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Review

The spread of *Tobacco mosaic virus* infection: insights into the cellular mechanism of RNA transport

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Abstract. Interactions of plant cells with pathogens or other biotic or abiotic environmental factors can give rise to systemic defense responses that rely upon the cell-to-cell and systemic transport of specific signals. A novel type of systemic signaling was revealed by recent evidence indicating the existence of RNA species that travel cell to cell and through the vasculature. The most compelling evidence for intercellular and systemic transport of RNA in plants is provided by viroids and viruses that apparently use the endogenous transport machinery to spread infection. The cell to cell movement of plant viruses occurs through small pores in the cell wall known as plasmodesmata and depends on virus-encoded 'movement proteins'. This review summarizes current knowledge of *Tobacco mosaic virus* infection with emphasis on the mechanism by which this virus targets its RNA genome from sites of replication to plasmodesmata to achieve intercellular spread.

Key words. TMV; movement protein; GFP; RNA transport; microtubules; gene silencing.

Introduction

Research on *Tobacco mosaic virus* (TMV) has played a leading role in the development of virology for more than a century [1–3]. In its design TMV is one of the simplest viruses known and therefore has served as a paradigm for studies of biological structure and macromolecular assembly as well as for the development and application of new techniques in X-ray analysis and electron microscopy. TMV also contributed to the development of molecular biology in its early phase, before its influence was replaced by that of *Escherichia coli* and its phages. When full-length complementary DNA (cDNA) clones of TMV were developed [4, 5], allowing the analysis of this virus by reverse genetics, TMV became the first plant virus for which structures and functions were known for its genes. Very important was the discovery that TMV encodes a 30-kd protein serving a cell-to-cell movement function [6]. Today, it is known that most, if not all, plant viruses encode one or more such 'movement proteins' (MPs) [7]. These proteins are essential for the spread of infection throughout the plant and have attracted much attention as potential targets for engineering virus resistance [8–16]. MPs act as major host range determinants [17, 18] and hence also became an important tool with which to investigate mechanisms involved in host-virus interactions and intercellular communication. It is the aim of this article to provide an overview of TMV infection and the role of MP in intercellular and systemic transport of the virus. Moreover, as TMV is able to spread its RNA genome from cell to cell in a non-encapsidated form, its potential role as a model for elucidating the cellular mechanism of RNA trafficking in plants [19] is discussed.

Structure and organization of the TMV genome

TMV consists of a single-stranded, messenger-sense RNA of about 6400 nucleotides that is encapsidated in a

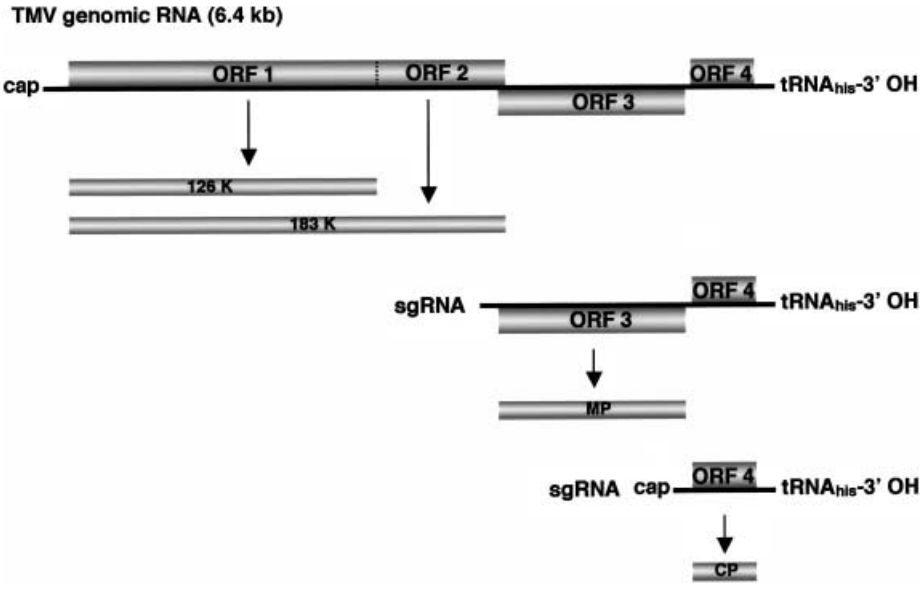


Figure 1. Organization and expression of the TMV genome. The 126-kd and 183-kd subunits of the replicase are translated from the genomic RNA of the virus; the 30-kd movement protein (MP) and the 17.5-kd coat protein (CP) are translated from subgenomic RNA molecules that are produced during replication. The genomic RNA and the CP subgenomic RNA, but not the MP subgenomic RNA, are capped. The viral RNA terminates with a t-RNA-like structure that accepts histidine.

helical coat of 2160 subunits of coat protein (CP). The RNA is capped at the 5′ end and folds into a histidine-accepting transfer RNA (t-RNA)-like structure at the 3′ end. An untranslated leader of 69 nucleotides is followed by several closely packed open reading frames (ORFs) which encode at least three nonstructural proteins (126-, 183-, 30-kd proteins) and a 17.5-kd coat protein. The 126-kd and 183-kd proteins represent the replicase function of the virus, whereby the longer polypeptide results from readthrough of the amber stop codon that terminates the synthesis of the shorter 126-kd polypeptide. The replicase is translated from the genomic RNA that enters a cell, whereas MP and CP are expressed from subgenomic RNAs that are produced during virus replication (fig. 1).

The infection cycle

Unlike many other viruses that depend on insects, nematodes, fungi or other vectors for transmission from plant to plant, TMV is transmitted mechanically by physical contact between plant tissue and virus-contaminated surfaces [20]. Infection of a cell (see fig. 2) is believed to be initiated by a process termed cotranslational disassembly in which the 5′ end of the virus is uncoated within 2–3 min, making the first ORF encoding replicase accessible for translation. This attractive hypothesis is based on experiments performed by Wilson (1984) who showed that treatment of virions with mild alkali greatly facilitated translation of replicase in cell-free in vitro translation systems [21]. Later it was shown that exposure of purified TMV particles to mild alkali or detergent treatment resulted in the rapid exposure of about 200 nucleotides at the 5′end of the viral RNA [22]. The mechanism that triggers disassembly in vivo is unknown. However, the pH and ionic conditions in a cell are similar to those applied in vitro by Mundry et al. (1991) [22], and may give rise to virus disassembly in the cytoplasm. It has also been suggested that the incoming virus may interact with a receptor-like structure that somehow induces instability required for uncoating the leader sequence. An unresolved question also is how virus uncoating can be completed when ribosomes disengage at the replicase ORF stop codons. Wilson (1985) suggested that uncoating may be bidirectional and involve a coreplicational disassembly mechanism initiated through binding of the replicase to the aminoacetylated t-RNA-like structure at the 3′ end of the viral RNA [23]. This view gained support from in vivo experiments performed by Wu and Shaw (1997) who showed that progeny negative-strand viral RNA begins to be produced at the same time as 3′→5′ disassembly is initiated and that disassembly depends on the presence of the 126-kd and 183-kd replicase proteins [24]. Viral disassembly, translation of replicase proteins and the initiation of replication may thus be coupled processes that occur in the same complex in vivo.

Once the negative strand copy of the viral RNA genome is synthesized, replication proceeds on negative-strand RNA templates to produce positive-strand full-length genomic RNA, as well as 3′-coterminal subgenomic MP- and CP-encoding RNAs that are driven by subgenomic

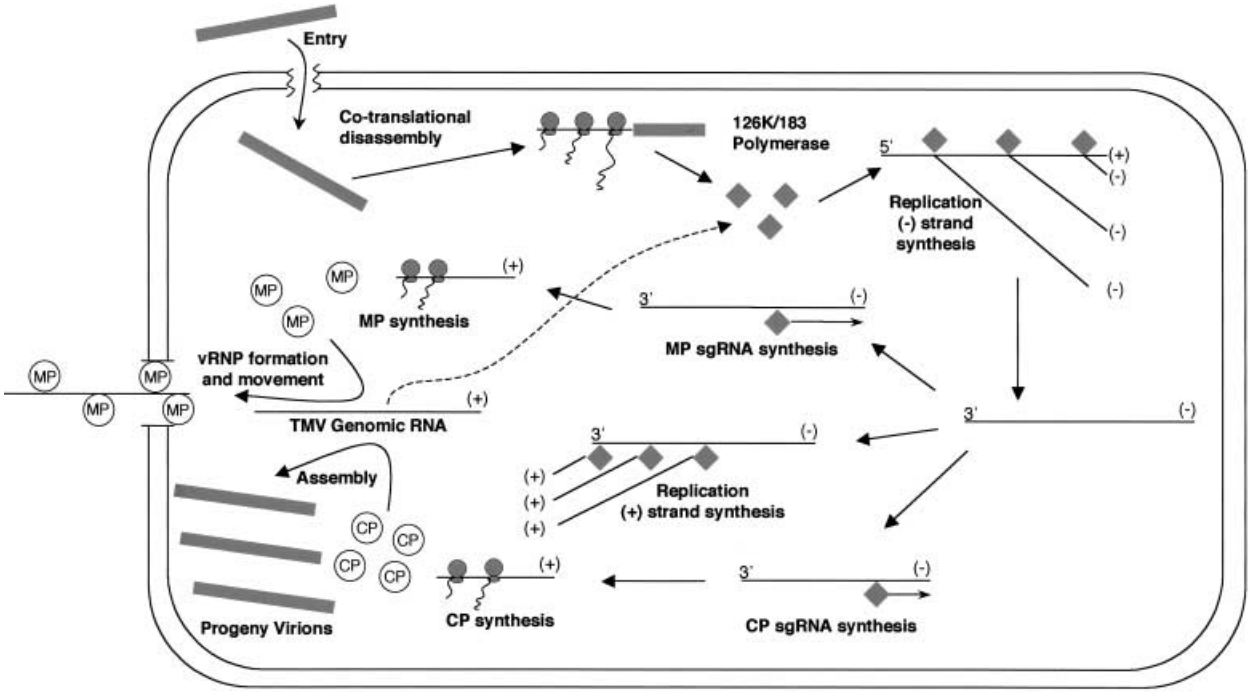


Figure 2. Different stages of TMV infection.

promoters [25]. In tobacco protoplasts, synthesis of TMV negative strands ceases 6–8 h after inoculation, whereas synthesis of positive strands continues for 16–18 h after inoculation [26]. Replication intermediates isolated from infected cells include completely double-stranded RNA (dsRNA) as well as molecules of partly double-stranded and partly single-stranded RNA (ssRNA). However, it is not known whether these structures are double-stranded or largely single-stranded in vivo. Since dsRNA can potentially trigger gene silencing [27] and the degradation of viral RNA [28, 29], it is conceivable that TMV and other RNA viruses have evolved mechanisms to largely avoid the formation or persistence of double-stranded replication intermediates. The MP is expressed transiently, early to mid-infection, and in relatively small amounts, whereas the coat protein is expressed in large amounts mainly late during the replication cycle [30–33]. Later in the replication cycle, after the synthesis of negative strand RNA has ceased and coat protein has accumulated, most of the progeny positive-strand RNAs are encapsidated to form virions [34, 35]. Neither MP nor CP is absolutely required for TMV RNA replication [36, 37]. However, evidence described in detail below suggests that in infected plants, viral replication, cell-to-cell movement and RNA encapsidation occur in a highly coordinated and interactive fashion.

Plasmodesmata

To spread infection from initially infected cells into adjacent cells, TMV and other viruses have to move across the plant cell wall. Therefore, viruses target plasmodesmata (Pd), plasma membrane-lined cell wall channels that provide symplastic continuity between adjacent cells throughout plant tissues, thus providing higher plants with a supracellular nature [38]. Pd are about 60 nm in diameter [38], i.e. about half in size compared to nuclear pore complexes. Usually, Pd form during culmination of cytokinesis by entrapment of endoplasmic reticulum (ER) membranes within the newly forming cell plate. These so-called primary Pd are distinguished from secondary Pd that form through preexisting walls in order to connect cells that initially were separate, e.g. in graft unions, postgenital carpel fusions or host-parasite connections (reviewed in [39]). High-resolution images of Pd produced by electron microscopy demonstrate that both ER and plasma membrane (PM) are continuous through the pore, the ER forming a furled, appressed 'desmotubule' in its center. The latter element stabilizes the internal structure of Pd and also limits their pore size. This is due to the fact that both the plasma membrane and the desmotubule are densely covered with globular particles which are interlinked with spoke-like elements [40–43] that restrict diffusion through the cytoplasmic sleeve. The size exclusion limit (SEL) of Pd is developmentally regulated and decreases when a leaf

undergoes the sink-to-source transition [44, 45]. Whereas Pd connecting cells in young sink leaves can permit the cell-to-cell trafficking of molecules up to a size of about 50-kd [45], Pd in mature source leaves are usually characterized by a basal SEL of approximately 1 kd [46–50], thus allowing transport of only small molecules, such as water, ions, hormones and metabolites. This change in the function of Pd during the sink-to-source transition is accompanied by changes in Pd structure, from 'simple' to 'branched' [45]. However, despite these overall changes in structure and conductivity, Pd in mature leaves permit the trafficking of viruses and other macromolecules, indicating that Pd are highly dynamic and able to transiently increase their SELs. The dynamic nature of Pd is emphasized by two recent reports [51, 52] describing that Pd connecting leaf epidermal cells can fluctuate between different states of dilation, permitting macromolecular movement [e.g. green fluorescent protein (GFP)] between about 30–70% of cells in sink leaves and between up to 50% of cells if mature leaves. These numbers reflect the overall reduction of Pd SELs upon maturation of the leaf, as described above. On the other hand, these new findings seem to indicate that there is no strict rule as to the SELs of individual Pds in mature and immature tissues. Moreover, the number of Pd permitting macromolecular (protein) movement may differ between plant species and depends on plant growth conditions [52] as well as on whether a given protein is actively targeted to Pd [51]. Collectively, these findings indicate that individual Pd are able to respond to diverse stimuli, potentiating the complexity by which intercellular communication can be regulated and macromolecular signalling gradients between individual cells formed [51].

The transport of both small and large molecules through Pd most likely occurs through the cytoplasmic sleeve (e.g. [38, 53, 54]). The fluidity of the ER may also permit the trafficking through the lipid bilayer of the desmotubule (especially hydrophobic molecules; [55]) or through the lumen of the desmotubule [43, 56–58].

The molecular nature of proteins associated with Pd remains elusive even after many years of intensive study. Nevertheless, recent advances in immunocytochemical techniques have allowed the identification of proteins enriched within Pd. These include calreticulin [59], an EF-hand centrin-like protein [60], calcium-dependent kinase [61], ubiquitin [62] and a 41-kd protein isolated from maize mesocotyl walls [63]. Importantly, Pd also contain actin [64, 65] and myosin [65–67], which is consistent with the dynamic nature of Pd. The possible involvement of actin and myosin in regulating the SEL of Pd is supported by the apparent dilation of Pd following depolymerization of F-actin by treatment or microinjection of plant cells with cytochalasin D [64, 68] or profilin [68]. Moreover, inhibition of myosin ATPase constricts Pd necks [66] and dilates ER elements near Pd [69]. ATP depletion opens Pd [70], suggesting that constriction of Pd may be an ATP-dependent process. Pd gating may also be regulated by calcium signaling, since intracellular calcium waves were shown to give rise to immediate, transient closure of Pd [71, 72]. Pd trafficking may also be regulated by mechanisms that act upon the exit and entrance areas of Pd. Electron-lucent collars (neck constrictions; [43, 73–75]) or rings of external particles called sphincters [76, 77] have been described that may function as constricting valves to increase or decrease the conductivity of Pd. Finally, Pd may be regulated by the deposition of callose in the surrounding cell wall microdomains. Experimental data showed that callose controls Pd permeability during aluminum toxicity [78]. Moreover, transgenic tobacco plants deficient in β-1,3-glucanase are characterized by increased callose deposition and a reduced Pd SEL [79]. The observation that callose deposition delays virus movement [79], and that degradation of callose in infected cells promotes virus movement [79a] supports the possible role of callose deposition as an in vivo Pd 'gatekeeper' [80]. However, since reabsorption of callose is a rather slow process [81], a direct role of callose in rapid closing and opening of Pd seems less likely. The cell wall microdomains around Pd are also characterized by a unique pectin composition (e.g. [82]). The cell wall enzyme pectin methylesterase, which is responsible for de-esterification of secreted proteins, localizes preferentially to microdomains surrounding Pd [83] and may influence the permeability of Pd by changing the quality of their cell wall environment.

TMV MP modifies Pd

The existence of a virus-encoded function required for cell-to-cell movement of TMV was demonstrated by early studies using temperature-sensitive (*ts*) strains that are unable to move cell-to-cell at a restrictive temperature. The most informative studies were performed with the Ls-1 strain of the closely related *Tomato mosaic virus* (strain L of TMV) [84]. At 32 °C, this virus can replicate and assemble normally in leaf cells or protoplasts but cannot move cell-to-cell in leaves; at 22 °C, virus spreads normally [84]. The defect was correlated with slight changes in the tryptic peptide map prepared from in vitro translated 30-kd protein [85] and was later determined to be due to a single base change in the Ls-1 genome which substituted a serine for a proline residue [86]. The role of the 30-kd protein in virus spread was further corroborated by the ability of MP-transgenic plants to complement for Ls1 at nonpermissive temperatures [6]. The 30-kd protein also complements for the defective cell-to-cell movement function of Ni2519 [87], another *ts*

mutant of TMV. Moreover, frame-shift mutations in the 30-kd gene gave rise to cell-to-cell movement-defective TMV phenotypes [36]. These studies established the role of the 30-kd MP as a viral pathogenicity factor, and further work concentrated on the molecular characterization of this protein.

Consistent with a function in viral movement, the MP was found to accumulate in Pd and to increase their SELs [50, 88–92]. The ability of MP to increase Pd SEL was first detected by injection of fluorescently labelled dextran into leaf mesophyll cells of transgenic tobacco plants expressing the protein [50]. Compared with Pd in non-transformed plants, which can traffic dextrans with molecular weights of 0.75–1.0 kd, Pd in the transgenic plants had a SEL of almost 10 kd [50]. Microinjection of MP expressed and purified from *E. coli* further demonstrated the ability of MP to increase Pd SEL. Microinjected wild-type tobacco mesophyll cells permitted the trafficking of dextrans with a molecular weight of 20 kd through Pd. The observation that the increase in Pd SEL occurred within very short time (3–5 min) indicated that MP interacts with an endogenous intercellular transport machinery [93]. Moreover, the finding that the coinjected dextrans traveled as far as 20–50 cells away from the site of injection suggested that the microinjected MP may itself traffic from cell to cell, leading to modification of Pd quite distant from the injected cell. This hypothesis was later confirmed by immunolocalization of MP following microinjection [94] and by transient expression of the protein in leaf epidermal cells [95].

The ability to modify Pd SEL appears to be a property of many, if not all, viral movement proteins. To date, the movement proteins of *Red clover necrotic mosaic dianthovirus* (RCNMV), *Alfalfa mosaic bromovirus* (AMV), *Cucumber mosaic bromovirus* (CMV), *Tobacco rattle tobravirus* (TRV), *Potato potexvirus X* (PVX) and the BL1 movement protein of *Bean dwarf mosaic geminivirus* (BDMV) have been shown to mediate transport of large fluorescent dextrans between plant cells [96–101]. Moreover, like TMV MP, the MPs of other viruses, including *Tomato mosaic tobamovirus* (ToMV), *Maize streak geminivirus* (MSV), *Apple chlorotic leaf spot trichovirus* (ACLSV), PVX, RCNMV, CMV and BDMV have also been shown to move from cell to cell themselves [97, 99, 102–107].

Transport as a RNP complex

Viruses use different mechanisms for movement through Pd. For instance, cell-to-cell transport of several ssRNA viruses (i.e. como-, nepo-, olea- and trichoviruses; [106, 108–111] and dsDNA plant viruses (i.e. caulimoviruses; [112, 113]) involves 'tubule-guided' transport of mature virions through Pd that are structurally modified by a tubule made of virus-encoded MP [114, 115]. In contrast, TMV and other RNA viruses are believed to move from cell to cell in the form of a ribonucleoprotein complex (vRNP). This process does not require CP [37, 116] and does not involve obvious changes in Pd structure. The Pd of TMV MP-transgenic plants contain fibrous material that can be labeled with anti-MP antibodies [89, 90]. These fibers may be comparable to the tubular arrangement of MP-containing fibers that have been observed to form across intercellular junctions in MP-transgenic cyanobacteria [117].

Consistent with movement of TMV as a vRNP, it has been demonstrated that MP binds both RNA and ssDNA in vitro resulting in the formation of unfolded and elongated protein: RNA complexes [118, 119]. The estimated diameter of the complex was determined to be 2.0–2.5 nm and to be compatible with the estimated dilated channel diameter of modified Pd [119]. It has been speculated that MP:RNA complexes represent a particular pool of viral RNA molecules which are destined for translocation and excluded from replication [118]. Such a complex would bind putative host cell Pd-targeting factors and receptors. Several studies support the in vivo formation of a vRNP. 'Informosome'-like RNP complexes could be isolated from TMV-infected plants but not from plants inoculated with virus encoding a temperature-sensitive MP and extracted at nonpermissive temperature, suggesting that vRNPs are formed in vivo and contain MP [120, 121]. vRNA complexed with MP in vitro was shown to be untranslatable both in vitro and also in vivo, upon electroporation into protoplasts. In contrast, translation and replication occurred in planta, suggesting that the vRNP undergoes modification upon passage through Pd [122]. In vitro phosphorylation by a protein kinase C or by cell-wall-associated kinase abrogated translational inhibition, suggesting the involvement of MP phosphorylation [123]. Further support for the in vivo formation of a vRNP was provided by experiments involving microinjection of *Nicotiana clevelandii* trichome cells. These experiments demonstrated that microinjected β-glucuronidase (GUS) can move between trichome cells when fused to MP but not when coinjected with MP as a free protein [94]. This finding seems to exclude the possibility that MP mediates macromolecular trafficking by activation of a trans-acting factor. Rather, MP functions as a cis-acting mediator of Pd transport, requiring the physical association of MP with the molecule to be transported (e.g. GUS or vRNA). Microinjected MPs do not only move themselves between cells but also mediate transport of co-injected nucleic acids [93, 97, 99, 103]. However, conclusive evidence that viral RNA indeed binds MP in vivo and traffics cell-to-cell as a vRNP complex rather than as naked RNA is still lacking and remains to be shown.

Specificity of transport

In order to function as a direct cis-acting mediator of Pd targeting and intercellular transport of RNA, the MP has to interact with specific cellular factors. This assumption has a direct bearing on the control of host range, since the lack of such a specific host factor in a given plant species will render the MP incompetent to function and will give rise to 'subliminal symtomless infection', a form of 'resistance' in which the virus can replicate in inoculated cells or in isolated protoplasts but is unable to spread between cells and throughout the plant. Typically, a plant that is 'resistant' to one virus can be infected by another whose MP is functionally active in that species. Indeed, incompetent viral movement functions could be complemented in many cases by coinfection with a helper virus [18], but also by foreign MP, either by infection of transgenic plants expressing functional MP [6, 124], by infection with hybrid viruses containing foreign MP genes [125, 126] or by cobombardment of plant tissues with cDNAs of a dependent virus genome and individual MP gene cDNAs [127]. These findings also indicate that MPs can facilitate the cell-to-cell transport of RNA molecules despite the lack of sequence homology between them. Complementation between MPs is therefore host specific rather than sequence specific, consistent with in vitro experiments which indicated that MPs bind nucleic acids in a non-sequence-specific fashion [118]. This apparent promiscuity of MPs with regard to their in vitro binding and in vivo transport substrates raises the intriguing and so far unresolved issue of the mechanism of specificity that prevents MPs from mediating unspecific intercellular transport of unrelated RNA molecules, such as cellular messenger RNAs (mRNAs). Evidence supporting the existence of such a mechanism is the absence of major growth defects of MP-transgenic plants that accumulate MP in Pd with increased SEL [128], indicating that gating of Pd is insufficient for RNA transport. Accordingly, it was observed that Pd in immature sink leaves do not transport vRNA (in the absence of MP), although they are able to traffic proteins nonspecifically [44, 45]. The ability of MP to transport vRNA (even in sink leaves) may rely upon the proximity of MP translation, vRNA and host factors required for Pd targeting. This situation is likely, given that MP translation occurs proximal to virus replication and in association with the cytoskeleton during virus infection (see below).

Host components involved in transport

The introduction of the GFP of *Aequorea victorea* into plant biology [129–131] allowed the development of TMV derivatives that express MP as a functional MP:GFP fusion protein (TMV-MP:GFP; [132, 133]).

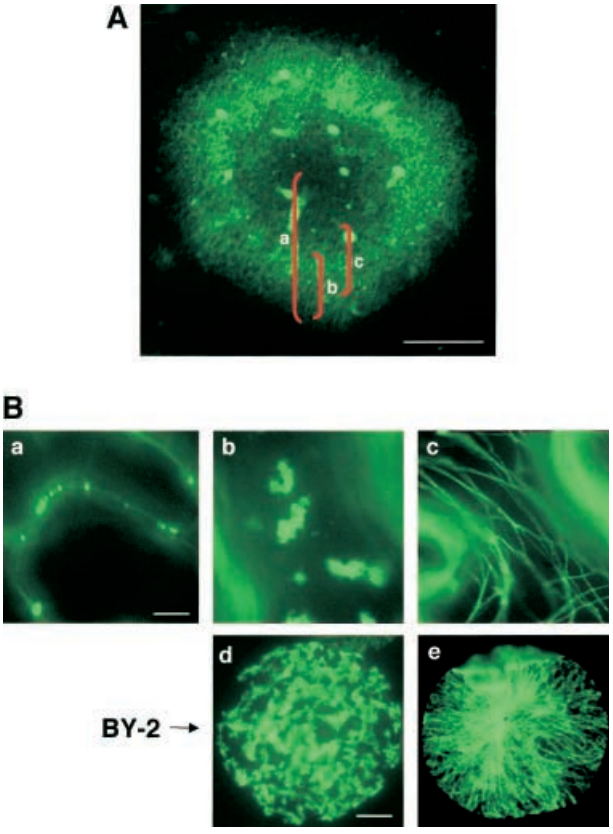


Figure 3. Analysis of infection in living plant tissue and protoplasts. (*A*) Infection site caused by TMV-MP:GFP in *N. benthamiana* (22 °C). Infection spreads radially, from infected cells into non-infected cells. The ring-shaped pattern of MP:GFP fluorescence reflects the time course of expression and subsequent degradation of MP in infected cells. Red brackets indicate regions within the infection site showing the subcellular distribution of MP:GFP referred to in (*B*). Size bar is 500 μm. (*B*) Subcellular distribution of MP:GFP during infection. The distribution of MP:GFP shown in figures *a, b and c* is seen in cells present in specific radial positions with the infection site, as indicated in (*A*). (*a*) Throughout the infection site, MP:GFP is localized to Pd. (*b*) In the highly fluorescent area, MP:GFP is present in irregularly shaped ER aggregates. (*c*) In cells at the trailing edge of the fluorescent 'ring', MP:GFP is associated with microtubules. The distribution of MP:GFP is temperature dependent and directly related to MP function in vRNA transport, as descibed in the text. (*d*) and (*e*) TMV-MP:GFP-infected BY-2 protoplasts showing MP:GFP distributions similar to those observed in planta. These were used for histochemical approaches that led to the identification of ER and microtubules as cellular components involved in infection (see text). Size bars are 10 μm.

This enabled the analysis of infection sites as well as the examination of the subcellular localization and function of MP in living plant leaf tissues and protoplasts [132, 134–137] (fig. 3). When inoculated on leaves of *Nicotiana benthamiana* or of other susceptible hosts, TMV-MP:GFP gives rise to fluorescent infection sites that usually appear at 3 days post infection (dpi) and expand radially. Since the virus construct does not encode CP, the observed radial growth of the infection site indicates cell-

to-cell movement of nonencapsidated vRNA. TMV-MP:GFP derivatives in which the coat protein gene was reintroduced produced similar rings of fluorescence, albeit with lower fluorescence intensity [138]. The leading edge of fluorescence appears to reflect the leading front of infection, as shown by experiments involving manual incisions to the leaf lamina [91]. Incisions made one epidermal cell diameter beyond the fluorescent leading edge of the infection front halted further virus movement, and no GFP fluorescence was detected on the opposite side of the incision for periods of up to 48 h. This apparent contradiction between the demonstrated ability of MP to move by itself from cell to cell over long distances following microinjection (MP, [93, 94]) or bombardment (MP:GFP, [95]), and the apparent inability of the protein to spread from infected cells into noninfected cells, led to the suggestion that MP can behave in different ways, depending on whether the protein is expressed normally during the course of infection or transiently from DNA in noninfected cells [91].

Unlike virus expressing GFP as a fusion to MP under the control of the MP subgenomic promoter, which gives rise to infection sites with ring-shaped fluorescence, virus constructs producing free GFP driven by the CP subgenomic promoter produce infection sites expanding in the form of slightly smaller but highly fluorescent disks [135, 138]. These differences in the appearance of infection sites are consistent with fluorimetric measurements using infected protoplasts, which demonstrated that MP:GFP accumulates transiently during infection, with a peak at about 22 h post infection (hpi), whereas GFP produced from the CP promoter accumulates constitutively to high levels [135]. In fact, during midstages of infection, MP is specifically degraded; treatment of virus-infected protoplasts with inhibitors of the 26S proteosome led to accumulation of ubiquitinylated MP, but not of ubiquitinylated replicase or CP [139].

Examination of infection sites produced by TMV-MP:GFP on *N. benthamiana* leaves allowed the cell-to-cell spread of vRNA and the growth of the fluorescent infection site to be directly correlated with the subcellular localization of the MP:GFP [91, 132, 134]. These studies demonstrated that during the course of infection, MP is first visualized in Pd and subsequently accumulates in irregularly shaped bodies. These bodies first increase in size and then diminish just at the time when MP:GFP becomes associated with filaments. Eventually, most of the fluorescent material is associated with filaments and, finally, all MP:GFP disappears except from Pd. Similar patterns of MP:GFP accumulation were also observed during a time course study of infection in BY-2 protoplasts, allowing identification of MP-interacting host components by applying specific antibody labeling and in situ hybridization procedures. It was shown that the irregularly shaped inclusion bodies are derived from ER and that the occurrence of fluorescent filaments is due to association of MP:GFP with microtubules [132, 140].

Role of inclusion bodies

Inclusion bodies observed in cells infected with TMV-MP:GFP contain replicase [134] and vRNA [137] in addition to MP, and thus are likely sites of virus replication and protein synthesis. These bodies contain ER and may be derived from cortical ER, where MP localizes very early during infection [134, 137]. Earlier studies have shown that TMV replication complexes copurify with membrane extracts from infected cells [141–146]. Moreover, electron microscopy studies indicated the association of TMV replication with cytoplasmic inclusions or viroplasms [147], which enlarge during the course of infection to form 'X bodies' composed of aggregates of tubules in a ribosome-rich matrix [148, 149] and which contain ER [150, 151]. Membranes are also the site of replication of other viruses, such as *Brome mosaic virus* [152], *Tobacco etch virus* [153] and poliovirus [154]. Association of virus replication with ER membranes and the formation of ER-derived inclusion bodies may provide a means of compartmentalization to coordinate and regulate efficient virus translation, replication and movement, and might also protect the virus against the innate defense responses of the host. Membranes may also play a role in the configuration of the replication complex. Osman and Buck [144] (1996) observed that RNA polymerase activity was associated only with membrane-bound enzyme; no template-dependent activity could be recovered after solubilization of the polymerase. Hence, membranes appear to be required for initiation of RNA synthesis. Other evidence suggests that the membrane configuration also affects the production of progeny ssRNA [155].

Reichel and Beachy (1998) reported observations suggesting that inclusion bodies in TMV-infected cells are formed transiently through recruitment of membranes from the ER network, and that the formation and subsequent dissipation of these 'ER aggregates' parallels the time course of accumulation and subsequent degradation of MP [156]. Moreover, ER aggregates were formed upon transient expression of MP:GFP in transfected cells, suggesting that the recruitment of ER is independent of virus infection and is a function of MP. Accordingly, it was found that vRNA did not localize to bodies when protoplasts were infected with TMV-ΔM, a virus derivative that lacks the capacity to encode MP [137]. vRNA-containing bodies were also not observed when protoplasts were treated with the actin-depolymerizing agent cytochalasin D, suggesting that MP and microfilaments participate in the formation and anchoring of the ER-derived structures [137]. However, based on earlier studies that demonstrated that TMV mutants lacking MP

can replicate normally [36], the formation or stabilization of ER bodies appears to be dispensable for replication. In fact, the observation that vRNA of TMV-ΔM localized to ER suggests that ER association is an intrinsic property of vRNA and/or replicase and does not require MP [137]. Recent studies demonstrated that MP:GFP lacking 55 C-terminal amino acids of MP does not accumulate in the form of fluorescent inclusion bodies but still maintains the ability to transport vRNA from cell to cell, albeit with slightly reduced efficiency [157]. Thus, although dispensable for replication and virus movement, aggregation of ER membranes into inclusion bodies by MP might have a role in sustaining the efficiency of virus infection.

Role of microtubules

Colocalization of MP with microtubules [132] and microfilaments [158] suggested the involvement of cytoskeletal elements in Pd targeting and cell-to-cell movement of complexes that contain vRNA and MP [132, 159, 160]. This hypothesis is consistent with observations in many different biological systems that the coordinated activities of cytoskeletal components are responsible for the specific transport of RNAs, as well as the anchoring of RNAs at their final locations (e.g. [161–170].

Whereas an active role of actin filaments in vRNA movement remains to be demonstrated (see below), a function for microtubule-associated MP is supported by recent studies. Más and Beachy (1999) used a combination of antibody labeling and in situ hybridization procedures to demonstrate the MP-dependent colocalization of vRNA with microtubules in protoplasts [137]. A subsequent study, again in protoplasts, demonstrated the mislocalization of vRNA in cells expressing a mutant MP (TAD5, [171]) that binds vRNA but fails to associate with microtubules [172]. These studies confirmed the role of MP in mediating the association of vRNA with microtubules but, unfortunately, protoplasts fall short as a system to verify the significance of these interactions during the spread of infection. Therefore, other approaches addressed the role of microtubules in the cell-to-cell progression of infection in living leaf tissues. For example, in planta observations demonstrated that the efficiency of vRNA cell-to-cell movement increases with the amount of MP:GFP associated with microtubules in cells at the leading front of infection [140]. Both events occur more efficiently at higher temperature [140] and thus are likely to contribute to the effects of temperature on TMV accumulation in protoplasts and leaf tissues [173, 174]. A direct correlation between the ability of MP to associate with microtubules and to transport vRNA was also demonstrated by the in planta analysis of TMV mutants carrying either internal (TAD5; [171, 172]) or C-terminal [157] deletion mutations in MP. A role for microtubules in vRNA transport is also supported by recent studies suggesting that virus resistance exhibited in plants expressing a dysfunctional MP (MP-derived resistance, [16]) is caused by interference with microtubule association of the MP [95]. The strongest evidence for a functional role of microtubules in intercellular transport of vRNA is based on amino acid exchange mutations in MP that were found to directly correlate its function in viral movement with microtubule binding in a temperature-sensitive manner [175]. One of these mutations, Ls1 (Pro154Ser), was shown previously to confer a movement-deficient phenotype to the related ToMV at an elevated temperature of 32 °C [84, 86]. Notably, these mutations map to a short structural amino acid sequence motif that is conserved between tobamovirus MPs and is also present in α-, β- and γ-tubulins as part of the M-loop that makes lateral contacts between microtubule protofilaments. Based on these intruiging findings, it has been proposed that MP mimics tubulin-tubulin interfaces to coassemble with microtubules and to mediate viral RNA transport as an integral or tightly associated component of the microtubule lattice [175] (see fig. 6B).

The presence of domains in MP that mediate microtubule association by direct interaction with tubulin is supported by transient expression experiments demonstrating that the association of MP with microtubules does not depend on virus infection [95, 134, 156] or on any plant-specific factor [175]. Binding of MP to microtubules also occurs in transfected mammalian cells, with disruption of the normal radial microtubule array and formation of stabilized microtubules that are independent of the centrosome [175]. These effects of MP expression in mammalian cells are reminiscent of cytopathic effects described for vaccinia infection in HeLa cells [176], suggesting that plant and animal viruses share similar mechanisms to optimize infection. In the case of MP-transfected cells, the formation of centrosome-independent microtubule arrays was correlated with the absence of centrosomal γ-tubulin, suggesting that MP may interact with microtubule-nucleating complexes to effect microtubule association and vRNA transport via a dynamic, microtubule polymerization-dependent mechanism [175].

Tubulin mimicry and the displacement of γ-tubulin from the centrosome are properties of MP so far unknown for other microtubule-associated proteins (MAPs). MAPs usually bind to the outside wall of microtubules by virtue of salt-sensitive electrostatic interactions between their basic microtubule-binding domains and the acidic C-terminal domain of tubulin, which lies on the outside of the microtubule (e.g. [177]). In contrast, the bond between MP and microtubules is insensitive to salt treatment, confirming that MP differs from other MAPs. Moreover, whereas MAP-associated microtubules are destabilized

by treatment with cold or moderate concentrations of microtubule-depolymerizing agents, MP-associated microtubules are resistant to such treatments and could only be disrupted by chaotropic agents [175]. These remarkable biochemical properties of MP-associated microtubules are consistent with tubulin mimicry and the formation of specialized microtubule complexes by MP (see fig. 6). It will be important to determine the ability of MP to coassemble with tubulin in vitro, to elucidate whether or not the complex requires vRNA and to analyze the structure of the complex by electron microscopy. Importantly, like tubulin, MP is a GTP-binding protein [178], and studies investigating the role of GTP in microtubule-association of MP are in progress [O. Sterthaus et al., unpublished]. A pertinent question is also whether the in vivo complex contains vRNA and whether specific host proteins are recruited into the complex.

Future experiments will also have to address the mechanism by which stable MP:microtubule complexes would transport vRNA to Pd. It is possible that stable microtubules form a scaffold for motor-mediated transport (fig. 6A). The involvement of microtubule motors in TMV RNA transport would be consistent with the proposed role of motor proteins in the cytoplasmic transport of certain animal viruses [179–182] and of mRNA [183–187]. However, microtubules are also known to segregate and distribute the elements of the ER [188, 189], and three possible mechanisms have been proposed [190]. The extension of membrane tubules by attachment to the growing plus end of microtubules ('TAC mechanism') is microtubule polymerization dependent, whereas mechanisms involving membrane sliding or microtubule movement depend on the activity of microtubule-associated motor proteins. Therefore, it is conceivable that motor proteins on MP-associated microtubules engage in the transport of vRNA-replicase complexes associated with ER, a model that is supported by the observed association and alignment of ER inclusion bodies with microtubules [117]. It is also conceivable that vRNA is transported past stationary MP in the microtubule wall (fig. 6C) or that motor proteins mediate the movement of MP- and vRNA-associated microtubules or protofilaments in toto (fig. 6D). The latter model might gain support from the observation of fluorescent elongated 'hairlike' structures that appear to protrude through the plasma membrane of TMV-MP:GFP-infected protoplasts and which could represent microtubule complexes sticking out of the protoplast surface (fig. 4a; [117]). Más and Beachy (1999) showed that the protrusions are associated with vRNA [137]. The observation that the protrusions are not affected by the microtubule-depolymerizing herbicide oryzalin [137] does not pose a contradiction to this model, since MP-associated microtubules can be resistant to microtubule-depolymerizing agents [175]. On the other hand, one has to caution that although MP-associated microtubules are definitely required for vRNA movement into the next cell, there is as yet no conclusive proof that MP-associated microtubules directly engage in vRNA transport. In particular, the type of interaction between MP:GFP and microtubules that produces highly stabilized complexes during later stages of infection could have specific roles beyond vRNA transport (for example in the suppression of plant defense responses; see below). Reported instances of microtubule disruption in infected cells by cold and depolymerizing agents [132,

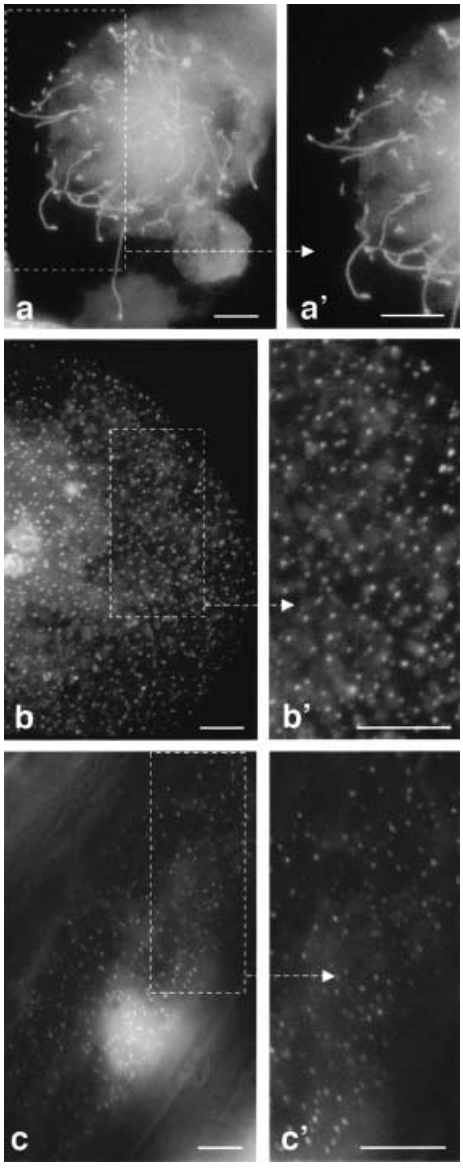


Figure 4. Subcellular distribution of MP:GFP during infection (cont.). (*a*) and (*a'*) MP:GFP in association with protrusions sticking out from the surface of an infected BY-2 protoplast. Size bars are 10 µm. (*b*) and (*b'*) Part of an infected protoplast showing MP:GFP in association with as yet non-identified punctate structures. Size bars are 5 µm. (*c*) and (*c'*) Leaf epidermal cell showing MP:GFP in association with punctate structures very similar to those observed in infected protoplasts. Size bars are 5 µm.

134, 136, 137] suggest that the structure and stability of MP-associated microtubules may alter during infection and that stabilized microtubule complexes might be preceded by more dynamic complexes. Based on the observation that only a fraction of the MP produced during infection is required for intercellular transport of TMV [191], it is conceivable that these complexes are of low fluorescence in MP:GFP-expressing cells. In fact, usually it is difficult to visualize MP:GFP-associated microtubules in the first cell at the leading front of infection. However, fluorescent microtubule complexes apparently engaged in vRNA transport can be visualized in the first cell at the leading edge of infection by using temperature-sensitive mutants that permit to experimentally increase the amount of MP:GFP (i.e. by accumulation of temperature-sensitive MP:GFP at nonpermissive conditions prior to permissive conditions [175]).

Role of microfilaments

TMV MP has been reported to interact with actin [158], and since actin is associated with Pd [64, 65, 68], it may be possible that microfilaments and microtubules share some functions of Pd targeting of the vRNP [159, 160] (see fig. 5). Synergistic and functional interrelationships between microtubules and actomyosin have been described in other cases [192–196], and several proteins bind both microtubules and F-actin [197, 198], including RNA-binding proteins involved in RNA transport [168].

Actin may also be involved in the MP-mediated formation of ER-derived inclusion bodies; treatment of infected protoplasts with the actin-depolymerizing agent cytochalasin D interfered with the formation of vRNA-containing ER bodies [137]. Treatment of protoplasts during later stages of infection altered the shape of the inclusion bodies and suggested a role of microfilaments in the stabilization and anchoring of the ER-derived structures [134]. These findings are consistent with observations in plant tissues indicating that microfilaments are often closely associated with ER and confer structural stability as well as motorforce to the network [199–203].

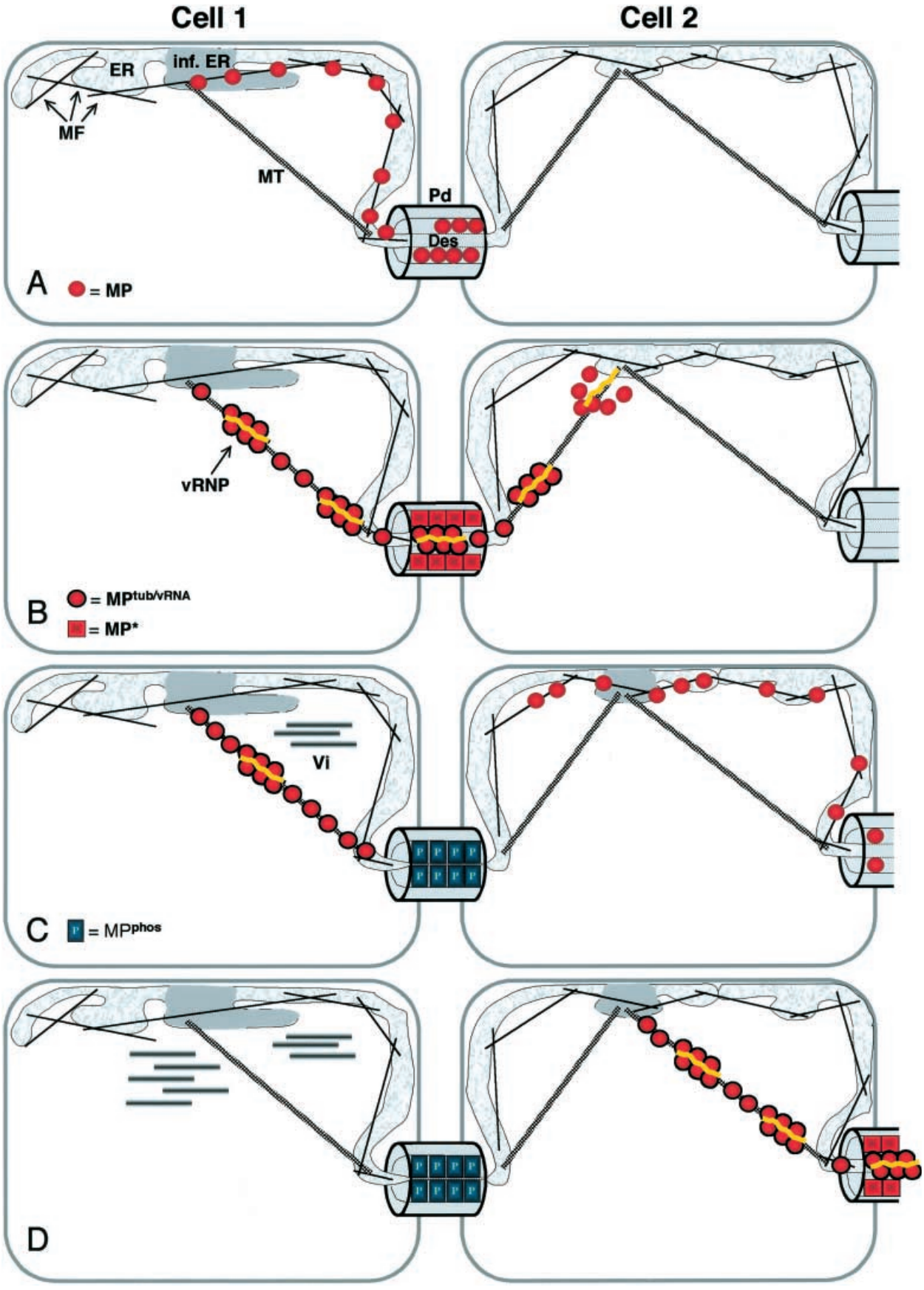
Role of other associations of MP:GFP with host components observed in protoplasts

In infected protoplasts, MP:GFP localized to ER and cytoskeleton as described above. In addition, MP:GFP was observed in association with regularly spaced 'puncta' located at the vertices of cortical ER close to the plasma membrane as well as to elongated structures protruding from the surface of the protoplasts (see above, fig. 4, and [134]). Punctate structures were also found in infected epidermal cells (fig. 4c, [156]), arguing against the hypothesis that the cellular components forming the puncta may be derived from Pd that were present before protoplast preparation [134]. Another hypothesis postulates that the puncta may represent attachment sites [134] at which cytoplasmic strands ('Hecht' strands, [204]) adhere to the ER, plasma membrane and cell wall. Cell wall attachment sites for Hecht strands are readily observed upon plasmolysis of onion epidermal cells [205–207]. Since a subset of these cytoplasmic strands attaches to sites of Pd and may continue through the channel, it is possible that MP targets puncta and Pd by the same mechanism. Brefeldin A, a fungal metabolite that disrupts the endomembrane system [208, 209], interfered with the targeting of MP to puncta in protoplasts early, but not late, during infection, suggesting that the establishment of, or the association of MP:GFP with, these structures may rely on components of the secretory pathway [134]. Cytochalasin D treatment reduced the amount of MP:GFP at these sites indicating that microfilaments may be involved in anchorage but not in targeting the MP to puncta [134].

Although the plasma membrane protrusions observed on the surface of infected protoplasts may represent moving microtubule complexes as suggested above, they are also reminiscent of the projections on the surface of protoplasts infected with tubule-forming viruses [108, 112, 114, 210, 211]. TMV MP thus may have a tubule-forming function that, although generally not yet observed in leaves, may be required in some specific hosts or during passage of the vRNA through specialized Pd. Recent observations by Más and Beachy [137] indicated that the protrusions contain vRNA and that MP is required for the localization of vRNA to these structures. The protrusions were not affected by oryzalin or cytochalasin D but were stained with $DIOC_6$, a dye used for visualization of ER membranes. These findings led to the proposal [137] that the protrusions are related to desmotubules, the central ER component of Pd [38]. Thus, these studies suggested that the final stages of viral movement may involve the translocation of vRNA along or within the desmotubule [137].

MP can target Pd independently from vRNA

So far, this review has addressed the role of ER and the cytoskeleton in the formation of replication complexes as well as in the targeting of vRNA to, and through, Pd. As pointed out earlier, the MP on its own has the ability to associate with ER, microtubules and Pd, and even seems to spread between cells independently of infection. Thus, does vRNA just take a ride on MP trafficking between cells? Certainly, it is not that simple. For example, at nonpermissive temperatures, the Ls1 mutation interferes with microtubule-association of MP and intercellular


Cell 1
Cell 2
ER
inf. ER
MF
MT
Pd
Des
A
= MP
vRNP
B
= MPtub/vRNA
= MP*
Vi
C
= MPphos
D

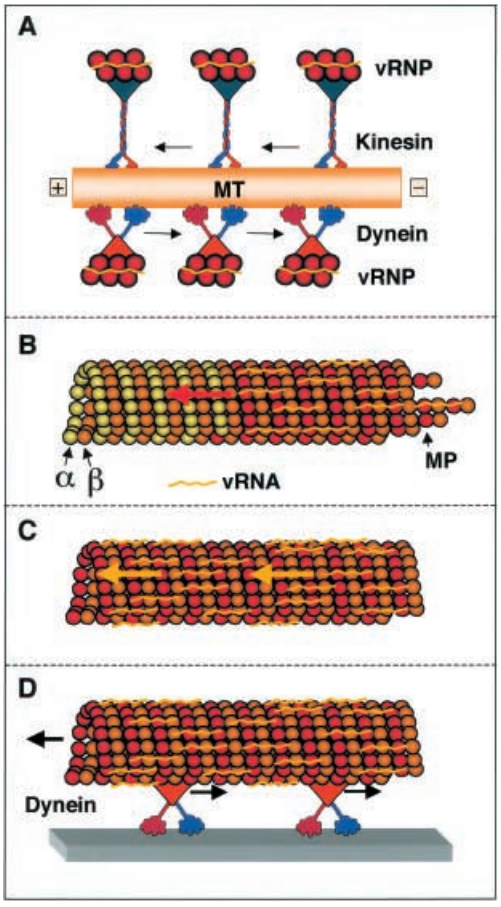


Figure 6. Examples of potential mechanisms of microtubule-dependent vRNA transport. (*A*) Transport of vRNP by a kinesin or dynein motor complex. (*B*) Coassembly of MP with tubulin and transport of the protein and associated vRNA along the microtubule by treadmilling. Tubulin mimicry may allow direct interactions between MP and tubulin:tubulin assembly surfaces. Thus, MP could replace a microtubule subunit during microtubule assembly (as shown), or could laterally associate with the microtubule lattice during or after assembly. MP could interact with the microtubule in monomeric (as shown) or multimeric form, e.g. as a vRNP. MP may associate with the microtubule with lower density than the saturating density shown. (*C*) Transport of vRNA past stationary microtubule-associated MP. (*D*) Motor protein-mediated in toto transport of MP- and vRNA- (or vRNP)-loaded microtubules.

transport of vRNA but not with Pd accumulation of MP. This finding demonstrates that targeting and accumulation of MP in Pd can occur independently of microtubule association and that the targeting and presence of MP in Pd is insufficient for intercellular transport of vRNA [175]. Studies to correlate N-and C-terminal deletion mutations in MP with the function and subcellular location of the protein led to the same conclusion [157]. Deletion of 66 amino acids from the C-terminus of MP disrupted the ability of the protein to associate with microtubules as well as its vRNA transport function but did not abolish Pd targeting. Similar results were obtained with a MP mutant from which internal amino acids 49–51 were deleted (TAD 5, [171, 172]). Moreover, although Pd in sink tissues have an SEL even larger than that measured in mature tissues that expressing MP and allow the trafficking of macromolecules of up to 50 kd in size, vRNA still requires the aid of MP for intercellular transport [45]. Collectively, these findings indicate that MP is capable of targeting Pd by a microtubule-independent pathway. This pathway, however, is insufficient for transporting vRNA, which rather depends on microtubule-associated MP. The dependence on microtubules may be expected, since the size of vRNA or vRNP amounts to several megadaltons and therefore is likely to require an active translocation mechanism for transport through the viscous cytoplasm (see [180]). Since visible levels of MP:GFP first accumulate in Pd during infection, it is proposed that microtubule-independent Pd targeting of MP early in infection supports the efficiency of viral movement by ensuring that Pd are gated for the incoming vRNA (see fig. 5).

Phosphorylation of MP

When localized to Pd, the MP may not necessarily be active in increasing the SEL of the channel. Dye-coupling experiments performed by Derrick et al. (1990) [212] were unable to detect any Pd SEL increase within the leaf epidermis of *Nicotiana clevelandii* infected with a range of different viruses. More recent experiments using infection sites in *N. tabacum* caused by GFP-tagged virus

Figure 5. Hypothetical model for MP functions during spread of TMV infection from an infected cell (cell 1) into a noninfected cell (cell 2). (*A*) Early infection. Replication and synthesis of viral protein occurs in association with ER (inf. ER, infected ER). Targeting of MP to Pd occurs via ER and microfilaments (MF), and is independent of microtubules (MT). Des, desmotubule. (*B*) Midstage of infection. Newly produced MP is in a configuration ($MP^{tub/vRNA}$) that binds vRNA, forms vRNP and associates with MT. MP that accumulated in Pd (or incoming vRNP?) increases Pd SEL (perhaps a conformation change is involved, MP*), allowing the passage of MP and vRNP into the next cell (cell 2). Upon passage of vRNP through Pd, the constituent MP is modified to release vRNA. vRNA associates with ER and establishes a new replication site. (*C*) Late infection. MP present in Pd is downregulated or replaced by yet another form of the protein that no more increases Pd SEL. This form is likely phosphorylated (MP^{phos}). Newly produced MP and remaining vRNP accumulate on MT, inactivating MT-dependent transport. Infection in cell 2 is initiated and delivers MP to Pd that link this cell to another, yet uninfected cell. Cell 1 produces CP and virions (Vi). (*D*) Very late infection. In cell 1, MP is degraded except in Pd. MP in Pd (MP^{phos}) is still inactive, blocking the reverse passage of infection from cell 2 into cell 1, as well as the forward passage of other macromolecules, e.g. of RNA-based gene-silencing signals. Cell 1 accumulates virions, whereas cell 2 already undergoes midstages of infection, spreading infection further into noninfected cells. Upon decomposition or mechanical damage of the plant, virions are released into the environment to eventually cause infection of a new plant.

indicated that the ability of MP to modify Pd is restricted to the leading front and the highly fluorescent halo of the infection site, and is inactivated in its center [91]. MP, therefore, may exist in two forms within Pd of infected tissues: an active form that transiently increases the SEL during early infection, and an inactive form that localizes to the channel but no longer increases the SEL during late infection (see fig. 5). The observation that a MP mutant lacking 66 amino acids (aa 203–268) from the C-terminus accumulated exclusively in Pd within the center of infection sites (in leaves of MP-transgenic plants, since the mutant MP was inactive) suggested the possibility that the inactive, Pd nonmodifying, form of MP may be independently targeted to Pd, thereby replacing the active form present in the channel [157]. On the other hand, it may also be possible that Pd-resident MP is downregulated late in infection. Since MP is phosphorylated in vivo [213–216], the ability of MP to modify the SEL of Pd may be modified by phosphorylation. Recent support for this hypothesis was provided by evidence that negatively charged amino acid substitutions that mimic phosphorylation of C-terminal amino acids inactivated the ability of MP to increase the SEL of Pd in a host-dependent manner [216]. However, it remains unclear whether in vivo phosphorylation of MP within its C-terminus is correlated in time with functional downregulation of MP during late stages of infection. Moreover, it will be important to determine whether the observed effects of the mutations are truly caused by mimicry of C-terminal phosphorylation or rather by other nonanticipated effects. For instance, mutations introduced into the 3′ end of the MP-coding region may indirectly affect movement by compromising the activity of the overlapping subgenomic coat protein promoter.

Phosphorylation may also be involved in other activities of this multifunctional protein. Mutation of Ser-37, which is phosphorylated and required for function in vivo, strongly affects protein stability as well as the ability of the protein to associate with host components [215]. However, the requirement of Ser-37 phosphorylation for function in vivo remains to be shown. Microinjection experiments indicating that the ability of MP to dilate Pd is independent of phosphorylation [216] may argue against this possibility. Other observations suggested that phosphorylation could have a role in the fine tuning of MP activities during infection. For example, deletion of MP sequences (aa 214–233) that overlap with yet another in vivo phosphorylated domain (aa 212–231) of the protein [214]) led to a shift in the distribution of MP from ER inclusion bodies to microtubules. This effect of the mutation was accompanied by a slight reduction in the effciency of vRNA movement, indicating that phosphorylation may be involved in coordinating the association of MP with host components to sustain the efficiency of infection [157]. Moreover, it has been reported that in vitro phosphorylation of MP abrogated the ability of the protein to repress translation of vRNA (see above; [123]). Taken together, these findings strongly suggest the involvement of phosphorylation in the orchestration of molecular interactions and functions of MP during infection.

Other host factors involved in infection

Besides the described interactions of MP with membranes and elements of the cytoskeleton, other molecular interactions between MP and host cell factors remain largely unknown. Although several groups have attempted to use the yeast two-hybrid system to identify MP-interacting factors, this approach seems to have been unsuccessful, presumably because of entrapment of the MP bait in the yeast cytoplasm. Far-Western approaches, however, were successful and led to the identification of cell wall-associated pectin methylesterase as a MP-binding protein [217, 218]. It is unknown how this extracellular enzyme would interact with intracellular MP. However, it has been proposed that MP might associate with this protein for ‘piggyback’ transport through the secretory pathway, or may just recognize it as a cell-wall associated receptor [218]. This enzyme could also be involved in increasing the SEL of Pd by rapidly changing the structural state of cell wall pectins that are particularly enriched in microdomains surrounding the channels [83]. A deletion mutation in MP (aa 130–185) abolishes the binding of MP to this enzyme as well as the function of MP in TMV cell-to-cell movement, thus providing in vivo significance of the interaction between the two proteins. However, it should be noted that the amino acids deleted are located in the central core of the MP and therefore might be essential for the proper structural folding of the protein. Additional MP-interacting factors are likely be isolated in the near future. One potential approach for the identification of such factors is the biochemical analysis of MP complexes isolated from infected cells. Another approach already taken by several laboratories is the identification and characterization of *Arabidopsis* mutants in which the cell-to-cell spread of infection is blocked. For instance, Yoshii et al. [219, 220] identified two mutations in *Arabidopsis*, *cum 1-1* and *cum 2-1*, that apparently affect the cell-to-cell spread of CMV within the inoculated leaf.

The involvement of host proteins in virus replication is indicated by the sensitivity of TMV multiplication to actinomycin D during early stages of infection [221, 222]. *Arabidopsis* mutants have been obtained in which accumulation of tobamovirus RNA was reduced to low levels [223, 224]. Two of these mutants were caused by unlinked, single, recessive mutations, termed *tom-1* and *tom-2*, indicating that at least two different host proteins are needed for TMV replication in *Arabidopsis*. Recently,

the *TOM 1* gene was cloned and seems to encode a 291-amino acid-long transmembrane protein that interacts with the helicase-domain of tobamovirus replicase and may serve as a membrane anchor to participate in the formation of replication complexes [225]. Biochemical isolation of TMV replicase from infected tomato plants led to the co-isolation of 56-, 54- and 50-kd proteins that crossreact with antibodies against eukaryotic translation initiation factors and one of which (56 kd) may be a genuine component of the TMV RNA polymerase [155]. Another putative candidate affecting virus replication is translation elongation factor eEF1α. This protein binds to conserved pseudoknots in the 3′UTR of TMV RNA in vitro [V. Zeyenko, et al., unpublished] and colocalizes to replicase-containing structures in infected tobacco leaves [226]. The role of eEF1α also has been analysed in the context of replication of other viruses, such as *Turnip yellow mosiac virus* [227], *Brome mosaic virus* [228], *West Nile virus* [229] and *Vesicular stomatitis virus* [230]. However, several detailed studies failed to demonstrate an active role of this protein in virus replication [231, 232]. Moreover, since EF1α is a translation factor, the presence of this protein in replication complexes may just reflect associated protein synthesis. On the other hand, EF-1α is a microtubule-associated protein in plants [233] and thus may play a role in linking membrane-associated vRNA synthesis with microtubules for Pd targeting and intercellular transport.

Another approach to identify proteins involved in TMV replication is based on the analysis of *Tm-1* gene mediated-resistance that is overcome by TMV strains with mutations in the helicase domain of the 126-kd or the 183-kd protein [234]. However, it is not known whether the Tm-1 protein directly interacts with the replicase or whether it inhibits virus replication indirectly as part of a non-virus-specific host defense response.

Long-distance transport

Systemic infection of a plant depends on the ability of the virus to spread infection beyond the initially infected leaf. Thus, the virus has to enter the phloem to allow long-distance spread into other plant organs. Although virus follows the transport pathway of solutes [235–237], the loading and unloading of viruses from the phloem is likely to involve specific mechanisms. For instance, in mature *N. tabacum* and *N. benthamiana* source leaves that load photoassimilates into the phloem via the apoplast, viruses are able to enter the phloem through Pd that separate the sieve element-companion cell (SE-CC) complex from surrounding cells and are impervious to solutes ([238], and citations herein). Despite the fact that this pathway is available, however, movement of TMV and other viruses from phloem parenchyma cells into CCs is inefficient [239–241]. Viruses may even completely fail to move across the bundle sheath-phloem boundary in some hosts [242–244]. Thus, infection of CCs appears to be a limiting step in systemic infection, consistent with evidence suggesting that the Pd connections between CC and parenchyma function as a tightly controlled gateway for entry of macromolecules into the phloem. Once in the CC, a virus potentially has direct access to the SE via Pd that are specialized to support the continuous transport of proteins to the enucleate SE, which lack protein synthesis [245]. These Pd possess a unique morphology that includes extensive branching on the CC side [246, 247], and appear to have a SEL substantially larger than that of Pd between other mature cell types [248]. Recently, it was shown that GFP expressed in CCs of minor veins of mature leaves was transported into major veins of young leaves where it unloaded from the phloem and moved into surrounding mesophyll [44, 45]. Thus, having entered the CC in the source leaves, viruses may be directly transported into post-phloem sink tissues, along with other macromolecules.

Entry into the CC-SE and protection of the viral genome against the alkaline conditions and ribonucleases present in the phloem sap is likely to depend on additional sets of viral and host factors. In fact, TMV and most other viruses that can move cell-to-cell in a nonencapsidated form, require coat protein for long-distance movement [116, 126, 249–252]. Although the precise role of coat protein in systemic infection remains to be established for many viruses, encapsidated virions appear to represent the functional long-distance movement complex [251, 253, 254]. Recent studies by Blackman et al. (1998) provided evidence that CMV enters the SE as a vRNP and separate coat protein, and subsequently assembles into virions within the SE, in a protected environment located between the SE plasma membrane and the parietal SE endoplasmic reticulum [255]. However, in the case of bipartite geminiviruses, the requirement for coat protein is host dependent, leading to the suggestion that long-distance movement can occur by both coat-protein-dependent and coat-protein-independent mechanisms [256]. Hordeiviruses represent an example of systemic phloem-dependent movement even without the requirement of coat protein [257]. Apparently, some viruses are capable of moving long distance in forms other than virions and additional factors, such as those provided by the host (see below), may be involved in mediating this movement. Nevertheless, viral coat protein plays an important role in phloem transport of many viruses. However, specific interactions between this protein and cellular factors necessary for long-distance movement have yet to be identified.

MP may also have specific functions in long-distance movement. For example, hybrid TMV genomes encoding the MP of *Odontoglossum ringspot tobamovirus*

(ORSV) in place of its own MP are competent for cell-to-cell movement in tobacco, but incompetent for phloem-dependent long-distance transport [126, 258]. Moreover, delayed or reduced production of MP reduces the ability of TMV to cause systemic infection [191, 259]. More recently, it was shown that certain mutations in the MP of RCNMV separate a function in long-distance movement from its function in local movement in a host-specific fashion [260]. Despite this evidence, however, the function of MP specifically required in long-distance transport remains to be elucidated. Experiments with grafted plants indicated that systemic movement of MP-deficient TMV can be complemented by MP expressed in the phloem [261]. A similar study led to the contradicting conclusion that MP, once in the phloem, does not require MP synthesis for long-distance movement [262]. The reason for these contrasting results of grafting experiments is unknown but could be based on apparent differences between plant genotypes, experimental conditions and the nature of the grafting procedure. Certainly, further studies are needed to determine the role of MP in systemic infection. Its function in long-distance movement may be restricted to phloem entry [262]. The observation that TMV MP does not increase the SEL of Pd connecting bundle sheath cells to phloem parenchyma cells [89] may argue against this possibility. However, this finding does not preclude that some additional property of MPs is required for TMV to efficiently enter the phloem. Moreover, the significance of this observation is questionable since MPs can occur in an inactivated state even in Pd connecting mesophyll cells (see above, [91]).

Several viruses produce specialized proteins that support long-distance transport but have no apparent role in cell-to-cell transport (e.g. gene VI protein of *Cauliflower mosaic caulimovirus* (CaMV) [263], p19 of *Tomato bushy stunt tombusvirus* (TBSV) [264], 126-kd and 183-kd replicase proteins of TMV, [265]). The 2b protein of CMV [266] and the potyvirus HC-pro protein [267] were recently characterized as efficient suppressors of gene silencing [266, 268–272] and thus likely promote infection through inhibition of vRNA degradation.

The requirement for specific host functions in long-distance movement is indicated by the fact that the ability of a virus to move systemically as well as cell to cell is often host dependent (e.g. [17, 273–275]. Genetic approaches led to the identification of *RTM1*, *RTM 2* and *VSM1* genes involved in controlling phloem-dependent virus movement in *Arabidopsis* [276–278]. *RTM1* and *RTM2* genes specifically restrict the long-distance movement of *Tobacco etch potyvirus* (TEV). Both genes have been cloned [279, 280] and encode predicted proteins with similarity to lectins and small heat-shock factors, respectively. The role of these proteins in restricting viral movement is not known. However, it has been speculated that they may have structural roles in binding and inhibiting the activity of TEV proteins involved in long-distance movement, or in promoting the activity of a movement-restricting factor [280]. Mutation of the *VSM1* gene inhibits long distance spread of *Turnip vein-clearing tobamovirus* (TVCV) [278] and therefore is likely to encode a protein essential for systemic transport of this virus.

Another important aspect of systemic movement is the mechanism by which viruses unload from the phloem to enter nonvascular tissues of uninoculated leaves. Phloem unloading appears to involve multiple pathways as was suggested by studies indicating that unloading of TVCV was inhibited by nontoxic concentrations of cadmium, whereas that of TEV was not affected [281]. The molecular basis for cadmium inhibition is not known. While it is possible that cadmium triggers a plant defense response that also affects viruses, an accumulation of salicylic acid or PR proteins, both which are known to be involved in mechanisms for induced plant resistance to systemic viral infection (see below, [282]), could not be detected. It will be important to elucidate whether cadmium affects specific viral functions or rather acts by inhibition of virus-specific host functions.

Virus : host compatibility, host defense strategies and viral counterstrategies

As stated above, the successful systemic infection of a plant by a virus is likely to depend on a multitude of compatible and precise interactions between viral and host factors [283]. A block in any of these interactions can affect virus replication, and either cell-to-cell or long-distance movement. In fact, most plants fail to be infected and are ‘nonhosts’ to most viruses. Only a few species may be partially or fully susceptible to any given virus. For instance, some virus:host interactions allow replication in initially infected cells but are incompatible to support cell-to-cell movement of the virus. In the case of TMV such ‘subliminal’ infections are found in cotton and soybean [284] whereas in other species such as tobacco, the virus can move systemically. Although specific genes responsible for such ‘resistance’ or lack of susceptibility for infection have been isolated (*VSM1*, *RTM1*, *RTM2*, *CUM1*, *TOM1*, and so on, as described above), their particular functions in virus:host interactions are not understood. Some compatible or incompatible host:virus interactions involved in cell-to-cell movement of TMV infection likely relate to the MP as a pathogenicity or host range factor. Some such interactions were recently addressed in a comparative approach analyzing the function and subcellular localization of GFP-tagged MP in susceptible and resistant hosts [95].

Some plant species encode dominant or semidominant resistance (R) genes that trigger hypersensitive cell death

responses (HR) to pathogens. The HR is an active response in which the plant recognizes the pathogen by a specific pathogen-derived elicitor and mounts a cascade of events that leads to confinement of the pathogen to the initial infection site. The most-studied genes for resistance to tobamoviruses are the *N* and *N′* genes of tobacco. The *N* gene from *Nicotiana glutinosa* has been bred into tobacco to confer resistance to TMV. Recently, the *N* gene has been isolated [285] and shown to confer resistance if expressed in transgenic tomato plants [286]. The corresponding avirulence gene of TMV has been mapped to the 126/183-kd replicase gene [287]. The *N′* gene of *N. sylvestris* and several varieties of tobacco acts against most tobamoviruses and some TMV mutants, but not against wild-type TMV. The elicitor is the TMV coat protein [288, 289], but for recognition by *N′*, the structure of the CP must be altered to expose the recognition site [290]. In tomato, HR-type resistance is mediated by *TM-2* and *TM-2*^{2} genes, and elicited by MP [291, 292]. The *TM-1* resistance referred to above is triggered by the replicase proteins of TMV [234]. The coat protein acts as an elicitor of HR in pepper [293] and eggplant [294]. Resistance gene-mediated HR induced by local infection by TMV and other pathogens is accompanied by systemic acquired resistance (SAR) [282], a non-cell-autonomous response that spreads throughout the plant and provides nonspecific resistance against a wide variety of pathogens. Neither the signal that spreads through the plant to mediate SAR nor the targets and mode of action of SAR are known. SAR depends on the synthesis of salicylic acid (SA) and can be triggered by exogenous SA even in the absence of a resistance gene and HR. Evidence that SA affects replication and/or systemic movement of viruses [295, 296] suggests its interference with essential interactions between the virus and its host. SA-induced proteins include pathogenesis-related (PR) proteins that have antifungal and antibacterial activities but apparently no role in the defense against viruses. The analysis of the transduction pathway and mechanism as well as the search for the ultimate targets of SA-induced resistance and SAR against viruses are still in an early stage (see [297] for review), but have strong potential to lead to new insights into virus:host interactions in resistant and susceptible hosts.

Another important type of resistance involves RNA silencing. Viruses can act as inducers and targets of homology-dependent RNA silencing which is mediated through recognition of viral nucleic acids and the adaptation of a sequence-specific RNA degradation response [298, 299]. A likely outcome of this process is the recovery phenomenon, in which initially infected and fully symptomatic plants recover from virus infection, showing only mild symptoms and containing only low virus titers [300–304]. Virus-induced RNA silencing is similar to posttranscriptional transgene silencing (PTGS) [303, 305] and includes the formation of a sequence-specific silencing signal that moves from cell to cell, directing sequence-specific RNA degradation in a non-cell-autonomous manner. If the virus used for inoculation carries sequences homologous to a nuclear gene, then RNA silencing triggered by the virus will also target the mRNA of that gene (virus-induced gene silencing or 'VIGS'). Once activated, VIGS and PTGS spread systemically [306–308], presumably because the mechanism of degradation of nuclear gene transcripts allows the silencing signal to be amplified in each cell it enters, spreading RNA silencing into noninfected tissues and throughout the plant.

Several reports indicated that RNA silencing functions as a natural defense mechanism against viruses [300, 302, 303, 305]. If this is the case, viruses must have ways to overcome this defense response of the plant to cause infection. Strong support for this assumption is provided by the finding that some viruses encode pathogenicity factors that act as trans-acting silencing suppressors, such as the 2b protein of CMV [266, 269] or the potyvirus HC-pro protein [270]. These proteins are able to reverse PTGS by affecting distinct targets in the silencing mechanism. PVX seems to follow another strategy. This virus does not give rise to PTGS reversal in infected plants and was initially thought not to encode a silencing suppressor. A recent report, however, demonstrated that one of the triple-gene block MPs (25-kd protein) of this virus may act as a suppressor by interfering with the production or spread of the silencing signal [309]. This finding may contribute to the new concept that some MPs might actually facilitate viral transport by prohibiting the cell-to-cell communication required for defense responses rather than by facilitation of active targeting and transport through Pd [310].

The mechanism evolved by TMV to suppress silencing is unknown as yet. It is possible that TMV avoids silencing by restricting the formation of dsRNA replication intermediates (e.g. through the helicase activity of the 126/183-kd replicase protein) or by just efficiently moving ahead of the silencing signal. TMV might also suppress the trafficking of the silencing signal via Pd [308], for example through downregulation of Pd SEL behind the infection front by MP (described above, [91]). The MP could also interfere with Pd targeting of the signal by decreasing microtubule dynamics, e.g. through the formation of stabilized, nondynamic microtubule complexes late in infection. The author proposes that the interaction of MP with microtubules and Pd could play two pathogenicity roles early and late during infection, as is shown in figure 5: early in infection when MP synthesis is initiated, the protein modifies Pd and mediates association of vRNA with microtubules for intercellular spread. Late in infection, however, when MP has accumulated to high levels, the protein ceases to modify Pd and inhibits RNA

trafficking by forming stable, nondynamic complexes with microtubules.

TMV as a model to study the role of the cellular cytoskeleton in RNA transport

The role of the cellular cytoskeleton in RNA trafficking has been described in a variety of eukaryotic systems including infection by viruses. It is known that certain animal viruses exploit the cytoskeleton to successfully enter the cell, to replicate and for transport through the viscous cytoplasm [180, 311]. The functional involvement of microtubules in the spread of TMV RNA supports the role of microtubules in viral pathogenesis and RNA transport in plants as well as in animals. In animal cells, the transport and localization of RNA functions as a determinant to produce asymmetric cellular functions by introducing intracellular asymmetries in mRNA distribution and localized concentrations of proteins or protein complexes. For example, due to their asymmetry, neurons can transmit directional signals. Moreover, by recruitment of certain mRNA species into stimulated synapses, neurons are able to establish synapse-specific protein expression that is pivotal for dendritic function and memory [312–314]. Another well-known example is the asymmetric distribution of mRNA in developing oocytes, which drives specific cell fates in the embryo [165]. However, despite these profound roles of cytoskeletal-based RNA transport in the determination of cellular identity and morphogenesis [168, 315, 316], understanding of the process at the molecular level is limited [161]. Research on viral RNA movement in plants has strong potential to contribute new insights into the cellular mechanism of RNA transport and may benefit from the advantage that the process can be observed and analyzed in whole live plants and tissues. GFP-tagged viruses allow the direct observation of infected cells and of cell-to-cell movement of vRNA. Infection of adjacent cells indicates successful microtubule-dependent transport of vRNA from sites of replication to Pd. Since TMV is likely to operate within preexisting intercellular transport pathways [93], it will be important to determine whether microtubules and other host factors involved in the cellular mechanism of cell-to-cell transport of vRNA also have roles in the transport of endogenous RNA species [19], e.g. of mRNA [317, 318], or of nucleic acid-based silencing signals [306–308]. Understanding the counterstrategies by which viruses are able to avoid or suppress antiviral RNA silencing responses of the plant will provide important new insights into plant pathogenesis, intercellular communication and the phenomenon of PTGS that is ubiquitous in both plant and animal kingdoms [319]. Moreover, studies on the interactions of MP with host components probably are also likely to feed into improved strategies for engineering broad-spectrum resistance. Plants expressing a functionally defective TMV MP showed resistance against a range of viruses belonging to diverse virus families [12, 16]. Recent observations suggest that wild-type and defective MP may undergo an incompatible interaction that prevents microtubule association of MP [95], thus identifying the MP-microtubule interaction as a potential target for resistance.

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