Plant Cell Monogr (7)
E. Waigmann · M. Heinlein: Viral Transport in Plants
DOI 10.1007/7089_2006_100/Published online: 1 February 2007

Tobacco Mosaic Virus – a Model for Macromolecular Cell-to-Cell Spread

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Abstract Macromolecular cell-to-cell transport in plants occurs through complex intercellular channels, the plasmodesmata. Plant viruses pirate these natural plant communication channels for their own spread from an infected cell to a neighboring healthy cell. Viral movement proteins are the major agents in promoting this process. *Tobacco mosaic virus* is the most extensively studied plant virus and can therefore be viewed as a model system for cell-to-cell transport. In this chapter we summarize knowledge about mechanistic properties of the movement protein of *Tobacco mosaic virus* and discuss the potential involvement of other viral and cellular components in the intercellular transport process.

1 Introduction

Tobacco mosaic virus (TMV), the first virus ever known, was detected more than a century ago and has served as a model for ground-braking research in virology and molecular biology. TMV was the first virus visualized in the electron microscope and henceforth was intensely studied to gather information that could also be applied to other viruses. As a consequence of these intense studies, the coat protein of the virus was the second protein fully sequenced (after insulin). Moreover, TMV RNA became the first plant viral genome to be sequenced completely (Goelet et al. 1982) and the second plant viral RNA (vRNA) that was completely cloned as cDNA. TMV also served as model to establish engineered resistance in transgenic plants (Powell-Abel et al. 1986). In 1987, the 30 kDa protein of the virus was the first protein to be functionally identified as a movement protein (TMV MP; Deom et al. 1987) and its requirement for the cell-to-cell progression of virus infection through plasmodesmata became established. Although in more recent years it became clear that most, if not all, plant viruses encode such proteins, the MP of TMV has remained at the forefront of research aimed at understanding the mechanism by which a virus moves from cell to cell. Moreover, since TMV spreads its RNA genome through plasmodesmata in non-virion form,

it serves as a model for studying the cellular mechanism by which RNA and protein macromolecules target plasmodesmata. Macromolecular communication through plasmodesmata has essential roles during plant development as well as in the orchestration of plant defense responses, and involves the controlled cell-to-cell and systemic trafficking of a whole range of RNA and protein macromolecules, including non-cell-autonomous transcription factors, RNA-based silencing signals, and messenger RNAs (Haywood et al. 2002; Heinlein 2002; Heinlein and Epel 2004; Huang et al. 2005; Jackson 2005; Kim 2005; Lucas et al. 2001; Tzfira et al. 2000; Wu et al. 2002; Yoo et al. 2004). Thus, research to determine the mechanism by which TMV MP and RNA target plasmodesmata and spread between cells holds the promise for new insights into the mechanisms involved in intercellular communication and plant development as well as in viral disease. Current and further studies also include analysis of the interface between virus and plant defense responses, such as RNA silencing. Given that viruses encode proteins that function as silencing suppressors and that several of these proteins have been previously implicated in movement, it will be important to address the question of whether virus movement is exclusively due to the interaction of the virus with cellular transport processes or whether movement may also result as a manifestation of successful suppression of defense responses by the virus (Carrington 1999). Although, so far, other viral systems have been more successful in contributing to the analysis of silencing pathway components and viral silencing suppressors (see chapter on silencing by Thomas Hohn, in this volume), the combination of established TMV cell biology with the cellular analysis of silencing pathway components will likely contribute important insights into those plant:virus interactions in the future.

In this book chapter we summarize our current understanding of TMV cell-to-cell movement and describe the role of various viral proteins and host factors in this process. Clearly, the movement protein TMV MP is the major actor and consequently the central part of this chapter is devoted to its manifold functions. Nonetheless, the emerging contributions of other viral proteins such as the coat protein and the replicase, as well as the contributions of host factors, whose number is steadily increasing, are also discussed.

2
Plasmodesmata: Structure and Composition

For cell-to-cell spread, *Tobacco mosaic virus* pirates plasmodesmata (Oparka 2005), complex cell-to-cell communication channels in the plant cell wall that provide cytoplasmic continuity between adjacent cells. The ultrastructure of plasmodesmata has been defined by numerous electron microscopy studies (for example Botha 1992; Ding et al. 1992). The plasma membrane delineates the plasmodesmal pore, which is traversed in its axial center by the ap-

pressed membrane of the endoplasmic reticulum (ER) termed desmotubule. Plasma membrane and desmotubule are densely covered with globular particles (Ding et al. 1992) that segment the region between plasma membrane and desmotubule, the cytoplasmic sleeve, into eight to ten channels (Ding et al. 1992). These channels are considered to function as conduits for diffusion of molecules between cells. The cell wall or neck region surrounding the plasmodesmal orifices is speculated to participate in the control of molecular traffic through the channel (Olesen 1979; Overall and Blackman 1996; White et al. 1994).

Unlike the ultrastructure, the molecular composition of plasmodesmata is poorly defined. Several strategies have been employed to elucidate plasmodesmal composition:

- Direct biochemical approaches aimed at extracting plasmodesmal proteins (for example, Epel et al. 1995; Kishi-Kaboshi et al. 2005; Turner et al. 1994)
- Use of antibodies against known proteins suspected to reside at plasmodesmata
- Expression of cDNA libraries to express random proteins fused to GFP and subsequent selection for punctuate localization of fusion proteins to the cell wall, a pattern indicating plasmodesmal localization
- Screening for host factors interacting with viral movement proteins

Here, we will focus on recent findings, as a more complete picture has been presented elsewhere (for example, Aaziz et al. 2001; Waigmann et al. 2004).

The biochemical approach to purifying plasmodesmal proteins from plant extracts led to identification of a 41 kDa protein within mesocotyl cell wall fractions of *Zea mays* (Epel et al. 1996b) and in plasmodesmal protein-enriched fractions from *Arabidopsis* (Sagi et al. 2005). Recently, this protein was shown to represent a member of the class 1 reversibly glycosylated polypeptides (^{C1}RGP) protein family (Sagi et al. 2005). ^{C1}RGPs localize to the Golgi and plasmodesmata, suggesting that these proteins are secretory proteins that are delivered to plasmodesmata via the Golgi apparatus. Within plasmodesmata, ^{C1}RGPs might be attached to the plasma membrane facing the cytoplasmic sleeve and could thus be involved in establishing the size exclusion limit of plasmodesmata (Sagi et al. 2005). Biochemical enrichment of a plasmodesmal protein fraction also led to purification of a casein kinase I (CKI) activity from *N. tabacum* suspension culture cells that was able to phosphorylate TMV MP, in line with previous findings that show TMV MP phosphorylation *in planta* (Lee et al. 2005; see also Sect. 3.4). Since the corresponding *N. tabacum* gene could not be identified conclusively, the 13 CKI-like (CKL) genes from *Arabidopsis* were analyzed for subcellular localization. Indeed, in transient expression assays in tobacco leaves, CKL6 co-localized with TMV MP in cell wall-associated puncta, suggesting its plasmodesmal localization (Lee et al. 2005).

The antibody-based strategy pinpointed cytoskeletal components (Overall et al. 2000; Reichelt et al. 1999; White et al. 1994) and calcium-binding proteins such as centrin and calreticulin as plasmodesmal proteins (Baluska et al. 1999; Blackman et al. 1999) Interestingly, calreticulin, an ER-resident protein reported to accumulate in plasmodesmata of the maize root apex (Baluska et al. 1999) was also shown to interact with TMV MP (Chen et al. 2005; see also Sect. 3.4).

Viral expression of a GFP-fused cDNA library derived from *N. benthamiana* roots revealed 12 GFP fusion proteins (PD01-12) potentially localizing to plasmodesmata (Escobar et al. 2003). Plasmodesmal localization was confirmed for one of these proteins, PD01, by immuno gold labeling with antiserum against GFP. Since some of these proteins might localize to plasmodesmata in response to the viral infection strategy used for their transient expression, only additional experiments can clarify the significance of these results (Escobar et al. 2003).

How far have we come on the road towards complete knowledge of plasmodesmal composition? Considering that plasmodesmata are dynamic entities, flexible in structure and permeability, plasmodesmal complexity may be similar to that of a nuclear pore with its more than 100 structural proteins. If so, the number of known plasmodesmal components represents only the beginning of a list that awaits completion in the future.

3
TMV MP, a Protein of Manifold Qualities

3.1
TMV MP Structure and Single-Stranded Nucleic Acid Binding

TMV MP consists of 268 amino acids and is the type member of a large group of viral MPs with a molecular mass of approximately 30 kDa, termed the "30K superfamily". The members of the 30K superfamily share only low sequence similarity (Koonin et al. 1991, Melcher 1990) but may share a common three-dimensional structure. Unfortunately, not even for the intensely studied TMV MP, has a three-dimensional structure been elucidated by X-ray crystallography, which is primarily due to the fact that recombinant TMV MP is not readily soluble and has a strong tendency to aggregate. Thus, structural models of TMV MP rely on bioinformatic predictions or on a combination of CD spectroscopy and biochemical methods. Bioinformatic prediction based on the family consensus sequences of MPs from 18 families revealed a common core consisting of four α-helices and seven β-elements flanked by variable N- and C-terminal domains (Melcher 2000). The N-terminal region was generally variable in range and structure, whereas the C-terminal region was predicted to be a predominantly random coil. For TMV MP, it has been shown

that deletion or mutation of sequences within the common core region affect the ability of the protein to bind RNA or to bind microtubules. This region of the protein also contains predicted transmembrane domains and functionally tested determinants for the interaction of the protein with microtubules and its targeting to plasmodesmata (see Sect. 3.3 and Fig. 1). Intramolecular complementation of a dysfunctional Pro81Ser amino acid exchange mutation by distant Thr104Ile and Arg167Lys exchange mutations indicates that the core region folds into a compact tertiary structure which allows distant primary sequence and secondary structure elements to interact (Deom and He 1997; Boyko et al. 2002, Fig. 1). The C-terminal random coil structure of the protein is dispensable for cell-to-cell movement (Berna et al. 1991), and may act as a flexible tail that regulates access to those functional domains. In line with this assumption, the carboxyterminus of TMV MP harbors three phosphorylation sites (Citovsky et al. 1993, Fig. 1), which have been shown to play a role in regulation of TMV MP subcellular localization and function in *N. tabacum* (Trutnyeva et al. 2005; Waigmann et al. 2000; see also Sect. 3.4)

Experimental evidence derived from studies using purified recombinant TMV MP (Brill et al. 2000) is in good agreement with the predicted core structure. CD spectroscopy of urea- and SDS-solubilized TMV MP demonstrated

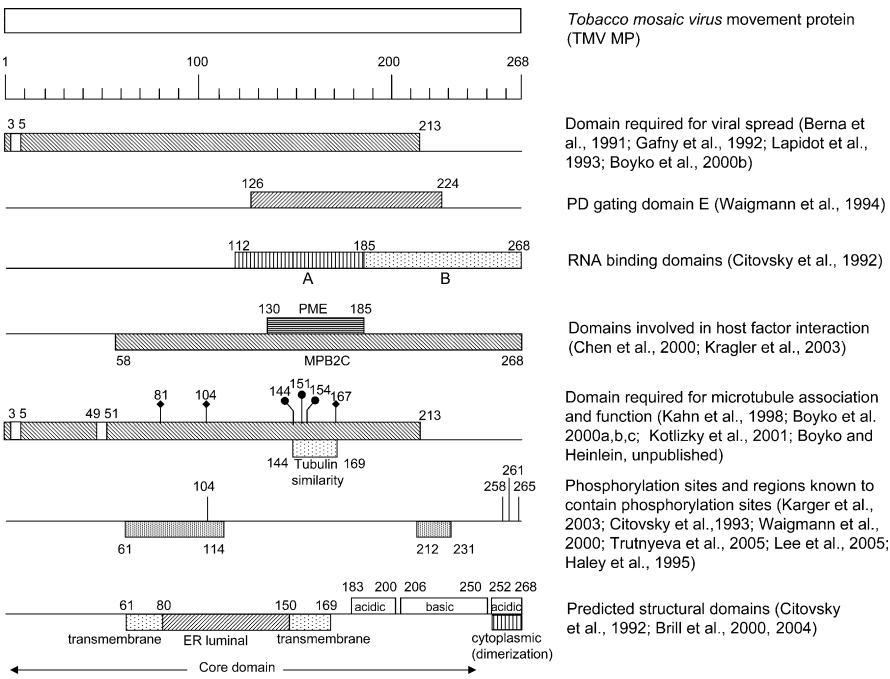

Fig. 1 Overview of TMV MP regions involved in various functions of the protein. For detailed description, see text; • ts mutations in MT association, ♦ required for function

a high α-helical content suggesting an ordered tertiary structure. Trypsin digestion followed by mass spectroscopy revealed two cleavage resistant, highly hydrophobic domains which might constitute two putative membrane spanning-regions. These regions could cause the TMV MP to behave as an integral membrane protein in biochemical fractionation experiments (Reichel and Beachy 1999). The C-terminal part of TMV MP (amino acids 250–268) was highly sensitive to trypsin treatment, indicating that this part of the protein is accessible to the solvent. Analytical ultracentrifugation suggested that the TMV MP forms homodimers. Upon trypsin digestion, dimers were converted into monomers, suggesting that the C-terminal part of TMV MP is involved in dimerization (Brill et al. 2004). In a topological model, the two potential transmembrane domains of TMV MP span the ER membrane, thereby imposing a U-like conformation onto the protein, whereas the short N- and longer C-terminal regions are exposed to the cytosol (N_{cyt}-C_{cyt} topology; Brill et al. 2000); the C-termini link two adjacent molecules into a dimer, potentially via charge–charge interactions (Brill et al. 2004).

MPs of other viral groups that are not part of the 30K superfamily may share structural features with TMV MP. For example, carmoviruses encode two small proteins, p7 and p9, involved in cell-to-cell movement of the virus (Hacker et al. 1992; Li et al. 1998). *Carnation mottle virus* (CarMV) p7 is a soluble protein that binds to RNA, whereas CarMV p9 is an intrinsic membrane protein with two transmembrane helices imposing a U-like conformation onto the protein. The short N- and long C-terminus of p9 are exposed to the cytosol enabling the C-terminal region to interact with CarMV p7, thereby providing membrane localization to the p7-RNA complex (Sauri et al. 2005; Vilar et al. 2002). The topology proposed for the CarMV p7/p9 is remarkably similar to that proposed for TMV MP, suggesting that despite sequence differences, some conserved structural and topological elements exist that may be important for movement protein function.

One of several functional hallmarks of MPs is their ability to bind nucleic acids, a feature first shown for TMV MP. TMV MP binds single-stranded (ss) RNA and DNA in a strong, cooperative, and sequence-non-specific manner (Citovsky et al. 1990, 1992). Mutational analysis of TMV MP revealed two independently active binding domains (Fig. 1) located between amino acid positions 112–185 (binding domain A) and 186–268 (binding domain B) of this 268-residue long MP (Citovsky et al. 1992). Nucleic acid binding domains have been identified in many viral MPs (for review see Waigmann et al. 2004); however, no conserved amino acid motif was revealed. Interestingly, nearly all MPs have only one RNA binding domain, the only exceptions being TMV MP and *apple chlorotic leaf spot trichovirus* (ACLSV) MP, which is also characterized by two adjacent independently active RNA binding domains (Isogai and Yoshikawa 2005).

What may be the role of MP binding to nucleic acids? The ultimate function of MPs is to mediate transport of the viral genome from cell-to-cell via

the size restrictive channels of plasmodesmata, thereby enabling the infection to spread within a plant. Binding of MP to ss nucleic acid is a direct means of physically associating the MP to its cognate viral genome. Based on electron microscopy and atomic force microscopy data, recombinant TMV MP seems to bind ss nucleic acids in a "bead-on-the-string"-like fashion thereby forming extended ribonucleoprotein particles (vRNP; Citovsky et al. 1992; Kiselyova et al. 2001) compatible with the size exclusion limit of dilated plasmodesmata (Waigmann et al. 1994). Thus, complexes between MPs and viral genomes most likely represent intermediates of the movement process, shaped in a transportable form. Interestingly, those complexes are non-translatable and non-replicable in vitro and in isolated plant protoplasts (Karpova et al. 1997), which indicates that they are diverted from translation and replication and dedicated to cell-to-cell movement. Inhibition of translation and replication of TMV RNA is eliminated in plant tissues (Karpova et al. 1997), potentially following TMV MP phosphorylation after passage through plasmodesmata (Karpova et al. 1999) by a cell wall-associated protein kinase (see Sect. 3.4; Citovsky et al. 1993, Lee et al. 2005).

3.2
Subcellular Localization of TMV MP

The cell-to-cell transport of the proposed vRNP is likely to depend on specific mechanisms that target and align the complex to plasmodesmata. Indeed, plasmodesmata in young (sink) leaves are characterized by a large size exclusion limit (SEL) and are able to transport protein macromolecules; yet MPs are required for virus spread (Oparka et al. 1999) suggesting that vRNA movement depends on MP and probably on additional MP-interacting host functions. First attempts to localize TMV MP in infected cells and to identify intercellular targets of the protein employed immuno-electron microscopy (Atkins et al. 1991; Meshi et al. 1992; Moore et al. 1992; Tomenius et al. 1987) and biochemical fractionation using virus-infected tissues and MP-transgenic plants (Deom et al. 1990; Moore et al. 1992; Moser et al. 1988). These studies indicated the presence of TMV MP in cell wall- and plasma membrane-rich fractions as well as in branched plasmodesmata. More recent biochemical analyses suggest that the TMV MP is associated with the microsomal fraction as an integral membrane protein (Reichel and Beachy 1998).

More insight into in vivo associations of TMV MP with host cell structures could be achieved by the analysis of infection sites caused by CP-deficient TMV derivatives expressing the TMV MP as a functional GFP fusion protein (TMV-MP:GFP; Heinlein et al. 1995, 1998; Epel et al. 1996a; Padgett et al. 1996). Infection in leaves of susceptible *Nicotiana* species with TMV-MP:GFP produces radially expanding fluorescent infection sites. The leading edge of these sites reflects the front of the spreading infection, as was shown by experiments involving manual incisions to the leaf lamina. These incisions, if

made just beyond the leading edge of fluorescence, interrupted further spread of infection but allowed further spread if made just behind the leading fluorescent cells (Oparka et al. 1997). These results also indicated that in tissue infected with TMV-MP:GFP visible amounts of MP:GFP or of MP:GFP-vRNA complexes occur only within the limits of infected cells and do not move cell-to-cell far ahead of infection. In contrast, in the absence of infection, when TMV MP was either microinjected (Waigmann and Zambryski 1995; Waigmann et al. 1994) or transiently expressed (Kotlizky et al. 2001), the TMV MP was shown to spread extensively cell-to-cell. This extensive movement underscores the role of TMV MP to modify plasmodesmata and to mediate macromolecular movement between cells. The lack of detection of extensive spread of virus-encoded MP:GFP between cells at the leading front of infection is likely due to the fact that cells at the leading front of infection just undergo the onset of infection during which only a very low amount of TMV MP is present.

The infection sites caused by TMV-MP:GFP appear in the form of fluorescent rings that surround a dark center. The fluorescent rings are continuously growing. Since at early stages of the infection MP:GFP fusion protein has been produced in areas of the ring that are later converted into the dark center, growth as a fluorescent ring indicates a short half life of the MP:GFP. This observation is consistent with fluorimetric measurements using infected protoplasts, which demonstrated that MP:GFP accumulated only transiently during infection, with a peak at about 24 h post-infection (hpi) (Epel et al. 1996a; Padgett et al. 1996). Similar timing of accumulation and subsequent degradation of the protein has also been observed in infected protoplasts using microscopy (Heinlein et al. 1998), immunoblot (Szécsi et al. 1999) or pulse-labeling (Hirashima and Watanabe 2001) analyses. This pattern of a short period of accumulation and subsequent degradation is probably specific for the TMV MP since treatment of virus-infected protoplasts with inhibitors of the 26S proteasome leads to accumulation of ubiquitinylated TMV MP, but not of ubiquitinylated replicase or CP (Reichel and Beachy 2000). Constructs in which the CP was reintroduced (TMV-MP:GFP-CP) were characterized by a drastic reduction in the TMV-MP:GFP expression level and infection sites appeared as faintly fluorescent disks (Heinlein et al. 1998; Szécsi et al. 1999). Since this much lower expression of MP:GFP does not impair the efficiency of vRNA spread, the transient accumulation of high levels of TMV-MP:GFP shown by TMV-MP:GFP and the even higher level of TMV MP accumulation during wild-type TMV infection (Szécsi et al. 1999) is not required for vRNA movement in *Nicotiana* species. Indeed, it was demonstrated earlier that only 2% of the wild-type level of TMV MP expressed during TMV infection is required for movement (Arce-Johnson et al. 1995). The role of TMV MP accumulation is not known. However, it is conceivable that high levels of TMV MP play accessory roles during TMV infection in *Nicotiana* species or in other hosts of TMV.

Since cells at the leading front of a radially expanding infection site represent the earliest stages of infection and since progressively more inner cell layers in the infection site represent progressively later stages of the infection (Heinlein et al. 1998; Oparka et al. 1997), the examination by fluorescence microscopy of cells within infection sites produced by TMV-MP:GFP in *N. benthamiana* leaves reveals a time course of accumulation and localization of TMV MP in infected cells. During early stages of the infection, TMV-MP:GFP accumulates in plasmodesmata and also associates with the ER. Later on, the protein is visualized in association with ER-associated inclusion bodies and microtubules. Finally, detectable MP:GFP fluorescence disappears from all locations except from plasmodesmata (Heinlein et al. 1998). Similar associations were observed in cells infected with the related *Tomato mosaic virus* strain Ob (Heinlein et al. 1995; Padgett et al. 1996). Infection sites of TMV-MP:GFP-CP that produce lower levels of TMV-MP:GFP showed strongly fluorescent plasmodesmata, whereas fluorescence associated with microtubules and bodies was observed with much reduced frequency (Heinlein et al. 1998). Thus, under low expression conditions, MP:GFP still accumulates in plasmodesmata but much less at microtubules and bodies. This implies that the accumulation of TMV-MP:GFP to visible levels in bodies and on microtubules is not required for the spread of infection (Heinlein et al. 1998).

3.3
TMV MP in ER-Derived Inclusion Bodies

The inclusion bodies derived from cortical ER (Heinlein et al. 1998; Mas and Beachy 1999; Reichel and Beachy 1998) likely represent sites of viral replication and protein synthesis, since they contain replicase (Heinlein et al. 1998) and vRNA (Mas and Beachy 1999), and also accumulate CP (Asurmendi et al. 2004). Thus, the inclusion bodies have lately also been referred to as virus-replication complexes (VRCs) (Asurmendi et al. 2004; Kawakami et al. 2004; Liu et al. 2005). Consistent with their membraneous nature, earlier studies have shown that TMV replication complexes, and also *Potato virus X*-replication complexes, co-purify with membrane extracts from infected cells (Doronin and Hemenway 1996; Nilsson-Tillgren et al. 1974; Osman and Buck 1996; Ralph et al. 1971; Watanabe and Okada 1986; Young and Zaitlin 1986; Young et al. 1987). Membranes are also the site of replication of other viruses, such as *Brome mosaic virus* (Restrepo-Hartwig and Ahlquist 1996), *Tobacco etch virus* (Schaad et al. 1997), *Peanut clump virus* (Dunoyer et al. 2002), *Grapevine fanleaf virus* (Ritzenthaler et al. 2002) and poliovirus (Bienz et al. 1994). Association of virus replication with membranes may support the configuration of the replication complex (Osman and Buck 1996, 1997), or may represent a means for compartmentalization, in order to coordinate and regulate efficient virus translation, replication and movement, and also to protect

the virus against the innate defense responses of the host. ER-aggregation leading to the formation of inclusion bodies is likely mediated by TMV MP (Ferralli et al. 2006; Reichel and Beachy 1998) and may imply a role of TMV MP in the enhancement of virus replication. On the other hand, ER aggregation may also be caused by protective defense responses of the plant. Such responses are exemplified by Mx proteins that function as mediators of innate resistance to RNA viruses in animals and humans by trapping and sorting viral components to subcellular locations, where they become unavailable for further virus propagation (Haller and Kochs 2002).

Several lines of evidence suggest that the formation of inclusion bodies from infected ER might be dispensable for replication and movement. For example, bodies do not form in the absence of TMV MP (Mas and Beachy 1999, Reichel and Beachy 1998). Yet, TMV mutants that lack TMV MP replicate normally (Meshi et al. 1987). Moreover, a TMV derivative encoding a mutant but functional TMV-MP:GFP was reported to cause infection in *N. benthamiana* leaves despite the absence of TMV-MP:GFP-containing inclusion bodies (Boyko et al. 2000c). This finding is consistent with the absence of TMV-MP:GFP-containing bodies in most cells infected with TMV-MP:GFP-CP (Heinlein et al. 1998).

3.3.1 Role of ER in TMV Spread

Independent of a potential role of the ER-derived inclusion bodies during infection, the ER network has important functions. In fact, viral RNA of an MP-deficient TMV construct was shown to be localized to ER, suggesting ER association as an intrinsic property of vRNA and/or replicase (Mas and Beachy 1999) and, thus, a critical role of ER in the initiation of cellular infection and virus replication. Recent studies indicate that the viral replicase coding region has a role in cell-to-cell spread of the virus (Hirashima and Watanabe 2001; see Sect. 4.2). One implication of this finding may be that the ER-resident replicase participates in movement by conveying replicated viral genomes to the MP for vRNP complex formation. However, since replicase also acts in the suppression of RNA silencing (Ding et al. 2004; Kubota et al. 2003), its effect on movement may be indirect and be founded on successful counter-defense against defense reactions of the host. Recent studies on initially infected cells of mechanically inoculated leaves suggested that TMV MP-associated inclusion bodies/VRCs may represent the form by which TMV moves in between cells (Kawakami et al. 2004; see Sect. 4.1). However, although the virus may move in association with membranes and replication factors, it remains obscure whether the observed spread of very large TMV-MP:GFP bodies into adjacent cells indeed represented plasmodesmata-mediated movement or rather leakage of cytoplasm, i.e., through gaps in the cell wall caused by mechanical inoculation.

3.3.2
Association of TMV MP with the Cytoskeleton

Although the accumulation of TMV-MP:GFP on microtubules is likely dispensable for infection as is discussed above, several in vivo studies indicated that the association of TMV MP with microtubules is nevertheless strongly correlated with the function of TMV MP in movement (Boyko et al. 2000a,b,c, 2002; Kotlizky et al. 2001). Moreover, using infected protoplasts and a combination of antibody labeling and in situ hybridization procedures, Más and Beachy (1999) showed that vRNA localizes to microtubules in a TMV MP-dependent manner (Mas and Beachy 1999). A subsequent study, again in protoplasts, demonstrated the mislocalization of vRNA in cells expressing a mutant, non-functional TMV MP (TAD5; Kahn et al. 1998; Fig. 1) that binds vRNA but fails to associate with microtubules (Mas and Beachy 2000). Thus, the MP:microtubule complexes observed during later stages of infection may reflect a functional interaction between TMV MP and tubulin during the movement process, even though such complexes are not routinely observed at the front of infection. This may be due to the small amount of TMV MP produced at the infection front, which may not be sufficient to allow visualization of the MP:microtubule interaction. Temperature-sensitive mutations in the TMV MP that simultaneously affect microtubule association and the function of TMV MP in vRNA movement were shown to map to a small domain in TMV MP with structural similarity to the M-loop of α, β, and γ-tubulin (Boyko et al. 2000a; Fig. 1). The tubulin M-loop is essential for the formation and stability of microtubules since it directly contacts the N-loop of tubulin molecules in the adjacent microtubule protofilament (Nogales et al. 1999). The mimicry of the tubulin M-loop may allow direct interaction of TMV MP with free or assembled tubulin of either isoform, including γ-tubulin, and may also pinpoint the TMV MP as a binding target for tubulin cofactors. Interestingly, when TMV MP is expressed in mammalian cells, it not only binds microtubules but also interferes with the recruitment of γ-tubulin to the centrosome (Boyko et al. 2000a; Ferralli et al. 2006). Although plant cells do not concentrate microtubule nucleation events in centrosomes but rather nucleate microtubules at dispersed cortical sites (Murata et al. 2005) and at the nuclear membrane (Schmit 2002; Seltzer et al. 2003), these findings may imply a possible interaction of TMV MP with microtubule organizing complexes or microtubule-organizing sites. In fact, at least in infected protoplasts, the TMV MP localizes to fixed peripheral punctate sites that are aligned to microtubules (Heinlein et al. 1998). Since the pattern of dispersed γ-tubulin sites in plants is also microtubule-aligned (Murata et al. 2005), it will be interesting to see whether or not TMV MP interacts with these γ-tubulin sites or with other markers of microtubule nucleation. An interaction of TMV MP with microtubule-nucleating sites would be reminiscent of the movement process recently described for retroviruses

such as Human-T-lymphotropic virus (HTLV-1), which involves a reorganization of microtubules and the relocation of the microtubule-organizing center to cell–cell contacts leading to the formation of a "virological synapse" (Derse and Heidecker 2003; Igakura et al. 2003). In fact, TMV-MP:GFP fluorescence in TMV-infected cells often concentrates in "paired bodies", i.e., in cell-wall associated localizations within infected cells that are aligned across the cell wall at sites of plasmodesmata (Padgett et al. 1996). It is tempting to speculate that a microtubule reorganizing activity of TMV MP might be involved in forming a virological synapse at the site of plasmodesmata. It should be noted, however, that the region of similarity with the tubulin M-loop overlaps with the region of TMV MP predicted to function as a transmembrane domain (Brill et al. 2000, 2004; Fig. 1). This could indicate that the TMV MP can assume different conformations and that microtubule and membrane association represent alternating events. Since TMV MP potentially forms a dimer (see Sect. 3.1), one could even speculate that one TMV MP subunit interacts with tubulin whereas the other subunit interacts with the ER. This way, TMV MP could form an ER-to-microtubule bridge that could align the ER and microtubules to facilitate microtubule- and TMV MP-mediated transport of vRNA-associated ER membranes (Ferralli et al. 2006). Preliminary observations in leading cells of infection sites suggest an association of TMV-MP:GFP with particles that translocate along cytoskeletal tracks (V. Boyko, A. Sambade, and M. Heinlein, unpublished observations). Whether these particles represent the proposed vRNA-associated ER membranes that are targeted to plasmodesmata and into non-infected cells remains to be shown.

Recent studies involving the treatment of plants with microtubule-disrupting agents led to the conclusion that microtubules are not required for the spread of TMV (Gillespie et al. 2002; Kawakami et al. 2004). However, since these studies did not provide unequivocal evidence that the treatments disrupted all microtubules and all tubulin-based activities, this conclusion is questionable (Seemanpillai et al. 2006). Although plants expressing GFP fused to *Arabidopsis* TUA6 (GFP-*tua*) demonstrate that the GFP-tagged microtubules are absent in drug-treated tissues (Gillespie et al. 2002), microtubules or microtubule fragments made of endogenous tubulin are still present (Seemanpillai et al. 2006). Thus, although an intact microtubule cytoskeleton seems not to be required for vRNA movement by TMV MP, a role of tubulin-based activities in the function of TMV MP cannot be ruled out (Seemanpillai et al. 2006).

The association of TMV MP with microtubules has been observed in protoplasts and mammalian cells transfected with TMV MP-encoding DNA constructs (Boyko et al. 2000a; Ferralli et al. 2006; Heinlein et al. 1998; Kotlizky et al. 2001) and, therefore, can occur independent of virus infection. Whereas in animal cells TMV MP seems to accumulate exclusively at microtubules, the accumulation of TMV MP on microtubules in plants appears

to be regulated and takes place only at certain stages of infection. Thus, it seems likely that specific plant host factors are involved in controlling TMV MP accumulation at microtubules in plants. The binding of TMV MP to microtubules also occurs in vitro (Ashby et al. 2006) and, as described above, in vivo experiments using temperature-sensitive mutants indicated a potential role of tubulin mimicry in this association (Boyko et al. 2000a). The highly fluorescent in vivo complexes that occur during late infection by TMV-MP:GFP appear to be in a non-dynamic state as they resist the treatment with cold, freezing and thawing, as well as with high concentrations of calcium and sodium salts (Boyko et al. 2000a). Similarly, in transfected mammalian cells, the TMV MP:microtubule complex resists treatment with cold as well as with high millimolar concentrations of microtubule-disrupting agents such as colchicine or nocodazole (Ferralli et al. 2006). As mentioned above, the role of these complexes is not known and may be the result of overaccumulation of TMV MP.

In addition to interaction with microtubules, TMV MP was also shown to interact with actin (McLean et al. 1995). This observation has received less attention and hence has not been expanded on. However, the general significance of intact actin filaments for cell-to-cell spread of TMV was addressed experimentally by drug studies (Gillespie et al. 2002; Liu et al. 2005) and actin silencing (Liu et al. 2005), which led to substantially reduced cell-to-cell movement of the virus (see also Sect. 4.2). These studies suggest that actin filaments may be involved in cell-to-cell transport, perhaps by targeting viral replication complexes towards plasmodesmata, as has been proposed in a recent model (Liu et al. 2005).

3.3.3
Is the Accumulation of TMV MP on Microtubules Connected to Degradation?

Since the TMV-MP:microtubule complexes occur before the level of cellular TMV MP diminishes, it has been suggested that the complex may target the TMV MP for degradation (Padgett et al. 1996). During infection the TMV MP occurs in high molecular weight forms, which is consistent with polyubiquitinylation of the protein (Reichel and Beachy 2000). A role of the ubiquitin/26S proteasome (Ub/26S) pathway in the degradation of TMV MP also gained support by the observation that treatments of plants with inhibitors of the 26S proteasome cause changes in the subcellular localization of MP (Gillespie et al. 2002). Recent studies, however, have shown that microtubule-associated TMV MP, in contrast to MP present in whole extract, is not ubiquitinylated. Moreover, treatments of infection sites with microtubule-disrupting agents do not transform the ring-like pattern of TMV-MP:GFP fluorescence of infection sites into a disk-shaped pattern. Thus, although these studies confirm that TMV MP is a substrate for ubiquitinylation and degradation by the 26S proteasome, the microtubules seem not to be involved in this process

(Ashby et al. 2006). However, it was also shown that microtubule-associated TMV MP interferes with motor-driven motility in vitro (Ashby et al. 2006). Therefore, it may be possible that these complexes interfere with the trafficking of signal molecules involved in antiviral plant defense responses, such as the non-cell-autonomous RNA silencing signal (Dunoyer et al. 2005; Heinlein 2005; Himber et al. 2003). In combination with the down-regulation of the SEL of plasmodesmata late in infection (Oparka et al. 1997) this activity may function in protecting the viral RNA in newly infected cells at the leading front of the infection site. The formation of the TMV MP:microtubule complex as well as the down-regulation of plasmodesmal SEL may also function in blocking the backward movement of vRNA into already infected cells, thus assuring that the virus spreads efficiently and unidirectionally into non-infected tissues.

3.3.4
TMV MP Targeting to Plasmodesmata

Another important question to answer is how the MP itself is targeted to plasmodesmata, since this process can be independent of microtubule association (Boyko et al. 2000a; Kahn et al. 1998). The subcellular localization of TMV MP in protoplasts is affected by treatment with the secretory pathway inhibitor Brefeldin A (Heinlein et al. 1998), which suggests a role of the secretory pathway. Only recently, a combination of drug treatments implicated the cortical ER and the actin cytoskeleton in targeting TMV MP to plasmodesmata (Wright et al. 2006). A potential role of secretory vesicles in the cell wall targeting of viral MP in the absence of virus infection has been demonstrated for the MP of tubule-forming *Grapevine fanleaf virus* (GFLV) in BY-2 cells. The protein targets newly formed plasmodesmata during cytokinesis by using a Brefeldin A-sensitive pathway that involves interactions with the t-SNARE syntaxin KNOLLE as well as phragmoplast microtubules (Laporte et al. 2003). Recent studies have demonstrated a role of the secretory actin-ER-driven and endocytic pathways in the plasmodesmal targeting of triple gene block (TGB) movement proteins of Hordei- and Potex-like viruses (Cowan et al. 2002; Haupt et al. 2005; Morozov and Solovyev 2003; Solovyev et al. 2000; Zamyatnin et al. 2004). Although TMV replicates in association with the ER network, a potential role of the secretory pathway in TMV movement has not yet been fully addressed. A role of secretory vesicles in the pathway that targets TMV MP to plasmodesmata may be suggested by results indicating that the TMV MP interacts with PME (Chen et al. 2000; Dorokhov et al. 1999), an enzyme that is targeted to the extracellular cell wall matrix. As suggested for the MP of GFLV (Laporte et al. 2003), the MP of TMV could interact with such secretory cargo to hitch a ride on secretory vesicles in order to reach the cell wall.

3.4 Gating and Cell-to-Cell Transport

Plasmodesmata can dynamically alter their size exclusion limit in response to intrinsic developmental and physiological signals and thus control communication within and between symplastic domains. The seminal discovery that the MP of TMV localizes to plasmodesmata and mediates the cell-to-cell trafficking of TMV RNA provided the first evidence for the existence of proteins able to manipulate plasmodesmata. Direct proof of the TMV MP's ability to "gate" plasmodesmata (i.e., to increase the plasmodesmal SEL) was obtained by a series of microinjection experiments. Purified TMV MP injected into plant cells enabled cell-to-cell trafficking of large fluorescently labeled dextrans that would otherwise have been confined to the microinjected cell (Waigmann et al. 1994). Movement occurred within minutes of microinjection; thus, these experiments allowed a glimpse into the dynamics of interaction between the TMV MP and plasmodesmata. Furthermore, microinjection of fluorescently labeled TMV MP protein provided proof for the TMV MP's ability to move between cells itself (Nguyen et al. 1996). We now know that most, if not all, viruses encode proteins with the ability to interact with plasmodesmal channels. Like the MP of TMV, many other MPs have been shown to alter the conductivity of plasmodesmata and to traffic between cells. Recent studies have shown that MPs share this capacity with endogenous non-cell-autonomous proteins (NCAPs) that are found in the phloem or which function non-cell-autonomously in cell fate determination during plant development (Lucas and Lee 2004). A non-cell-autonomous pathway protein (NCAPP1) was isolated that binds phloem NCAPs and is located to the ER in close proximity to the plasmodesmal orifice. A mutant form of this protein was shown to block the trafficking of NCAPs including MPs. Based on this finding it is speculated that the interaction of MPs and other NCAPs with plasmodesmata involves proteins such as NCAPP1, which assist in the interaction with cellular constituents that lead the way to the channel (Lee et al. 2003; Lucas and Lee 2004). The mechanism by which TMV MP modifies the SEL of plasmodesmata and how the vRNA is transported through the channel is unknown. In vitro association between single stranded nucleic acids and TMV MP result in the formation of elongated and unfolded RNA:protein complexes (see Sect. 3.1), indicating that the movement process may involve the unfolding and linearization of vRNA. There is also evidence that NCAPs must unfold in order to move from cell to cell (Kragler et al. 1998). The fact that MP accumulates in plasmodesmata may suggest that the movement process involves a transport structure formed by MP within the plasmodesmal channel. In fact, TMV forms MP-containing membrane protrusions in infected protoplasts (Heinlein 1998, 2002a), which is very reminiscent of the "tubule-forming" viruses that are known to assemble tubules made of MP within plasmodesmata to allow the cell-to-cell transport of virions (Kasteel

et al. 1997; van Lent et al. 1991; Wellink et al. 1993). However, unlike the MPs of tubule-forming viruses, the MP of TMV has been correlated with the deposition of a MP-associated fibrous substructure in the central cavity of plasmodesmata rather than with the formation of a tubule.

3.5 Host Factors

Plant viruses probably utilize endogenous pathways for their intra- and intercellular transport processes, thereby relying on interaction of viral movement proteins with plant endogenous proteins. TMV MP has been shown to interact with an ever-increasing number of host factors such as actin (McLean et al. 1995) and tubulin (Heinlein et al. 1995; McLean et al. 1995; discussed in Sect. 3.3.3), a cell-wall associated pectin methylesterase, microtubule-associated protein MPB2C, calreticulin, and plasmodesmal associated kinase (PAPK1).

The cell-wall localized enzyme pectin methylesterase (PME; Dorokhov et al. 1999; Chen et al. 2000) was isolated as an interaction partner of TMV MP by a renatured blot overlay assay from tobacco cell wall protein fractions. PME enzymatically affects cell wall porosity, pH, and ion balance (Nairn et al. 1998; Pressey 1984) and may utilize the ER as a transport pathway to the cell wall (Gaffe et al. 1997). By yeast two-hybrid analysis it was shown that TMV MP binds PME with a domain spanning amino acids 130–185 (Fig. 1). TMV encoding a TMV MP mutant lacking this region failed to move cell-to-cell *in planta*, suggesting that interaction of TMV MP with PME is required for viral cell-to-cell movement (Chen et al. 2000). However, deletion of more than 50 amino acids from the core region of the protein may also have caused inactivation by disrupting the overall tertiary structure of the protein. Nevertheless, several mechanisms by which PME may participate in TMV cell-to-cell movement are under discussion (Chen et al. 2000; Waigmann et al. 2004):

1. PME might provide ER localization to TMV MP in trans, thereby mediating its transport along the ER to plasmodesmata
2. PME may act as a cellular receptor for TMV MP, thereby mediating TMV MP localization at the host cell wall
3. TMV MP binding may interfere with PME activity, altering the cell wall ion balance, which could in turn induce changes in plasmodesmal permeability and enable viral cell-to-cell movement

Another TMV MP interacting protein, MPB2C, has been isolated using a membrane-based yeast screening system (Kragler et al. 2003). MPB2C represents a novel plant-specific protein localizing at microtubules in a discrete punctuate pattern that also co-localizes with TMV MP at microtubule-associated sites (Kragler et al. 2003). Transient expression of MPB2C mediated increased accumulation of TMV MP at microtubules coupled to a de-

crease in cell-to-cell transport activity of TMV MP. Also, MPB2C did not interfere with cell-to-cell transport of a movement-enhanced TMV MP mutant, TMV MP^{R3}, reported to show restricted microtubule localization (Gillespie et al. 2002). Collectively, these results suggest that MPB2C is not required for, but plays a negative role in, TMV movement (Kragler et al. 2003). To strengthen this concept, the MPB2C gene was silenced in *Nicotiana* plants (Curin et al. 2006). Indeed, upon MPB2C silencing, cell-to-cell movement of transiently expressed TMV MP and spread of TMV were unimpaired. Strikingly, a nearly complete loss of accumulation of transiently expressed TMV MP on microtubules was observed in silenced plants indicating that MPB2C is involved in accumulating TMV MP at microtubules (Curin et al. 2006). These findings also further support the concept that the accumulation of high levels of TMV MP on microtubules in late stages of infection is dispensable for movement. These findings do not exclude the possibility that microtubules may have a more active role in movement in early infection.

A biochemical approach where TMV MP was used as a specific ligand resulted in identification of calreticulin as a TMV MP interacting host factor (Chen et al. 2005). Calreticulin is a calcium-sequestering ER-resident protein which accumulates in ER-containing plasmodesmata (Chen et al. 2005; see also Sect. 2). In transgenic plants overexpressing calreticulin, TMV spread was substantially reduced. Also, the subcellular distribution of transiently expressed TMV MP was changed, with TMV MP being accumulated at microtubules (Chen et al. 2005). What could be the underlying cause for the changed TMV MP accumulation pattern in calreticulin-overexpressing cells? Plasmodesmata might be overloaded by calreticulin, which prevents TMV MP from reaching its ultimate plasmodesmal destination. Consequently, TMV MP can no longer exit the cell through plasmodesmata, and may therefore accumulate on microtubules, either because they constitute a component of the pathway that targets plasmodesmata or because they represent just another natural cellular interaction structure for the MP. Since the subcellular distribution was addressed in the absence of virus infection, it has not yet been elucidated whether the change in TMV MP subcellular distribution triggered by calreticulin overexpression in the context of transient expression is related to the inhibitory effect of calreticulin overexpression on TMV spread (Chen et al. 2005).

Protein phosphorylation represents a mechanism for regulating protein function. Since phosphorylation of TMV MP has been demonstrated in vivo and in vitro (Citovsky et al. 1993; Haley et al. 1995; Karpova et al. 1999; Waigmann et al. 2000; Watanabe et al. 1992), the quest to reveal the exact function of these phosphorylation events and to isolate the responsible kinase(s) has been ongoing. The best-studied phosphorylation event is phosphorylation of the three Ser/Thr residues at the C-terminus of TMV MP (Citovsky et al. 1993; Trutnyeva et al. 2005; Waigmann et al. 2000; Fig. 1). Since a TMV MP mutant lacking this phosphorylatable region retained movement ability in its

host plants (Berna et al. 1991; Boyko et al. 2000c), C-terminal phosphorylation of TMV MP is obviously not essential for viral spread. Moreover, a TMV phosphorylation mimicking mutant, with all three phosphorylatable residues replaced by negatively charged Asp or Glu, showed reduced viral spread in *N. tabacum* but not in other tested *Nicotiana* species (Trutnyeva et al. 2005; Waigmann et al. 2000). Thus, C-terminal phosphorylation probably represents a mechanism to negatively regulate TMV spread in a host-dependant manner (Waigmann et al. 2000). Interestingly, transient expression studies aimed to clarify the role of each single phosphorylation site for TMV MP movement in *N. tabacum* revealed that phosphorylation on one of the three Ser/Thr residues may even positively affect intercellular movement of the protein, while a negative effect on cell-to-cell transport can only be observed after phosphorylation on two or all three Ser/Thr residues of the protein (Trutnyeva et al. 2005). Potentially, during viral infection, TMV MP phosphorylation might be a sequential event. The virus might use the first phosphorylation event to promote TMV MP transport and thus the transport of its vRNA, while subsequent phosphorylation events inactivating the TMV MP transport function might limit the negative effect of TMV infection for the host plant at later stages (Trutnyeva et al. 2005). A kinase possibly responsible for these phosphorylation events has been recently isolated biochemically using TMV MP as a bait (Lee et al. 2005). This kinase, termed plasmodesmal-associated protein kinase (PAPK), resides at plasmodesmata and specifically phosphorylates TMV MP at its C-terminus in vitro. Further studies utilizing this kinase will likely provide new insights into the role and regulational properties of the C-terminal phosphorylation sites of TMV MP.

4
Role of Coat Protein and Replicase in TMV Cell-to-Cell Transport

Besides the MP, TMV also encodes a coat protein and a replicase that is produced in a long 183 kDa and a short 126 kDa form. While numerous transport studies have focused on TMV MP, the role of the other two viral components in cell-to-cell transport has been more thoroughly investigated only in the last few years, and will be summarized below.

4.1
Coat Protein

Previous observations reported that the presence of the TMV coat protein (CP) is not required for TMV replication or cell-to-cell movement (Siegel et al. 1962; Takamatsu et al. 1987). However, more recent studies suggest a connection between CP and the formation of movement competent complexes involved in spread of TMV. TMV might spread between cells in the form of

VRCs composed of MP, replicase, and genomic viral RNA (Asurmendi et al. 2004; Kawakami et al. 2004). VRCs were observed to rapidly move through the cytoplasm and to associate with plasmodesmata. Since VRC movement was sensitive for actin disrupting agents, the VRCs were proposed to migrate via the actin cytoskeleton towards plasmodesmata, where they move into neighboring cells as viral movement complexes (VMC). CP was speculated to perform a regulatory role in VRC establishment, thereby also influencing the generation of VMCs and hence, cell-to-cell movement (Asurmendi et al. 2004). In addition, studies based on CP^{T42W}, a coat protein mutant that cannot form infectious particles but shows increased subunit interaction, suggest a connection between CP and the amount of MP production (Bendahmane et al. 1997, 2002). Plants expressing CP^{T42W} are more resistant to TMV infections than non-transgenic and wild-type CP-transgenic plants. Resistance is characterized by smaller TMV infection sites and reduced levels of TMV MP. Supporting studies in BY2 cells confirm that CP^{T42W} transgenic BY2 protoplasts infected with TMV accumulate less MP than infected wild-type BY2 protoplasts, whereas CP transgenic BY2 protoplasts produce even more MP. Potentially, CP positively influences the production of MP, perhaps by enhancing the level of subgenomic mRNA encoding the MP (Asurmendi et al. 2004; Bendahmane et al. 2002). On the other hand, when the CP gene was reintroduced into a viral CP-lacking TMV-MP:GFP construct a strong reduction of MP:GFP production was observed in infected cells (Heinlein et al. 1998; Szécsi et al. 1999). Nevertheless, these results suggest that the CP might interface with MP-mediated transport at two levels: by regulating the amount of TMV MP production, and by regulating the establishment of VRCs. Thus, although the CP is dispensable for cell-to-cell movement of the viral RNA, the protein may play a regulatory role in influencing TMV MP expression and activity.

4.2 Replicase

As common for viral RNA replicases, TMV replicase consists of a N-terminal methyl transferase domain, a helicase domain, and an RNA-dependent RNA polymerase domain, the latter of which is only present in the 183 kDa form but not in the shorter 126 kDa form of the protein. The N-terminal methyl transferase domain is separated by a non-conserved region from the helicase domain. The two virus-encoded replicase proteins interact (Goregaoker et al. 2001) and are found in replication complexes isolated from infected plants (Osman and Buck 1996; Watanabe et al. 1999). The 183 kD protein alone is sufficient for replication in protoplasts, although replication efficiency is strongly increased if both replicase proteins are expressed (Ishikawa et al. 1986; Lewandowski and Dawson 2000). A chimeric virus, TMV-hel, consisting of TMV-U1 and the helicase domain of TMV-R, showed defects

in cell-to-cell transport even though genome replication as well as synthesis and accumulation of TMV MP in protoplasts were similar to TMV-U1 (Hirashima and Watanabe 2001). The defect in cell-to-cell movement could not be complemented in transgenic plants expressing TMV MP, but could be complemented when the non-conserved region of the replicase was also encoded by the chimeric virus (Hirashima and Watanabe 2003). Naturally occurring revertants of TMV-hel that regained cell-to-cell movement capacity showed amino acid changes either in the helicase region or in the neighboring non-conserved region of the replicase. Overall, these results indicated an involvement of the replicase in TMV cell-to-cell transport (Hirashima and Watanabe 2001, 2003). Since the movement protein is essential for cell-to-cell transport it is likely that replicase interacts with TMV MP. However, the nature of this interaction remains unknown, as well as the mechanism by which replicase would support cell-to-cell transport.

Another approach to the study of the involvement of replicase in TMV cell-to-cell transport utilizes a defective RNA (dRNA)/helper virus system. In this system, the dRNAs encoded various truncated versions of the replicase whereas the helper virus encoded both the full length 126 kDa and 183 kDa forms of the replicase. For efficient movement of dRNAs, expression *in cis* of the 126 kDa replicase or the N-terminal 258 amino acids of the replicase was required, whereas smaller versions of the replicase were unable to support cell-to-cell movement of the dRNA. This failure could not be complemented *in trans* by the full length replicase proteins produced by the helper virus, suggesting that the nascent dRNA encoded replicase might be required to bind to dRNA *in cis* to facilitate intercellular movement (Knapp et al. 2005).

Yet another line of evidence argues for involvement of the 126-kDa form of the replicase in viral transport. The 126-kDa protein is a component of VRCs, which were reported to align with and traffic along microfilaments (Kawakami et al. 2004; Liu et al. 2005). Based on their observations, Liu et al. (2005) suggested that the 126 kDa replicase not only influences the size of VRCs, potentially via its ability to form oligomers, but also mediates the interaction between VRCs and microfilaments, and movement of VRCs along the microfilament network. Furthermore, disassembly of microfilaments by drugs or an actin-silencing strategy resulted in substantially reduced cell-to-cell movement of the virus. Although the effects of actin disruption can be manifold, these results led to a model that implicated the 126 kDa replicase directly in cell-to-cell movement: the replicase may mediate intracellular transport of VRCs via microfilaments towards plasmodesmata, where the TMV MP may then take over and move the viral RNA through plasmodesmata (Liu et al. 2005).

Overall, we may have to significantly broaden our view on how cell-to-cell movement of TMV RNA is achieved. If VRCs, which physically integrate the viral RNA with the CP, the MP, and the replicase, constitute the actual translocation unit for intercellular transport, either a direct involvement or

a modulating influence of the CP and replicase in cell-to-cell transport is a distinct possibility.

5 Discussion

Within this book chapter, we provide an overview on current knowledge of cell-to-cell movement of TMV genomic RNA. Even though an ever-growing number of host factors and other viral proteins have been found to participate in or modulate the transport process, the key player was and is the movement protein, TMV MP. Over the years, the number of known functions of the TMV MP has been steadily increasing. At present, TMV MP is known to bind to RNA, to localize to the cell wall, to gate plasmodesmata, to bind to microtubules, to interact with the ER (potentially by integrating into the ER membrane), and to interact with several host factors. In many cases, domains or regions within the TMV MP sequence that are required or involved in a particular function have been experimentally defined. Figure 1 presents an overview of the TMV MP regions involved in the various functions. Generally, to render TMV MP fully functional in viral spread, only the carboxyterminal 55 amino acids are dispensable, whereas amino acids 1–213 are required. However, when looking at the molecular requirements for individual TMV MP functions, a more detailed picture emerges. RNA binding is mediated by two independently acting domains, A and B (Citovsky et al. 1992), but only domain A is required in its intact form for viral spread. Gating domain E, which is required to increase plasmodesmal size exclusion limit (Waigmann et al. 1994), overlaps with RNA binding domain A and partially with RNA binding domain B (Fig. 1). This close molecular link between gating and RNA binding might indicate that functional interaction with plasmodesmata is only possible when TMV MP is bound to RNA. It is not known yet which part of the TMV MP confers onto the protein itself the capacity to move between cells. However, a hint may be obtained from the series of microinjection experiments performed to define domain E (Waigmann et al. 1994). In these experiments, TMV MP-mediated movement of fluorescently labeled dextrans between cells was scored. Dextrans were able to spread into numerous cells not directly connected to the microinjected cell, which implied that not only the dextrans, but also the microinjected TMV MP, moved between cells in order to gate plasmodesmata in more distant cells. Therefore, domain E is most likely not only required for gating plasmodesmata but also for mediating cell-to-cell movement of TMV MP itself.

Also overlapping with RNA binding domain A and gating domain E is a region that interacts with host factor PME, a pectin methyl esterase that modulates pH and ion balance and alters cell wall porosity (Chen et al. 2000). Interaction between TMV MP and PME seems to be required for cell-to-cell

spread of TMV. PME might function in transporting the TMV MP towards plasmodesmata, and/or by altering cell wall porosity at the sites of plasmodesmata, thereby inducing changes in plasmodesmal permeability (see Sect. 3.4). In particular, the latter hypothesis is very attractive in the light of the direct overlap between gating domain E and the binding region between TMV MP and PME (Fig. 1), since it could imply that binding of TMV MP to PME is necessary to achieve gating.

Interaction between microtubules and TMV MP has received a lot of attention and was consequently studied in detail. This interaction not only occurs in plant cells but is conserved upon expression of TMV MP in mammalian cells and in vitro, indicating that MP can function as a genuine microtubule-associated protein (MAP) that binds to microtubules through direct interactions (Ashby et al. 2006). The analysis of a series of TMV derivatives encoding progressive aminoterminal and carboxyterminal deletion mutations in TMV MP fused GFP indicated that the interaction of microtubules requires amino acids 1–213 of the TMV MP, i.e.,the same major part of TMV MP that is also required for its function in TMV movement (Boyko et al. 2000c, Fig. 1). The analysis of a series of internal three amino acid deletion mutations indicated that amino acids 49–51 are important for microtubule association and function of the TMV MP (Kahn et al. 1998, Fig. 1). Moreover, deletion of amino acids 3–5 inactivates the protein (Gafny et al. 1992; Lapidot et al. 1993) and causes its constitutive accumulation on microtubules (Kotlizky et al. 2001; Fig. 1). Interestingly, expression of this dysfunctional MP in transgenic *N. benthamiana* plants reduces microtubule association of virus-encoded MP (Kotlizky et al. 2001), a finding that may provide a partial explanation for virus resistance observed in *N. tabacum* plants expressing this dysfunctional protein (Cooper et al. 1995; Lapidot et al. 1993). Amino acid residues Pro 81, Thr 104, and Arg 167 have been genetically shown to functionally interact and to be required for interaction between microtubules and TMV MP (Boyko et al. 2002). Interestingly, amino acid residue Thr 104 was also identified as a phosphorylated residue (Karger et al. 2003; Fig. 1). Phosphorylation at this site is not essential for MP function, since replacement of Thr 104 by the non-phosphorylatable Ala does not affect viral movement. However, substituting Thr 104 with negatively-charged phosphorylation-mimicking amino acid residue Asp strongly inhibits cell-to-cell spread of the mutant virus in *N. tabacum* plants; thus, phosphorylation at Thr 104 may serve as an inactivation mechanism. However, it has not yet been revealed whether phosphorylation at Thr 104 may also influence the MP–microtubular interaction. In vivo assays using TMV derivatives encoding temperature-sensitive MPs (with mutations in amino acid positions 144, 151, and 154 as depicted in Fig. 1) fused to GFP have provided direct correlations between the association of MP:GFP with microtubules and its activity in viral RNA cell-to-cell movement (Boyko et al. 2000a; Boyko and Heinlein, unpublished results). Interestingly, temperature-sensitivity of

all three MP mutants is caused by specific single-amino acid exchange mutations (Ls1: Pro154Ser; Ni2519: Arg144Gly; GV1: Gly151Val) in a domain with structural similarity to the M-loop of α-, β-, and γ-tubulins (Boyko et al. 2000a; Boyko and Heinlein, unpublished results). The M-loop is involved in microtubule assembly and stabilizes the microtubule by forming M-loop/N-loop bridges between adjacent microtubule protofilaments (Nogales et al. 1999). Therefore, it is possible that the TMV MP mimics the M-loop to facilitate direct contacts with tubulin, to interfere with its assembly, or to be by itself recognized as a binding target of tubulin, tubulin cofactors, or microtubule-associated proteins.

Host factor MPB2C is a microtubule-associated protein involved in accumulating TMV MP at microtubules (Kragler et al. 2003; Curin et al. 2006). Interaction between MPB2C and TMV MP is not required for cell-to-cell movement, but exerts a negative effect on TMV MP cell-to-cell transport in a transient expression assay. A TMV MP region stretching from amino acid 58 to the carboxyterminus of the TMV MP is sufficient for binding to MPB2C (Fig. 1; Kragler et al. 2003); however, no minimal interacting region has yet been defined.

To a certain degree, functional studies have been complemented by structural studies even though the success of structural studies has been hampered by the TMV MP's low solubility and tendency to aggregate. Using a combination of various biophysical and biochemical methods, two transmembrane regions have been identified that may mediate TMV MP integration into the ER membrane (Brill et al. 2000). The carboxyterminus of TMV MP probably forms a flexible tail that might be involved in dimerization. Interestingly, the region implicated in interaction with microtubules (amino acids 144–169) overlaps nearly precisely with one of the transmembrane regions spanning from amino acid 150 to amino acid 169 (Fig. 1; see Sect. 3.3.2). Potentially, integration of TMV MP protein into the ER membranes and binding to microtubules are two mutually exclusive events. How the TMV MP might choose between the two localizations is not known yet. However, the influence of host factors such as MPB2C or kinases that could modulate the conformation of the TMV MP is a possibility.

Indeed, the TMV MP is known to be phosphorylated *in planta* at several Ser/Thr sites (Fig. 1). Phosphorylation might provide a regulatory level that allows the TMV MP to engage in its various functions in a coordinated manner even though domains assigned to individual functions show a considerable overlap. With the recent identification of plant kinases that recognize TMV MP as a substrate for phosphorylation (see Sect. 3.4), this important aspect will be revealed in the near future.

The role of microtubules in the TMV transport process has been heavily discussed. So far it remains unclear whether microtubules have indeed a direct causal role in the cell-to-cell movement process, or whether the interaction may occur as a consequence or in parallel to this process. To gain

further insight it would be important to investigate the localization and function of wild-type MP as well as the location of viral RNA in newly infected cells at the front of the spreading infection site. However, such in vivo analysis in newly infected cells is at present hampered by the low level of TMV MP expression as well as by the fact that infection spreads from one cell into the next within only 4 h, which provides a very limited time frame for MP:GFP to accumulate to detectable levels. A new approach to visualize MP:GFP in relation to the movement process has recently been taken by Kawakami et al. (2004) by concentrating their analysis on primary infected cells of mechanically inoculated leaves. Unlike movement between cells at the front of spreading infection sites, movement from primary infected cells into adjacent cells occurs at a rather late stage of virus replication. Thus, movement occurs in the presence of detectable amounts of TMV MP. However, while this system offers the advantage that TMV MP can be visualized, it may be possible that initial virus spread occurs through gaps in the cell wall produced upon mechanical inoculation, rather than through plasmodesmata. Moreover, since only very little TMV MP is required for movement (Arce-Johnson et al. 1995) it may be difficult to dissect the fraction of TMV MP actually involved in the movement process. With these limitations, new insights will have to await the development of novel experimental approaches or more sensitive fluorescence detection techniques.

While microtubules have a potential role during early stages of infection, it seems clear that a fully intact microtubule cytoskeleton is not required for the spread of infection. Several studies have shown that plant leaves treated with microtubule-disrupting agents still allow TMV movement (Ashby et al. 2006; Gillespie et al. 2002; Kawakami et al. 2004). Although the inhibitors do not disrupt all microtubules (Seemanpillai et al. 2006), these studies have shown that movement continues when the microtubular cytoskeleton is greatly affected. In contrast, treatment with drugs that disrupt the actin cytoskeleton led to a reduction in the cell-to-cell spread of TMV infections (Gillespie et al. 2002; Liu et al. 2005) and may point to a major involvement of actin filaments in the movement process, perhaps in addition to a role of microtubules. The ability of TMV to move between cells in which the majority of microtubules are disrupted may reflect the possibility that localized tubulin-dependent processes at plasmodesmata-proximal sites suffice for TMV RNA movement and the spread of infection into adjacent cells. In fact, TMV establishes several ER-associated infection sites in direct vicinity to plasmodesmata (Padgett et al. 1996). Given that:

Both the MP and the viral genome are expressed to high levels during infection (Arce-Johnson et al. 1995; Padgett et al. 1996).

Virus movement requires very few virus particles (Li et al. 2002; Sacristan et al. 2003).

Local events at one of the many plasmodesmata that connect a cell with adjacent cells may suffice for virus movement the inhibition of virus move-

ment may indeed be very difficult to achieve unless a full disruption of the transport mechanism can be established.

Further studies are also required to uncover the role of the accumulation of TMV MP on microtubules late in infection. The finding that microtubule-associated TMV MP is not ubiquitinylated argues against the proposal that the late microtubule complex is involved in proteasome-mediated degradation (Ashby et al. 2006). However, the complex is probably not, at least not directly, involved in TMV movement either, since the complex occurs in cells behind the infection front (Heinlein et al. 1998) and TMV variants that develop the late complex in only few cells can still move cell-to-cell (Gillespie et al. 2002, Heinlein et al. 1998). Also, increased accumulation of TMV MP on microtubules by overexpression of host factor MPB2C resulted in significant reduction of TMV MP cell-to-cell movement (Kragler et al. 2003). Based on results of in vitro experiments it seems possible that the complex interferes with motor-dependent motility along microtubules (Ashby et al. 2006). Blocking the microtubule-dependent pathway may help to ensure that viral movement occurs forward into non-infected cells and not backward into already infected cells. Alternatively, accumulation of TMV MP at microtubules might serve as an effective means to limit the availability of TMV MP for entry into the plasmodesmata trafficking pathway as well as binding to viral RNA. The latter may be particularly important at late stages of infection, where packaging of viral RNA by coat protein into viral particles may be more important than cell-to-cell trafficking, and removal of the competing TMV MP may be desirable (Waigmann et al. 2004). Yet another proposal to be further tested is that microtubule-associated TMV MP is involved in the down-regulation of viral replication and subsequent pathogenesis at late stages of infection. This could be achieved, for example, by the sequestration of host factors to microtubules that are involved in TMV replication and/or translation (Ashby et al. 2006).

Our understanding of the TMV MP-mediated transport process of viral RNA through plasmodesmata has come a long way since its study began more than 20 years ago. Still, the actual mechanism of translocation through the plasmodesmal channel remains obscure. Open questions such as the mechanism of translocation through plasmodesmal channels, or the biological role of microtubular accumulation of TMV MP, will entice researchers to strive to elucidate the remaining mysteries of the multifunctional TMV MP. TMV as a model system will thus remain at the forefront of plant viral research.

Acknowledgements TMV-related research by E.W is funded by grants from the Austrian Science Foundation (SFB 17, project part 08) and the Wiener Wissenschafts-, Forschungs-, und Technologiefonds WWTF (grant no. LS123). TMV-related research by M.H. is funded by grants form the Swiss National Science Foundation (grant no. 631-065953 and grant no. 3100A0-111916), le ministère délégué à la recherche et aux nouvelles technologies (France, grant BCMS187), the European Community (QLG2-CT-2002-01673), and the Human Frontier Science Program Organization (RGP22/2006).

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