, including miRNAs, to move between cells, suggests that there are some yet unknown mechanisms that keep the majority of miRNAs within the cell where they are produced.

There are also secondary mechanisms that act to restrain miRNA activity to specific cells in specific times or conditions. One restrictive mechanism, which is currently limited to a single example, is an endogenous miRNA-target mimic (Franco-Zorrilla et al. 2007). The non-protein coding gene *INDUCED BY PHOSPHATE STARVATION (IPS)* is up-regulated in response to phosphate deficiency. *IPS* contains a recognition sequence for miR399, which also down-regulates *PHO2*, an important phosphate regulator (Franco-Zorrilla et al. 2007). The *IPS* miR399 recognition sequence has a mismatched loop at the normal miRNA cleavage site, which prevents miR399-guided cleavage of the *IPS* transcript. Instead, the *IPS* transcript sequesters miR399-containing AGOs, resulting in increased expression of *PHO2* due to decreased availability of miR399 (Franco-Zorrilla et al. 2007). The expression of similar types of mimic transcripts may be a common mechanism to attenuate miRNA activity and protect essential genes required for

cell or tissue identity. Indeed, some predictions of miRNA targets specifically exclude sequences that would fit the requirements for these target-mimic sites because they would be unable to be cut between nucleotides 8 and 12 of the predicted match (German et al. 2008; Alves et al. 2009).

Secondary mechanisms that prevent uncontrolled dispersal of miRNAs through the plant most likely also regulate siRNAs but there may be some siRNA-specific factors. Initial reports implied that the key requirement for production of mobile sRNAs was DCL4 processing. This hypothesis was based on initial evidence that ta-siRNAs and transgene inverted repeat hairpin-derived siRNAs are preferentially processed by DCL4 and that artificial miRNAs (processed by DCL1) could not be detected moving cell-to-cell (Schwab et al. 2006; Tretter et al. 2008). However, it has now been shown that many miRNAs and siRNAs not derived from DCL4 have the capacity for cell-to-cell and systemic mobility (Yoo et al. 2004; Buhtz et al. 2010; Carlsbecker et al. 2010; Dunoyer et al. 2010a, b; Molnar et al. 2010; Felippes et al. 2011).

Interestingly, there is recent evidence that synthetic, fluorescently labelled 21 nt sRNA introduced into living *Arabidopsis* leaf cells by particle bombardment are able to move between cells and enter the vascular system (Dunoyer et al. 2010b). These mobile sRNAs are also reported to be able to direct silencing of a target transgene in recipient cells (Dunoyer et al. 2010b) (Fig. 5.4). In these experiments, bombardment of fluorescently labelled double-stranded, but not single-stranded 21 nt siRNAs was able to induce non-cell-autonomous transgene silencing (Dunoyer et al. 2010b). The authors concluded that siRNA duplexes but not single-stranded siRNAs are mobile between cells (Dunoyer et al. 2010a, b). However, the results could reflect a difference in the stability of bombarded double-stranded versus single-stranded siRNAs.

Indeed, these results appear to contradict earlier experiments wherein a synthetic, labelled 25 nt double-stranded siRNA microinjected into *N. benthamiana* leaf epidermal cells was not mobile. In contrast, 25 nt single-stranded siRNA moved between cells but only when co-injected with the phloem-localized small RNA binding protein CmPSRP1 (Yoo et al. 2004). These earlier results in *N. benthamiana* are consistent with protein-assisted, active intercellular transport of single-stranded but not double-stranded siRNA (Yoo et al. 2004).

Another mechanism that may influence intercellular movement of miRNAs, siRNAs and ta-siRNAs throughout plants is the action of a family of small RNA specific ribonuclease genes called *SMALL RNA DEGRADING NUCLEASES* (*SDN*) (Ramachandran and Chen 2008). *SDN1* is a 3′-5′ exonuclease that specifically degrades single-stranded sRNAs larger than 17 nt and up to at least 27 nt (Ramachandran and Chen 2008). When all three *SDN1* homologues are knocked down in *Arabidopsis* using artificial miRNAs there is a strong over-accumulation of miRNAs and corresponding increase in developmental defects (Ramachandran and Chen 2008). Although it has not yet been demonstrated, it is likely that sRNAs that are not bound by an AGO protein are more susceptible to degradation by the SDN proteins.

4 Systemic and Graft-Transmissible Movement of siRNAs

There is good evidence that subsets of miRNAs are able to move systemically in response to nutrient starvation or other environmental triggers (Buhtz et al. 2008, 2010), but it is generally accepted that the majority of miRNAs are cell autonomous (Kidner and Martienssen 2004). Indeed, considering the strong regulatory effects of miRNAs and as mentioned earlier, it would seem necessary to tightly control their movement to prevent mis-regulation of genes essential for the identity and function of neighbouring cells and tissues (Valoczi et al. 2006).

The primary evolutionary role of siRNAs, on the other hand, is in genome defence against viruses and transposons. Numerous studies have therefore attempted to define the genetic requirements for production, systemic transmission and reception of siRNA-mediated silencing signals. Experimental systems involving reporter transgenes and endogenous sequences have been used to study systemic spreading of gene silencing (Palauqui et al. 1997; Brosnan et al. 2007; Dunoyer et al. 2010a; Molnar et al. 2010). Collectively, these various approaches have provided convincing evidence that siRNA are mobile and can act as systemic regulators of gene silencing in plants.

Demonstration of graft-transmissible gene silencing was first reported before the molecular mechanisms of sRNA-mediated gene silencing were discovered. Using tobacco (*N. tabacum*), Palauqui and co-workers in 1997 (Palauqui et al. 1997) showed that a transgene-specific silencing signal could not only travel across a graft junction but also through 30 cm of intervening non-transgenic stem to mediate graft-transmissible transgene silencing. Grafted *N. benthamiana* have been used to show that systemic transgene silencing is able to spread to most cells, except into symplastically-isolated guard cells (Wille and Lucas 1984; Voinnet et al. 1998). Interestingly, in leaves that developed after silencing spread to near the shoot apex, guard cells were also silent. This indicated that some silencing is maintained within a cell in the absence of reinforcement by any external signals. Furthermore, it shows that plasmodesmata are essential for entry of silencing signals into cells that are not associated with the vascular system.

Various other non-grafting systems including transient expression mediated by *Agrobacterium* infiltration of leaves and viral transgene expression have also been used in tobacco to demonstrate that silencing can be induced in a single leaf and spread systemic throughout the plant (Fagard and Vaucheret 2000). More recently, micrografting of *Arabidopsis* has proved to be a powerful tool for identifying the genetic determinants required for graft-transmissible gene silencing (Brosnan et al. 2007) and to demonstrate that siRNAs are graft-transmissible silencing signals (Dunoyer et al. 2010a; Molnar et al. 2010).

4.1 Reception of Graft-Transmissible Silencing Signals in Recipient Tissues

The development of micrografting system in *Arabidopsis* has enabled the identification of key genetic requirements for production (Molnar et al. 2010) and reception (Brosnan et al. 2007) of systemic gene silencing signals in recipient tissues. The initial system developed in *Arabidopsis* involved using a *GFP* hairpin silencing-inducing transgene expressed in rootstocks that were grafted to GFP reporter-expressing scions (shoots) (Brosnan et al. 2007). Several genes required for TGS, including *DCL3*, *RDR2*, *AGO4* and *NRPD1A*, were identified to be required for the reception of a graft-transmissible silencing signal into naïve, recipient cells in the shoot. Additionally, components of the PTGS pathway, namely *RDR6* (Schwach et al. 2005; Brosnan et al. 2007) and *AGO1* (Brosnan et al. 2007), were also required for reception of silencing in new tissue. While both TGS and PTGS pathways were required for reception of silencing, large amounts of 21 nt siRNAs homologous to the transgene coding sequence accumulated in the scions. This indicated that PTGS was largely responsible for this example of systemic silencing of a transgene, but that the TGS pathway may have been acting upstream to initiate PTGS in the scion (Brosnan et al. 2007).

Although DNA methylation and histone modification of the *GFP* transgene were not detected in systemically silenced scion tissue, there was evidence of transcriptional down-regulation of a *BAR* transgene linked on the GFP T-DNA (Brosnan et al. 2007). This transcriptional down-regulation was dependent on AGO4 and POL V, confirming that the TGS pathway was involved in perception of systemic silencing transmitted from the rootstock (Brosnan et al. 2007). Thus, these data confirmed that reception of the systemic GFP silencing signal in naïve recipient cells in the scion involved cross-talk between the TGS and PTGS pathways (Brosnan et al. 2007; Smith et al. 2007).

Another member of the chromatin modification pathway, JMJ14, a histone H3 Lys4 (H3K4) trimethyl demethylase acts downstream of RDR2 and AGO4, and is probably also required for reception of the silencing signal in naïve cells. However, these findings were based on cell-to-cell based transgene reporter system (Searle et al. 2010) and need to be confirmed in a grafting system.

In the initial grafting study of systemic transgene silencing in *Arabidopsis* (Brosnan et al. 2007), none of the genes involved reception of systemic gene silencing in scion tissue were required for production or transmission of a silencing signal from rootstocks. Furthermore, when a *dcl2-dcl3-dcl4* triple mutant, in which GFP-specific siRNAs were greatly reduced, was used as a rootstock, it was still able to transmit silencing to the shoot (Brosnan et al. 2007). This suggested that siRNA may not be a graft-transmissible silencing signal responsible for systemic spreading of gene silencing. However, when interpreted in the light of subsequent grafting studies in *Arabidopsis* (Dunoyer et al. 2010a; Molnar et al. 2010), the silencing signal transmitted from *dcl2-dcl3-dcl4* triple mutant rootstocks was most likely siRNAs produced by DCL1 (even though its predominant role is in miRNA biogenesis; see Sect. 1.2).

4.2 siRNAs Are Graft-Transmissible Silencing Signals

More recent grafting studies with *Arabidopsis* using highly sensitive deep sequencing to detect siRNAs, have demonstrated that siRNAs are indeed a graft-transmissible gene silencing signal (Dunoyer et al. 2010a; Molnar et al. 2010). These studies have shown that all size classes of siRNAs can move between the root and shoot system, and that mobile 24-nt siRNAs can trigger sequence-specific *de novo* methylation (Dunoyer et al. 2010a; Molnar et al. 2010).

Given shoot-to-root transmission of gene silencing is more efficient than root-to-shoot (Molnar et al. 2010), Molnar and co-workers initially concentrated on the mobility of transgene-specific siRNAs from *GFP* inverted repeat (IR) hairpin scions into non-transgenic rootstocks. They found that *GFP*-specific small RNAs were detectable in the non-transgenic rootstocks, although the siRNAs were 1,000-fold less abundant in the recipient root tissue compared to the transgenic scion (Molnar et al. 2010). They also examined production and mobility of endogenous siRNAs from wild-type transgenic scions to mutant roots defective for siRNA production. In roots of a *dcl2-dcl3-dcl4* triple mutant (unable to produce 22 or 24 nt siRNAs) grafted under wild-type *GFP* IR shoots, there were almost wild-type levels of 24 nt siRNAs in grafted rootstocks (Molnar et al. 2010). This suggested that 24 nt siRNAs were more graft-transmissible than the 21 or 22 nt counterparts.

These authors also used different *Arabidopsis* ecotypes as scions and rootstocks, and DNA polymorphisms between the ecotypes allowed them to detect graft-transmissible movement of siRNAs produced from endogenous loci (Molnar et al. 2010). Using this approach, 795 unique endogenous loci (and over 7,000 endogenous loci when including repetitive ones) were identified that produced mobile siRNAs (Molnar et al. 2010). When a *sde4* mutant (mutated in the gene that encodes Pol IV, which is required for DCL3-dependent 24 nt siRNA biogenesis) was used as the scion, the levels of 24 nt siRNAs transmitted to the rootstock were greatly diminished (Molnar et al. 2010). These results confirmed that 24 nt siRNAs were more mobile than 21 or 22 nt siRNAs.

Most of the mobile siRNA loci did not correspond to any genes. One exception was the *PAI* tryptophan biosynthetic genes of the *Arabidopsis* C24 ecotype. This ecotype has a tail-to-tail inverted repeat (PAI1-PAI4) that substitutes for a single copy of PAI1 in the Columbia (Col) ecotype. As expected, Molnar and co-workers found that 21 to 24 nt *PAI* siRNAs are present in C24 but are undetectable in the Col genotype. When C24 shoots were grafted over Col rootstocks, however, 24 nt PAI siRNAs were detected in the Col roots. Furthermore, when the shoot was a C24 *sde4* mutant incapable of producing 24 nt siRNAs, these PAI siRNAs were absent from the grafted Col roots (Molnar et al. 2010). Although C24 *sde4* shoots accumulate normal or higher levels of 21 nt PAI siRNAs, these were almost undetected in Col roots. Collectively, their results indicate preferential mobility of 24 nt siRNAs (Molnar et al. 2010). The authors suggested that the enhanced mobility of 24 nt siRNAs may be related to Pol IV transcription of the locus, but this was not demonstrated.

Finally, Molnar and co-workers (Molnar et al. 2010) showed that 24 nt siRNAs produced from three out of eight tested endogenous loci could move from wild-type shoots to confer hypermethylation of the loci in *dcl2-dcl3-dcl4* triple mutant grafted roots. Thus, mobile 24 nt siRNAs are able to induce DNA methylation in recipient tissues (Molnar et al. 2010).

Using a similar approach, Dunoyer and co-workers (Dunoyer et al. 2010a) examined siRNAs from two endogenous inverted repeat elements in *Arabidopsis* that produce dsRNA with near-perfect base pairing. These loci were called *IR71* and *IR2039*. The authors (Dunoyer et al. 2010a) used deep sequencing of small RNA from grafted plants to demonstrate that siRNAs from *IR71* are systemically mobile and appear to have a preference to move from the shoot to the root, although movement from root-to-shoot was also detected. Mobile 24 nt *IR71* siRNAs produced in the shoots were also shown to induce hypermethylation of *IR71* sequences in grafted roots (Dunoyer et al. 2010a).

The majority of siRNAs in *Arabidopsis* are 24 nt DCL3-dependent siRNAs derived from transposons and repetitive elements (Kasschau et al. 2007; Zhang et al. 2007; Mosher et al. 2009). As mentioned earlier, these siRNAs play an important role in maintaining the repressive epigenetic marks that prevent transposon activation (Zilberman et al. 2003; Chan et al. 2006; Law and Jacobsen 2010). Given the capacity of 24 nt siRNAs to move long distances and direct DNA methylation in recipient tissues (Dunoyer et al. 2010a; Molnar et al. 2010), this graft-transmissible siRNA transport pathway could contribute significantly to systemic silencing of transposable elements in plants.

4.3 Root-to-Shoot Versus Shoot-to-Root siRNA Signalling

In the case of host plant recovery to viral infection (Wingard 1928), viral siRNAs most likely move from the older infected leaves toward the apical meristem and newly developing leaves to confer resistance to the virus. This is consistent with the hypothesis that siRNAs are transported in the phloem from source tissue to a sink in the growing shoot apex. If this model is correct, then presumably, viral siRNAs would move down to sink tissue in the roots as well. Early work on *Tobacco Mosaic Virus* infectivity in plants, demonstrated that viral particles can move both downward to the roots as well as upward to the shoot (Samuel 1934).

The recent reports using micrografting in *Arabidopsis* have demonstrated movement of endogenous and transgene siRNAs from both shoot-to-root and root-to-shoot (Dunoyer et al. 2010a; Molnar et al. 2010). More efficient transmission of transgene silencing occurs from shoot-to-root (Dunoyer et al. 2010a; Molnar et al. 2010), but nevertheless, root-to-shoot transmission of transgene silencing does occur (Brosnan et al. 2007; Dunoyer et al. 2010a; Molnar et al. 2010), apparently countering the directional flow of the phloem (Turgeon and Wolf 2009). Small (or large) RNA has not been found in xylem sap of plants (Buhtz et al. 2008) and as mentioned earlier, systemic transgene silencing is not able to spread to symplasmically-isolated

guard cells (Wille and Lucas 1984; Voinnet et al. 1998). Collectively, these data suggests that siRNAs move bi-directionally in the phloem, or alternatively, movement of siRNAs from roots to shoots occurs via a separate symplasmic route distinct from the phloem. This critical question in graft-transmissible gene silencing remains to be resolved.

4.4 Intracellular Location of siRNA Silencing Processes and Relevance to Systemic Gene Silencing

Given the nuclear localisation of numerous TGS proteins required for reception of siRNA mediated silencing in naïve recipient cells (Brosnan et al. 2007; Smith et al. 2007; Searle et al. 2010), it seems likely that a major part of the reception process involves transport of silencing signals into the nucleus. In *C. elegans* this function may be at least partially fulfilled by an Argonaute protein, NRDE3 (Guang et al. 2008). In the absence of small RNAs, NRDE3 is localized in the cytoplasm, but in the presence of siRNAs derived from RDR-dependent dsRNA, NRDE3 relocates to the nucleus. Relocation of NRDE3 associated with siRNAs to the nucleus results in the silencing of nuclear-localized, complementary transcripts (Guang et al. 2008). It is not clear if a similar mechanism occurs in plants, but it is likely that some mechanism exists to transport siRNAs, especially 24 nt siRNAs, into the nucleus.

There is a growing body of evidence that 21 nt siRNAs (PTGS) as well as 24 nt siRNAs (TGS) are active in the nucleus. As part of the PTGS pathway, DCL4 produces 21 nt siRNAs and forms a complex with DRB4 that is nuclear-localised (Hiraguri et al. 2005). The *Cauliflower Mosaic Virus* (CaMV) P6 viral suppressor of PTGS must be imported into the nucleus to inhibit the function of DRB4, suggesting nuclear-localised PTGS is important in antiviral defence (Haas et al. 2008). Consistent with PTGS occurring in the nucleus, RDR6 has been reported to localize there (Luo and Chen 2007). RDR6 has also been shown to interact in cytoplasmic SGS3-RDR6 bodies (Kumakura et al. 2009) suggesting that siRNA-based PTGS also occurs in the cytoplasm. In plants, miRNAs and most likely siRNAs formed in the nucleus are exported to the cytoplasm by HASTY, the *Arabidopsis* orthologue of human Exportin 5 (Park et al. 2005).

Thus, trafficking of RNA, siRNA and riboprotein complexes between intracellular compartments is required for some aspects of siRNA-mediated silencing. This is supported by the role of the putative RNA trafficking protein SDE5 that is required by both the *trans*-acting small interfering RNA (ta-siRNA) pathway and for siRNA based PTGS (Jauvion et al. 2010). Furthermore, members of the widely conserved RNA trafficking THO/TREX have been shown to be required for ta-siRNA biogenesis and siRNA-based PTGS (Jauvion et al. 2010). It has been suggested that these proteins are involved in transporting the siRNA precursors within the cell to the Argonaute catalytic centre (Jauvion et al. 2010). Whether this pathway is required for movement of siRNAs or their precursors between neighbouring cells or beyond into other tissues, remains to be seen.

4.5 Systemic siRNA Transport and Trans-generational Epigenetic Inheritance

Transgenerational maintenance of newly-established epigenetic marks is a potential mechanism to allow plants to survive, reproduce and evolve under adverse conditions or a changing environment (Boyko et al. 2010). It is well documented that RNA-mediated epigenetic modification can be induced by both biotic (viral, fungal or bacterial infection) and abiotic stress (temperature extremes, drought, flood, or nutrient deficiency) (Boyko et al. 2010; Lang-Mladek et al. 2010).

As described in Sect. 2.2.1, intercellular movement of siRNAs is crucial for both male and female gametogenesis (Slotkin et al. 2009; Olmedo-Monfil et al. 2010). In view of graft-transmissible movement of siRNAs (Dunoyer et al. 2010a; Molnar et al. 2010), it remains a distinct possibility that siRNAs may move from vegetative tissue into the germline to confer inheritance of somatically-initiated epigenetic states in the subsequent generation. Indeed, transgenerational epigenetic inheritance has been shown to occur after virus-induced silencing and subsequent RNA-directed DNA methylation of a transgene. The new epigenetic marks on the transgene were inherited without transgenerational inheritance of the viral silencing trigger (Jones et al. 2001).

5 Summary

Intercellular and systemic movement of sRNAs are of fundamental importance to many processes in higher plants. miRNAs and ta-siRNAs have been identified that require intercellular movement to execute their function in leaf and root development. Furthermore, cell-to-cell movement of siRNAs is required for restricting the number of female gametophytic precursor cells to one in ovules (Olmedo-Monfil et al. 2010), and for protecting the male gamete genome from transposon activity (Slotkin et al. 2009; Olmedo-Monfil et al. 2010).

Systemic movement of siRNAs is also fundamentally important in higher plants. The evidence is very strong that long-distance signalling involving siRNAs is pivotal to systemic virus resistance. However, questions remain regarding the involvement and biological significance of long-distance sRNA movement in plant development, response to environmental cues and transgenerational inheritance of new epigenetic states of genes.

Another distinct gap in our knowledge is the mechanism of intercellular movement of sRNAs between cells and over longer distances. There are conflicting reports regarding whether double-stranded (Dunoyer et al. 2010a, b) or single-stranded molecules (Yoo et al. 2004) are the predominant intercellular sRNA signal in plants. It is also unresolved whether they move by simple diffusion or by a process that is facilitated by other factors such as proteins or ribonucleo-protein complexes (Yoo et al. 2004). If sRNAs move only by simple diffusion, we would expect

that intercellular movement of individual sRNA could be simply controlled by their abundance and stability. On the other hand, if regulatory proteins or ribonucleoprotein complexes are involved in regulating intercellular movement of sRNA, their discovery and description of their function would greatly enhance our understanding of many aspects of plant biology.

Acknowledgements The authors thank Dr. Uwe Dressel and Dr Iain Searle for comments on the manuscript. The authors are also grateful for research support from the Australian Research Council to BJC, and a Grains Research and Development Corporation scholarship to NRG.

References

Agorio A, Vera P (2007) ARGONAUTE4 is required for resistance to *Pseudomonas syringae* in *Arabidopsis*. Plant Cell 19:3778–3790

Allen E, Howell MD (2010) miRNAs in the biogenesis of *trans*-acting siRNAs in higher plants. Semin Cell Dev Biol 21:798–804

Allen E, Xie Z, Gustafson AM, Carrington JC (2005) microRNA-directed phasing during *trans*-acting siRNA biogenesis in plants. Cell 121:207–221

Alves L Jr, Niemeier S, Hauenschild A, Rehmsmeier M, Merkle T (2009) Comprehensive prediction of novel microRNA targets in *Arabidopsis thaliana*. Nucleic Acids Res 37:4010–4021

Ambros V, Bartel B, Bartel DP, Burge CB, Carrington JC, Chen X, Dreyfuss G, Eddy SR, Griffiths-Jones S, Marshall M, Matzke M, Ruvkun G, Tuschl T (2003) A uniform system for microRNA annotation. RNA 9:277–279

Aufsatz W, Stoiber T, Rakic B, Naumann K (2007) *Arabidopsis* histone deacetylase 6: a green link to RNA silencing. Oncogene 26:5477–5488

Aukerman MJ, Sakai H (2003) Regulation of flowering time and floral organ identity by a MicroRNA and its APETALA2-like target genes. Plant Cell 15:2730–2741

Aung K, Lin SI, Wu CC, Huang YT, Su CL, Chiou TJ (2006) pho2, a phosphate overaccumulator, is caused by a nonsense mutation in a microRNA399 target gene. Plant Physiol 141:1000–1011

Axtell MJ, Jan C, Rajagopalan R, Bartel DP (2006) A two-hit trigger for siRNA biogenesis in plants. Cell 127:565–577

Baker CC, Sieber P, Wellmer F, Meyerowitz EM (2005) The early extra petals1 mutant uncovers a role for microRNA miR164c in regulating petal number in *Arabidopsis*. Curr Biol 15:303–315

Balachandran S, Xiang Y, Schobert C, Thompson GA, Lucas WJ (1997) Phloem sap proteins from *Cucurbita maxima* and *Ricinus communis* have the capacity to traffic cell to cell through plasmodesmata. Proc Natl Acad Sci USA 94:14150–14155

Baumberger N, Baulcombe DC (2005) *Arabidopsis* ARGONAUTE1 is an RNA Slicer that selectively recruits microRNAs and short interfering RNAs. Proc Natl Acad Sci USA 102: 11928–11933

Blevins T, Rajeswaran R, Shivaprasad PV, Beknazariants D, Si-Ammour A, Park HS, Vazquez F, Robertson D, Meins F Jr, Hohn T, Pooggin MM (2006) Four plant dicers mediate viral small RNA biogenesis and DNA virus induced silencing. Nucleic Acids Res 34:6233–6246

Borsani O, Zhu J, Verslues PE, Sunkar R, Zhu JK (2005) Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in *Arabidopsis*. Cell 123:1279–1291

Boutet S, Vazquez F, Liu J, Beclin C, Fagard M, Gratias A, Morel JB, Crete P, Chen X, Vaucheret H (2003) *Arabidopsis* HEN1: a genetic link between endogenous miRNA controlling development and siRNA controlling transgene silencing and virus resistance. Curr Biol 13:843–848

Boyko A, Blevins T, Yao Y, Golubov A, Bilichak A, Ilnytskyy Y, Hollander J, Meins F Jr, Kovalchuk I (2010) Transgenerational adaptation of *Arabidopsis* to stress requires DNA methylation and the function of dicer-Like proteins. PLoS One 5:e9514

Brodersen P, Sakvarelidze-Achard L, Bruun-Rasmussen M, Dunoyer P, Yamamoto YY, Sieburth L, Voinnet O (2008) Widespread translational inhibition by plant miRNAs and siRNAs. Science 320:1185–1190

Brosnan CA, Mitter N, Christie M, Smith NA, Waterhouse PM, Carroll BJ (2007) Nuclear gene silencing directs reception of long-distance mRNA silencing in *Arabidopsis*. Proc Natl Acad Sci USA 104:14741–14746

Buhtz A, Springer F, Chappell L, Baulcombe DC, Kehr J (2008) Identification and characterization of small RNAs from the phloem of *Brassica napus*. Plant J 53:739–749

Buhtz A, Pieritz J, Springer F, Kehr J (2010) Phloem small RNAs, nutrient stress responses, and systemic mobility. BMC Plant Biol 10:64

Cao X, Aufsatz W, Zilberman D, Mette MF, Huang MS, Matzke M, Jacobsen SE (2003) Role of the DRM and CMT3 methyltransferases in RNA-directed DNA methylation. Curr Biol 13:2212–2217

Carlsbecker A, Lee JY, Roberts CJ, Dettmer J, Lehesranta S, Zhou J, Lindgren O, Moreno-Risueno MA, Vaten A, Thitamadee S, Campilho A, Sebastian J, Bowman JL, Helariutta Y, Benfey PN (2010) Cell signalling by microRNA165/6 directs gene dose-dependent root cell fate. Nature 465:316–321

Chan SW, Henderson IR, Zhang X, Shah G, Chien JS, Jacobsen SE (2006) RNAi, DRD1, and histone methylation actively target developmentally important non-CG DNA methylation in *Arabidopsis*. PLoS Genet 2:e83

Chen X (2004) A microRNA as a translational repressor of APETALA2 in *Arabidopsis* flower development. Science 303:2022–2025

Chen HM, Chen LT, Patel K, Li YH, Baulcombe DC, Wu SH (2010) 22-Nucleotide RNAs trigger secondary siRNA biogenesis in plants. Proc Natl Acad Sci USA 107:15269–15274

Chiou TJ (2007) The role of microRNAs in sensing nutrient stress. Plant Cell Environ 30:323–332

Chitwood DH, Guo M, Nogueira FT, Timmermans MC (2007) Establishing leaf polarity: the role of small RNAs and positional signals in the shoot apex. Development 134:813–823

Chitwood DH, Nogueira FT, Howell MD, Montgomery TA, Carrington JC, Timmermans MC (2009) Pattern formation via small RNA mobility. Genes Dev 23:549–554

Cuperus JT, Carbonell A, Fahlgren N, Garcia-Ruiz H, Burke RT, Takeda A, Sullivan CM, Gilbert SD, Montgomery TA, Carrington JC (2010) Unique functionality of 22-nt miRNAs in triggering RDR6-dependent siRNA biogenesis from target transcripts in *Arabidopsis*. Nat Struct Mol Biol 17:997–1003

Daxinger L, Kanno T, Bucher E, van der Winden J, Naumann U, Matzke AJ, Matzke M (2009) A stepwise pathway for biogenesis of 24-nt secondary siRNAs and spreading of DNA methylation. EMBO J 28:48–57

Deleris A, Gallego-Bartolome J, Bao J, Kasschau KD, Carrington JC, Voinnet O (2006) Hierarchical action and inhibition of plant dicer-like proteins in antiviral defense. Science 313:68–71

Ding SW, Voinnet O (2007) Antiviral immunity directed by small RNAs. Cell 130:413–426

Dong Z, Han MH, Fedoroff N (2008) The RNA-binding proteins HYL1 and SE promote accurate *in vitro* processing of pri-miRNA by DCL1. Proc Natl Acad Sci USA 105:9970–9975

Dunoyer P, Lecellier CH, Parizotto EA, Himber C, Voinnet O (2004) Probing the microRNA and small interfering RNA pathways with virus-encoded suppressors of RNA silencing. Plant Cell 16:1235–1250

Dunoyer P, Himber C, Voinnet O (2005) Dicer-like 4 is required for RNA interference and produces the 21-nucleotide small interfering RNA component of the plant cell-to-cell silencing signal. Nat Genet 37:1356–1360

Dunoyer P, Himber C, Voinnet O (2006) Induction, suppression and requirement of RNA silencing pathways in virulent *Agrobacterium tumefaciens* infections. Nat Genet 38:258–263

Dunoyer P, Himber C, Ruiz-Ferrer V, Alioua A, Voinnet O (2007) Intra- and intercellular RNA interference in *Arabidopsis thaliana* requires components of the microRNA and heterochromatic silencing pathways. Nat Genet 39:848–856

Dunoyer P, Brosnan CA, Schott G, Wang Y, Jay F, Alioua A, Himber C, Voinnet O (2010a) An endogenous, systemic RNAi pathway in plants. EMBO J 29:1699–1712

Dunoyer P, Schott G, Himber C, Meyer D, Takeda A, Carrington JC, Voinnet O (2010b) Small RNA duplexes function as mobile silencing signals between plant cells. Science 328:912–916

Eamens AL, Smith NA, Curtin SJ, Wang MB, Waterhouse PM (2009) The *Arabidopsis thaliana* double-stranded RNA binding protein DRB1 directs guide strand selection from microRNA duplexes. RNA 15:2219–2235

Ellendorff U, Fradin EF, de Jonge R, Thomma BP (2009) RNA silencing is required for *Arabidopsis* defence against *Verticillium* wilt disease. J Exp Bot 60:591–602

Fagard M, Vaucheret H (2000) Systemic silencing signal(s). Plant Mol Biol 43:285–293

Fahlgren N, Montgomery TA, Howell MD, Allen E, Dvorak SK, Alexander AL, Carrington JC (2006) Regulation of AUXIN RESPONSE FACTOR3 by TAS3 ta-siRNA affects developmental timing and patterning in *Arabidopsis*. Curr Biol 16:939–944

Fang Y, Spector DL (2007) Identification of nuclear dicing bodies containing proteins for microRNA biogenesis in living *Arabidopsis* plants. Curr Biol 17:818–823

Felippes FF, Ott F, Weigel D (2011) Comparative analysis of non-autonomous effects of tasiRNAs and miRNAs in *Arabidopsis thaliana*. Nucleic Acids Res 39:2880–2889

Floyd SK, Bowman JL (2004) Gene regulation: ancient microRNA target sequences in plants. Nature 428:485–486

Franco-Zorrilla JM, Valli A, Todesco M, Mateos I, Puga MI, Rubio-Somoza I, Leyva A, Weigel D, Garcia JA, Paz-Ares J (2007) Target mimicry provides a new mechanism for regulation of microRNA activity. Nat Genet 39:1033–1037

Gandikota M, Birkenbihl RP, Hohmann S, Cardon GH, Saedler H, Huijser P (2007) The miRNA156/157 recognition element in the 3′ UTR of the *Arabidopsis* SBP box gene SPL3 prevents early flowering by translational inhibition in seedlings. Plant J 49:683–693

Garcia D, Collier SA, Byrne ME, Martienssen RA (2006) Specification of leaf polarity in *Arabidopsis* via the *trans*-acting siRNA pathway. Curr Biol 16:933–938

Garcia-Ruiz H, Takeda A, Chapman EJ, Sullivan CM, Fahlgren N, Brempelis KJ, Carrington JC (2010) *Arabidopsis* RNA-dependent RNA polymerases and dicer-like proteins in antiviral defense and small interfering RNA biogenesis during turnip mosaic virus infection. Plant Cell 22:481–496

Gasciolli V, Mallory AC, Bartel DP, Vaucheret H (2005) Partially redundant functions of *Arabidopsis* dicer-like enzymes and a role for DCL4 in producing *trans*-acting siRNAs. Curr Biol 15:1494–1500

Gazzani S, Lawrenson T, Woodward C, Headon D, Sablowski R (2004) A link between mRNA turnover and RNA interference in *Arabidopsis*. Science 306:1046–1048

German MA, Pillay M, Jeong DH, Hetawal A, Luo S, Janardhanan P, Kannan V, Rymarquis LA, Nobuta K, German R, De Paoli E, Lu C, Schroth G, Meyers BC, Green PJ (2008) Global identification of microRNA-target RNA pairs by parallel analysis of RNA ends. Nat Biotechnol 26:941–946

Gregory BD, O'Malley RC, Lister R, Urich MA, Tonti-Filippini J, Chen H, Millar AH, Ecker JR (2008) A link between RNA metabolism and silencing affecting *Arabidopsis* development. Dev Cell 14:854–866

Guang S, Bochner AF, Pavelec DM, Burkhart KB, Harding S, Lachowiec J, Kennedy S (2008) An Argonaute transports siRNAs from the cytoplasm to the nucleus. Science 321:537–541

Guo HS, Ding SW (2002) A viral protein inhibits the long range signaling activity of the gene silencing signal. EMBO J 21:398–407

Haas G, Azevedo J, Moissiard G, Geldreich A, Himber C, Bureau M, Fukuhara T, Keller M, Voinnet O (2008) Nuclear import of CaMV P6 is required for infection and suppression of the RNA silencing factor DRB4. EMBO J 27:2102–2112

Havecker ER, Wallbridge LM, Hardcastle TJ, Bush MS, Kelly KA, Dunn RM, Schwach F, Doonan JH, Baulcombe DC (2010) The *Arabidopsis* RNA-directed DNA methylation argonautes functionally diverge based on their expression and interaction with target loci. Plant Cell 22: 321–334

Havelda Z, Hornyik C, Crescenzi A, Burgyan J (2003) In situ characterization of *Cymbidium Ringspot Tombusvirus* infection-induced posttranscriptional gene silencing in *Nicotiana benthamiana*. J Virol 77:6082–6086

Henderson IR, Zhang X, Lu C, Johnson L, Meyers BC, Green PJ, Jacobsen SE (2006) Dissecting *Arabidopsis thaliana* DICER function in small RNA processing, gene silencing and DNA methylation patterning. Nat Genet 38:721–725

Himber C, Dunoyer P, Moissiard G, Ritzenthaler C, Voinnet O (2003) Transitivity-dependent and -independent cell-to-cell movement of RNA silencing. EMBO J 22:4523–4533

Hiraguri A, Itoh R, Kondo N, Nomura Y, Aizawa D, Murai Y, Koiwa H, Seki M, Shinozaki K, Fukuhara T (2005) Specific interactions between dicer-like proteins and HYL1/DRB-family dsRNA-binding proteins in *Arabidopsis thaliana*. Plant Mol Biol 57:173–188

Imlau A, Truernit E, Sauer N (1999) Cell-to-cell and long-distance trafficking of the green fluorescent protein in the phloem and symplastic unloading of the protein into sink tissues. Plant Cell 11:309–322

Jagadeeswaran G, Saini A, Sunkar R (2009) Biotic and abiotic stress down-regulate miR398 expression in *Arabidopsis*. Planta 229:1009–1014

Jauvion V, Elmayan T, Vaucheret H (2010) The conserved RNA trafficking proteins HPR1 and TEX1 are involved in the production of endogenous and exogenous small interfering RNA in *Arabidopsis*. Plant Cell 22:2697–2709

Jones L, Ratcliff F, Baulcombe DC (2001) RNA-directed transcriptional gene silencing in plants can be inherited independently of the RNA trigger and requires Met1 for maintenance. Curr Biol 11:747–757

Kantar M, Unver T, Budak H (2010) Regulation of barley miRNAs upon dehydration stress correlated with target gene expression. Funct Integr Genomics 10:493–507

Kasschau KD, Fahlgren N, Chapman EJ, Sullivan CM, Cumbie JS, Givan SA, Carrington JC (2007) Genome-wide profiling and analysis of *Arabidopsis* siRNAs. PLoS Biol 5:e57

Kidner CA, Martienssen RA (2004) Spatially restricted microRNA directs leaf polarity through ARGONAUTE1. Nature 428:81–84

Kim YJ, Zheng B, Yu Y, Won SY, Mo B, Chen X (2011) The role of mediator in small and long noncoding RNA production in *Arabidopsis thaliana*. EMBO J 30:814–822

Kragler F, Monzer J, Xoconostle-Cazares B, Lucas WJ (2000) Peptide antagonists of the plasmodesmal macromolecular trafficking pathway. EMBO J 19:2856–2868

Kumakura N, Takeda A, Fujioka Y, Motose H, Takano R, Watanabe Y (2009) SGS3 and RDR6 interact and colocalize in cytoplasmic SGS3/RDR6-bodies. FEBS Lett 583:1261–1266

Kurihara Y, Watanabe Y (2004) *Arabidopsis* micro-RNA biogenesis through dicer-like 1 protein functions. Proc Natl Acad Sci USA 101:12753–12758

Kurihara Y, Takashi Y, Watanabe Y (2006) The interaction between DCL1 and HYL1 is important for efficient and precise processing of pri-miRNA in plant microRNA biogenesis. RNA 12:206–212

Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T (2001) Identification of novel genes coding for small expressed RNAs. Science 294:853–858

Lakatos L, Csorba T, Pantaleo V, Chapman EJ, Carrington JC, Liu YP, Dolja VV, Calvino LF, Lopez-Moya JJ, Burgyan J (2006) Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. EMBO J 25:2768–2780

Lang-Mladek C, Popova O, Kiok K, Berlinger M, Rakic B, Aufsatz W, Jonak C, Hauser MT, Luschnig C (2010) Transgenerational inheritance and resetting of stress-induced loss of epigenetic gene silencing in *Arabidopsis*. Mol Plant 3:594–602

Lau NC, Lim LP, Weinstein EG, Bartel DP (2001) An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. Science 294:858–862

Law JA, Jacobsen SE (2010) Establishing, maintaining and modifying DNA methylation patterns in plants and animals. Nat Rev Genet 11:204–220

Lee RC, Ambros V (2001) An extensive class of small RNAs in *Caenorhabditis elegans*. Science 294:862–864

Lee RC, Feinbaum RL, Ambros V (1993) The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75:843–854

Lee JY, Yoo BC, Rojas MR, Gomez-Ospina N, Staehelin LA, Lucas WJ (2003) Selective trafficking of non-cell-autonomous proteins mediated by NtNCAPP1. Science 299:392–396

Li J, Yang Z, Yu B, Liu J, Chen X (2005) Methylation protects miRNAs and siRNAs from a 3′-end uridylation activity in *Arabidopsis*. Curr Biol 15:1501–1507

Lister R, O'Malley RC, Tonti-Filippini J, Gregory BD, Berry CC, Millar AH, Ecker JR (2008) Highly integrated single-base resolution maps of the epigenome in *Arabidopsis*. Cell 133:523–536

Lucas WJ, Lee JY (2004) Plasmodesmata as a supracellular control network in plants. Nat Rev Mol Cell Biol 5:712–726

Luo Z, Chen Z (2007) Improperly terminated, unpolyadenylated mRNA of sense transgenes is targeted by RDR6-mediated RNA silencing in *Arabidopsis*. Plant Cell 19:943–958

Mallory AC, Ely L, Smith TH, Marathe R, Anandalakshmi R, Fagard M, Vaucheret H, Pruss G, Bowman L, Vance VB (2001) HC-Pro suppression of transgene silencing eliminates the small RNAs but not transgene methylation or the mobile signal. Plant Cell 13:571–583

Marin E, Jouannet V, Herz A, Lokerse AS, Weijers D, Vaucheret H, Nussaume L, Crespi MD, Maizel A (2010) miR390, *Arabidopsis* TAS3 ta-siRNAs, and their AUXIN RESPONSE FACTOR targets define an autoregulatory network quantitatively regulating lateral root growth. Plant Cell 22:1104–1117

Matzke MA, Birchler JA (2005) RNAi-mediated pathways in the nucleus. Nat Rev Genet 6: 24–35

McConnell JR, Emery J, Eshed Y, Bao N, Bowman J, Barton MK (2001) Role of PHABULOSA and PHAVOLUTA in determining radial patterning in shoots. Nature 411:709–713

Mi S, Cai T, Hu Y, Chen Y, Hodges E, Ni F, Wu L, Li S, Zhou H, Long C, Chen S, Hannon GJ, Qi Y (2008) Sorting of small RNAs into *Arabidopsis* argonaute complexes is directed by the 5′ terminal nucleotide. Cell 133:116–127

Mlotshwa S, Pruss GJ, Peragine A, Endres MW, Li J, Chen X, Poethig RS, Bowman LH, Vance V (2008) DICER-LIKE2 plays a primary role in transitive silencing of transgenes in *Arabidopsis*. PLoS One 3:e1755

Moissiard G, Parizotto EA, Himber C, Voinnet O (2007) Transitivity in *Arabidopsis* can be primed, requires the redundant action of the antiviral dicer-like 4 and dicer-like 2, and is compromised by viral-encoded suppressor proteins. RNA 13:1268–1278

Moldovan D, Spriggs A, Yang J, Pogson BJ, Dennis ES, Wilson IW (2010) Hypoxia-responsive microRNAs and *trans*-acting small interfering RNAs in *Arabidopsis*. J Exp Bot 61:165–177

Molnar A, Schwach F, Studholme DJ, Thuenemann EC, Baulcombe DC (2007) miRNAs control gene expression in the single-cell alga *Chlamydomonas reinhardtii*. Nature 447:1126–1129

Molnar A, Melnyk CW, Bassett A, Hardcastle TJ, Dunn R, Baulcombe DC (2010) Small silencing RNAs in plants are mobile and direct epigenetic modification in recipient cells. Science 328: 872–875

Montgomery TA, Howell MD, Cuperus JT, Li D, Hansen JE, Alexander AL, Chapman EJ, Fahlgren N, Allen E, Carrington JC (2008a) Specificity of ARGONAUTE7-miR390 interaction and dual functionality in TAS3 *trans*-acting siRNA formation. Cell 133:128–141

Montgomery TA, Yoo SJ, Fahlgren N, Gilbert SD, Howell MD, Sullivan CM, Alexander A, Nguyen G, Allen E, Ahn JH, Carrington JC (2008b) AGO1-miR173 complex initiates phased siRNA formation in plants. Proc Natl Acad Sci USA 105:20055–20062

Mosher RA, Melnyk CW, Kelly KA, Dunn RM, Studholme DJ, Baulcombe DC (2009) Uniparental expression of PolIV-dependent siRNAs in developing endosperm of *Arabidopsis*. Nature 460:283–286

Napoli C, Lemieux C, Jorgensen R (1990) Introduction of a chimeric chalcone synthase gene into *Petunia* results in reversible co-suppression of homologous genes in *trans*. Plant Cell 2:279–289

Navarro L, Dunoyer P, Jay F, Arnold B, Dharmasiri N, Estelle M, Voinnet O, Jones JD (2006) A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. Science 312:436–439

Nikovics K, Blein T, Peaucelle A, Ishida T, Morin H, Aida M, Laufs P (2006) The balance between the MIR164A and CUC2 genes controls leaf margin serration in *Arabidopsis*. Plant Cell 18:2929–2945

Nogueira FT, Madi S, Chitwood DH, Juarez MT, Timmermans MC (2007) Two small regulatory RNAs establish opposing fates of a developmental axis. Genes Dev 21:750–755

Nogueira FT, Chitwood DH, Madi S, Ohtsu K, Schnable PS, Scanlon MJ, Timmermans MC (2009) Regulation of small RNA accumulation in the maize shoot apex. PLoS Genet 5:e1000320

Numa H, Kim JM, Matsui A, Kurihara Y, Morosawa T, Ishida J, Mochizuki Y, Kimura H, Shinozaki K, Toyoda T, Seki M, Yoshikawa M, Habu Y (2010) Transduction of RNA-directed DNA methylation signals to repressive histone marks in *Arabidopsis thaliana*. EMBO J 29: 352–362

Olmedo-Monfil V, Duran-Figueroa N, Arteaga-Vazquez M, Demesa-Arevalo E, Autran D, Grimanelli D, Slotkin RK, Martienssen RA, Vielle-Calzada JP (2010) Control of female gamete formation by a small RNA pathway in *Arabidopsis*. Nature 464:628–632

Oparka KJ (2004) Getting the message across: how do plant cells exchange macromolecular complexes? Trends Plant Sci 9:33–41

Palatnik JF, Allen E, Wu X, Schommer C, Schwab R, Carrington JC, Weigel D (2003) Control of leaf morphogenesis by microRNAs. Nature 425:257–263

Palauqui JC, Elmayan T, Pollien JM, Vaucheret H (1997) Systemic acquired silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. EMBO J 16:4738–4745

Parizotto EA, Dunoyer P, Rahm N, Himber C, Voinnet O (2004) *In vivo* investigation of the transcription, processing, endonucleolytic activity, and functional relevance of the spatial distribution of a plant miRNA. Genes Dev 18:2237–2242

Park MY, Wu G, Gonzalez-Sulser A, Vaucheret H, Poethig RS (2005) Nuclear processing and export of microRNAs in *Arabidopsis*. Proc Natl Acad Sci USA 102:3691–3696

Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, Maller B, Hayward DC, Ball EE, Degnan B, Muller P, Spring J, Srinivasan A, Fishman M, Finnerty J, Corbo J, Levine M, Leahy P, Davidson E, Ruvkun G (2000) Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature 408:86–89

Pekker I, Alvarez JP, Eshed Y (2005) Auxin response factors mediate *Arabidopsis* organ asymmetry via modulation of KANADI activity. Plant Cell 17:2899–2910

Peragine A, Yoshikawa M, Wu G, Albrecht HL, Poethig RS (2004) SGS3 and SGS2/SDE1/RDR6 are required for juvenile development and the production of *trans*-acting siRNAs in *Arabidopsis*. Genes Dev 18:2368–2379

Raja P, Sanville BC, Buchmann RC, Bisaro DM (2008) Viral genome methylation as an epigenetic defense against geminiviruses. J Virol 82:8997–9007

Raja P, Wolf JN, Bisaro DM (2010) RNA silencing directed against geminiviruses: post-transcriptional and epigenetic components. Biochim Biophys Acta 1799:337–351

Rajagopalan R, Vaucheret H, Trejo J, Bartel DP (2006) A diverse and evolutionarily fluid set of microRNAs in *Arabidopsis thaliana*. Genes Dev 20:3407–3425

Ramachandran V, Chen X (2008) Degradation of microRNAs by a family of exoribonucleases in *Arabidopsis*. Science 321:1490–1492

Raman S, Greb T, Peaucelle A, Blein T, Laufs P, Theres K (2008) Interplay of miR164. CUP-SHAPED COTYLEDON genes and LATERAL SUPPRESSOR controls axillary meristem formation in *Arabidopsis thaliana*. Plant J 55:65–76

Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G (2000) The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. Nature 403:901–906

Reinhart BJ, Weinstein EG, Rhoades MW, Bartel B, Bartel DP (2002) MicroRNAs in plants. Genes Dev 16:1616–1626

Ruiz-Medrano R, Xoconostle-Cazares B, Kragler F (2004) The plasmodesmatal transport pathway for homeotic proteins, silencing signals and viruses. Curr Opin Plant Biol 7:641–650

Samuel G (1934) The movement of tobacco mosaic virus within the plant. Ann Appl Biol 21: 90–111

Scholthof HB (2005) Plant virus transport: motions of functional equivalence. Trends Plant Sci 10:376–382

Schwab R, Voinnet O (2009) miRNA processing turned upside down. EMBO J 28:3633–3634

Schwab R, Palatnik JF, Riester M, Schommer C, Schmid M, Weigel D (2005) Specific effects of microRNAs on the plant transcriptome. Dev Cell 8:517–527

Schwab R, Ossowski S, Riester M, Warthmann N, Weigel D (2006) Highly specific gene silencing by artificial microRNAs in *Arabidopsis*. Plant Cell 18:1121–1133

Schwab R, Maizel A, Ruiz-Ferrer V, Garcia D, Bayer M, Crespi M, Voinnet O, Martienssen RA (2009) Endogenous ta-siRNAs mediate non-cell autonomous effects on gene regulation in *Arabidopsis thaliana*. PLoS One 4:e5980

Schwach F, Vaistij FE, Jones L, Baulcombe DC (2005) An RNA-dependent RNA polymerase prevents meristem invasion by potato virus X and is required for the activity but not the production of a systemic silencing signal. Plant Physiol 138:1842–1852

Searle IR, Pontes O, Melnyk CW, Smith LM, Baulcombe DC (2010) JMJ14, a JmjC domain protein, is required for RNA silencing and cell-to-cell movement of an RNA silencing signal in *Arabidopsis*. Genes Dev 24:986–991

Sieber P, Wellmer F, Gheyselinck J, Riechmann JL, Meyerowitz EM (2007) Redundancy and specialization among plant microRNAs: role of the MIR164 family in developmental robustness. Development 134:1051–1060

Slotkin RK, Vaughn M, Borges F, Tanurdzic M, Becker JD, Feijo JA, Martienssen RA (2009) Epigenetic reprogramming and small RNA silencing of transposable elements in pollen. Cell 136:461–472

Smith LM, Pontes O, Searle I, Yelina N, Yousafzai FK, Herr AJ, Pikaard CS, Baulcombe DC (2007) An SNF2 protein associated with nuclear RNA silencing and the spread of a silencing signal between cells in *Arabidopsis*. Plant Cell 19:1507–1521

Stadler R, Wright KM, Lauterbach C, Amon G, Gahrtz M, Feuerstein A, Oparka KJ, Sauer N (2005) Expression of GFP-fusions in *Arabidopsis* companion cells reveals non-specific protein trafficking into sieve elements and identifies a novel post-phloem domain in roots. Plant J 41:319–331

Sussex I (1954) Experiments on the cause of dorsiventrality in leaves. Nature 174:352–353

Takeda A, Iwasaki S, Watanabe T, Utsumi M, Watanabe Y (2008) The mechanism selecting the guide strand from small RNA duplexes is different among argonaute proteins. Plant Cell Physiol 49:493–500

Taunton J, Hassig CA, Schreiber SL (1996) A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. Science 272:408–411

Tian L, Chen ZJ (2001) Blocking histone deacetylation in *Arabidopsis* induces pleiotropic effects on plant gene regulation and development. Proc Natl Acad Sci USA 98:200–205

Tretter EM, Alvarez JP, Eshed Y, Bowman JL (2008) Activity range of *Arabidopsis* small RNAs derived from different biogenesis pathways. Plant Physiol 147:58–62

Turgeon R, Wolf S (2009) Phloem transport: cellular pathways and molecular trafficking. Annu Rev Plant Biol 60:207–221

Valoczi A, Varallyay E, Kauppinen S, Burgyan J, Havelda Z (2006) Spatio-temporal accumulation of microRNAs is highly coordinated in developing plant tissues. Plant J 47:140–151

van der Krol AR, Mur LA, Beld M, Mol JN, Stuitje AR (1990) Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell 2:291–299

Vaucheret H (2008) Plant ARGONAUTES. Trends Plant Sci 13:350–358

Vaucheret H, Vazquez F, Crete P, Bartel DP (2004) The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. Genes Dev 18:1187–1197

Voinnet O (2005) Induction and suppression of RNA silencing: insights from viral infections. Nat Rev Genet 6:206–220
Voinnet O, Vain P, Angell S, Baulcombe DC (1998) Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. Cell 95:177–187
Walbot V, Evans MMS (2003) Unique features of the plant life cycle and their consequences. Nat Rev Genet 4:369–379
Wierzbicki AT, Haag JR, Pikaard CS (2008) Noncoding transcription by RNA polymerase Pol IVb/Pol V mediates transcriptional silencing of overlapping and adjacent genes. Cell 135: 635–648
Wierzbicki AT, Ream TS, Haag JR, Pikaard CS (2009) RNA polymerase V transcription guides ARGONAUTE4 to chromatin. Nat Genet 41:630–634
Wightman B, Ha I, Ruvkun G (1993) Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell 75:855–862
Wille AC, Lucas WJ (1984) Ultrastructural and histochemical studies on guard cells. Planta 160:129–142
Williams L, Carles CC, Osmont KS, Fletcher JC (2005) A database analysis method identifies an endogenous *trans*-acting short-interfering RNA that targets the *Arabidopsis* ARF2, ARF3, and ARF4 genes. Proc Natl Acad Sci USA 102:9703–9708
Wingard SA (1928) Hosts and symptoms of ring spot, a virus disease of plants. J Agric Res 37:127–154
Wolf S, Deom CM, Beachy RN, Lucas WJ (1989) Movement protein of tobacco mosaic virus modifies plasmodesmatal size exclusion limit. Science 246:377–379
Wong CE, Zhao YT, Wang XJ, Croft L, Wang ZH, Haerizadeh F, Mattick JS, Singh MB, Carroll BJ, Bhalla PL (2011) MicroRNAs in the shoot apical meristem of soybean. J Exp Bot. doi:10.1093/jxb/erq437
Xie Z, Qi X (2008) Diverse small RNA-directed silencing pathways in plants. Biochim Biophys Acta 1779:720–724
Xie Z, Johansen LK, Gustafson AM, Kasschau KD, Lellis AD, Zilberman D, Jacobsen SE, Carrington JC (2004) Genetic and functional diversification of small RNA pathways in plants. PLoS Biol 2:E104
Xie Z, Allen E, Wilken A, Carrington JC (2005) DICER-LIKE 4 functions in *trans*-acting small interfering RNA biogenesis and vegetative phase change in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 102:12984–12989
Yang L, Liu Z, Lu F, Dong A, Huang H (2006a) SERRATE is a novel nuclear regulator in primary microRNA processing in *Arabidopsis*. Plant J 47:841–850
Yang Z, Ebright YW, Yu B, Chen X (2006b) HEN1 recognizes 21–24 nt small RNA duplexes and deposits a methyl group onto the 2′ OH of the 3′ terminal nucleotide. Nucleic Acids Res 34: 667–675
Yoo BC, Kragler F, Varkonyi-Gasic E, Haywood V, Archer-Evans S, Lee YM, Lough TJ, Lucas WJ (2004) A systemic small RNA signaling system in plants. Plant Cell 16:1979–2000
Yu B, Yang Z, Li J, Minakhina S, Yang M, Padgett RW, Steward R, Chen X (2005) Methylation as a crucial step in plant microRNA biogenesis. Science 307:932–935
Yu B, Bi L, Zheng B, Ji L, Chevalier D, Agarwal M, Ramachandran V, Li W, Lagrange T, Walker JC, Chen X (2008) The FHA domain proteins DAWDLE in *Arabidopsis* and SNIP1 in humans act in small RNA biogenesis. Proc Natl Acad Sci USA 105:10073–10078
Zhang B, Pan X, Cannon CH, Cobb GP, Anderson TA (2006a) Conservation and divergence of plant microRNA genes. Plant J 46:243–259
Zhang B, Pan X, Cobb GP, Anderson TA (2006b) Plant microRNA: a small regulatory molecule with big impact. Dev Biol 289:3–16
Zhang X, Henderson IR, Lu C, Green PJ, Jacobsen SE (2007) Role of RNA polymerase IV in plant small RNA metabolism. Proc Natl Acad Sci USA 104:4536–4541
Zhao B, Liang R, Ge L, Li W, Xiao H, Lin H, Ruan K, Jin Y (2007a) Identification of drought-induced microRNAs in rice. Biochem Biophys Res Commun 354:585–590

Zhao T, Li G, Mi S, Li S, Hannon GJ, Wang XJ, Qi Y (2007b) A complex system of small RNAs in the unicellular green alga *Chlamydomonas reinhardtii*. Genes Dev 21:1190–1203

Zhao B, Ge L, Liang R, Li W, Ruan K, Lin H, Jin Y (2009) Members of miR-169 family are induced by high salinity and transiently inhibit the NF-YA transcription factor. BMC Mol Biol 10:29

Zheng X, Zhu J, Kapoor A, Zhu JK (2007) Role of *Arabidopsis* AGO6 in siRNA accumulation. DNA methylation and transcriptional gene silencing. EMBO J 26:1691–1701

Zhou L, Liu Y, Liu Z, Kong D, Duan M, Luo L (2010) Genome-wide identification and analysis of drought-responsive microRNAs in *Oryza sativa*. J Exp Bot 61:4157–4168

Zilberman D, Cao X, Jacobsen SE (2003) ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation. Science 299:716–719

Zilberman D, Cao X, Johansen LK, Xie Z, Carrington JC, Jacobsen SE (2004) Role of *Arabidopsis* ARGONAUTE4 in RNA-directed DNA methylation triggered by inverted repeats. Curr Biol 14:1214–1220

Chapter 6
Long-Distance Signaling by Small RNAs

Julia Kehr

1 Introduction

Systemic signaling through the phloem has long ago been discovered and it is meanwhile well established that it plays important roles in regulating many essential physiological processes in development, nutrient allocation, and stress and defense responses. The possible involvement of macromolecules like peptides, proteins or RNAs in long-range signaling has long been overlooked, since they were regarded as being too large to act as information transmitters. However, it is meanwhile well accepted that ribonucleic acids (RNAs) are among the important information transmitters in all eukaryotes (Müller et al. 2007). It was shown that directional, asymmetric mRNA transport plays essential roles in, for example, mating-time switching in budding yeast (Müller et al. 2007) or segmentation in Drosophila (Irion and St Johnston 2007). Also in plants mRNA transport was found to be a crucial component of cellular communication that coordinates developmental processes (Haywood et al. 2002; Ding et al. 2003; Kim and Pai 2009). It is, for example, known that several plant transcription factors (TFs) have the ability to move between cells and are involved in meristem initiation and maintenance (Lucas et al. 1995; Sessions et al. 2000). Moreover, mRNAs can move to even distant plant organs (Ruiz-Medrano et al. 1999). Generally, sieve elements (SEs) seem to provide an ideal environment for RNA transport, since a lack of RNase activity seems to be a common feature of phloem samples in different species (Sasaki et al. 1998; Doering-Saad et al. 2002; Gaupels et al. 2008; Zhang et al. 2009). In contrast to xylem sap where RNAs could not be detected (Buhtz et al. 2008), RNA has been

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F. Kragler and M. Hülskamp (eds.), *Short and Long Distance Signaling*,
Advances in Plant Biology 3, DOI 10.1007/978-1-4419-1532-0_6,

found in phloem sap more than 40 years ago (Kollmann et al. 1970; Ziegler and Kluge 1962). For a long time, however, their unexpected presence was attributed to contaminations introduced by the sampling procedure. Meanwhile several studies have convincingly demonstrated that phloem samples obtained by different sampling strategies from distinct, unrelated plant species indeed contain a high number RNAs, and it has been shown that they represent authentic components of the phloem stream. Phloem samples seem to contain a set of diverse RNAs, including viral RNAs, cellular mRNAs, tRNAs, and a fraction of small RNAs (smRNAs) in the size range of 20–26 nucleotides (nt) (reviewed in: Kragler 2010; Kehr and Buhtz 2008). The occurrence of smRNAs has meanwhile been established in different cucurbits, white lupin, castor bean, yucca (Yoo et al. 2004), oilseed rape (Buhtz et al. 2008), and recently apple (Varkonyi-Gasic et al. 2010).

During the last decade small, non-coding RNAs have emerged as important transcriptional and post-transcriptional regulators of gene expression. The two major classes of small regulatory RNAs, short interfering (si) RNAs and micro (mi) RNAs, seem to have many similarities, but can be distinguished by differences in their modes of biosynthesis and action. Both types of small RNAs interact with the multi-component RNA-induced silencing complex (RISC) and regulate gene expression by directing the cleavage of their target messenger RNAs (mRNAs). siRNAs medicate a process called post-transcriptional gene silencing (PTGS), an innate, widespread plant defense mechanism against foreign DNA, transgenes, the activity of transposable elements, and viruses (Waterhouse et al. 2001), by restricting their expression. In contrast, miRNAs affect the expression of endogenous genes, and are involved in regulating a number of different processes ranging from development to nutrient allocation.

This chapter will summarize the current knowledge about how phloem samples can be obtained, how smRNAs can be analyzed, and which smRNAs have been detected in the phloem. In addition, the evidence on whether smRNAs can really be transported over long-distances in living plants and what functional important such a transport could have will be discussed.

2 Phloem Sampling Methods

One of the major difficulties with studying phloem long-distance signal transduction in plants is to access the content of phloem transport tubes without causing significant artifacts or contaminations. Several different methods to obtain phloem samples from woody and herbaceous plants have been developed that mainly differ in the amount of sap that can be obtained and in the degree of purity and dilution of the samples. The feasibility of the individual sampling strategies is largely dependent on the plant species in the focus of interest. Some characteristics of the different sampling methods are summarized in Table 6.1. Despite of the fact that all the different methods can introduce (different kinds of) artifacts and can therefore result in quite unequal types of samples, all are normally summarized under the terms “phloem sap” or “phloem sample”, masking their different origins and characteristics.

Table 6.1 Summary of phloem sampling methods

Method	Sample volume	Plant species	Advantages	Disadvantages	Reference(s)
Spontaneous exudation	High µl–ml	Trees, cucurbits, brassica, ricinus, lupin, yucca	Easy, large volumes	Significant injury, prone to contamination (cut non-phloem cells, adjacent cells, structural components), turgor drop	Bose 1947, Milburn 1970, Yoo et al. 2004, Giavalisco et al. 2006
Chelator-facilitated exudation	ml, diluted	Can be adapted to almost all species	Easy	Significant injury, contamination (cut non-phloem cells, adjacent cells, structural components, apoplast), sample dilution, turgor drop	King and Zeevaart 1974
Insect stylectomy	<nl–µl	Most plants, sample volume is species-and insect dependent	Minimal invasive, not prone to significant contamination, no turgor drop	Extremely low volumes, difficult, requires special equipment, contamination by injected aphid proteins, artificial sinks, feeding site is dependent on insect-plant combination	Kennedy and Mittler 1953
Microcapillaries	µl	Only tested for a few species (potato, maize)	Intermediate invasive	Small volumes, difficult, requires special equipment, possible contamination by cells destroyed by the capillary, turgor drop	Brandt et al. 1999, Raps et al. 2001

One of the most traditional methods is the collection of phloem exudate from incisions into the bark of woody plants (Bose 1947; Zimmermann and Ziegler 1975). A similar approach can be used in a few herbaceous species, for example *Ricinus*, *Cucurbitaceae*, *Yucca*, and *Brassica*, where phloem sap exudes upon vascular damage after excising whole organs (Milburn 1970; Alosi et al. 1988; Yoo et al. 2004) or making small punctures (Walz et al. 2004; Giavalisco et al. 2006; Buhtz et al. 2008). All exudation methods allow a relatively easy collection of samples enriched in SE contents. Sample volumes can reach the milliliter scale and in some cases, for example in palms or yucca, even liters of phloem exudate can be collected (Turgeon and Wolf 2009). However, all exudation techniques are prone to contamination by non-phloem cells injured during sampling. Indeed, it was shown that phloem exudates from incisions can be contaminated by cell contents of injured tissues surrounding the phloem (Ruiz-Medrano et al. 1999). Moreover, the quite extensive injury of SEs results in a rapid drop in turgor pressure, what might cause a significant dilution of samples, or an influx of components from associated cells, e.g. companion cells. In addition, also a release of structural components normally attached to the cytoplasmic lining of SEs has been observed (Knoblauch and van Bel 1998; Turgeon and Wolf 2009; Dinant et al. 2010). To minimize contaminations, the first exuding droplets are normally discarded. However, to demonstrate sample purity additional evidence is required. This can, for example, be obtained by measuring sugar composition. Here, a high content of sucrose and the absence of reducing sugars like glucose and fructose are regarded as being indicative of SE content. Another option is the detection of mRNAs or proteins that should not occur in SE as e.g. photosynthesis-related compounds. In this case their absence indicates acceptable low levels of contamination (Doering-Saad et al. 2006; Giavalisco et al. 2006; Buhtz et al. 2008). More recent studies also compared phloem samples to the surrounding tissue damaged upon sampling to demonstrate that phloem sap specifically accumulates certain components and others are absent, what indicates sample specificity (Buhtz et al. 2010; Varkonyi-Gasic et al. 2010).

In most plant species, however, spontaneous exudation cannot be employed, since phloem bleeding ceases shortly after wounding to avoid assimilate loss. This process is probably initiated by a rapid release of calcium that induces accumulation of specific phloem proteins what leads to a reversible blockage of sieve pores that can be followed by the likewise calcium-dependent irreversible occlusion by the polysaccharide callose (Kehr 2006). Plants that do not exude spontaneously can be anyhow sampled by chelator-facilitated exudation (King and Zeevaart 1974; Marentes and Grusak 1998; Hoffmann-Benning et al. 2002). Here, petioles are cut under water and subsequently placed in a collection solution containing the chelator ethylene diamine tetraacetic acid (EDTA) or similar substances that inhibit the wounding response by binding calcium. This method allows the easy collection of phloem exudate, but samples are dilute and not useful for compound quantification. In addition, caused by the long collection times, contamination by destroyed cells and apoplastic fluid, and degradation of phloem components are severe concerns. Therefore, the EDTA exudation technique can be useful to answer certain, focused questions, but is not suitable for the reliable identification or quantification of phloem constituents (Table 6.1).

In contrast to all exudation methods that are rather invasive, phloem-feeding insects such as aphids or planthoppers are able to precisely target individual SEs as their feeding source. Researchers are taking advantage of this skill by cutting their feeding instruments, called stylets, to sample SE content in a minimally-invasive manner. The so-called insect stylectomy was first described by Kennedy and Mittler (1953) and is regarded to yield pure phloem sap and to minimize sampling artifacts (Fisher et al. 1992). It is based on cutting the stylets that are inserted into a sieve tube while the insects are ingesting phloem sap. In early experiments with big aphids feeding on woody plants, the stylets were severed by razor-blades or microscissors. In herbaceous plants where the sucking insects are significantly smaller, excision of the stylets is normally achieved by high-frequency or laser microcautery (Fisher and Frame 1984; Dinant et al. 2010). Because of the small diameter of the stylets and their slow and directed mode of insertion into the phloem, they do not induce a significant loss of turgor pressure that could cause artifacts. However, also aphids can cause unnatural alterations of phloem composition by acting as additional sinks and, moreover, by injecting insect proteins that probably modulate plant defense responses (Kehr 2006). In addition, the technique is difficult to perform and can result in extremely small sample volumes. The amount of sap obtainable by this method from a single plant of the model species *Arabidopsis thaliana*, for example, was reported to be restricted to a few nanolitres (Zhu et al. 2005) what not only requires to take measures against evaporation but obviously limits the analyses that can be performed. An alternative technique partially imitating insect feeding is sampling by microcapillaries under the microscope after SEs have been visualized by the phloem-mobile fluorochrome carboxyfluorescein diacetate (Brandt et al. 1999; Raps et al. 2001). However, this technique was as yet seldom used, probably because it comparably difficult to apply and also yields only small sample amounts (Table 6.1).

Most data on phloem sap macromolecules in general and RNAs in particular have been obtained from samples collected by exudation-based methods from different plant species including different cucurbits and oilseed rape (Yoo et al. 2004; Omid et al. 2007; Buhtz et al. 2008; Pant et al. 2009; Zhang et al. 2009; Buhtz et al. 2010). Here, RNAs of all size ranges from large transcripts to small tRNAs and even si and miRNAs have been found. However, also aphid stylectomy has more recently been used to characterize proteins and to detect specific mRNAs in the SEs of, for example, rice and wheat (Sasaki et al. 1998; Doering-Saad et al. 2002; Gaupels et al. 2008), and one study also demonstrated its applicability to phloem small RNA studies in apple (Varkonyi-Gasic et al. 2010).

3 Small RNAs in Phloem Samples

A number of recent studies concentrated on the discovery of RNAs in phloem sap and of their functional characterization as long-range information transmitters. Most studies focused on the analysis of large RNAs and besides ribosomal RNAs a number of transcripts were detected. Some of these mRNAs could be shown to be

phloem-mobile and to induce phenotypic changes in their target tissue, reviewed in: (Kehr and Buhtz 2008; Kehr 2009; Kragler 2010). In addition, one publication described the analysis of RNAs in the size range of 30–90 bases. In this study tRNAs, ribosomal RNAs and spliceosomal RNAs, whose functions are so far not fully understood, could be identified (Zhang et al. 2009).

This chapter will, however, focus on the results obtained for smRNAs in the size range of 20–26 nt, what includes miRNAs and siRNAs that have both emerged as important post-transcriptional regulators of gene expression during the last years. The smRNA complement of phloem sap of different species was analyzed by different attempts. First evidence that specific smRNAs accumulate in phloem tissue came from in situ hybridization experiments that, however, did not allow a clear localization in sieve elements (Valoczi et al. 2006; Juarez et al. 2004). That smRNAs are indeed present in the phloem transport stream was indicated by radioactive labeling of total RNA populations isolated from phloem samples and subsequent RNA gel blot analysis (Yoo et al. 2004). Cloning and conventional sequencing of smRNAs from pumpkin phloem sap led to a limited number of smRNA identifications. Nowadays, also microarrays containing all known plant miRNAs and quantitative miRNA PCR platforms have been used to study phloem miRNA levels under different growth conditions (Pant et al. 2009; Buhtz et al. 2010). However, different high-throughput sequencing technologies have allowed the most comprehensive overview of smRNAs present in sieve elements of undisturbed plants and their responses to different environmental stresses (Buhtz et al. 2008; Pant et al. 2009). These experiments resulted in several thousand unique sequences, and led to the identification of mature miRNAs from many different families and some potential new siRNAs, but also left a confusing number of sequences with unclear assignment and function (Buhtz et al. 2008). Interestingly, many of these unknown sequences accumulated specifically in phloem samples as compared to leaves or roots (Buhtz et al. 2008, 2010), suggesting a specific phloem function. In addition to mature smRNAs, some complementary strands could also be detected (Buhtz et al. 2008; Pant et al. 2009), suggesting that phloem smRNAs might occur double stranded.

3.1 Phloem siRNAs

siRNAs are 21- and 24 nt long molecules that mainly act in defense of the genome. They are processed by DICER-like enzymes from long and perfectly complementary double-stranded RNA molecules that can stem from viruses, erroneous RNAs or endogenous natural antisense transcripts. These double-stranded molecules are themselves produced by the activity of RNA-dependent RNA polymerases. The resultant mature siRNAs are subsequently incorporated into the RISC, where they guide the cleavage of complementary target mRNAs resulting in their down-regulation (Sunkar and Zhu 2007).

It has been known for a long time that gene silencing can spread over long distances via the phloem (Voinnet and Baulcombe 1997). However, for a long time it remained unknown, which kind of RNAs could be transmitted and in addition to siRNAs themselves aberrant larger RNAs were proposed as potential mobile silencing signals (Mlotshwa et al. 2002). Only recently, siRNAs have been detected in phloem samples from different species including cucurbits and lupin. Yoo et al. (2004) sequenced hundreds of small phloem RNAs from pumpkin exudate samples and identified a number of 21 nt short RNAs complementary to cucurbit transposon-like (*TnL*) genes. Also both sense and antisense strands of a transgene-derived *Squash mosaic virus coat protein* (*CP SqMV*) siRNA accumulated in phloem sap of spontaneously silenced pumpkin plants. Similarly, phloem samples from *Cucumber yellow closterovirus* (CuYV)-infected pumpkin plants accumulated high levels of viral-derived siRNAs. Interestingly, siRNA originating from a transgene or a virus infection could be detected only in the phloem of silenced, but not unsilenced plants (Yoo et al. 2004). Another recent study found preferentially 22-nt smRNAs derived from *Hop stunt viroid* (vd) infection in cucumber phloem exudates (Martinez et al. 2010). All these results strongly suggested that siRNAs themselves could be the phloem-mobile molecules, and the results of two new studies recently reinforced this assumption (Dunoyer et al. 2010b; Molnar et al. 2010). These studies demonstrated that siRNAs derived from inverted repeat (IR) loci like *IR71* or phosphoribosylanthranilate isomerase (*PAI*) are contained in the phloem stream (Dunoyer et al. 2010a; Molnar et al. 2010). It is believed that endogenous as well as transgene-derived siRNAs are graft transmittable, probably with bias toward the 24 nt species (Molnar et al. 2010). Other data suggest that the silencing signals are probably duplexes of mature siRNAs (Dunoyer et al. 2010b). However, only very few siRNAs, namely *CP SqMV*, *IR71* and *PAI*, have actually been identified to be present in phloem samples or translocated over graft unions, respectively, so far (Yoo et al. 2004; Dunoyer et al. 2010b; Molnar et al. 2010). However, recent results obtained from grafting studies between Arabidopsis wildtype and multiple dicer-like (*dcl*) mutants showed that siRNAs of all sizes from thousands of genetic loci can be translocated long distance, suggesting that phloem translocatability is a general feature of siRNAs (Molnar et al. 2010).

3.2 Phloem miRNAs

miRNAs are mostly 21 nt long molecules that mainly guide growth and development by down-regulating endogenous target mRNAs. miRNAs are derived from longer single-stranded precursor molecules that exhibit a characteristic hairpin structure. These precursors are processed to duplexes of mature miRNAs and miRNA*s that, in contrast to siRNAs, do not show perfect sequence complementarity. This step is catalyzed by the enzyme Dicer-like 1, in plants presumably in the nucleus (Jones-Rhoades et al. 2006). After export to the cytosol, a methyl group is

post-synthetically added by the methyltransferase HEN1-1. miRNA* strands are subsequently degraded, whereas mature miRNAs enter the RISC to down-regulate the expression of target genes (Jones-Rhoades et al. 2006). In plants, mature miRNAs generally show near perfect sequence complementarity to their target mRNAs (Rhoades et al. 2002). The majority of the known plant miRNA target transcripts are transcription factors, and therefore miRNAs play important roles in coordinating different aspects of developmental pathways (Rhoades et al. 2002). However, some miRNAs have recently been shown to be also specifically involved in the regulation of the responses to e.g. nutrient deficiency (Chiou 2007; Meng et al. 2010).

Earlier in situ hybridization studies indicated the presence of several specific miRNAs, for example miR166, in vascular tissue of different plant species, although these studies did not allow a clear attribution to sieve elements (Valoczi et al. 2006; Juarez et al. 2004). Meanwhile a large number of miRNAs has been identified by classical cloning as well as by high-throughput sequencing, microarray hybridization and PCR-based approaches in phloem samples from different plant species including pumpkin (Yoo et al. 2004), lupin (Atkins and Smith 2007) and oilseed rape (Buhtz et al. 2008; Pant et al. 2009; Buhtz et al. 2010), where phloem sap exudes after wounding. In oilseed rape grown under control conditions, for example, members of 37 miRNA families could be detected in phloem samples (Buhtz et al. 2008; Pant et al. 2009; Buhtz et al. 2010). More recently, also phloem sap obtained from apple by the less-invasive aphid stylet technique has been analyzed by PCR regarding their miRNA levels (Varkonyi-Gasic et al. 2010).

These studies confirmed phloem localization of all miRNAs that was earlier suggested by the in situ hybridization studies. Interestingly, in addition to mature miRNAs also complementary miRNA* strands were found in phloem sap samples of oilseed rape (Buhtz et al. 2008; Pant et al. 2009), indicating that miRNAs might travel long-distance as double strands.

A large overlap between the miRNA complements of phloem samples obtained from diverse plants with different sampling methods was observed (Yoo et al. 2004; Buhtz et al. 2008, 2010; Varkonyi-Gasic et al. 2010). For instance miR156, miR159, miR166 and miR167 were detectable in all species analyzed and therefore seem to belong to a group of conserved phloem miRNAs (Table 6.2). Many of the miRNAs detected in phloem samples are known to regulate development of vegetative and floral organs, for instance miR159, miR160, miR164, miR166, miR167, and miR319 (Mallory et al. 2004). Some of the phloem miRNAs (e.g. miR395, miR397, miR398, miR399, miR408, miR2111) are also known to be nutrient starvation-responsive in non-phloem tissues. These nutrient-responsive miRNAs are expected to be only found when plants are nutrient-deprived and have therefore not been detected in all available studies. However, four of them have been shown to accumulate to exceptionally high levels in phloem sap upon the respective nutrient deficiencies (Buhtz et al. 2008). These were the sulfate deficiency-dependent miR395, the low copper-induced miR398, or miR399 and miR2111 that accumulated under phosphate starvation (Pant et al. 2009; Buhtz et al. 2008, 2010). Interestingly, also miRNAs responsive to other abiotic and biotic stress conditions in non-phloem tissues were found in phloem sap. Significant examples are the well phloem-represented miR169 family, whose members have been described to respond to abcsisic acid (ABA),

Table 6.2 miRNAs detected in phloem sap with different methods

miRNA	Species	Sampling method	Identification method	Observations	References[a]
miR156	*B.napus, C.maxima, M. domestica*	Exudation, aphid stylectomy	Sequencing, RNA gel blot, array, PCR		1, 2, 4, 5, 6
miR157	*B.napus*	Exudation	Sequencing, array		2, 5
miR158	*B.napus*	Exudation	array, PCR	Responsive to -Fe	5
miR159	*B.napus, C.maxima, M. domestica*	Exudation, aphid stylectomy	Sequencing, RNA gel blot, array, PCR		1, 2, 4, 5, 6
miR160	*B.napus, C.maxima, M. domestica*	Exudation, aphid stylectomy	Sequencing, RNA gel blot, array, PCR		2, 5, 6
miR162	*B.napus, C.maxima, M. domestica*	Exudation, aphid stylectomy	Sequencing, RNA gel blot, array, PCR		2, 5, 6
miR164	*B.napus, C.maxima*	Exudation	Sequencing, RNA gel blot, array		2, 5
miR166	*B.napus, L. albus*	Exudation	Sequencing, RNA gel blot, array		2, 7
miR167	*B.napus, C.maxima, M. domestica*	Exudation, aphid stylectomy	Sequencing, RNA gel blot, array, PCR		1, 2, 4, 5, 6
miR168	*B.napus, C.maxima*	Exudation	Sequencing, RNA gel blot, array		2, 5
miR169	*B.napus, C.maxima, M. domestica*	Exudation, aphid stylectomy	Sequencing, RNA gel blot, array	Responsive to +P, +N (4)	2, 4, 5, 6
miR172	*B.napus, C.maxima, M. domestica*	Exudation, aphid stylectomy	Sequencing, RNA gel blot, array, PCR	Phloem-mobile (8)	2, 3, 5, 6
miR319	*B.napus*	Exudation	Sequencing, RNA gel blot, array		2, 5
miR390	*B.napus, C.maxima, M. domestica*	Exudation, aphid stylectomy	Sequencing, RNA gel blot, array, PCR		2, 5, 6
miR393	*C. maxima, B.napus, M.domestica*	Exudation, aphid stylectomy	RNA gel blot, PCR		5, 6
miR395	*B.napus*	Exudation	Sequencing, RNA gel blot, array	Responsive to -S (5), phloem-mobile (5)	2, 5
miR397	*C.maxima*	Exudation	PCR, array	Responsive to -Cu, +S (5)	
miR398	*B.napus, C. maxima, M.domestica*	Exudation, aphid stylectomy	RNA gel blot, array, PCR	Responsive to -Cu, +Fe (5)	2, 5, 6
miR399	*B.napus, C.maxima*	Exudation	Sequencing, RNA gel blot, array, PCR	Responsive to -P (2,3,4,5), responsive to -Cu,+Fe (5), phloem-mobile (3,5,7)	2, 3, 4, 5
miR400	*B.napus*	Exudation	Sequencing, RNA gel blot		2
miR403	*B.napus*	Exudation	Array		5
miR408	*B.napus*	Exudation	RNA gel blot, PCR	Responsive to -Cu, +Fe (5)	2, 5
827	*B.napus*	Exudation	Sequencing	Responsive to -P (4), absent in (5)	4
miR2111	*B.napus*	Exudation	Sequencing, array	Responsive to -Cu, +Fe (5), -P (4)	2, 4

[a] (1) Yoo et al. 2004, (2) Buhtz et al. 2008, (3) Pant et al. 2008, (4) Pant et al. 2009, (5) Buhtz et al. 2010, (6) Varkonyi-Gasic et al. 2010, (7) Atkins and Smith 2007

heat and drought (Li et al. 2008; Zhao et al. 2009; Li et al. 2010) or miR393 that has been shown to be induced by nitrate (Vidal et al. 2010) and bacterial elicitors (Navarro et al. 2006). However, a stress-induced accumulation or reduction of these miRNAs in the phloem as a response to stress has as yet not been described.

More detailed analyses of spatial miRNA distribution showed that many miRNAs accumulated differentially when phloem samples were compared to extracts from other organs. miR169, for example, accumulated to much higher levels in phloem exudates than in other tissues (Buhtz et al. 2008, 2010; Varkonyi-Gasic et al. 2010). Another example is miR168 that was not detected in apple phloem samples obtained by aphid stylectomy, but was enriched in pumpkin and *Brassica* phloem exudate samples as compared to other tissues (Buhtz et al. 2010; Varkonyi-Gasic et al. 2010). This suggests that this miRNA is either artificially enriched by the exudate sampling method, or that its phloem accumulation is specific to herbaceous plants or strongly dependent on the growth conditions applied.

Other miRNAs, like miR172 or miR400, were clearly underrepresented in phloem sap samples, and miR171 was never detectable in phloem sap of any plant species analyzed, providing evidence that this miRNA acts in a strictly cell-autonomous manner as other results had earlier suggested (Parizotto et al. 2004).

4 Evidence for Phloem Mobility and Potential Functions of Translocated smRNAs

To demonstrate a signaling function of a specific phloem smRNA it is essential to not only show its presence in phloem samples, but to also provide evidence that it can indeed move over long-distance *in vivo*. It can be assumed that not all phloem smRNAs are mobile, as has been shown for larger phloem transcripts (Omid et al. 2007). However, most siRNAs are thought to be phloem translocatable and also mobility of specific miRNAs could meanwhile be demonstrated, while others seem to act only locally restricted. Evidence for phloem mobility of smRNAs and possible functions of this translocation will be discussed in the following paragraphs.

4.1 siRNA Mobility and Possible Functions

It has long been accepted that gene silencing can propagate between cells and it has also been known for a long time that gene silencing can spread over long distances via the phloem (Voinnet and Baulcombe 1997). In the case of gene silencing, mobility can often easily be followed if the expression of visible marker proteins like, for example, the Green Fluorescent Protein (GFP) or glucoronidase (GUS) is silenced. Early experiments using grafting experiments on transgenic plants demonstrated

that signals from silenced rootstocks are transmitted to non-silenced scions expressing the respective transgene, leading to post-transcriptional gene silencing (PTGS) in scions (Palauqui et al. 1997). Using a different system with GUS expression as the visual marker, it could also be shown that silencing signals can be translocated from silenced hosts to parasitic plants that form vascular connections with their hosts (Tomilov et al. 2008). The observed silencing signals were always sequence-specific. Therefore, since the process of gene silencing was first discovered, RNAs were thought to be involved in the transmission of the silencing trigger (Hamilton and Baulcombe 1999; Lucas et al. 2001; Tournier et al. 2006). However, until recently it remained unknown which kind of RNAs could transmit the gene silencing signal. In addition to siRNAs longer, aberrant RNA molecules were proposed as the potential phloem-mobile signals (Mlotshwa et al. 2002). Using GFP silencing as the marker Himber et al. (2003) could show that silencing can spread short-distance through a limited number of 10–15 cells, probably in the form of 21-nt siRNAs. In addition, it could also be demonstrated in the same study that a long-range cell-to-cell movement process exists that is supposed to occur after signal amplification and probably involves larger 24–26-nt siRNAs. Only recently, GFP siRNAs were bombarded into *Arabidopsis* leaves that constitutively expressed GFP to investigate whether siRNAs can move from cell to cell. Here, silencing spread from the bombarded cells into the surrounding tissue. In parallel, fluorescently-labeled siRNAs moved into neighboring cells, what demonstrated that indeed siRNAs and not intermediates generated from the target transcript were mobile (Dunoyer et al. 2010b). Moreover, bombardment near veins led to long-distance movement of siRNAs. Long-distance translocation of siRNAs was recently further corroborated using grafting experiments between wildtype and a T-DNA insertion mutant at the siRNA generating inverted repeat *IR71* that cannot produce certain siRNAs. When a wildtype scion was grafted onto a mutant rootstock, siRNAs from the entire inverted repeat could be detected in roots, demonstrating that endogenous siRNAs or siRNA precursor transcripts can move from shoot to root (Dunoyer et al. 2010a). A recent publication by Molnar et al. (2010) also performed analysis of *Arabidopsis* grafts to show that small RNAs can be translocated over long distances. Grafting wildtype scions onto *dcl2 dcl3 dcl4* triple mutant rootstocks combined with deep sequencing demonstrated that siRNAs from thousands genetic loci could move to distal tissues. Mobile siRNAs were, moreover, functionally indistinguishable from locally synthesized siRNAs in that they caused DNA methylation in target cells (Dunoyer et al. 2010a; Molnar et al. 2010).

The spontaneous propagation of silencing between cells and organs following virus infection or transgene expression most likely functions as a systemic protection mechanism against foreign DNA. In addition, because endogenous mobile siRNAs can induce epigenetic effects in their target tissues, it has been suggested that they might induce silencing of transposons in meristems. Moreover their transport into developing reproductive tissue could be required to pass epigenetic modifications to the next generation to increase the stress adaptation potential of the offspring (Molnar et al. 2010).

4.2 miRNA Mobility and Possible Functions

In contrast to siRNAs it was long believed that miRNAs in general can only act locally restricted (Dunoyer et al. 2007). This was suggested by the close correlation of the patterns of miRNA transcription and activity (Parizotto et al. 2004), the spatial restriction of miRNA gene expression (Alvarez et al. 2006), and the limited localization of mature miRNAs (Valoczi et al. 2006) observed for a number of specific miRNAs that were subjected to detailed analysis. However, it was on the other hand suggested by grafting studies that the miR399-mediated response to low phosphate contains a systemic component (Bari et al. 2006). Moreover, the identification of a large number of miRNAs in phloem samples obtained by different sampling methods from various plant species and even more the phloem accumulation of specific miRNAs under stress suggested a potential role of miRNAs as long-distance signaling molecules (Buhtz et al. 2008, 2010; Pant et al. 2009). As for all other phloem sap components, however, the detection of a miRNA in the phloem alone is no proof that this miRNA can be phloem translocated *in vivo*. Recently, grafting experiments were performed that showed that the -S-responsive miR395 and the miP-responsive miR399 can indeed move through graft unions. Interestingly, both miRNAs have been earlier detected in phloem sap of *Brassica napus* and the nutrient deficiency-responsive accumulation was specifically pronounced in phloem samples when compared to other tissues (Buhtz et al. 2008, 2010). miR395 controls sulfate assimilation and allocation by regulating the expression of ATP sulfurylases and the low-affinity sulfate transporter SULTR2.1 (Jones-Rhoades and Bartel 2004). miR395 accumulation under sulfate limitation suppresses the expression of its targets, which leads to sulfate accumulation in shoots (Liang et al. 2010). miR399 participates in the maintenance of phosphate homeostasis by regulating the expression of PHO2, an ubiquitin-conjugating E2 enzyme (Aung et al. 2006; Bari et al. 2006). Similar to miR395, accumulation of miR399 under phosphate limitation suppresses PHO2 expression what leads to an accumulation of more phosphate in shoots. In particular, reciprocal graftings between wildtype and miR399-overexpressing Arabidopsis or tobacco plants have shown that miR399 can be translocated from scions across the graft union to wildtype rootstocks under full nutrition to down-regulate target mRNA expression (Lin et al. 2008; Pant et al. 2008). The same has been demonstrated for miR395 and miR399 using grafts between wildtype Arabidopsis and the miRNA processing mutant *hen1-1* that is impaired in mature miRNA generation (Park et al. 2002). When such grafts were grown under the respective starvation conditions, the deficiency-responsive miRNAs could be detected in the mutant rootstocks, while they were absent in non-grafted mutant roots. In parallel with this shoot-to-root translocation, the levels of target mRNAs were significantly decreased in roots (Buhtz et al. 2010). In the same study, it has also been shown that not all miRNAs are phloem translocatable, as for example miR171 movement could not be observed. In the grafting systems employed, miR395 and miR399 could both only move from shoot-to-root and not in the opposite direction, what is most likely caused by the small seedlings used for grafting, where roots represent the only efficient sink.

However, it cannot be excluded that translocation of these miRNAs might occur in other directions when plants have different source-sink relations.

The physiological importance of long-distance movement of the two nutrient-responsive miRNAs is still a matter of debate, since both miRNAs can be synthesized in roots themselves (Aung et al. 2006; Kawashima et al. 2009). However, as already mentioned it was demonstrated that the translocation of miR395 and miR399 in both grafting systems led to a significant down-regulation of their respective target genes (Pant et al. 2008; Buhtz et al. 2010), providing the first direct evidence that they could indeed act as long-distance signals during the regulation of nutrient homeostasis. During nutrient stress, for example, the translocation of miRNAs from shoots to roots could lead to the degradation of target mRNAs in roots, and thus to an initiation of nutrient starvation responses, at a time-point where roots are not yet stressed. It was therefore proposed that this transport could be essential to coordinate deficiency responses and nutrient allocation between organs in times of low availability (Buhtz et al. 2010).

But not only nutrient-dependent miRNAs can move through the phloem, but also developmental decisions seem to be controlled via this pathway. Actually evidence for the systemic control of development by long-distance messengers has been existent since the first experiments on the onset of flowering and its photoperiodic control that led to the postulation of a ubiquitous "florigen" that can determine flowering time (Chailakhyan 1936). Similar to floral induction, early grafting experiments also showed that potato plants grown under inductive short-day photoperiods can synthesize a tuber-inducing stimulus, the so called "tuberigen", in leaves that can be translocated over long-distance to stolons to induce tuberization. Martin et al. (2009) reported that miR172 mainly accumulated in the phloem of potato stems and showed that grafting of miR172 overexpressor scions onto wildtype plants increased tuber formation in the rootstock, suggesting that miR172 or a miR172-regulated long-distance signal might be phloem-mobile. Further evidence that miR172 itself might be the information transmitter came from grafting experiments using *Nicotiana benthamiana* (Kasai et al. 2010). Based on these results it has been proposed that miRNA translocation over long-distance through the phloem may play a role in coordinating developmental processes. In addition to miR172 also a larger phloem transcript, *BEL5* mRNA, has been shown to be phloem-mobile and to participate in long-distance signaling influencing the time point of tuberization (Lee and Cui 2009). How these different RNA-based long-distance signaling pathways interact is currently unknown.

In situ hybridization experiments also demonstrated that miR166, a miRNA regulating the development of lateral organs by targeting *HD-ZIP III* mRNAs, is expressed during leaf development and accumulates within phloem cells of maize. This accumulation led to the suggestion that also miR166 might represent a mobile signal that can move through the phloem (Juarez et al. 2004), although its transport could as yet not be validated. Indeed, for most of the smRNAs found in phloem sap up to now no transport studies have been performed and future experiments will be required to confirm or disprove their long-range mobility and thus their possible involvement in systemic signaling pathways.

4.3 Mechanisms of Phloem Translocation

To date, there is convincing evidence that phloem transport of RNAs of plant, virus and viroid origin in general is dependent on specific proteins that are able to bind RNAs and function as chaperones by assisting folding of their macromolecular structure. This step is probably essential to allow an import from the adjacent companion cells into sieve elements through the connecting plasmodesmata (Lough and Lucas 2006). Accordingly, an endogenous pumpkin RNA-binding phloem protein, the viral movement protein analog CmPP16, has been shown to be capable of mediating the movement of its own and foreign mRNAs between cells by increasing the size exclusion limit of plasmodesmata (Xoconostle-Cazares et al. 1999). Also one of the major cucurbit phloem proteins, PP2, can apparently bind RNA and could be involved in its graft transmission (Gomez and Pallas 2004), but binds RNA in a rather non-sequence-specific manner (Gomez et al. 2005). In addition, phloem sap from different species contains many more proteins with RNA-binding capacity (Barnes et al. 2004; Gomez et al. 2005; Giavalisco et al. 2006) that could potentially be involved in RNA translocation. Recently, mRNA movement in pumpkin was characterized in more detail and a model for the movement of mRNA in the form of ribonucleoprotein complexes within pumpkin phloem has been proposed (Ham et al. 2009). It is believed that the RNA-binding phloem protein RBP50 is an essential mediator of RNA translocation in pumpkin. It is shown that RBP50 could be translocated from source to sink tissues in heterografting experiments between pumpkin and cucumber, and at least six mRNAs were bound to the RBP50 complex, including the known phloem-mobile transcripts *CmPP16* and *GAIP* (Xoconostle-Cazares et al. 1999; Haywood et al. 2005). The binding of RBP50 seemed to be sequence-specific, using a mechanism supposedly involving interaction with the polypyrimidine tract binding (PTB) motifs RBP50 contains (Ham et al. 2009). According to the model proposed from the results of this study, RBP50 aggregates to homooligomers that complex with mRNAs and several interacting proteins to form phloem-mobile ribonucleoprotein complexes (Ham et al. 2009).

Although smRNA transport in particular is far from being understood, there is general consent that cell-to-cell and phloem long-distance translocation of siRNAs and miRNAs most likely also depend on chaperone proteins that fold and stabilize them. Microinjection experiments demonstrated that pure diffusion through plasmodesmata is not sufficient to move even these small RNAs between cells. However, a small RNA-binding protein was identified that was able to facilitate miRNA movement between cells (Yoo et al. 2004). This protein, called CmPSRP1, had specificity for binding and trafficking single-stranded molecules and preferentially bound small RNA molecules in the size range of 20–40 nt (Yoo et al. 2004), making it a good candidate as a phloem smRNA transport mediator. However, so far no homologues or functional analogues have been found in species other than pumpkin.

siRNAs have recently been shown to travel as double-stranded molecules (Dunoyer et al. 2010b). Likewise, miRNAs occur in the phloem as sense and the complementary star* strands (Buhtz et al. 2008). This suggests that miRNAs are also translocated as duplexes and that components of the phloem transport mechanism

for siRNAs and miRNAs might be at least partially conserved (Kehr and Buhtz 2008). The seemingly contradicting results from earlier studies using strand-specific RNase digests that indicated that phloem miRNAs occur single stranded (Yoo et al. 2004; Buhtz et al. 2008) can probably be explained by a strand separation induced during RNA isolation.

Once inside sieve elements, according to current knowledge all RNAs seem to travel following the metabolic flux from source to sink. It could, for example, be shown that systemic spread of silencing could be altered by manipulating source-sink relationships (Tournier et al. 2006) and there is so far no conclusive example contradicting this passive mode of RNA movement. Target selectivity could, however, be achieved by selective unloading into sink tissues, as has been earlier demonstrated to exist for different types of RNAs (Foster et al. 2002).

5 Conclusions and Perspectives

A growing number of smRNAs is identified in the phloem of different plant species, what suggests that they could constitute a common class of long-range signaling molecules. Moreover, recent results have shown that a wide range of siRNAs and some, but not all, miRNAs are indeed phloem-mobile. While phloem siRNAs spread gene silencing, miRNAs are probably involved in integrating nutrient deficiency responses between organs and transmitting developmental decisions. However, for most smRNAs found in phloem samples, phloem-mobility has as yet not been confirmed and future studies using grafting experiments and other techniques will be required to demonstrate which smRNAs are phloem-mobile *in vivo* and which are not. Furthermore, it will be essential to unravel the molecular mechanism of smRNA long-distance transport in model and semi-model species, and to define the exact physiological functions of smRNA translocation. To this end precise time-course experiments monitoring precursors, mature smRNA molecules, and target mRNA levels at the sites of smRNA synthesis and in target tissues, or even cell types, will be indispensible. In addition, future research should focus on interactions between different long-distance signaling pathways to understand how the phloem can integrate nutrient allocation with defense responses, growth, and development of the different organs.

Acknowledgments The author is grateful for the financial support from the Spanish Ministry of Science and Innovation (MICINN, grant BIO2008-03432 and the I3 program).

References

Alosi MC, Melroy DL, Park RB (1988) The regulation of gelation of phloem exudate from *Cucurbita* fruit by dilution, glutathione, and glutathione reductase. Plant Physiol 86:1089–1094

Alvarez JP, Pekker I, Goldshmidt A, Blum E, Amsellem Z, Eshed Y (2006) Endogenous and synthetic microRNAs stimulate simultaneous, efficient, and localized regulation of multiple targets in diverse species. Plant Cell 18:1134–1151

Atkins CA, Smith PMC (2007) Translocation in legumes: assimilates, nutrients, and signaling molecules. Plant Physiol 144:550–561

Aung K, Lin S, Wu C, Huang Y, Su C, Chiou T (2006) Pho2, a phosphate overaccumulator, is caused by a nonsense mutation in a microRNA399 target gene. Plant Physiol 141:1000–1011

Bari R, Pant BD, Stitt M, Scheible W (2006) PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. Plant Physiol 141:988–999

Barnes A, Bale J, Constantinidou C, Ashton P, Jones A, Pritchard J (2004) Determining protein identity from sieve element sap in *Ricinus communis* L. by quadrupole time of flight (Q-TOF) mass spectrometry. J Exp Bot 55:1473–1481

Bose JC (1947) Plants and their autographs. Longman Green & Co, London, p 154

Brandt S, Kehr J, Walz C, Imlau A, Willmitzer L, Fisahn J (1999) A rapid method for detection of plant gene transcripts from single epidermal, mesophyll and campanion cells of intact leaves. Plant J 20:245–250

Buhtz A, Springer F, Chappell L, Baulcombe DC, Kehr J (2008) Identification and characterization of small RNAs from the phloem of *Brassica napus*. Plant J 53:739–749

Buhtz A, Pieritz J, Springer F, Kehr J (2010) Phloem small RNAs, nutrient stress responses, and systemic mobility. BMC Plant Biol 10:64

Chailakhyan MK (1936) New facts in support of the hormonal theory of plant development. C. R. (Doklady) Acad. Sci URSS 13:79–83

Chiou TJ (2007) The role of microRNAs in sensing nutrient stress. Plant Cell Environ 30: 323–332

Dinant S, Bonnemain JL, Girousse C, Kehr J (2010) Phloem sap intricacy and interplay with aphid feeding. C R Biol 333:504–515

Ding B, Itaya A, Qi Y (2003) Symplasmic protein and RNA traffic: regulatory points and regulatory factors. Curr Opin Plant Biol 6:596–602

Doering-Saad C, Newbury HJ, Bale JS, Pritchard J (2002) Use of aphid stylectomy and RT-PCR for the detection of transporter mRNAs in sieve elements. J Exp Bot 53:631–637

Doering-Saad C, Newbury HJ, Couldridge CE, Bale JS, Pritchard J (2006) A phloem-enriched cDNA library from *Ricinus*: insights into phloem function. J Exp Bot 57:3183–3193

Dunoyer P, Himber C, Ruiz-Ferrer V, Alioua A, Voinnet O (2007) Intra- and intercellular RNA interference in *Arabidopsis thaliana* requires components of the microRNA and heterochromatic silencing pathways. Nat Genet 39:848–856

Dunoyer P, Brosnan CA, Schott G, Wang Y, Jay F, Alioua A, Himber C, Voinnet O (2010a) An endogenous, systemic RNAi pathway in plants. EMBO J 29:1699–1712

Dunoyer P, Schott G, Himber C, Meyer D, Takeda A, Carrington JC, Voinnet O (2010b) Small RNA duplexes function as mobile silencing signals between plant cells. Science 328:912–916

Fisher DB, Frame JM (1984) A guide to the use of the exuding-stylet technique in phloem physiology. Planta 161:385–393

Fisher DB, Wu Y, Ku MSB (1992) Turnover of soluble proteins in the wheat sieve tube. Plant Physiol 100:1433–1441

Foster TM, Lough TJ, Emerson SJ, Lee RH, Bowman JL, Forster RLS, Lucas WJ (2002) A surveillance system regulates selective entry of RNA into the shoot apex. Plant Cell 14:1497–1508

Gaupels F, Buhtz A, Knauer T, Deshmukh S, Waller F, van Bel AJE (2008) Adaptation of aphid stylectomy for analyses of proteins and mRNAs in barley phloem sap. J Exp Bot 59: 3297–3306

Giavalisco P, Kapitza K, Kolasa A, Buhtz A, Kehr J (2006) Towards the proteome of *Brassica napus* phloem sap. Proteomics 6:896–909

Gomez G, Pallas V (2004) A long-distance translocatable phloem protein from cucumber forms a ribonucleoprotein complex *in vivo* with *Hop Stunt Viroid* RNA. J Virol 78:10104–10110

Gomez G, Torres H, Pallas V (2005) Identification of translocatable RNA-binding phloem proteins from melon, potential components of the long-distance RNA transport system. Plant J 41: 107–116

Ham BK, Brandom JL, Xoconostle-Cazares B, Ringgold V, Lough TJ, Lucas WJ (2009) A polypyrimidine tract binding protein, pumpkin RBP50, forms the basis of a phloem-mobile ribonucleoprotein complex. Plant Cell 21:197–215

Hamilton AJ, Baulcombe DC (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 286:950–952
Haywood V, Kragler F, Lucas WJ (2002) Plasmodesmata: pathways for protein and ribonucleoprotein signaling. Plant Cell 14:S303–S325
Haywood V, Yu T, Huang N, Lucas WJ (2005) Phloem long-distance trafficking of *GIBBERELLIC ACID-INSENSITIVE* RNA regulates leaf development. Plant J 42:49–68
Himber C, Dunoyer P, Moissiard G, Ritzenthaler C, Voinnet O (2003) Transitivity-dependent and -independent cell-to-cell movement of RNA silencing. EMBO J 22:4523–4533
Hoffmann-Benning S, Gage DA, McIntosh L, Kende H, Zeevaart JAD (2002) Comparison of peptides in the phloem sap of flowering and non-flowering Perilla and lupine plants using microbore HPLC followed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. Planta 216:140–147
Irion U, St JD (2007) *bicoid* RNA localization requires specific binding of an endosomal sorting complex. Nature 445:554–558
Jones-Rhoades MW, Bartel DP (2004) Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. Mol Cell 14:787–799
Jones-Rhoades MW, Bartel DP, Bartel B (2006) MicroRNAs and their regulatory roles in plants. Annu Rev Plant Biol 57:19–53
Juarez MT, Kui JS, Thomas J, Heller BA, Timmermans MCP (2004) microRNA-mediated repression of rolled leaf1 specifies maize leaf polarity. Nature 428:84–88
Kasai A, Kanehira A, Harada T (2010) miR172 can move long distances in *Nicotiana benthamiana*. Open Plant Sci J 4:1–6
Kawashima CG, Yoshimoto N, Maruyama-Nakashita A, Tsuchiya YN, Saito K, Takahashi H, Dalmay T (2009) Sulphur starvation induces the expression of microRNA-395 and one of its target genes but in different cell types. Plant J 57:313–321
Kehr J (2006) Phloem sap proteins: their identities and potential roles in the interaction between plants and phloem-feeding insects. J Exp Bot 57:767–774
Kehr J (2009) Long-distance transport of macromolecules through the phloem. F1000 Biol Rep **1**, 31
Kehr J, Buhtz A (2008) Long distance transport and movement of RNA through the phloem. J Exp Bot 59:85–92
Kennedy JS, Mittler TE (1953) A method of obtaining phloem sap via the mouth parts of aphids. Nature 171:528
Kim I, Pai H (2009) Mobile macromolecules in plant development. J Plant Biol 52:186–192
King RW, Zeevaart JAD (1974) Enhancement of phloem exudation from cut petioles by chelating agents. Plant Physiol 53:96–103
Knoblauch M, van Bel AJE (1998) Sieve tubes in action. Plant Cell 10:35–50
Kollmann R, Dörr I, Kleinig H (1970) Protein filaments – structural components of the phloem exudate. Planta 95:86–94
Kragler F (2010) RNA in the phloem: a crisis or a return on investment? Plant Sci 178:99–104
Lee J, Cui W (2009) Non-cell autonomous RNA trafficking and long-distance signaling. J Plant Biol 52:10–18
Li WX, Oono Y, Zhu J, He XJ, Wu JM, Iida K, Lu XY, Cui X, Jin H, Zhu JK (2008) The Arabidopsis NFYA5 transcription factor is regulated transcriptionally and posttranscriptionally to promote drought resistance. Plant Cell 20:2238–2251
Li Y, Fu Y, Ji L, Wu C, Zheng C (2010) Characterization and expression analysis of the Arabidopsis mir169 family. Plant Sci 178:271–280
Liang G, Yang F, Yu D (2010) MicroRNA395 mediates regulation of sulfate accumulation and allocation in *Arabidopsis thaliana*. Plant J 62:1046–1057
Lin SI, Chiang SF, Lin WY, Chen JW, Tseng CY, Wu PC, Chiou TJ (2008) Regulatory network of microRNA399 and PHO2 by systemic signaling. Plant Physiol 147:732–746
Lough TJ, Lucas WJ (2006) Integrative plant biology: role of phloem long-distance macromolecular trafficking. Annu Rev Plant Biol 57:203–232
Lucas WJ, Bouché-Pillon S, Jackson DP, Nguyen L, Baker L, Ding B, Hake S (1995) Selective trafficking of KNOTTED1 homeodomain protein and its mRNA through plasmodesmata. Science 270:1980–1983

Lucas WJ, Yoo B, Kragler F (2001) RNA as a long-distance information macromolecule in plants. Nat Rev Mol Cell Biol 2:849–857

Mallory AC, Dugas DV, Bartel DP, Bartel B (2004) MicroRNA regulation of NAC-domain targets is required for proper formation and separation of adjacent embryonic, vegetative, and floral organs. Curr Biol 14:1035–1046

Marentes E, Grusak MA (1998) Mass determination of low-molecular-weight proteins in phloem sap using matrix-assisted laser desorption/ionization time of flight mass spectrometry. J Exp Bot 49:903–911

Martin A, Adam H, Díaz-Mendoza M, Zurczak M, González-Schain ND, Suárez-López P (2009) Graft-transmissible induction of potato tuberization by the microRNA *miR172*. Development 136:2873–2881

Martinez G, Donaire L, Llave C, Pallas V, Gomez G (2010) High-throughput sequencing of Hop stunt viroid-derived small RNAs from cucumber leaves and phloem. Mol Plant Pathol 11: 347–359

Meng Y, Ma X, Chen D, Wu P, Chen M (2010) MicroRNA-mediated signaling involved in plant root development. Biochem Biophys Res Commun 393:345–349

Milburn JA (1970) Phloem exudation from castor bean: induction by massage. Planta 95: 272–276

Mlotshwa S, Voinnet O, Mette MF, Matzke M, Vaucheret H, Ding SW, Pruss G, Vance VB (2002) RNA silencing and the mobile silencing signal. Plant Cell 14:S289–301

Molnar A, Melnyk CW, Bassett A, Hardcastle TJ, Dunn R, Baulcombe DC (2010) Small silencing RNAs in plants are mobile and direct epigenetic modification in recipient cells. Science 328: 872–875

Müller M, Heuck A, Niessing D (2007) Directional mRNA transport in eukaryotes: lessons from yeast. Cell Mol Life Sci 64:171–180

Navarro L, Dunoyer P, Jay F, Arnold B, Dharmasiri N, Estelle M, Voinnet O, Jones JDG (2006) A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. Science 312:436–439

Omid A, Keilin T, Glass A, Leshkowitz D, Wolf S (2007) Characterization of phloem-sap transcription profile in melon plants. J Exp Bot 58:3645–3656

Palauqui J, Elmayan T, Pollien J, Vaucheret H (1997) Systemic acquired silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. EMBO J 16:4738–4745

Pant BD, Buhtz A, Kehr J, Scheible WR (2008) MicroRNA399 is a long-distance signal for the regulation of plant phosphate homeostasis. Plant J 53:731–738

Pant BD, Musialak-Lange M, Nuc P, May P, Buhtz A, Kehr J, Walther D, Scheible W (2009) Identification of nutrient-responsive Arabidopsis and rapeseed microRNAs by comprehensive real-time polymerase chain reaction profiling and small RNA sequencing. Plant Physiol 150: 1541–1555

Parizotto EA, Dunoyer P, Rahm N, Himber C, Voinnet O (2004) *In vivo* investigation of the transcription, processing, endonucleolytic activity, and functional relevance of the spatial distribution of a plant miRNA. Genes Dev 18:2237–2242

Park W, Li J, Song R, Messing J, Chen X (2002) CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in *Arabidopsis thaliana*. Curr Biol 12: 1484–1495

Raps A, Kehr J, Gugerli P, Moar WJ, Bigler F, Hilbeck A (2001) Immunological analysis of phloem sap of Bacillus thuringiensis corn and of the nontarget herbivore Rhopalosiphum padi (Homoptera: Aphididae) for the presence of Cry1Ab. Mol Ecol 10:525–533

Rhoades MW, Reinhart BJ, Lim LP, Burge CB, Bartel B, Bartel DP (2002) Prediction of plant microRNA targets. Cell 110:513–520

Ruiz-Medrano R, Xoconostle-Cázares B, Lucas WJ (1999) Phloem long-distance transport of *CmNACP* mRNA: implications for supracellular regulation in plants. Development 126:4405–4419

Sasaki T, Chino M, Hayashi H, Fujiwara T (1998) Detection of several mRNA species in rice phloem sap. Plant Cell Physiol 39:895–897

Sessions A, Yanofsky MF, Weigel D (2000) Cell-cell signaling and movement by the floral transcription factors LEAFY and APETALA1. Science 289:779–781

Sunkar R, Zhu JK (2007) Micro RNAs and short-interfering RNAs in plants. J Integr Plant Biol 49:817–826

Tomilov AA, Tomilova NB, Wroblewski T, Michelmore R, Yoder JI (2008) Trans-specific gene silencing between host and parasitic plants. Plant J 56:389–397

Tournier B, Tabler M, Kalantidis K (2006) Phloem flow strongly influences the systemic spread of silencing in GFP *Nicotiana benthamiana* plants. Plant J 47:383–394

Turgeon R, Wolf S (2009) Phloem transport: cellular pathways and molecular trafficking. Annu Rev Plant Biol 60:207–221

Valoczi A, Varallyay E, Kauppinen S, Burgyan J, Havelda Z (2006) Spatio-temporal accumulation of microRNAs is highly coordinated in developing plant tissues. Plant J 47:140–151

Varkonyi-Gasic E, Gould N, Sandanayaka M, Sutherland P, MacDiarmid RM (2010) Characterisation of microRNAs from apple (*Malus domestica* 'Royal Gala') vascular tissue and phloem sap. BMC Plant Biol 10:159

Vidal EA, Araus V, Lu C, Parry G, Green PJ, Coruzzi GM, Gutierrez RA (2010) Nitrate-responsive miR393/AFB3 regulatory module controls root system architecture in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 107:4477–4482

Voinnet O, Baulcombe DC (1997) Systemic signalling in gene silencing. Nature 389:553–553

Walz C, Giavalisco P, Schad M, Juenger M, Klose J, Kehr J (2004) Proteomics of curcurbit phloem exudate reveals a network of defence proteins. Phytochemistry 65:1795–1804

Waterhouse PM, Wang M, Lough T (2001) Gene silencing as an adaptive defence against viruses. Nature 411:834–842

Xoconostle-Cazares B, Xiang Y, Ruiz-Medrano R, Wang HL, Monzer J, Yoo BC, McFarland KC, Franceschi VR, Lucas WJ (1999) Plant paralog to viral movement protein that potentiates transport of mRNA into the phloem. Science 283:94–98

Yoo B, Kragler F, Varkonyi-Gasic E, Haywood V, Archer-Evans S, Lee YM, Lough TJ, Lucas WJ (2004) A systemic small RNA signaling system in plants. Plant Cell 16:1979–2000

Zhang S, Sun L, Kragler F (2009) The phloem-delivered RNA pool contains small noncoding RNAs and interferes with translation. Plant Physiol 150:378–387

Zhao B, Ge L, Liang R, Li W, Ruan K, Lin H, Jin Y (2009) Members of miR-169 family are induced by high salinity and transiently inhibit the NF-YA transcription factor. BMC Mol Biol 10:29

Zhu X, Shaw PN, Pritchard J, Newbury J, Hunt EJ, Barrett DA (2005) Amino acid analysis by micellar electrokinetic chromatography with laser-induced fluorescence detection: application to nanolitre-volume biological samples from *Arabidopsis thaliana* and *Myzus persicae*. Electrophoresis 26:911–919

Ziegler H, Kluge M (1962) Die Nucleinsäuren und ihre Bausteine im Siebröhrensaft von *Robinia pseudoacacia* L. Planta 58:144–153

Zimmermann MH, Ziegler H (1975) List of sugars and sugar alcohols in sieve-tube exudates. In: Zimmermann MH, Milburn JA (eds) Encyclopedia of plant physiology, vol 1. Springer, Berlin, pp 480–503

Chapter 7
Signaling and Phloem-Mobile Transcripts

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1 Introduction

The systemic allocation of non-coding RNA (ncRNA) and messenger RNA (mRNA) molecules regulating physiological and developmental decisions in distant organs is an emerging concept in plant biology. The presence of an intercellular communication system coincides with cellular specialization and higher plants evolved a specialized vascular phloem system to transfer signals from leaves to apical tissues. The angiosperm phloem, besides its well-known role in transport of fixed carbon from photosynthetic to heterotrophic tissues also delivers hormones and amino acids. It is well established that such small phloem-allocated factors act as signals, which determine photosynthate distribution, coordinate the growth rate and differentiation of distant organs, and confer stress responses.

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F. Kragler and M. Hülskamp (eds.), *Short and Long Distance Signaling*,
Advances in Plant Biology 3, DOI 10.1007/978-1-4419-1532-0_7,

The phloem stretches throughout the plant body and consists of files of connected cells termed sieve elements (SE). By large cytoplasmic connections (=sieve plates) at their ends, these living cells form continuous symplasmic conduits termed sieve tubes. The SEs are living cells devoid of nuclei. They are kept alive by neighboring companion cells (CC) connected via specialized micro-channels termed plasmodesmata. These intercellular conduits stretch the cell walls and form a membranous and cytoplasmic continuum (symplasm) between the phloem cells and the surrounding tissue (Lucas et al. 1993). Plasmodesmata allow diffusion of small molecules and facilitate trafficking of specific subsets of proteins and RNAs into the phloem system (Ruiz-Medrano et al. 2001; Ruiz-Medrano et al. 2004).

Studies on the phloem content revealed an abundant presence of macromolecules such as proteins and RNAs. The proteinaceous components include phloem specific structural phloem proteins, enzymes, and transcription factors, and the nucleic acid components include specific mRNAs, non-coding housekeeping RNAs, small regulatory microRNAs (miRNA), and silencing-induced/short interfering RNAs (siRNA). One important feature of these phloem mobile macromolecules is that they can act as specific signals integrating plant growth and development to external stimuli (Lough and Lucas 2006; Kehr and Buhtz 2008; Turgeon and Wolf 2009; Atkins et al. 2011). Such responses include, but are not restricted to, flower induction, apical dominance, and stresses. Of course signals can be conveyed by classical phytohormones found in phloem sap. However, diverse subsets of macromolecules including proteins and transcripts such as mRNAs, siRNA, miRNAs and other non-coding RNAs seem to act as highly specific phloem transmitted signals (Lough and Lucas 2006; Kehr and Buhtz 2008; Kragler 2010; Atkins et al. 2011).

2 Transport of RNA

2.1 Cellular Structures Mediating RNA Transport

The classical view is that intercellular exchange of macromolecules such as proteins or viral components between cells is facilitated by a membrane-bound secretion-internalization mechanism. In animals a peptide exposing a signal motif can be secreted into the extracellular space (exocytosis) and adjacent cells can internalize such extracellular peptides by receptor-mediated endocytosis. Originally it was thought that plants lack endocytosis due to the high turgor present within the cell wall embedded mature plant cell. However, endocytosis was observed in protoplasts and membrane bound structures resembling endocytotic vesicles are formed at the plasma membrane (Saxton and Breidenbach 1988; Aniento and Robinson 2005). In general such an exo-endocytotic transport system does not impose a size constraint on the cargo that is exemplified by the unspecific uptake of highly abundant foreign complexes such as BSA-gold in plant cells (Griffing et al. 1995). Thus, intercellular transport of large molecules such as transcription factors, and even larger RNA

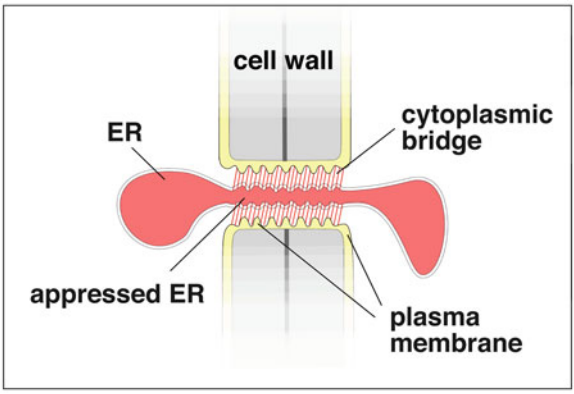

Fig. 7.1 Schematic structure of a simple (non-branched) plasmodesma embedded in the cell wall

molecules mediated by vesicles might occur in plants. However, it has not yet been shown that a classical secretory transport pathway plays a crucial role in transferring RNA between plant cells. Rather, plants seem to overcome the restrictions imposed by the internal pressure and the tight cellulosic cell wall by special intercellular bridges formed by plasmodesmata.

Most plant cells are connected by cell wall stretching tubular structures described more than a century ago by the Austrian/Galician botanist Eduard Tangl (Tangl 1880), which were named plasmodesmata. A single plasmodesma has a complex membranous and proteinaceous structure and forms a channel through the cellulosic cell wall (Fig. 7.1). Two nested membranous tubes outline the connection. The inner tube is associated to the endoplasmic reticulum (ER) and is named either appressed ER or desmotubule; the outer membranous tube is continuous with the plasma membrane outlining the connected cells. The space between the two tubular membrane systems, named cytoplasmic annulus, connects the cytoplasm of two coupled cells. Plasmodesmata are highly dynamic structures. They are initially formed during cytokinesis (primary plasmodesmata) or later between existing cells (secondary plasmodesmata) (Kragler et al. 1998). Plasmodesmata can have an open or closed state and facilitate active transport of viral proteins (see Chap. 1, M. Heinlein, and Chap. 2, P. Zambryski) and subsets of proteins such as homeodomain transcription factors (see Chap. 4, D. Jackson). Via the intercellular bridges formed by plasmodesmata the cytoplasm, endoplasmic rediculum (ER), and plasma membrane of two neighboring cells are connected and allow the exchange of membrane-bound and cytosolic macromolecules (Haywood et al. 2002; Oparka 2004; Maule 2008; Lucas et al. 2009; Barton et al. 2011; Genoves et al. 2011; Kaido et al. 2011; Tilsner et al. 2011). Similar to the intercellular bridges formed by plasmodesmata also human cells form complex membranous tubular connection named tunneling nanotubes (TNTs) facilitating the symplasmic exchange of proteins and membrane-bound material (Rustom et al. 2004; Gerdes et al. 2007). In plants it was shown that relatively large proteins with a size of up to 50 kDa

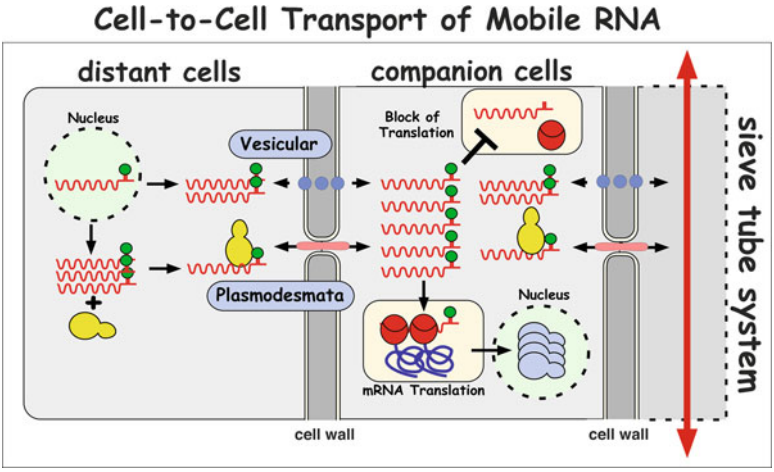

Fig. 7.2 Cell-to-cell transport of mobile RNA and allocation into the phloem stream. Distinct RNA molecules produced in cells distant from or neighboring (companion cells) the sieve tube system can be allocated into the phloem stream. Mobile RNA molecules (*red lines*) expose a transport motif (*green*) and are transported across the cell wall either via the symplasmic route established by plasmodesmata or an alternative, yet unknown, vesicular route (e.g., by exo-endocytosis). Intercellular RNA transport via plasmodesmata is enabled by specific RNA-binding proteins (*yellow*), which provide access to the plasmodesmatal transport machinery and, facilitate the transfer to connect cells. In neighboring or distant cells mobile mRNA can be translated into proteins initiating a signal cascade. Mobile small RNAs such as siRNA or miRNA are negative regulators of protein production, which also can trigger a signal cascade or protect the cells against aberrant or foreign RNA. Note that currently no evidence for a vesicular transport of RNA independent from the plasmodesmatal route exists in plants

diffuse via plasmodesmata in sink tissues (Oparka et al. 1999a). But sufficient support for the notion exists that a receptor-based plasmodesmatal transport system mediates the specific transport of a subset of proteins and RNA to neighboring cells (Haywood et al. 2002; Ruiz-Medrano et al. 2004) (see Chap. 1, M. Heinlein) and facilitates their entry into the long-distance transport pathway established by the phloem tissue (Fig. 7.2). Collectively, the emerging picture is that RNA molecules entering the phloem transport pathway contain a specific motif(s), which are recognized by phloem proteins and mediate their transfer into and out of the sieve tube system.

2.2 Proteins Mediating RNA Transport

The first insights on the mechanism by which RNA molecules are delivered into the phloem stream were provided by studies on RNA binding proteins present in the

pumpkin phloem sap. A protein named *Cucurbita maxima* PHLOEM SMALL RNA BINDING 1 (CmPSRB1) was shown to interact with high affinity with small single-stranded RNA (ssRNA) molecules. In microinjection experiments this protein specifically triggered the intercellular transfer of small ssRNA molecules resembling siRNA (Yoo et al. 2004). Another phloem protein, the small 16 kDa phloem protein initially found in *Cucurbita maxima* (CmPP16) was shown to bind cooperatively to large RNA, to alter the size exclusion limit plasmodesmatal channels, and to mediate intercellular transport of RNA molecules (Xoconostle-Cazares et al. 1999). Identification of CmPP16 interaction partners revealed the complexity of the phloem RNA transport system. An ER-associated NON-CELL-AUTONOMOUS PHLOEM PROTEIN 1 (NCAPP1) (Lee et al. 2003) and polypyrimidine track RNA-BINDING PROTEIN 50 (RBP50) (Ham et al. 2009) form a ribonucleic acid protein complex with a subclass of phloem-mobile, polyadenylated mRNAs. RBP50 also binds to a phloem specific heat shock 70 protein (HSP70) chaperone complex whose function was suggested to keep phloem-allocated proteins in transport competent unfolded state (Aoki et al. 2002). This chaperone-associated RNP complex seems to facilitate the allocation of bound mRNAs through the plasmodesmata present between companion cell and sieve elements, and consequently, mRNA transport along the phloem tissue (Ham et al. 2009).

To limit access of RNA molecules to the phloem stream, plants may employ a cell wall modifying glucanase found in association with plasmodesmata. Callose deposition at plasmodesmata restricts the transport capacity of the intercellular connection and is regulated by beta-1,3-glucan synthase versus glucanase activities (Ueki and Citovsky 2005; Levy et al. 2007; Benitez-Alfonso et al. 2009; Zavaliev et al. 2011). Such an unspecific down-regulation of intercellular connectivity can be triggered by stress factors or occurs in specific tissues, which in turn could limit transfer of RNA into and out of the phloem.

2.3 The Phloem Transport System: Systemic Signals and RNA

As with all multicellular organisms, intercellular and long-distance communication play a vital role in coordinating a genetic program to maintain homeostasis and to signal response(s) to environmental cues. In plants, several well-studied examples of such signaling and the function underlying such processes are emerging. Flower induction, post-transcriptional gene silencing (PTGS) based on siRNAs, systemic acquired resistance (SAR) to pathogens and drought response, tuber induction, among many others, rely on the synthesis of signaling molecules in tissue exposed to a given stimulus, and their transport to naive tissues and/or organs, which respond to or re-direct plant growth and development accordingly.

Flower induction is perhaps the phenomenon in which long-distance signaling has been more thoroughly studied. While the role of chromatin remodeling in flower producing tissue has gained ample attention, it became clear that in day-length sensitive

species inductive stimuli are perceived in mature leaves and not in shoot apices. Changes in the light cycle induce the synthesis in leaves and transport of a phloem-mobile signal(s) to the shoot apex, which in turn initiates the flowering program. Recent evidence has shown that in a number of plant species such as Arabidopsis, rice, and pumpkin the likeliest signal derived from leaves and transported via the phloem to the apex is the FT protein. This phloem mobile phosphoethanolamine-binding protein acts in conjunction with a bZIP factor upstream of *CONSTANS* in leaves and *MADS* box factors in apical meristems (Lifschitz et al. 2006; Corbesier et al. 2007; Jaeger and Wigge 2007; Lin et al. 2007b; Mathieu et al. 2007; Tamaki et al. 2007). However, there is some evidence for the potential involvement of RNA as part of the mobile proteinaceous FT signal. Indeed, inactivation of the phloem glycine-rich RNA-binding protein GRP7 from *Arabidopsis thaliana* results in inhibition of flowering (Streitner et al. 2008). Although it is not clear whether the flower induction function is related to the suggested GRP7 RNA-binding capacity, a regulatory role of GRP7 gene product in pathogen defense has been confirmed (Fu et al. 2007).

In the case of SAR long-distance macromolecular signals have been proposed, and, were identified in plants infected by necrotrophic pathogens,. Here a lipid transfer protein, and by extension, a lipid, has been shown to be involved in signaling SAR in response to bacterial infection (Maldonado et al. 2002). However, salicylate appears to be involved in SAR to biotrophic pathogens (Vlot et al. 2008a; Vlot et al. 2008b), while other reports indicate that neither methyl salicylate nor jasmonic acid is required for the establishment of SAR (Attaran et al. 2009). Taken together no consensus on the nature of the mobile signal seems to be found and it seems that as with other signaling systems the systemic SAR response depends on more than one signaling component. Maybe such additional signals are based on mobile RNA molecules. The pattern of RNA-based gene silencing and the presence of siRNA molecules in the phloem stream suggests that these small RNAs are the phloem-mobile signal that triggers silencing in distant tissues (Hamilton and Baulcombe 1999; Yoo et al. 2004; Dunoyer et al. 2010) (see also Chap. 5, B. Carrol; Chap. 6, J. Kehr). It is well established that the siRNAs targeting viral RNAs are produced upon infection and move systemically, however, a set of phloem mobile mRNAs are also produced in infected tissues and might act as SAR-related signal(s) (Ruiz-Medrano et al. 2007).

It has been proposed that a long-term response to drought depends on a signal transported from mature leaves, or even roots, to shoot apices. Conceivably, such a signal has to decrease the rate of proliferation in above ground tissue, while allowing root growth (Ruiz-Medrano et al. 2001). Unfortunately, to date the identity of such a systemic drought signal(s) remains unknown. Nevertheless, a local acting mobile inhibitor of a cyclin-dependent protein kinase (ICK1/KRP1) was identified to response to drought. This kinase regulator was shown to move from cell to cell within the shoot apex suggesting that it could act as a local signal (Weinl et al. 2005).

Responses to low inorganic phosphate have been linked to the transport of a signal from source leaves to roots, the nature of which remained unknown until recently (see also Chap. 6, J. Kehr). Produced in leaves, miR399 was shown to be

involved in the systemic response to phosphate starvation, (Pant et al. 2008). The mechanism by which this miRNA acts was recently explored. miR399 moving from leaves to root tissue inhibits translation of its mRNA target *PHO2*, which is a negative regulator of phosphate accumulation (Pant et al. 2008; Hsieh et al. 2009). This example suggest that subsets of small miRNAs can be transported to distant organs in a functional state and act as long-distance signals.

3 Motifs Mediating RNA Transport

Scant information is available on sequence motifs or the structural requirements rendering plant produced RNA molecules phloem mobile. Nevertheless, it is well documented that RNA viruses and viroids establish a systemic infection by entering the phloem stream. Since the genome of most viruses and of all viroids is known, the analysis of their structure – function relation with respect to intercellular and long-distance movement can provide important insights on motifs required for RNA trafficking. Obviously, viruses and in particular viroid RNAs, which do not encode proteins, depend on the host system to move to distant tissues.

The initial evidence that specific proteins facilitate RNA transfer between cells and over long distances was found in studies on plant viruses (see Chap. 1, M. Heinlein). Viruses produce specialized nucleic acid binding proteins increasing the size exclusion limit of plasmodesmata, which in turn facilitates the transfer of the viral nucleic acid (vRNA or vDNA) – protein complex into neighboring cells and consequently into the phloem system. Another example of specific delivery of RNA sequences is found in viroids. These infectious agents consist of a small circular RNA molecule, encode no proteins, and, thus, are strictly depending on the proteins provided by the host cells to propagate. They have a complex secondary structure determining their host range and intercellular spread (Qi et al. 2004; Zhong et al. 2008). As spread of viroid RNAs strictly depend on host proteins they provide a remarkable useful system to study the requirements of RNA structures mediating transport.

3.1 Viral RNA Delivery Motifs

Viruses and viroids use various mechanisms to access plants and can be roughly grouped into two types: vector-transmitted or mechanically transmitted. Most viruses are transmitted through animal or fungal vectors; insect vectors can introduce the virus by chewing on plant tissue, or by sucking on the phloem content with the help of a stylet (e.g., aphids and whiteflies). In general, viruses transmitted by phloem-feeding insects are limited to the phloem tissue, while those transmitted via mechanical damage are able to infect most other tissues. Via phloem feeding insects the virus can reach the phloem stream directly without trafficking from outer cell

layers. The viral infection is mediated by viral-encoded proteins, which interact with the viral genome and facilitate the transport via plasmodesmata to neighboring cells. Although, these movement enabling viral proteins have a quite divergent structure, they appear to function by a common basic mechanism. Through interaction with the endoplasmic reticulum (ER) and/or cytoskeletal components a mobile nucleoprotein complex or virions containing the viral genome are formed competent to move via plasmodesmata to neighboring cells (see Chap. 1, M. Heinlein) (Lucas 2006; Guenoune-Gelbart et al. 2008; Sambade et al. 2008; Harries and Ding 2011; Niehl and Heinlein 2011).

Phloem-mediated long-distance transport of viruses has not been studied as thoroughly as their cell-to-cell transport, but a growing body of evidence suggests that a number of plant endogenous and viral-encoded proteins play a role in this process. As observed in local cell-to-cell transport, some viruses move long-distance as virions, some move as nucleoprotein complexes, and some seem to move in both ways (Gilbertson and Lucas 1996; Simon-Buela and Garcia-Arenal 1999). This indicates already that the complexity of viral phloem transport system is greater than one would anticipate. Indeed, genes from diverse viruses that suppress virus-induced gene silencing (VIGS) were initially thought to be involved in long-distance transport. More recent work indicates, however, that the *Cucumber mosaic virus 2b* gene product is essential for long distance transport and also interferes with the VIGS machinery (Diaz-Pendon et al. 2007; Diaz-Pendon and Ding 2008). The viral interference with the VIGS system evidently results in higher levels of viruses but this does not explain the observed effect on long-distance transport of the infectious material. Factors associated to the systemic transport of viruses are found in the three plant genes named *RTM1*, *RTM2*, and *RTM3,* which restrict the long-distance transport of potyviruses. The *RTM1* gene product belongs to the jacalin-like plant lectin protein family with potential sugar binding activity, *RTM2* encodes a protein with similarities to small heat shock proteins, and *RTM3* belongs to a large uncharacterized gene family. All three factors restrict long-distance transport of the *Tobacco etch virus* in *A. thaliana*. However, their precise role and the mechanisms underlying the restriction of viral movement are not known (Whitham et al. 2000; Chisholm et al. 2001; Cosson et al. 2010). With an umbravirus an interaction of the ORF3 gene product with the nucleolar factor fibrillarin, an essential component of the translocatable viral ribonucleoprotein complex, was shown to facilitate systemic infection (Kim et al. 2007a; Kim et al. 2007b). These findings support the view that some viral silencing-suppressor genes are also long-distance transport factors (Ding and Voinnet 2007; Rajamaki and Valkonen 2009). Given that gene silencing initiates in Cajal bodies present in the nucleoli, it might be possible that the viral produced long-distance movement factor plays two roles beneficial for the virus. The first role could be that the viral gene product facilitates the entrance of the virus into the intercellular transport system used to spread siRNA. The second beneficial role could be that this hijacking interferes with the transport and/or production of siRNA targeting viral RNA signal (Dunoyer et al. 2004; Ding and Voinnet 2007; Rajamaki and Valkonen 2009; Bivalkar-Mehla et al. 2011).

Recently, the *Brome mosaic virus* RNA3 has been shown to be capable of moving long-distance independently of the presence of viral coat and movement proteins in *Nicotiana benthamiana* (Gopinath and Kao 2007). This indicates that the movement machinery provided by the host is sufficient to mediate long-distance transport of viral RNA. It seems that the endogenous phloem RNA binding proteins such as the aforementioned CmPP16, PP1, PP2, and CmRBP50 (Xoconostle-Cazares et al. 1999; Gomez et al. 2005; Ham et al. 2009) are sufficient to facilitate delivery of transport competent RNA into and via the phloem.

In some instances viral RNA sequences acting in *cis* seem to be required for long-distance transport. For example, the *Bamboo mosaic potexvirus* requires a pseudoknot structure in the 3′ untranslated region (UTR) of its RNA for its transport into other tissues (Chen et al. 2003b). This RNA motif mediates binding to a chloroplastic enzyme, phosphoglycerate kinase, and it was shown that viral levels are decreased in plants in which this gene has been knocked down (Lin et al. 2007a). Whether the enzyme is involved in mediating transport or also affects other viruses is not known, but the pervasive presence of these pseudoknot structures that resemble transfer RNAs and can be amino-acylated in many RNA viruses, may indicate that, besides replication, the structure is involved in systemic transport (Dreher and Goodwin 1998; Dreher 2009). Additional evidence that RNA motifs are involved in viral transport is found in the 5′ UTR essential for replication and cell-to-cell movement of the *Potato virus X potexvirus* (Lough et al. 2006). Similar functional results have been obtained with the 5′ UTR of the unrelated *Turnip yellow mosaic virus* (Shin et al. 2008). In addition, a phloem protein binding to viral particles was suggested to play role for viral long distance transport. Indeed, the RNA binding phloem protein PP1 seems to associate to *Cucumber mosaic virus* particles and might facilitate its transport (Requena et al. 2006). Unfortunately, there is scant information regarding additional viral RNA sequences involved in phloem transport (see Table 7.1). To date we do not know which viral and/or host factors bind predicted viral RNA motifs in a specific manner, and whether such a host - virus interaction might be crucial for transport.

3.2 Viroid RNA Delivery Motifs

Viroids are small RNA molecules with a size between 250 and 400 nucleotides. These ubiquitous pathogenic RNAs are devoid of protein-coding regions and, as already mentioned, host factors must provide all necessary proteins for replication and cell-to-cell and long-distance transport (Ding 2009). However, to date only one melon phloem protein, PP2, has been identified to bind viroid RNA (Gomez et al. 2005). This protein is related to the pumpkin phloem lectin CmPP2 and harbors a dsRNA-binding domain, but the significance of the viroid RNA interaction for long-distance trafficking remains to be shown.

Table 7.1 Potential RNA motifs/sequences facilitating cell-to-cell and long-distance trafficking of RNAs

RNA	Motif(s)	Structure	Length (bases)	Proteins involved	Tropism	Reference
PSTVd	Two motifs	Loops & WC	21, 8	n.d.	Bundle sheath to mesophyll	Qi et al. (2004)
PSTVd	One motif	Loop & WC	11	n.d.	Bundle sheath to phloem	Zhong et al. (2007)
PSTVd	One motif	Loop & WC	9	n.d.	Palisade to spongy mesophyll	Takeda et al. (2011)
HSVd	Full-length	n.d.	297	CsPP2	Phloem	Gómez and Pallás (2004)
HSVd	Full-length	n.d.	297	CmPP2, CmLec17	Phloem	Gomez et al. (2005)
ASBVd	Full-length	n.d.	247	CmPP2, CmLec17	Phloem	Gomez et al. (2005)
PVX	5′UTR	n.d.	107	PVX TGB	Cell-to-cell	Lough et al. (2006)
BMV	RNA 1, 2 & 3	n.d.	3,234, 2,865, 2,108	n.d.	Leaf to sink leaf	Gopinath and Kao (2007)
miR172	Full-length	n.d.	21	n.d.	Leaf to root	Martin et al. (2009)
miR399	Full-length	n.d.	21	n.d.	Leaf to root	Pant et al. (2008)
FT	1–102 ORF	n.d.	102	n.d.	Leaf to apex	Li et al. (2009)
CmPP16	Full-length	n.d.	673	CmPP16	Phloem to shoot apex	Xoconostle-Cázares et al. (1999)
CmNACP1	Full-length	n.d.	1,263	n.d.	Phloem to shoot apex	Ruiz-Medrano et al. (1999)
StBEL5	5′ & 3′ UTR	n.d.	148, 521	n.d.	Leaf to root	Banerjee et al. (2009)
GAI	ORF & 3′UTR	n.d.	2,202	n.d.	Leaf to apex	Huang and Yu (2009)

PSTVd potato spindle tuber viroid, *HSVd* hop stunt viroid, *ASBVd* avocado sunblotch viroid, *miR172* microRNA 172, *miR399* microRNA399, *FT* floral locus T, *PVX* potato virus x, *CmPP16* cucurbita maxima phloem protein 1, *CmNACP1* cucurbita maxima NAC domain phloem protein 1, *StBEL5* solanum tuberosum BEL1-like protein 5, *GAI* gibberellic acid insensitive, *BMV* brome mosaic virus, *CsPP2* cucumis sativus phloem protein 2, *CmmPP2* cucumis melo phloem protein 2, *CmmLec17* cucumis melo phloem lectin 17, *TGB* viral triple gene block proteins, *WC* Watson-Crick nucleotide interaction, *n.d.* not determined, *ORF* open reading frame, *UTR* untranslated region

It was shown that structural RNA motifs, rather than specific nucleotides, are necessary to facilitate transport of viroid RNA between different cell types (Table 7.1). These rather small RNA molecules consist of nucleotide stretches encompassing loops, bulges and double-stranded RNA that form distinct structures. Of particular interest is the motif essential for trafficking between mesophyll and phloem cells, which was shown to be distinct from the structure necessary for transport between palisade and spongy mesophyll (Qi et al. 2004; Zhong et al. 2007; Zhong et al. 2008; Takeda et al. 2011) and most likely functions in binding host factors that mediate transport through plasmodesmata specifically in the phloem tissue (Zhong et al. 2008). Mutations in distinct loops present in the *Potato spindle tuber viroid* (PSTVd) RNA decreased replication levels as well as systemic trafficking, so these motifs appear to have a dual function. In contrast to previous considerations, the loops between double-stranded sequences seem to be highly conserved, which facilitates the prediction of structures for a wide variety of viroid RNAs (Takeda et al. 2011). One short RNA stretch of approx. nine nucleotides was predicted to fold similar to a stem – loop motif found in *Escherichia coli* 23S RNA. This particular structure and not individual nucleotides in the viroid RNA was essential to trigger trafficking from palisade to spongy mesophyll, and, thus, providing access to the phloem tissue (Takeda et al. 2011).

3.3 Small RNA Delivery Motifs

As mentioned, several miRNA have been found to be present in the phloem sap, and a few have been shown to act as signals (see Chap. 5, B. Carrol). These mobile miRNAs might prove useful to determine common motifs or RNA binding proteins involved in long-distance trafficking of other mobile RNAs. However, given the small size of miRNAs of approx. 21 nucleotides there would be a strong constraint for structural conservation and it is more likely that their transport depends either on secondary modifications or on specific miRNA interacting proteins forming mature miRNAs, or on both. Regarding the sequence of mature miRNA the most stable structure is formed, according to *Caenorhabditis elegans let-7* miRNA, when the small RNA is bound to its target mRNA. This heteromeric RNA complex builds a partial duplex structure through Watson-Crick (WC) base pairing (Cevec et al. 2010). This is quite different from the loop structures found in the *Potato spindle tuber viroid* necessary for systemic trafficking. Here WC-, and non-WC pairs surround small loops and bulges (Takeda et al. 2011). In addition, the constraints on the WC pairings appear not to be very stringent, since changes that maintain the WC pairings do not necessarily disrupt the motif (Takeda et al. 2011). Also it should be noted that bonds between RNA bases are of a more diverse nature than for DNA (Leontis and Westhof 2001). Hydrogen bonds can be formed between bases and sugars in 12 different arrangements, and bases that do not form stable hydrogen bonds in DNA can readily do so in particular structural arrangements.

3.4 mRNA Delivery Motifs

As with viral and miRNAs our insights regarding motifs necessary or sufficient to mediate protein encoding transcripts transport from cell to cell and via the phloem is limited. Evidence for an RNA motif facilitating transport of mRNA was provided again by experiments conducted with viral RNA. A cis-element in the 59-untranslated region (UTR) of potexviral RNA, which plays a role in replication (Miller et al. 2007), was found to trigger cell-to-cell transport of a green fluorescent protein (GFP) mRNA (Lough et al. 2006). A GFP reporter system was also used to show the presence of mRNA trafficking signals in nucleotides 1–102 of the *Arabidopsis FT* mRNA (Li et al. 2009) and in 39 nucleotides of the 3′ UTR present in *GIBBERELLIC ACID-INSENSITIVE* (*GAI*) mRNA (Huang and Yu 2009). Also the 5′ and 3′ UTRs of potato *StBEL5* mRNA seem to be important for mRNA stability and long-distance movement (Banerjee et al. 2009). These examples suggest that endogenous phloem mobile mRNAs harbor structural motifs required for their intercellular transport and, consequently, entry into the phloem stream. However, in these few mRNAs, which were studied in detail, no common sequence motifs were found. In general, it seems that untranslated regions (5′ UTRs and 3′ UTRs) are involved in gaining access into the phloem tissue and/or required for phloem transport and that these sequences are rich in CT/GA repeats (R. R.-M; unpublished) . However, these repeats appear to be overrepresented in most analyzed plant UTRs. Also no obvious sequences or motifs are present in the phloem allocated *CmPP16, CmNACP, CmGAIP* mRNAs and several other analyzed melon mRNAs reported to move long distance.

In general it will be necessary to map the RNA motifs/regions allowing long distance transport case by case. Once this is achieved by a sufficient number of mobile RNAs a structural analysis similar to those performed in PSTVd might shed light on the requirements to render an RNA mobile and facilitate the identification of cellular components involved in triggering the transport. Currently it seems likely that no simple conserved RNA sequence stretch is used to mediate transport. What we know is that several classes of phloem mobile RNAs exist and that transport of foreign mRNAs can be triggered by fusing these with viral RNAs and mobile plant endogenous mRNAs.

4 Destination Selective Phloem-Allocation of RNA

It is generally assumed that the phloem flows passively from mature, photosynthetic leaves to heterotrophic tissues. Nutrients and small signaling molecules seem to follow the unidirectional flow and are unloaded in sink tissues. With GFP expressed within the CC-SE complex, it was shown that this small protein (27 kDa), which has no interaction partner in plant cells, could be transported into and along the SE and unloaded in meristematic- and actively-growing tissues (Imlau et al. 1999; Oparka et al. 1999b). Pioneering work of Fisher on wheat suggested that the mobile phloem

Table 7.2 List of representative plant endogenous mRNA molecules identified to move long-distance

Gene	Species	Suggested function	Reference
PFP-LeT6	*Solanum lycopersicon*	Leaf development	Kim et al. (2001)
CmNACP	*Cucurbita maxima*	Meristem maintenance Floral development Leaf senescence	Ruiz-Medrano et al. (1999)
CmRINGP	*Cucurbita maxima*	Transcriptional regulation	Ruiz-Medrano et al. (1999)
CmRABP	*Cucurbita maxima*	Intracellular vesicular trafficking	Ruiz-Medrano et al. (1999)
CmGAIP	*Cucurbita maxima*	Leaf development	Haywood et al. (2005)
CmWRKYP	*Cucurbita maxima*	Defense response	Ruiz-Medrano et al. (1999)
CmSTMP	*Cucurbita maxima*	Cell fate in meristems	Ruiz-Medrano et al. (1999)
CmCYCLINP	*Cucurbita maxima*	Cell cycle	Ruiz-Medrano et al. (1999)
CmPP16	*Cucurbita maxima*	RNA transport	Xoconostle-Cazares et al. (1999)
DELLA-GAI	*Arabidopsis thaliana*	Leaf development	Haywood et al. (2005)
StBEL5	*Solanum tuberosum*	Tuber development	Banerjee et al. (2006)
F-308	*Cucumis melo*	*Aux/IAA* – Auxin signaling	Omid et al. (2007)
F-571	*Cucumis melo*	*Aux/IAA* – Auxin signaling	Omid et al. (2007)
Un-131	*Cucumis melo*	*SAUR* – Auxin signaling	Omid et al. (2007)
F-266	*Cucumis melo*	Unknown	Omid et al. (2007)
Un-37	*Cucumis melo*	Unknown	Omid et al. (2007)
F-162	*Cucumis melo*	Unknown	Omid et al. (2007)
MpSLR/IAA14	*Malus prunifolia*	*Aux/IAA* – Auxin signaling	Kanehira et al. (2010)
FT	*Nicotiana benthamiana*	Floral development	Li et al. (2009)

proteome has a distinct profile depending on the site of sampling (Fisher et al. 1992). It seems that distinct proteins traveled short distances, move to apical tissues, or join the phloem flow in sub-apical tissues. This suggests that mobile phloem proteins, particularly those that would appear to have a non-cell-autonomous function and are not required for the maintenance of the sieve tubes, contain a motif allowing them to enter or exit the phloem at different locations. This notion is supported by the observation that the transport of viral movement protein of *Cucumber mosaic virus* (CMV) (*Blackman* et al. 1998), the allocation of *Bean dwarf mosaic virus* (Sudarshana et al. 1998), or viroids (Zhu et al. 2001) depends on plant age or flower induction. That the phloem unloading of macromolecules in developing tissues is highly regulated is also suggested by the seasonal closure of the symplasmic connectivity of the shoot apical meristem established by plasmodesmata.

Using an elaborative and sophisticated aphid approach Aoki et al. (2005) demonstrated that a labeled phloem protein (PP16) re-introduced into the phloem of rice plants was transported preferentially towards the root. However, destination-selective phloem transport seems also to occur for phloem-allocated RNAs. The profile of phloem sap RNAs varies significantly at different sampling sites or times (Yoo et al. 2004). Also the phloem mobility of mRNAs seems to be conserved among different species (see Table 7.2). It seems that the phloem transported miRNAs and mRNAs are part of a fail-safe mechanism ensuring the induction of specific pathways. In such a scenario, in certain circumstances the cell- or

tissue- autonomous levels of apical root or shoot RNAs might be insufficient to trigger physiological responses, thus phloem-allocated RNAs might ensure an accurate adaptation to environmental changes sensed by leaves.

Several regulatory phenomena requiring phloem-delivered signals could well be based on an mRNA or miRNA transport system. One of the first documented examples of phloem transport of mRNA across a graft union and unloading was documented with the aforementioned *CmNACP* RNA; however, the highest level of this RNA is found in roots. While some members of this transcription factor family are necessary for meristem maintenance and organ formation, others are expressed in roots and are required for vascular cell differentiation (Ruiz-Medrano et al. 1999; Olsen et al. 2005). Whether *CmNACP* mRNA is transported to roots remains to be shown, but the absence of *CmNACP* protein in phloem sap underlines the selectivity of phloem content allocation. It should be mentioned that in heterograft studies (cucumber on pumpkin) *CmNACP* RNA was detected mostly in the extrafascicular phloem distinct from the bicolateral phloem bundles (Ruiz-Medrano et al. 1999).

Recently it has been shown that most of the sucrose present in phloem sap is translocated through the intrafascicular phloem, which explains the paradox of the low concentration of fixed carbon found in cucurbit phloem sap (Zhang et al. 2010). A fascinating possibility is that, at least in cucurbits, the extrafascicular phloem is involved in the delivery of long-range macromolecular signals, while the intrafascicular phloem mediates the transport of the majority of fixed carbon. However, it is not known whether a similar functional specialization of different types of phloem occurs in other plant species.

Another important question is where mobile mRNAs exit the phloem in sink tissues. In heterografted scions, in contrast to some phloem-allocated RNAs of *CmRINGP*, *CmRABP*, *CmWRKYP*, *CmSTMP,* and *CmCYCLINP*, the mRNAs of *CmNACP*, *CmPP16,* and *CmGAIP* are transported to meristematic tissues (Ruiz-Medrano et al. 1999). A similar observation was made in melon. Here a number of abundant phloem sap mRNAs did not traffic across a graft union in a melon/pumpkin heterografting system (Omid et al. 2007). Interestingly, no correlation exists between transcript abundance and ability to move through a graft union. It seems that, the entry and exit as well as the transport along the phloem vessels of mRNA, are tightly regulated and selective processes.

Also the analysis of the phloem sap transcript profile in response to biotic or abiotic stress yields information on the mechanisms regulating mRNA transport into the phloem. Interestingly, CMV infection alters the melon phloem sap proteome and transcriptome only marginally, which suggests that the import of macromolecules into the phloem stream is quite robust (Ruiz-Medrano et al. 2007; Malter and Wolf 2011). The identity of some of the induced transcripts and proteins suggests that the presence of a viral infection has a profound impact. For example, the altered levels of phloem mobile TRANSLATIONALLY – CONTROLLED TUMOR PROTEIN (TCTP) could affect the growth rate of distant tissues (Malter and Wolf 2011).

4.1 Identifying Phloem Expressed mRNAs

Transcripts such as mRNAs have traditionally been perceived as cell-autonomous molecules. It was logical to assume that these molecules are produced in cells where they fulfill their biological function. The general notion was that 'mobile' mRNA molecules detected in the phloem sap should be considered as contaminants deriving from neighboring cells or from the harvesting process.

However, advances in analytical tools together with the expansion of genomic databases have enabled detailed analyses of the transcripts identified in phloem tissues. For example, mRNAs encoding proteins involved in metal homeostasis, stress responses, protein degradation or turnover, metabolism, and in building and maintaining phloem vessels were found in phloem-enriched tissues of *Apium graveolens* (Vilaine et al. 2003). Also, laser-capture microdissection (LCM) technique enabled more accurate characterization of the transcript profile in specific phloem cells of rice (Asano et al. 2002) and maize (Nakazono et al. 2003). Transcripts encoding transporters, metal-binding and metal-homeostasis proteins, lignin biosynthesis and proteolysis are preferentially expressed in the vascular tissue (Nakazono et al. 2003). Nevertheless, isolation of specific phloem cells cannot yet be achieved using the LCM technique and the transcript profile obtained reflects expression within various phloem cell types.

4.2 Classification of Mobile mRNAs in the Sieve Tube System

The observation that some plants, including cucurbits, exude copiously from severed phloem (Crafts 1932) provided the first means for a thorough analysis of the RNA profile within the sieve tube. Indeed, the long distance transport of mRNA molecules was shown by analyzing the extent of RNA transport from pumpkin stock into cucumber scions (Ruiz-Medrano et al. 1999). Cloning and sequencing of 100 randomly selected pumpkin phloem sap transcripts revealed numerous unique sequences. Several of the identified mRNAs encoded factors putatively involved in signal transduction, and, as mentioned before, only a subset was able to enter meristematic tissue of scion heterograft (see Table 7.2).

Another detailed analysis of the phloem transcriptome is based on a cDNA library constructed from mRNAs extracted from melon phloem sap (Omid et al. 2007). Random sequencing of about 1,900 clones resulted in 986 unique transcripts. Annotation of the expressed sequence tags (ESTs) revealed 666 significant matches, while 320 of them were unique. Somewhat surprisingly, among the sequenced ESTs, almost 80% of the identified genes were singletons. These results suggested that several thousands of mRNA molecules are present in the sieve tube. The ability of a number of phloem sap transcript to traffic long distances via the phloem was examined in an experimental melon/pumpkin heterografting system. Of 47 randomly selected melon phloem sap transcripts, only six were identified in pumpkin

scions growing on melon rootstocks (see Table 7.2). More importantly, the capacity for long-distance movement was not associated with the abundance of specific transcripts in the phloem sap. The most abundant transcript (60 ESTs out of the 1,900 identified sequences) not identified in the pumpkin scion was the melon *phloem protein 1* (PP1) mRNA. In contrast, low abundant transcripts (one to six ESTs of the 1,900 identified sequences) were detected in pumpkin scions.

Functional classification based on BLAST descriptions of the melon phloem sap transcripts showed that they fall into the following categories: cellular response to hormones and stress (40%); signal transduction, transcriptional control and nucleic acid binding (15%); defense-associated responses (10%). Interestingly, only 2% of the transcripts were related to metabolism (Omid et al. 2007). In another analysis, cells from Arabidopsis plants expressing GFP in the CCs were submitted to fluorescence assisted cell sorting (FACS) and the RNA found in companion cells was harvested, sequenced, and analyzed (Ivashikina et al. 2003). A comparison between the transcriptome profiles of melon phloem sap and *Arabidopsis* CCs revealed significant differences. In the CCs 16% of the annotated transcripts were associated with metabolism and 29% with redox regulation. In a marked contrast the proportions of phloem sap transcripts were 2.3% associated to metabolism and 3.5% to redox regulation. Thus, the phloem sap transcript profile does not reflect the profile of the neighboring CCs, which are symplasmically connected to the sieve tubes and keep the sieve tube system alive. Therefore, movement of transcripts from CCs into the sieve tube is selective and controlled.

4.3 Mobile mRNAs and Apical Growth

The first experimental demonstration of a biological role for a long-distance-trafficking mRNA molecule was provided by Kim et al. (2001). Movement of *mouse ears*, a mutant homeobox fusion transcript, from the mutant tomato rootstock into the apical meristem of a wild-type scion was associated with the formation of octapinnate compound leaves. This phenotypic alteration suggested that long-distance movement of mRNA molecules might be involved in controlling developmental processes. A few years later, it was demonstrated that transcript encoding GRAS family proteins also acts as a phloem-mobile information molecule (Haywood et al. 2005). Gibberellic acid insensitive (GAI) is a GRAS family protein that acts as a negative regulator of the gibberellic acid (GA) response. A deletion mutation in the conserved DELLA domain of GAI resulted in a gain-of-function mutant in which the GA-signaling pathway is impaired (Peng et al. 1997). Presence of the *gai* mutation in wild-type tomato and *Arabidopsis* scions grafted on their respective *gai* mutants established that this transcript is capable of long-distance movement. Moreover, the mutated transcript exited the scion phloem and moved cell-to-cell into the shoot apex, resulting in an altered leaf morphology that resembled the phenotype characteristic of impaired GA signaling (Haywood et al. 2005).

Long-distance movement of mRNA molecules involved in GA- and/or cytokinin-mediated signaling was also found in association with potato tuber formation (Banerjee et al. 2006, 2009; Chatterjee et al. 2007). Transgenic potato plants in which the potato BEL1-like transcription factor (*StBEL5*) was overexpressed exhibited enhanced tuber formation and increased cytokinin levels (Chen et al. 2003a). A further study established that StBEL5 and its KNOX partner (*POTH1*) bind, in tandem, specific DNA sequences of the promoter of ga20 oxidase1 (*ga20ox1*) to repress its activity (Chen et al. 2004). These studies indicated that both transcription factors regulate tuber formation by mediating hormone levels in the stolon tip (Rosin et al. 2003; Chen et al. 2004). Interestingly, trafficking of the *StBEL5* transcript across a graft union to the stolons of potato plants was found to be associated with enhanced tuber formation (Banerjee et al. 2006). In addition, accumulation of *StBEL5* in stolon tips was increased under short days, a condition which promotes tuber formation, whereas overexpression of the transcript in transgenic potato plants overcame the inhibitory effect of long days on tuber formation.

Also another RNA molecule is transported from leaves to root tissue where it induces tuber formation. The mobile microRNA 172 (*miR172*) promotes, in contrast to the photoperiodic regulated *StBEL5*, tuberization and flowering under non-inductive conditions (Martin et al. 2009). As with *StBEL5* mRNA, *miR172* accumulates in leaves, roots and stems at a basal level even in the absence of tuberization stimulus. An apparent destination-selective transport has been observed for another miRNA, *miR399*, which regulates phosphate uptake in roots and is allocated over graft junctions (Buhtz et al. 2008). Highlighting the complexity of the RNA signaling system the authors indicated that expression of the phloem-transported miRNA is also induced by phosphate starvation in roots. These examples provide the foundation for the new emerging paradigm that specific endogenous mRNA molecules operate as long-distance signals in a whole-plant communication network.

5 Phloem Proteins and RNA

5.1 Signaling Phloem Proteins and RNA

A number of phloem mobile proteins acting as housekeeping factors are allocated into and detected within the assimilate-transporting SE. For example phloem specific thioredoxin and cytochrome b5 reductase are thought to maintain the redox balance of the phloem content. Other phloem specific proteins such as the phloem proteins, PP1 and PP2 (Clark et al. 1997), or the *FORISOME* gene product (Noll et al. 2007) seem to protect the tissue or regulate the massive flow occurring in the SEs. Other phloem allocated proteins such as the cytoskeleton factors F-actin, profilin, or actin-depolymerizing factor (ADF) likely function in creating and keeping a low-resistance pathway in the phloem translocation stream (Turgeon and Wolf 2009). On the other hand the identity of many other phloem-allocated proteins

highlight their role as tissue differentiation inducing signals. In day-length sensitive species a flower-inducing protein named FT is produced in mature leaves and transported via the phloem conduit into the shoot apices, where it induces the flower-forming program in apical shoots (Corbesier et al. 2007; Lin et al. 2007b; Tamaki et al. 2007). The first insights are already available in form of the phloem proteome, which has been elucidated for a few species such as pumpkin, melon, *Brassica napus*, lupines, and, partially, from castor bean (*Ricinus communis*) and the monocotyledonous barley and rice from which analytical amounts of phloem sap can be routinely obtained (Walz et al. 2004; Giavalisco et al. 2006; Lough and Lucas 2006; Kehr and Rep 2007; Aki et al. 2008; Gaupels et al. 2008; Lin et al. 2009; Turgeon and Wolf 2009; Atkins et al. 2011; Rodriguez-Medina et al. 2011). Although we know the phloem proteome of several plants, more detailed studies on phloem-transported proteins have to be performed to determine which proteins may act as specific signals produced in mature, photosynthetic leaves and inform distant tissues such as apical meristems, young leaves, and roots to changing environmental conditions.

Although apical tissues harboring the plant stem cells follow a tightly regulated developmental program, it has become increasingly clear that the meristems are subject to environmental regulation. The aforementioned FT protein is an obvious example highlighting the strong dependence of developing tissues, and in particular meristems, on environmental cues sensed in distant organs. Another example of potential control of meristem development and function is bud dormancy in pines, which is triggered by both low-light conditions and low temperature (Rinne et al. 2001). Interestingly, these conditions induce a decrease in the permeability of plasmodesmata that connect the meristem proper to the cells immediately underneath (Rinne and van der Schoot 1998; Rinne et al. 2001, 2011). A similar phenomenon has been observed during the floral transition in Arabidopsis (Gisel et al. 1999, 2002). It has been suggested that regulation of symplasmic connectivity occurs mainly through sealing of plasmodesmata by glucan deposition (Xu and Jackson 2010; Zavaliev et al. 2011). An analysis of the proteome in the apex of a pinus species undergoing the transition from dormancy to growth has revealed the presence of an RNA-binding glycine-rich related protein (GRP7) also found in the phloem proteome of different species, such as castor bean, lupin, rice and pumpkin (Barnes et al. 2004; Giavalisco et al. 2006; Aki et al. 2008; Lin et al. 2009; Rodriguez-Medina et al. 2011). Interestingly, this protein is a circadian oscillator and is involved in floral induction, as well as in response to pathogens (Fu et al. 2007). Although the precise biochemical function of FT and GRP7 in the meristem is unknown, it was shown in different plant species that both proteins are transported via the phloem and change the developmental program of the apical meristem. Another example of long-distance regulation via a phloem-mobile protein is found in the TRANSLATIONALLY – CONTROLLED TUMOR PROTEIN (TCTP). This phloem protein belongs to a large family of exclusively eukaryotic proteins with diverse functions. It has been shown that TCTP is a central mitotic regulator in both plant and animals (Brioudes et al. 2010) and it has been found in the phloem sap of melon, pumpkin, rice, and *Brassica napus*, and is thought to be involved in the long-distance transport of the RNA-binding phloem protein CmPP16 (Aoki et al. 2005). In general a complex signaling system based on phloem-allocated proteins seems to

exist in plants regulating a wide array of pathways. However, for most identified phloem-allocated macromolecules it remains to be shown when, how, and to which extent they are unloaded in apical tissues and exert a specific signaling function.

5.2 Translation of Mobile mRNA

As indicated, numerous mobile transcripts including mRNA, si/miRNA and housekeeping non-coding RNAs such as ribosomal RNA (rRNA), transfer RNA (tRNA) and small nucleolar RNA (snoRNA) were detected in the phloem sap of pumpkin and *Brassica napus*. Several mRNAs were shown to be allocated via the phloem from source to sink tissues, however, we still lack conclusive insights where and when they are serving as templates for translation into proteins. The generally accepted textbook knowledge is that the conducting enucleated sieve elements are living cells in which no translational (ribosomal) activity takes place. On the other hand, a highly sensitive proteomic assay on the protein content of *C. maxima* phloem sap revealed the presence of relative high amounts of ribosomal factors. For example ribosomal subunits, initiation and elongation factors (Lin et al. 2009), all rRNAs, and most tRNAs (Zhang et al. 2009) were detected in the phloem. In theory, mobile mRNAs might be recognized by translational components within the sieve elements and, with the help of the phloem-allocated ribosomal factors and tRNAs, translated during transport. On the other hand, *in vitro* translation assays suggest the presence of a strong translatory inhibition activity in the phloem sap (Zhang et al. 2009). Isolated native phloem sap RNA in contrast to native RNA harvested from leaves inhibited effectively *in vitro* translation of viral RNA, plant mRNA, and firefly mRNA. According to tRNA fragmentation assays the unspecific inhibitory activity can most likely be assigned to tRNA halves accumulating in the phloem sap. Also the composition of the tRNAs detected in the phloem sap suggests a biased distribution and function. Some tRNAs are highly abundant (e.g.; Asp-tRNA), some are present in very low amounts (e.g.; Ala-tRNA), and some are not detected at all (e.g., isoforms of Ile-tRNA) [(Zhang et al. 2009); F.K., unpublished results]. In contrast to cell extracts from green tissue, it was not possible to detect any translational activity in phloem exudates used under various conditions (Zhang et al. 2009; Kragler 2010). Thus, although the presence of ribosomal factors in the phloem stream suggests otherwise, protein synthesis seems not to take place or is very limited in the sieve tubes. However, these studies are based on the mobile phloem protein content harvested by bleeding, and, thus, it might be possible that essential components facilitating translation were not present in the used extracts. It seems that a conclusive answer regarding translation within the phloem stream can only be given once we will be able to detect *in vivo* translation in sieve tubes, which seems currently not experimental feasible.

On the other hand several lines of evidence exist that mRNA is translated after transport. For example it was shown that *KNOTTED1* mRNA encoding a homeodomain transcription factor is translated after transfer via plasmodesmata into epidermal cells (Kim et al. 2005). Another mutant tomato homeodomain transcript named *Mouse-ears* (*Me*) was found to move over graft junction in tomato and to accumulate

in leaf primordia where the translational product seems to alter leaf shape (Kim et al. 2001). Also the already mentioned homeodomain transcript StBEL1 was shown to move from leaves towards roots where its translation product seems to increases the size of potato tubers (Banerjee et al. 2006). The agricultural important dominant acting mutant GIBBERELIC ACID INSENSITVE (GAI) protein lacking the DELLA domain is produced after transport from its mobile mRNA (Haywood et al. 2005). Otherwise a phenotype would not be established in wild-type scions. The transcript was shown to move via graft junctions and induce a phenotype in pumpkin, tomato, and Arabidopsis. It is relative easy to employ grafting techniques to establish that a particular RNA is mobile, however, the challenge will be to determine in which cell type the allocated transcript is allocated and, where it might be translated into an active protein.

6 Conclusions and Perspectives

Although we know that RNA molecules can move from cell to cell and are allocated via the phloem to distant organs a number of important questions remain to be answered. For example it is assumed, but not yet shown, that phloem mobile mRNAs are translated in target tissues. Mobile mRNAs could function in such that they bind to signaling proteins and facilitate their transfer over long distances. Alternatively mobile mRNAs could function by binding to and interfering with regulatory proteins in target tissues. Also the sole presence of distinct RNA molecules such as tRNA or other small RNAs in the phloem exudates does not necessarily mean that these have a signaling function. Isolation of phloem specific transcripts is a demanding experimental process, thus, some rare transcripts could be contaminants from the harvesting procedure used. Also some highly abundant phloem transcripts such as some tRNA molecules could just serve as source material recycled in sink tissues. Therefore it will be necessary to determine experimentally the assumed individual signaling function of phloem-allocated RNAs.

Nevertheless, it is well established that the meristem receives cues from mature leaves re-directing plant growth and developmental programs. It has been proposed and shown that the transport of specific RNAs to apical tissues plays a signaling role in the response to environmental stimuli. Here a still open question is why a number of phloem – allocated transcripts are expressed in both, the source and the sink tissues. On a hypothetical line a tissue receiving a mobile RNA (although it already produces this particular RNA) requires “priming” by an additional dosage of exogenous (imported) mRNA to reach a threshold level triggering a signaling cascade. Such a signaling mechanism has been suggested to occur during pathogen defense responses in plants (Conrath et al. 2006).

However, elucidating the signaling function of phloem-transported RNA is technically challenging. Once long – distance transport of a particular RNA is established, e.g., through grafting experiments, its non-cell-autonomous function must be distinguished from its cell-autonomous function. This may prove difficult, especially

when mobile RNAs are produced and functional within both, the source and the target tissues.

Over the last decades the concept that mRNA molecules act as signals for the coordination of developmental processes at the whole-plant level has gained some experimental support. Current studies are aimed at identifying the sites of synthesis of the macromolecules observed in the phloem stream and the sequence motifs (or chaperoning mechanisms) required for transcripts to enter, exit, and traffic long distances in sieve tubes. Other important questions relate to the translation of the mRNA, and the synthesis and possible movement and function of the respective proteins in target tissues. A special challenge will be to develop experimental systems in which phenotypic changes can be evaluated following specific inhibition of the long-distance movement of each transcript.

Acknowledgment(s) Work in the lab of R.R.-M. was supported by funds from CONACyT (Grant No. 50769) and UC-MEXUS; FK was supported by the Project P 19682-B03 from the Austrian Science Funds (FWF). Research in the lab of S.W. was supported by the Israel Science Foundation (ISF grant number 386/06).

References

Aki T, Shigyo M, Nakano R, Yoneyama T, Yanagisawa S (2008) Nano scale proteomics revealed the presence of regulatory proteins including three FT-Like proteins in phloem and xylem saps from rice. Plant Cell Physiol 49:767–790

Aniento F, Robinson DG (2005) Testing for endocytosis in plants. Protoplasma 226:3–11

Aoki K, Kragler F, Xoconostle-Cazares B, Lucas WJ (2002) A subclass of plant heat shock cognate 70 chaperones carries a motif that facilitates trafficking through plasmodesmata. Proc Natl Acad Sci USA 99:16342–16347

Aoki K, Suzui N, Fujimaki S, Dohmae N, Yonekura-Sakakibara K, Fujiwara T, Hayashi H, Yamaya T, Sakakibara H (2005) Destination-selective long-distance movement of phloem proteins. Plant Cell 17:1801–1814

Asano T, Masumura T, Kusano H, Kikuchi S, Kurita A, Shimada H, Kadowaki K (2002) Construction of a specialized cDNA library from plant cells isolated by laser capture microdissection: toward comprehensive analysis of the genes expressed in the rice phloem. Plant J 32: 401–408

Atkins CA, Smith PM, Rodriguez-Medina C (2011) Macromolecules in phloem exudates – a review. Protoplasma 248:165–172

Attaran E, Zeier TE, Griebel T, Zeier J (2009) Methyl salicylate production and jasmonate signaling are not essential for systemic acquired resistance in *Arabidopsis*. Plant Cell 21: 954–971

Banerjee AK, Chatterjee M, Yu Y, Suh SG, Miller WA, Hannapel DJ (2006) Dynamics of a mobile RNA of potato involved in a long-distance signaling pathway. Plant Cell 18:3443–3457

Banerjee AK, Lin T, Hannapel DJ (2009) Untranslated regions of a mobile transcript mediate RNA metabolism. Plant Physiol 151:1831–1843

Barnes A, Bale J, Constantinidou C, Ashton P, Jones A, Pritchard J (2004) Determining protein identity from sieve element sap in *Ricinus communis* L. by quadrupole time of flight (Q-TOF) mass spectrometry. J Exp Bot 55:1473–1481

Barton DA, Cole L, Collings DA, Liu DY, Smith PM, Day DA, Overall RL (2011) Cell-to-cell transport via the lumen of the endoplasmic reticulum. Plant J 66:806–817

Benitez-Alfonso Y, Cilia M, San Roman A, Thomas C, Maule A, Hearn S, Jackson D (2009) Control of Arabidopsis meristem development by thioredoxin-dependent regulation of intercellular transport. Proc Natl Acad Sci USA 106:3615–3620

Bivalkar-Mehla S, Vakharia J, Mehla R, Abreha M, Kanwar JR, Tikoo A, Chauhan A (2011) Viral RNA silencing suppressors (RSS): novel strategy of viruses to ablate the host RNA interference (RNAi) defense system. Virus Res 155:1–9

Blackman LM, Boevink P, Cruz SS, Palukaitis P, Oparka KJ (1998) The movement protein of cucumber mosaic virus traffics into sieve elements in minor veins of *Nicotiana clevelandii*. Plant Cell 10:525–537

Brioudes F, Thierry AM, Chambrier P, Mollereau B, Bendahmane M (2010) Translationally controlled tumor protein is a conserved mitotic growth integrator in animals and plants. Proc Natl Acad Sci USA 107:16384–16389

Buhtz A, Springer F, Chappell L, Baulcombe DC, Kehr J (2008) Identification and characterization of small RNAs from the phloem of *Brassica napus*. Plant J 53:739–749

Cevec M, Thibaudeau C, Plavec J (2010) NMR structure of the let-7 miRNA interacting with the site LCS1 of lin-41 mRNA from Caenorhabditis elegans. Nucleic Acids Res 38:7814–7821

Chatterjee M, Banerjee AK, Hannapel DJ (2007) A BELL1-like gene of potato is light activated and wound inducible. Plant Physiol 145:1435–1443

Chen H, Rosin FM, Prat S, Hannapel DJ (2003a) Interacting transcription factors from the three-amino acid loop extension superclass regulate tuber formation. Plant Physiol 132:1391–1404

Chen IH, Meng M, Hsu YH, Tsai CH (2003b) Functional analysis of the cloverleaf-like structure in the 3′ untranslated region of bamboo mosaic potexvirus RNA revealed dual roles in viral RNA replication and long distance movement. Virology 315:415–424

Chen H, Banerjee AK, Hannapel DJ (2004) The tandem complex of BEL and KNOX partners is required for transcriptional repression of ga20ox1. Plant J 38:276–284

Chisholm ST, Parra MA, Anderberg RJ, Carrington JC (2001) *Arabidopsis* RTM1 and RTM2 genes function in phloem to restrict long-distance movement of tobacco etch virus. Plant Physiol 127:1667–1675

Clark AM, Jacobsen KR, Bostwick DE, Dannenhoffer JM, Skaggs MI, Thompson GA (1997) Molecular characterization of a phloem-specific gene encoding the filament protein, phloem protein 1 (PP1), from *Cucurbita maxima*. Plant J 12:49–61

Conrath U, Beckers GJ, Flors V, Garcia-Agustin P, Jakab G, Mauch F, Newman MA, Pieterse CM, Poinssot B, Pozo MJ, Pugin A, Schaffrath U, Ton J, Wendehenne D, Zimmerli L, Mauch-Mani B (2006) Priming: getting ready for battle. Mol Plant Microbe Interact 19:1062–1071

Corbesier L, Vincent C, Jang S, Fornara F, Fan Q, Searle I, Giakountis A, Farrona S, Gissot L, Turnbull C, Coupland G (2007) FT protein movement contributes to long-distance signaling in floral induction of *Arabidopsis*. Science 316:1030–1033

Cosson P, Sofer L, Le QH, Leger V, Schurdi-Levraud V, Whitham SA, Yamamoto ML, Gopalan S, Le Gall O, Candresse T, Carrington JC, Revers F (2010) RTM3, which controls long-distance movement of potyviruses, is a member of a new plant gene family encoding a meprin and TRAF homology domain-containing protein. Plant Physiol 154:222–232

Crafts AS (1932) Phloem anatomy, exudation, and transport of organic nutrients in Cucurbits. Plant Physiol 7:i4–i225

Diaz-Pendon JA, Ding SW (2008) Direct and indirect roles of viral suppressors of RNA silencing in pathogenesis. Annu Rev Phytopathol 46:303–326

Diaz-Pendon JA, Li F, Li WX, Ding SW (2007) Suppression of antiviral silencing by cucumber mosaic virus 2b protein in *Arabidopsis* is associated with drastically reduced accumulation of three classes of viral small interfering RNAs. Plant Cell 19:2053–2063

Ding B (2009) The biology of viroid-host interactions. Annu Rev Phytopathol 47:105–131

Ding SW, Voinnet O (2007) Antiviral immunity directed by small RNAs. Cell 130:413–426

Dreher TW (2009) Role of tRNA-like structures in controlling plant virus replication. Virus Res 139:217–229

Dreher TW, Goodwin JB (1998) Transfer RNA mimicry among tymoviral genomic RNAs ranges from highly efficient to vestigial. Nucleic Acids Res 26:4356–4364

Dunoyer P, Lecellier CH, Parizotto EA, Himber C, Voinnet O (2004) Probing the microRNA and small interfering RNA pathways with virus-encoded suppressors of RNA silencing. Plant Cell 16:1235–1250

Dunoyer P, Brosnan CA, Schott G, Wang Y, Jay F, Alioua A, Himber C, Voinnet O (2010) An endogenous, systemic RNAi pathway in plants. EMBO J 29:1699–1712

Fisher DB, Wu Y, Ku MS (1992) Turnover of soluble proteins in the wheat sieve tube. Plant Physiol 100:1433–1441

Fu ZQ, Guo M, Jeong BR, Tian F, Elthon TE, Cerny RL, Staiger D, Alfano JR (2007) A type III effector ADP-ribosylates RNA-binding proteins and quells plant immunity. Nature 447:284–288

Gaupels F, Buhtz A, Knauer T, Deshmukh S, Waller F, van Bel AJ, Kogel KH, Kehr J (2008) Adaptation of aphid stylectomy for analyses of proteins and mRNAs in barley phloem sap. J Exp Bot 59:3297–3306

Genoves A, Pallas V, Navarro JA (2011) Contribution of topology determinants of a viral movement protein to its membrane association, intracellular traffic and viral cell-to-cell movement. J Virol 85(15):7797–7809

Gerdes HH, Bukoreshtliev NV, Barroso JF (2007) Tunneling nanotubes: a new route for the exchange of components between animal cells. FEBS Lett 581:2194–2201

Giavalisco P, Kapitza K, Kolasa A, Buhtz A, Kehr J (2006) Towards the proteome of *Brassica napus* phloem sap. Proteomics 6:896–909

Gilbertson RL, Lucas WJ (1996) How do viruses traffic on the vascular highway. Trends Plant Sci 1:260–268

Gisel A, Barella S, Hempel FD, Zambryski PC (1999) Temporal and spatial regulation of symplastic trafficking during development in Arabidopsis thaliana apices. Development 126:1879–1889

Gisel A, Hempel FD, Barella S, Zambryski P (2002) Leaf-to-shoot apex movement of symplastic tracer is restricted coincident with flowering in *Arabidopsis*. Proc Natl Acad Sci USA 99: 1713–1717

Gomez G, Pallas V (2004) A long-distance translocatable phloem protein from cucumber forms a ribonucleoprotein complex *in vivo* with Hop stunt viroid RNA. J Virol 78: 10104–10110

Gomez G, Torres H, Pallas V (2005) Identification of translocatable RNA-binding phloem proteins from melon, potential components of the long-distance RNA transport system. Plant J 41: 107–116

Gopinath K, Kao CC (2007) Replication-independent long-distance trafficking by viral RNAs in *Nicotiana benthamiana*. Plant Cell 19:1179–1191

Griffing LR, Villanueva MA, Taylor J, Moon S (1995) Methods in cell biology. Academic, San Diego, pp 109–121

Guenoune-Gelbart D, Elbaum M, Sagi G, Levy A, Epel BL (2008) Tobacco mosaic virus (TMV) replicase and movement protein function synergistically in facilitating TMV spread by lateral diffusion in the plasmodesmal desmotubule of *Nicotiana benthamiana*. Mol Plant Microbe Interact 21:335–345

Ham BK, Brandom JL, Xoconostle-Cazares B, Ringgold V, Lough TJ, Lucas WJ (2009) A polypyrimidine tract binding protein, pumpkin RBP50, forms the basis of a phloem-mobile ribonucleoprotein complex. Plant Cell 21(1):197–215

Hamilton AJ, Baulcombe DC (1999) A species of small antisense RNA in postranscriptional gene silencing in plants. Science 286:950–951

Harries P, Ding B (2011) Cellular factors in plant virus movement: at the leading edge of macromolecular trafficking in plants. Virology 411:237–243

Haywood V, Kragler F, Lucas WJ (2002) Plasmodesmata: pathways for protein and ribonucleoprotein signaling. Plant Cell 14(Suppl):S303–S325

Haywood V, Yu TS, Huang NC, Lucas WJ (2005) Phloem long-distance trafficking of GIBBERELLIC ACID-INSENSITIVE RNA regulates leaf development. Plant J 42:49–68

Hsieh LC, Lin SI, Shih AC, Chen JW, Lin WY, Tseng CY, Li WH, Chiou TJ (2009) Uncovering small RNA-mediated responses to phosphate deficiency in Arabidopsis by deep sequencing. Plant Physiol 151:2120–2132

Huang NC, Yu TS (2009) The sequences of Arabidopsis GA-INSENSITIVE RNA constitute the motifs that are necessary and sufficient for RNA long-distance trafficking. Plant J 59(6): 921–929

Imlau A, Truernit E, Sauer N (1999) Cell-to-cell and long-distance trafficking of the green fluorescent protein in the phloem and symplastic unloading of the protein into sink tissues. Plant Cell 11:309–322

Ivashikina N, Deeken R, Ache P, Kranz E, Pommerrenig B, Sauer N, Hedrich R (2003) Isolation of AtSUC2 promoter-GFP-marked companion cells for patch-clamp studies and expression profiling. Plant J 36:931–945

Jaeger KE, Wigge PA (2007) FT protein acts as a long-range signal in Arabidopsis. Curr Biol 17:1050–1054

Kaido M, Funatsu N, Tsuno Y, Mise K, Okuno T (2011) Viral cell-to-cell movement requires formation of cortical punctate structures containing Red clover necrotic mosaic virus movement protein. Virology 413:205–215

Kanehira A, Yamada K, Iwaya T, Tuswamoto R, Kasai A, Nakazono M, Harada T (2010) Apple phloem cells contain some mRNAs transported over long distances. Tree Genet Genomes 6:635–642

Kehr J, Buhtz A (2008) Long distance transport and movement of RNA through the phloem. J Exp Bot 59:85–92

Kehr J, Rep M (2007) Protein extraction from xylem and phloem sap. Methods Mol Biol 355: 27–35

Kim M, Canio W, Kessler S, Sinha N (2001) Developmental changes due to long-distance movement of a homeobox fusion transcript in tomato. Science 293:287–289

Kim JY, Rim Y, Wang J, Jackson D (2005) A novel cell-to-cell trafficking assay indicates that the KNOX homeodomain is necessary and sufficient for intercellular protein and mRNA trafficking. Genes Dev 19:788–793

Kim SH, Macfarlane S, Kalinina NO, Rakitina DV, Ryabov EV, Gillespie T, Haupt S, Brown JW, Taliansky M (2007a) Interaction of a plant virus-encoded protein with the major nucleolar protein fibrillarin is required for systemic virus infection. Proc Natl Acad Sci USA 104:11115–11120

Kim SH, Ryabov EV, Kalinina NO, Rakitina DV, Gillespie T, MacFarlane S, Haupt S, Brown JW, Taliansky M (2007b) Cajal bodies and the nucleolus are required for a plant virus systemic infection. EMBO J 26:2169–2179

Kragler F (2010) RNA in the phloem: a crisis or a return on investment? Plant Sci 178:99–104

Kragler F, Lucas WJ, Monzer J (1998) Plasmodesmata: dynamics, domains and patterning. Ann Bot (London) 81:1–10

Lee JY, Yoo BC, Rojas MR, Gomez-Ospina N, Staehelin LA, Lucas WJ (2003) Selective trafficking of non-cell-autonomous proteins mediated by NtNCAPP1. Science 299:392–396

Leontis NB, Westhof E (2001) Geometric nomenclature and classification of RNA base pairs. RNA 7:499–512

Levy A, Erlanger M, Rosenthal M, Epel BL (2007) A plasmodesmata-associated beta-1,3-glucanase in *Arabidopsis*. Plant J 49:669–682

Li C, Zhang K, Zeng X, Jackson S, Zhou Y, Hong Y (2009) A cis element within flowering locus T mRNA determines its mobility and facilitates trafficking of heterologous viral RNA. J Virol 83:3540–3548

Lifschitz E, Eviatar T, Rozman A, Shalit A, Goldshmidt A, Amsellem Z, Alvarez JP, Eshed Y (2006) The tomato FT ortholog triggers systemic signals that regulate growth and flowering and substitute for diverse environmental stimuli. Proc Natl Acad Sci USA 103:6398–6403

Lin JW, Ding MP, Hsu YH, Tsai CH (2007a) Chloroplast phosphoglycerate kinase, a gluconeogenetic enzyme, is required for efficient accumulation of Bamboo mosaic virus. Nucleic Acids Res 35:424–432

Lin MK, Belanger H, Lee YJ, Varkonyi-Gasic E, Taoka K, Miura E, Xoconostle-Cazares B, Gendler K, Jorgensen RA, Phinney B, Lough TJ, Lucas WJ (2007b) FLOWERING LOCUS T protein may act as the long-distance florigenic signal in the cucurbits. Plant Cell 19: 1488–1506

Lin MK, Lee YJ, Lough TJ, Phinney BS, Lucas WJ (2009) Analysis of the pumpkin phloem proteome provides insights into angiosperm sieve tube function. Mol Cell Proteomics 8:343–356

Lough TJ, Lucas WJ (2006) Integrative plant biology: role of phloem long-distance macromolecular trafficking. Annu Rev Plant Biol 57:203–232

Lough TJ, Lee RH, Emerson SJ, Forster RL, Lucas WJ (2006) Functional analysis of the 5′ untranslated region of potexvirus RNA reveals a role in viral replication and cell-to-cell movement. Virology 351:455–465

Lucas WJ (2006) Plant viral movement proteins: agents for cell-to-cell trafficking of viral genomes. Virology 344:169–184

Lucas WJ, Ding B, Van Der Schoot C (1993) Tansley review No. 58: plasmodesmata and the supracellular nature of plants. New Phytol 125:435–476

Lucas WJ, Ham BK, Kim JY (2009) Plasmodesmata – bridging the gap between neighboring plant cells. Trends Cell Biol 19:495–503

Maldonado AM, Doerner P, Dixon RA, Lamb CJ, Cameron RK (2002) A putative lipid transfer protein involved in systemic resistance signalling in *Arabidopsis*. Nature 419:399–403

Malter D, Wolf S (2011) Melon phloem-sap proteome: developmental control and response to viral infection. Protoplasma 248:217–224

Martin A, Adam H, Diaz-Mendoza M, Zurczak M, Gonzalez-Schain ND, Suarez-Lopez P (2009) Graft-transmissible induction of potato tuberization by the microRNA miR172. Development 136:2873–2881

Mathieu J, Warthmann N, Kuttner F, Schmid M (2007) Export of FT protein from phloem companion cells is sufficient for floral induction in *Arabidopsis*. Curr Biol 17:1055–1060

Maule AJ (2008) Plasmodesmata: structure, function and biogenesis. Curr Opin Plant Biol 11:680–686

Miller WA, Wang Z, Treder K (2007) The amazing diversity of cap-independent translation elements in the 3′-untranslated regions of plant viral RNAs. Biochem Soc Trans 35:1629–1633

Nakazono M, Qiu F, Borsuk LA, Schnable PS (2003) Laser-capture microdissection, a tool for the global analysis of gene expression in specific plant cell types: identification of genes expressed differentially in epidermal cells or vascular tissues of maize. Plant Cell 15:583–596

Niehl A, Heinlein M (2011) Cellular pathways for viral transport through plasmodesmata. Protoplasma 248:75–99

Noll GA, Fontanellaz ME, Ruping B, Ashoub A, van Bel AJ, Fischer R, Knoblauch M, Prufer D (2007) Spatial and temporal regulation of the forisome gene for1 in the phloem during plant development. Plant Mol Biol 65:285–294

Olsen AN, Ernst HA, Leggio LL, Skriver K (2005) NAC transcription factors: structurally distinct, functionally diverse. Trends Plant Sci 10:79–87

Omid A, Keilin T, Glass A, Leshkowitz D, Wolf S (2007) Characterization of phloem-sap transcription profile in melon plants. J Exp Bot 58:3645–3656

Oparka KJ (2004) Getting the message across: how do plant cells exchange macromolecular complexes? Trends Plant Sci 9:33–41

Oparka KJ, Roberts AG, Boevink P, Santa Cruz S, Roberts IM, Kotlizky G, Sauer N, Epel B (1999a) Simple, but not branched, plasmodesmata allow the nonspecific trafficking of proteins in developing tobacco leaves. Cell 97:743–754

Oparka KJ, Roberts AG, Boevink P, Santa Cruz S, Roberts I, Pradel KS, Imlau A, Kotlizky G, Sauer N, Epel B (1999b) Simple, but not branched, plasmodesmata allow the nonspecific trafficking of proteins in developing tobacco leaves [see comments]. Cell 97:743–754

Pant BD, Buhtz A, Kehr J, Scheible WR (2008) MicroRNA399 is a long-distance signal for the regulation of plant phosphate homeostasis. Plant J 53:731–738

Peng J, Carol P, Richards DE, King KE, Cowling RJ, Murphy GP, Harberd NP (1997) The *Arabidopsis* GAI gene defines a signaling pathway that negatively regulates gibberellin responses. Genes Dev 11:3194–3205

Qi Y, Pelissier T, Itaya A, Hunt E, Wassenegger M, Ding B (2004) Direct role of a viroid RNA motif in mediating directional RNA trafficking across a specific cellular boundary. Plant Cell 16:1741–1752

Rajamaki ML, Valkonen JP (2009) Control of nuclear and nucleolar localization of nuclear inclusion protein a of picorna-like Potato virus A in *Nicotiana* species. Plant Cell 21: 2485–2502

Requena A, Simon-Buela L, Salcedo G, Garcia-Arenal F (2006) Potential involvement of a cucumber homolog of phloem protein 1 in the long-distance movement of cucumber mosaic virus particles. Mol Plant Microbe Interact 19:734–746

Rinne PL, van der Schoot C (1998) Symplasmic fields in the tunica of the shoot apical meristem coordinate morphogenetic events. Development 125:1477–1485

Rinne PL, Kaikuranta PM, van der Schoot C (2001) The shoot apical meristem restores its symplasmic organization during chilling-induced release from dormancy. Plant J 26:249–264

Rinne PL, Welling A, Vahala J, Ripel L, Ruonala R, Kangasjarvi J, van der Schoot C (2011) Chilling of dormant buds hyperinduces FLOWERING LOCUS T and recruits GA-inducible 1,3-beta-glucanases to reopen signal conduits and release dormancy in *Populus*. Plant Cell 23:130–146

Rodriguez-Medina C, Atkins CA, Mann AJ, Jordan ME, Smith PM (2011) Macromolecular composition of phloem exudate from white lupin (*Lupinus albus* L). BMC Plant Biol 11:36

Rosin FM, Hart JK, Horner HT, Davies PJ, Hannapel DJ (2003) Overexpression of a knotted-like homeobox gene of potato alters vegetative development by decreasing gibberellin accumulation. Plant Physiol 132:106–117

Ruiz-Medrano R, Xoconostle-Cazares B, Lucas WJ (1999) Phloem long-distance transport of CmNACP mRNA: implications for supracellular regulation in plants. Development 126: 4405–4419

Ruiz-Medrano R, Xoconostle-Cazares B, Lucas WJ (2001) The phloem as a conduit for interorgan communication. Curr Opin Plant Biol 4:202–209

Ruiz-Medrano R, Xoconostle-Cazares B, Kragler F (2004) The plasmodesmatal transport pathway for homeotic proteins, silencing signals and viruses. Curr Opin Plant Biol 7:641–650

Ruiz-Medrano R, Hinojosa-Moya JJ, Xoconostle-Cázares B, Lucas WJ (2007) Influence of cucumber mosaic virus infection on the mRNA population present in the phloem translocation stream of pumpkin plants. Funct Plant Biol 34:292–301

Rustom A, Saffrich R, Markovic I, Walther P, Gerdes HH (2004) Nanotubular highways for intercellular organelle transport. Science 303:1007–1010

Sambade A, Brandner K, Hofmann C, Seemanpillai M, Mutterer J, Heinlein M (2008) Transport of TMV movement protein particles associated with the targeting of RNA to plasmodesmata. Traffic 9:2073–2088

Saxton MJ, Breidenbach RW (1988) Receptor-mediated endocytosis in plants is energetically possible. Plant Physiol 86:993–995

Shin HI, Cho NJ, Cho TJ (2008) Role of 5′-UTR hairpins of the Turnip yellow mosaic virus RNA in replication and systemic movement. BMB Rep 41:778–783

Simon-Buela L, Garcia-Arenal F (1999) Virus particles of cucumber green mottle mosaic tobamovirus move systemically in the phloem of infected cucumber plants. Mol Plant Microbe Interact 12:112–118

Streitner C, Danisman S, Wehrle F, Schoning JC, Alfano JR, Staiger D (2008) The small glycine-rich RNA binding protein AtGRP7 promotes floral transition in *Arabidopsis thaliana*. Plant J 56:239–250

Sudarshana MR, Wang HL, Lucas WJ, Gilbertson RL (1998) Dynamics of bean dwarf mosaic geminivirus cell-to-cell and long-distance movement in Phaseolus vulgaris revealed, using the green fluorescent protein. Mol Plant Microbe Interact 11:277–291

Takeda R, Petrov AI, Leontis NB, Ding B (2011) A three-dimensional RNA motif in Potato spindle tuber viroid mediates trafficking from palisade mesophyll to spongy mesophyll in Nicotiana benthamiana. Plant Cell 23:258–272

Tamaki S, Matsuo S, Wong HL, Yokoi S, Shimamoto K (2007) Hd3a protein is a mobile flowering signal in rice. Science 316:1033–1036

Tangl E (1880) Ueber offene Communicationen zwischen den Zellen des Endosperms einiger Samen. Jahrbücher für Wissenschaftliche Botanik 12:170–190

Tilsner J, Amari K, Torrance L (2011) Plasmodesmata viewed as specialised membrane adhesion sites. Protoplasma 248:39–60

Turgeon R, Wolf S (2009) Phloem transport: cellular pathways and molecular trafficking. Annu Rev Plant Biol 60:207–221

Ueki S, Citovsky V (2005) Control improves with age: intercellular transport in plant embryos and adults. Proc Natl Acad Sci USA 102:1817–1818

Vilaine F, Palauqui JC, Amselem J, Kusiak C, Lemoine R, Dinant S (2003) Towards deciphering phloem: a transcriptome analysis of the phloem of *Apium graveolens*. Plant J 36:67–81

Vlot AC, Klessig DF, Park SW (2008a) Systemic acquired resistance: the elusive signal(s). Curr Opin Plant Biol 11:436–442

Vlot AC, Liu PP, Cameron RK, Park SW, Yang Y, Kumar D, Zhou F, Padukkavidana T, Gustafsson C, Pichersky E, Klessig DF (2008b) Identification of likely orthologs of tobacco salicylic acid-binding protein 2 and their role in systemic acquired resistance in *Arabidopsis thaliana*. Plant J 56:445–456

Walz C, Giavalisco P, Schad M, Juenger M, Klose J, Kehr J (2004) Proteomics of curcurbit phloem exudate reveals a network of defence proteins. Phytochemistry 65:1795–1804

Weinl C, Marquardt S, Kuijt SJ, Nowack MK, Jakoby MJ, Hulskamp M, Schnittger A (2005) Novel functions of plant cyclin-dependent kinase inhibitors, ICK1/KRP1, can act non-cell-autonomously and inhibit entry into mitosis. Plant Cell 17:1704–1722

Whitham SA, Anderberg RJ, Chisholm ST, Carrington JC (2000) Arabidopsis RTM2 gene is necessary for specific restriction of tobacco etch virus and encodes an unusual small heat shock-like protein. Plant Cell 12:569–582

Xoconostle-Cazares B, Xiang Y, Ruiz-Medrano R, Wang HL, Monzer J, Yoo BC, McFarland KC, Franceschi VR, Lucas WJ (1999) Plant paralog to viral movement protein that potentiates transport of mRNA into the phloem. Science 283:94–98

Xu XM, Jackson D (2010) Lights at the end of the tunnel: new views of plasmodesmal structure and function. Curr Opin Plant Biol 13:684–692

Yoo B-C, Kragler F, Varkonyi-Gasic E, Haywood V, Archer-Evans S, Lee Y-M, Lough TJ, Lucas WJ (2004) A systemic small RNA signaling system in plants. Plant Cell 16:1979–2000

Zavaliev R, Ueki S, Epel BL, Citovsky V (2011) Biology of callose (beta-1,3-glucan) turnover at plasmodesmata. Protoplasma 248:117–130

Zhang S, Sun L, Kragler F (2009) The phloem-delivered RNA pool contains small noncoding RNAs and interferes with translation. Plant Physiol 150:378–387

Zhang B, Tolstikov V, Turnbull C, Hicks LM, Fiehn O (2010) Divergent metabolome and proteome suggest functional independence of dual phloem transport systems in cucurbits. Proc Natl Acad Sci USA 107:13532–13537

Zhong X, Tao X, Stombaugh J, Leontis N, Ding B (2007) Tertiary structure and function of an RNA motif required for plant vascular entry to initiate systemic trafficking. EMBO J 26:3836–3846

Zhong X, Archual AJ, Amin AA, Ding B (2008) A genomic map of viroid RNA motifs critical for replication and systemic trafficking. Plant Cell 20:35–47

Zhu Y, Green L, Woo YM, Owens R, Ding B (2001) Cellular basis of potato spindle tuber viroid systemic movement. Virology 279:69–77

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F. Kragler and M. Hülskamp (eds.), *Short and Long Distance Signaling*,
Advances in Plant Biology 3, DOI 10.1007/978-1-4419-1532-0,