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Short and Long Distance Signaling

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Short and Long Distance Signaling

Foreword by William J. Lucas

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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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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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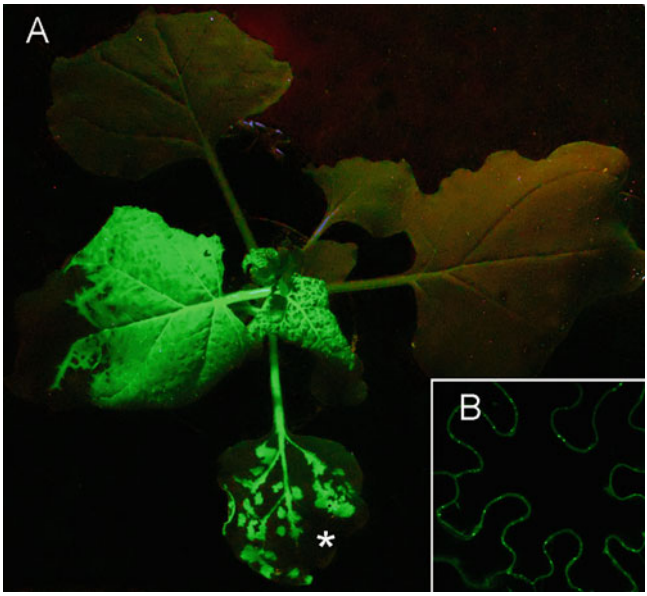

Fig. 1.1 TMV spread and the localization of MP to PD (**a**) GFP-tagged TMV infection in *Nicotiana benthamiana*. After local inoculation of one leaf (labelled with an asterisk) infection spreads cell-to-cell within the inoculated leaf and systemically into other leaves. (**b**) In infected cells, MP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