MOLECULAR PLANT PATHOLOGY (2000) **1**(1), 33–39

Cell-to-cell movement of tobacco mosaic virus: enigmas and explanations

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SUMMARY

Tobacco mosaic virus (TMV) spreads between cells through plant intercellular connections, the plasmodesmata. This transport process is mediated by a specialized virus-encoded movement protein, TMV MP. Recent advances in two major aspects of TMV MP function highlight the limits of our current knowledge and promise exciting future developments. First, findings that TMV MP interacts with cytoskeletal elements and cell wall proteins suggest potential mechanisms for TMV MP targeting from the cell cytoplasm to plasmodesmal channels. Second, indications that TMV MP phosphorylation plays a regulatory role in several activities of TMV MP begin to unravel molecular pathways that control TMV cell-to-cell transport. TMV systemic movement that follows its initial cell-to-cell spread, on the other hand, may be controlled through two different pathways used for viral entry into and exit from the host plant vascular tissue.

INTRODUCTION

Tobacco mosaic virus (TMV) was once thought to move between cells as a whole viral particle by mechanically displacing the desmotubule, the core impermeable component of plasmodesmata (reviewed by Leisner and Howell, 1993). However, data accumulated over the past two decades have changed this view, indicating that TMV actively and specifically alters plasmodesmal permeability, potentially utilizing an endogenous pathway for macromolecular transport between plant cells. Thus, the cell-to-cell movement of TMV is coming under increasingly intense scrutiny as one of the best experimental systems to study intercellular transport in plants. The inevitable consequence of progress in this field is that, while the essential developments could be distilled into one review article in 1989 (Hull, 1989), only selected topics representing a small fraction of the total can now be discussed in the same space. Here, we have chosen to focus attention on two of the most interesting and yet least explored aspects of TMV movement: (i) targeting to plasmodesmata and (ii) regulation of plasmodesmal transport.

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FROM CYTOPLASM TO PLASMODESMATA

Following initial infection, usually by mechanical inoculation, TMV spreads from cell to cell through plasmodesmata until it reaches the vascular system; the virus is then transported systemically through the vasculature. Presumably, viral spread through the vascular tissue is a passive process, occurring with the flow of photoassimilates (Lartey *et al*., 1998); in contrast, cell-to-cell movement is an active function, requiring a specific interaction between the virus and plasmodesmata. For TMV and several other viruses, this interaction is mediated by virus-encoded nonstructural movement proteins (MP, reviewed by Carrington *et al*., 1996; Ghoshroy *et al*., 1997; Lucas and Gilbertson, 1994).

TMV MP is a 30-kDa protein (Deom *et al*., 1987) with at least five functional features: (i) TMV MP binds TMV RNA, forming an extended TMV MP-RNA transport complex that can penetrate the plasmodesmal channel (Citovsky *et al*., 1990; Citovsky *et al*., 1992); (ii) TMV MP interacts with cytoskeletal elements possibly to facilitate transport of the MP-TMV RNA complexes from the cell cytoplasm to plasmodesmata (Heinlein *et al*., 1995; McLean *et al*., 1995); (iii) TMV MP binds to a cell wall-associated pectin methylesterase (PME) (Fig. 1); (iv) TMV MP increases the size exclusion limit of plasmodesmata (Wolf *et al*., 1989) to allow intercellular movement of TMV MP-RNA complexes as well as TMV MP itself; and (v) TMV MP interaction with plasmodesmata is negatively regulated by phosphorylation (Fig. 1).

TMV MP is synthesized in the cytoplasm (Palukaitis and Zaitlin, 1986) where its journey begins to the cell wall, through plasmodesmata, and into a neighbouring, uninfected cell. Although TMV MP targeting to the cell wall and its cognate plasmodesmata is one of the central events in viral cell-to-cell movement, studies examining the mechanism of this process

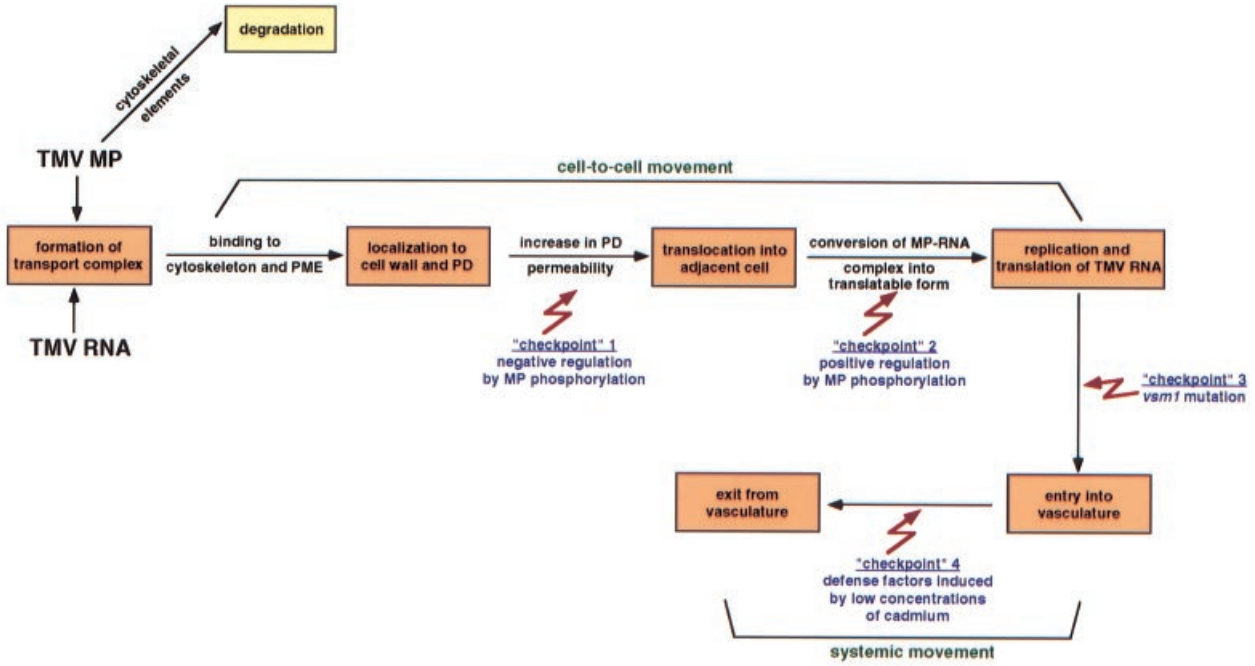

Fig. 1 TMV cell-to-cell and systemic movement and its regulatory points. TMV cell-to-cell movement initiates with the formation of MP-TMV RNA transport complexes. These complexes are then targeted to the cell wall and its resident plasmodesmata (PD) by the association of TMV MP with cytoskeletal elements and/or cell wall-associated pectin methyl esterase (PME). In addition, the cytoskeleton may be involved in targeting some of the (presumably free) TMV MP for degradation. Following targeting to plasmodesmata, TMV MP increases plasmodesmal permeability to allow translocation of the transport complexes into the neighbouring cell. This step may be negatively controlled by TMV MP phosphorylation, which blocks its ability to dilate plasmodesmata in some plant hosts. Passage through plasmodesmata also converts the MP-TMV RNA complexes into a translatable form, allowing its replication and translation. This conversion is most likely induced by TMV MP phosphorylation which, in this case, positively regulates viral spread by rendering the transport complexes infectious. When cell-to-cell spreading virions enter the vascular tissue, TMV systemic movement commences. Viral entry into and exit from the vasculature probably occurs by different mechanisms. The entry process can be inhibited by a *vsm1* mutation in *Arabidopsis*, whereas viral exit is blocked by treatment with nontoxic concentrations of cadmium. For further details, see text.

are only just beginning. Recent data suggest that TMV MP interacts with microtubules and, to a lesser extent, with actin microfilaments (Heinlein *et al.*, 1995; McLean *et al.*, 1995). This interaction was inferred from the co-localization of TMV MP tagged with the green fluorescent protein (GFP) of *Aequorea victoria*, with tubulin as well as with actin filaments in virus-infected protoplasts (Heinlein *et al.*, 1995; McLean *et al.*, 1995) or in protoplasts that transiently express TMV MP (McLean *et al.*, 1995). The association of TMV MP with actin and tubulin was also demonstrated using *in vitro* binding assays (McLean *et al.*, 1995). Cytoskeletal elements, especially actin (Ding and Kwon, 1996; White *et al.*, 1994), may co-localize with plasmodesmata and were thus suggested to target TMV MP and, by implication, MP-TMV RNA complexes to plasmodesmata (Fig. 1). However, TMV MP association with the cytoskeleton may have additional or even alternative functions. For example, the biological role of interaction between a tobamoviral MP and cytoskeletal elements was suggested to be host-dependent (Padgett *et al.*, 1996). Specifically, in *Nicotiana tabacum*, MP-GFP interacting with the cytoskeleton formed a characteristic pattern of MP-GFP filaments within cells at the leading edge of infection, consistent with a role in plasmodesmal targeting of the transported MP and/or MP-RNA complexes. Conversely, in *N. benthamiana*, MP-GFP filaments were only observed in the inner areas of the infected tissue; furthermore, a loss of MP-GFP fluorescence was coincident with MP degradation, suggesting that cytoskeletal components may target MP for degradation (Padgett *et al.*, 1996; Fig. 1). Indeed, microtubules have been shown to participate in the induced degradation of cellular proteins in cultured mammalian cells (Aplin *et al.*, 1992). Consistent with this idea, disruption of microtubules by oryzalin did not affect the accumulation of TMV MP-GFP in the punctate sites in the cell periphery, suggesting that microtubules do not play a direct role in TMV MP targeting to these presumably plasmodesmal locations (Heinlein *et al.*, 1998).

The cellular endoplasmic reticulum (ER) may serve as another potential route for transport of TMV MP to plasmodesmata. TMV MP-GFP (as well as viral replicase) has been shown to associate with the cytoplasmic face of cortical ER (Heinlein *et al.*, 1998). This association was suggested to initiate the formation

of 'viral factories' and, at later stages of infection, direct TMV MP to plasmodesmata (Heinlein *et al.*, 1998) that associate with cortical ER (Oparka *et al.*, 1994). This targeting pathway may be also aided by actin, which is known to form a complex with endoplasmic reticulum (Lichtscheidl *et al.*, 1990).

Clearly, the role of the cytoskeleton in TMV MP transport is still open to interpretation. For example, binding to the cytoskeleton may negatively regulate TMV MP movement by anchoring this protein to the cell cytoplasm. Similar mechanisms of cytoplasmic anchoring have been described in the regulation of protein nuclear import (reviewed by Nigg, 1997). One reason for the difficulties in interpreting experiments using TMV MP-GFP is the intrinsic complexity of the system. First, TMV MP-GFP fusions often form insoluble aggregates which probably lack any biological role (V.C., unpublished results). Second, because only minute quantities of TMV MP-RNA complexes are sufficient for the cell-to-cell spread of infection, the bulk of TMV MP that is visualized using GFP fusions may not directly participate in viral movement. Finally, analyses of TMV MP-GFP data may be complicated by the length of time that is required for the maturation of GFP into a fluorescent state (Cubitt *et al.*, 1995), which may preclude the detection of very early cellular events taking place immediately following TMV MP synthesis.

Cytoskeletal elements were the first cellular proteins that were shown to interact with TMV MP. The search for additional host cell factors that recognize TMV MP continues. Because TMV MP has been shown to localize to plasmodesmata within plant cell walls (Ding *et al.*, 1992; Tomenius *et al.*, 1987), it may specifically interact directly with cell wall-associated proteins. To detect such interactions, a renatured blot overlay-binding assay was developed, in which a protein mixture containing a putative TMV MP interactor is separated by SDS-polyacrylamide gel electrophoresis and electrotransferred on to a membrane, followed by guanidine hydrochloride extraction of SDS from the blotted proteins. The membrane is then renatured, incubated with purified TMV MP, washed, and TMV MP binding is detected using anti-MP antibodies. These experiments identified a 36–38 kDa protein band within cell wall fractions of mature leaves of *N. tabacum*, but not in the soluble fractions of tobacco leaf tissue (M.H.C. and V.C., unpublished data).

Purification of the cell wall-associated TMV MP interactor followed by protein microsequencing analysis, identified it as a member of the pectin methyl esterase (PME) multigene family. PME is involved in cell wall turnover and appears to have a role in plant growth and development. PME activity is thought to modulate pH and ion balance and affect cell wall porosity (Narin *et al.*, 1998; Pressey, 1984; and references therein). In addition, PME has been implicated in more specialized cellular processes such as plant responses to pathogen attack (Markovic and Jornvall, 1986).

TMV MP–PME interaction was confirmed using the yeast two-hybrid system and PME cDNA from tomato leaves. Immunoelectron microscopy studies using anti-PME antibodies have shown that this protein is localized throughout the cell wall, including the plasmodesmata (M.H.C. and V.C., unpublished data). Potentially, binding to PME may function to initially target and/or anchor TMV MP to the host cell wall (Fig. 1). In this scenario, TMV MP association with PME in the vicinity of plasmodesmata will commence the cell-to-cell transport process. In contrast, binding to PME in cell wall areas that do not contain plasmodesmata will result in abortive movement, with TMV MP either being degraded or redirected back into the cell cytoplasm. This model assumes that TMV MP targeting to the cell periphery may occur irrespective of the presence of plasmodesmata. Indeed, recent data suggest that TMV MP expressed in tobacco protoplasts that do not possess plasmodesmata forms protrusions on the cell surface (Heinlein *et al.*, 1998); in these cells, TMV MP may recognize the cell surface via binding to PME which is likely to be present within the residual cell wall of the protoplasts. In addition, a more active role for PME in viral movement cannot be ruled out. For example, TMV MP binding may interfere with PME activity, altering the cell wall ion balance and, consequently, inducing changes in plasmodesmal permeability. Supporting the role of PME in TMV MP transport, the TMV MP–PME interaction was shown to involve TMV MP domains required for its function *in vivo* (M.H. Chen and V. Citovsky, unpublished data).

REGULATED TRANSPORT OF TMV MP THROUGH PLASMODESMATA

Although TMV MP is present within the plasmodesmata of all infected cells, it only increases the plasmodesmal permeability at the leading edge of the expanding infection site (Oparka *et al.*, 1997). Thus, TMV MP activity that takes place within cells behind the leading infection edge may be negatively regulated to prevent its continuous interference with host plant intercellular communication. It is possible that the post-translational modification of TMV MP—such as phosphorylation—may perform this regulatory function. Indeed, TMV MP is known to undergo phosphorylation at multiple sites (Citovsky *et al.*, 1993; Haley *et al.*, 1995; Kawakami *et al.*, 1999; Watanabe *et al.*, 1992). The best characterized TMV MP phosphorylation site comprises the carboxyl terminal serine-258, threonine-261 and serine-265 residues which are specifically phosphorylated by a host cell wall-associated protein kinase (Citovsky *et al.*, 1993). Phosphorylation occurs both *in vitro* (Citovsky *et al.*, 1993) and *in vivo*, requiring Mg^{2+} but not Ca^{2+} cations (M.H.C., E.W. and V.C., unpublished data). Independence from Ca^{2+} distinguished the TMV MP-phosphorylating enzyme activity from several known protein kinases that associate with plant cell walls (He *et al.*, 1996; Yahalom *et al.*, 1998).

The biological function of TMV MP carboxyl terminal phosphorylation was examined using negatively charged amino acid substitutions within the phosphorylation site. Substitutions with aspartate or glutamate are known to reveal the electrostatic effects of phosphorylation (Dean and Koshland, 1990). For example, the replacement of serine by aspartate in the HPr protein has been shown to cause shifts in its two-dimensional NMR spectra which are similar to those elicited by phosphorylation (Wittekind *et al.*, 1989). Moreover, the inactivation by phosphorylation of Ser-113 in isocitrate dehydrogenase is mimicked when aspartate is substituted at this site (Thorsness and Koshland, 1987). In microinjection studies, substituting the serine-258, threonine-261 and serine-265 of TMV MP with aspartate residues inactivated MP ability to increase plasmodesmal permeability (M.H. Chen, E. Waigmann and V. Citovsky, unpublished data). Furthermore, the negative regulation of the TMV MP interaction with plasmodesmata was host-dependent. In *N. tabacum*, mimicking phosphorylation blocked the ability of TMV MP interact efficiently with plasmodesmata whereas, in *N. benthamiana*, the same TMV MP derivative remained fully active as compared to nonphosphorylated TMV MP. Thus, *N. benthamiana*, one of the most susceptible hosts for plant viruses (Dawson and Hilf, 1992; Gibbs *et al.*, 1977), may lack the mechanism for TMV MP inactivation by phosphorylation.

It is tempting to speculate that the regulatory activity of Mp phosphorylation may underlie the well-known but previously unexplained differences between various tobacco species in their susceptibility to viral infection (Dawson and Hilf, 1992; Gibbs *et al.*, 1977). Besides TMV MP, a few other plant viral MPs have been tested for phosphorylation. For example, phosphorylation of the 17-kDa MP of the potato leafroll virus was recently reported and also proposed as negatively regulating the function of this transport protein (Sokolova *et al.*, 1997).

Because the carboxyl terminal phosphorylation of TMV MP blocks its interaction with plasmodesmata, it should also inhibit the ability of the virus to spread *in vivo* from cell to cell. Indeed, TMV carrying the negatively charged derivative of TMV MP within its genomic RNA was unable to move locally and systemically within *N. tabacum* (M.H.C., E.W. and V.C., unpublished data). The mutant virus was fully capable of movement in transgenic plants expressing wild-type TMV MP, indicating that the mutation did not interfere with the replication and translation of viral gene products. Thus, the mimicking of TMV MP phosphorylation had blocked TMV movement through plasmodesmata, probably by preventing the TMV MP-induced increase in the permeability of these intercellular channels (Fig. 1, checkpoint 1). Similarly to microinjection experiments, the effect of mimicking TMV MP phosphorylation on TMV movement *in vivo* was host-dependent, occurring in *N. tabacum* but not in a more promiscuous *N. benthamiana* host (M.H. Chen, E. Waigmann and V. Citovsky, unpublished data).

The negative regulation of TMV MP interaction with plasmodesmata makes biological sense. Unlike other TMV-encoded proteins expressed throughout the course of viral infection, MP is synthesized only transiently (reviewed in Hull, 1989). However, the protein product is not apparently subjected to rapid turnover; instead, it persists in the infected cells, accumulating within their plasmodesmata (Ding *et al.*, 1992; Tomenius *et al.*, 1987). The continuous presence of active TMV MP may elevate plasmodesmal permeability and alter intercellular communication, an important biological process. It is possible that phosphorylation minimizes TMV MP interference with plasmodesmal transport during viral infection. Thus, viral cell-to-cell movement may involve plasmodesmal 'gating' by the newly synthesized, unphosphorylated TMV MP, followed by the phosphorylation of TMV MP that has already performed its function within the plasmodesmata, preventing it from further action.

Because viruses often adapt existing cellular machinery for their own needs, TMV most likely employs an endogenous pathway for the cell-to-cell transport of proteins and nucleic acids. Thus, TMV MP phosphorylation may represent a general mechanism by which plants regulate their intercellular transport of macromolecules. Indeed, in addition to large molecules such as viral MPs and MP-nucleic acid complexes (reviewed by Carrington *et al.*, 1996; Ghoshroy *et al.*, 1997; Lazarowitz and Beachy, 1999), plasmodesmata traffic transcription factors (Lucas *et al.*, 1995) and gene silencing signals (Palauqui *et al.*, 1997). The plasmodesmal transport of these biologically active factors should be tightly regulated.

In addition to regulating TMV MP–plasmodesmata interactions, TMV MP phosphorylation may control other events in the viral life cycle. For example, MPs of tobamoviruses act as efficient translational repressors of viral RNAs in MP-RNA complexes *in vitro* and in isolated plant protoplasts lacking plasmodesmata (Karpova *et al.*, 1997). However, these complexes become infectious in plant tissues (Karpova *et al.*, 1997). In addition, when TMV MP was phosphorylated *in vitro* by protein kinase C before or after binding to RNA, the resulting TMV MP–RNA complexes became translatable *in vitro* and infectious in protoplasts (Karpova *et al.*, 1999). Taken together, these data suggest that in plant tissue TMV MP–RNA complexes are converted into a translatable form by TMV MP phosphorylation, which possibly takes place during or after passage through the plasmodesmata. Thus, TMV MP phosphorylation may act as a molecular switch between viral spread and translation/replication (Fig. 1, checkpoint 2).

Besides its carboxyl terminal phosphorylation, TMV MP is phosphorylated at other serine residues (Haley *et al.*, 1995; Kawakami *et al.*, 1999; Watanabe *et al.*, 1992), possibly by soluble protein kinases of the host cell. While the biological role of these phosphorylation sites is less clear, at least some of them may be critical for the function of TMV MP. For example, recent

studies (Kawakami *et al.*, 1999) suggest that serine-37 and serine-238 within the MP of tomato mosaic tobamovirus (ToMV) are phosphorylated in tobacco protoplasts. While mutations of serine-238 did not affect viral infectivity, amino acid changes at position 37 resulted in an altered intracellular localization of TMV MP and its decreased stability (Kawakami *et al.*, 1999). Interestingly, a substitution of serine-37 with another phosphorylatable residue, threonine, still resulted in a TMV MP mutant with decreased stability in plant protoplasts (Kawakami *et al.*, 1999). Thus, the mere presence of serine-37 rather than its phosphorylation is likely to be responsible for these effects.

Finally, besides phosphorylation, other types of post-translational modification may affect the function of TMV MP. For example, in *Arabidopsis thaliana*, TMV MP has been shown to be proteolytically processed at its amino terminus. The processed TMV MP was nonfunctional, suggesting that proteolytic cleavage may represent an alternative strategy inn the deactivation of TMV MP (Hughes *et al.*, 1995).

Another regulatory 'checkpoint' for TMV spread may occur during viral entry into and exit from the vascular system of the host plant (Fig. 1, checkpoints 3 and 4). Following an initial infection, usually by mechanical inoculation, TMV moves from cell to cell through plasmodesmata until it reaches the vascular system; the virions are then transported systemically through the vasculature (reviewed by Leisner and Howell, 1993; Fig. 1). Evidence is increasingly suggesting that TMV and other tobamoviruses enter the host plant vasculature by a mechanism that is different from that used for their egress back into nonvascular tissues. Specifically, a recessive mutation, *vsm1*, in a single *Arabidopsis* gene, has been shown to block viral entry into the host plant vasculature (Lartey *et al.*, 1998; Fig. 1, checkpoint 3). On the other hand, the exposure of tobacco plants to nontoxic concentrations of the heavy metal cadmium prevented tobamoviral disease (Ghoshroy *et al.*, 1998) by blocking viral exit from the vascular tissue into the noninoculated, systemic organs, but it did not affect viral entry into the vasculature (Citovsky *et al.*, 1998; Fig. 1, checkpoint 4). The inhibitory effect of cadmium was independent of classical plant defence pathways involving salicylic acid and the induction of pathogenesis-related (PR) genes (Citovsky *et al.*, 1998). The treatment of plants with cadmium did not interfere with viral replication and local movement within the inoculated leaf. Furthermore, higher, toxic levels of cadmium did not inhibit viral movement, allowing the systemic spread of infection and development of the viral disease (Ghoshroy *et al.*, 1998). These observations suggest that cadmium-induced viral protection requires a metabolically active healthy plant. Therefore, nontoxic levels of cadmium may trigger the synthesis of cellular factors interfering with viral systemic movement. Potentially, the production of these compounds may represent another level of regulation of macromolecular transport throughout the plant (Fig. 1).

WHAT NEXT?

Recent developments reviewed here illustrate some of the deficiencies in our understanding of TMV MP activity and its regulation. For instance, the putative cellular receptor for TMV MP mediating the specific recognition of plasmodesmata and the onset of intercellular movement has yet to be identified. In addition, potential signal sequences within TMV MP that determine its specific localization and transport functions remain to be characterized. To better understand the regulation of plasmodesmal transport, it will be critical to identify cellular factors that are directly involved in the regulatory pathway(s), e.g. the cell wall-associated protein kinase phosphorylating TMV MP. Perhaps the best way to achieve these goals is to combine biochemical, molecular and cellular biological techniques with a genetic approach aimed at identifying and characterizing plant mutants with altered plasmodesmal functions. For example, *Arabidopsis* mutants resistant to the systemic spread of tobamoviruses (Lartey *et al.*, 1998) and ecotypes (Lee *et al.*, 1994; Leisner *et al.*, 1993; Simon, 1994) or mutants with an altered systemic movement of other plant viruses have been reported (Mahajan *et al.*, 1998). Owing to the fundamental importance of plant intercellular connections, advances in our knowledge in the area of TMV MP–plasmodesmata interactions will have significant consequences for understanding many basic cellular processes in plants.

ACKNOWLEDGEMENTS

The work in our laboratories is supported by grants from National Institutes of Health (Grant no. R01-GM50224), US Department of Agriculture (Grant no. 94-02564), and US-Israel Binational Research and Development Fund (BARD) (Grant no. US-2247-93) to V.C. and a grant from the Austrian Science Foundation (Grant no. P12614-MOB) to E.W. E.W. is supported by an APART fellowship (APART 441) from the Austrian Academy of Science and T.T. is supported by a BARD Postdoctoral Fellowship.

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