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

YOON RHEE, TZVI TZFIRA, MIN-HUEI CHEN, ELISABETH WAIGMANN* AND VITALY CITOVSKY*

Department of Biochemistry and Cell Biology, State University of New York, Stony Brook, NY 11794-5215, and *Institute of Biochemistry, University of Vienna, Dr Bohrgasse 9, A-1030, Vienna, Austria

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 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.

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 systematically through the vasculature. Presumably, viral spread through the vascular tissue is a passive process, occurring with the flow of phloem transport (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 non-structural 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 methyl esterase (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...
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 Kwok, 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

---

**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 vvm1 mutation in *Arabidopsis*, whereas viral exit is blocked by treatment with nontoxic concentrations of cadmium. For further details, see text.
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 assayed by blocking 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. Immuno-electron 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 HP 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, probably by preventing the TMV MP-induced increase in permeability of these intercellular channels (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 unpublished data).
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 modifications 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 in 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 (Larrety 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 (Larrety 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.

REFERENCES


