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Domains of the TMV movement protein involved in subcellular localization

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Summary

To identify and map functionally important regions of the tobacco mosaic virus movement protein, deletions of three amino acids were introduced at intervals of 10 amino acids throughout the protein. Mutations located between amino acids 1 and 160 abolished the capacity of the protein to transport virus from cell to cell, while some of the mutations in the C-terminal third of the protein permitted function. Despite extensive tests, no examples were found of intermolecular complementation between mutants, suggesting that function requires each movement protein molecule to be fully competent. Many of the mutants were fused to green fluorescent protein, and their subcellular localizations were determined by fluorescence microscopy in infected plants and protoplasts. Most mutants lost the ability to accumulate in one or more of the multiple subcellular sites targeted by wild-type movement protein, suggesting that specific functional domains were disrupted. The order in which accumulation at subcellular sites occurs during infection does not represent a targeting pathway. Association of the movement protein with microtubules or with plasmodesmata can occur in the absence of other associations. The region of the protein around amino acids 9–11 may be involved in targeting the protein to cortical bodies (probably associated with the endoplasmic reticulum) and to plasmodesmata. The region around residues 49–51 may be involved in co-alignment of the protein with microtubules. The region around residues 88–101 appears to play a role in targeting to both the cortical bodies and microtubules. Thus, the movement protein contains independently functional domains.

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Introduction

The spread of plant virus infections from cell to cell requires virus-encoded proteins commonly referred to as movement proteins (MPs). The viral genome is transported, in some cases as an encapsidated virion or in others as unencapsidated nucleic acid, through plasmodesmata (McLean *et al.*, 1997; Robards and Lucas, 1990). Some MPs form tubular structures that extend through plasmodesmata, and through which virions pass, while others apparently do not form new structures, but instead modify plasmodesmata in a way that allows virus transport. MPs are typically not required for viral replication, but can be important determinants of virus host range.

Tobacco mosaic virus (TMV), the type member of the tobamoviruses, encodes a single MP with a molecular mass of about 30 kDa (Deom *et al.*, 1987; Meshi *et al.*, 1987). Transgenic plants that accumulate the MP of TMV can complement mutants of TMV that do not produce functional MP (Deom *et al.*, 1987; Holt and Beachy, 1991). The coat protein (CP) is not required for cell-to-cell movement (Dawson *et al.*, 1988), although it is required for systemic spread in most hosts (Dawson, 1992). Although MPs from different tobamoviruses show relatively little sequence similarity, they perform similar functions, and can in some cases function properly when switched between tobamoviruses (Deom *et al.*, 1994; Nejidat *et al.*, 1991; Padgett and Beachy, 1993). Furthermore, MPs can in some cases substitute for those of unrelated viruses (Cooper *et al.*, 1996; De Jong and Ahlquist, 1992; Giesman-Cookmeyer *et al.*, 1995) suggesting that dissimilar MPs function in similar ways.

The mechanism by which the MP of TMV potentiates virus movement from cell to cell is not yet understood. However, observations made *in vitro* and *in vivo* have allowed the assignment of several activities and characteristics to the protein. The MP, when expressed in and purified from *E. coli*, binds single-stranded nucleic acid cooperatively but with no sequence specificity (Citovsky *et al.*, 1990, 1992), as do MPs of several other viruses (Citovsky *et al.*, 1991; Ivanov *et al.*, 1994; Osman *et al.*, 1992; Rouleau *et al.*, 1994; Schoumacher *et al.*, 1992). The MP binds GTP specifically *in vitro* (Li and Palukaitis, 1996). MP translated *in vitro* was found to bind to both monomeric and polymerized tubulin and actin (McLean *et al.*, 1995).

In vivo, the MP associates with cell walls and plasma membranes, and is also found in a soluble high-molecular-mass complex, the other components of which have not been determined (Deom *et al.*, 1990; Fenczik, 1994; Moore *et al.*, 1992). The protein localizes to plasmodesmata in

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infected plants (Tomenius *et al.*, 1987) and in transgenic plants that accumulate the MP, and induces the formation of fibrous material within the plasmodesmata (Ding *et al.*, 1992; Moore *et al.*, 1992). The MP alters the molecular size exclusion limit of plasmodesmata, increasing the limit from about 750 Da in non-transgenic plants to greater than 10 kDa in transgenic plants (Wolf *et al.*, 1989). When MP expressed in and isolated from bacteria is injected into plant cells, it increases the apparent size exclusion limit of plasmodesmata (Waigmann *et al.*, 1994) and mediates its own transport through plasmodesmata (Waigmann and Zambryski, 1995). The MP is phosphorylated *in vivo* (Citovsky *et al.*, 1993; Haley *et al.*, 1995; Watanabe *et al.*, 1992), although the role of phosphorylation is not known.

Fluorescence microscopy of plants and protoplasts infected with TMV producing the MP fused to the green fluorescent protein (GFP) allowed the subcellular localization of the MP to be determined. The MP associates with plasmodesmata in plants, with punctate structures at the periphery of protoplasts, and with cortical microtubules and bodies (probably endoplasmic reticulum (ER)-associated) in both plants and protoplasts (Heinlein *et al.*, 1995, 1998; Oparka *et al.*, 1997; Padgett *et al.*, 1996; C. Reichel, unpublished results). Based on immunofluorescent microscopy studies, the MP may also associate with actin filaments *in vivo* (McLean *et al.*, 1995).

Scanning deletion mutagenesis has been used to identify regions of the cauliflower mosaic virus MP that are required for function (Thomas and Maule, 1995). Alanine scanning mutagenesis of the red clover necrotic mosaic virus MP revealed regions important for function and for RNA binding *in vitro* (Giesman-Cookmeyer and Lommel, 1993). Most previous mutational studies of the TMV MP employed large deletions within the protein sequence (e.g. Gafny *et al.*, 1992; Waigmann *et al.*, 1994). In this report, scanning deletion mutagenesis was carried out in order to identify and map structurally or functionally important regions of the TMV MP. At intervals of 10 amino acids throughout the protein, three-amino acid deletions (TADs) were introduced using a cDNA clone of TMV from which infectious transcripts could be produced. GFP was fused to many of the mutants so that the effects of the mutations on the subcellular localization of the protein could be directly observed in living tissue. These observations allow the assignment of targeting functions to different parts of the MP sequence, and suggest that the MP contains domains that function independently of each other.

Results and discussion

Functionality of the mutants in virus infection

Deletions of three amino acids were introduced into the MP in a cloned cDNA of TMV from which infectious RNA transcripts could be prepared. Figure 1 shows the position of each mutation in the amino acid sequence, along with the predicted hydropathy profile of the protein, which is similar for all tobamovirus MPs despite their dissimilar sequences (not shown). The functionality of each mutant was tested by inoculating transcripts to *Nicotiana tabacum* plants (Figure 1), using both non-transgenic plants and plants that accumulate the wild-type (wt) MP (MP(+) plants). Two cultivars were used: Xanthi nn, a systemic host for TMV, and Xanthi NN, a hypersensitive host that produces necrotic local lesions in response to infection. These and other experiments described below were also carried out using preparations of virions purified from infected plants, using transgenic MP(+) plants to propagate non-functional mutants. To test whether propagation on transgenic plants might lead to the accumulation of new mutations in the *MP* gene of the virus, one of the non-functional mutant viruses (TAD 10) was passaged 10 times on transgenic plants. The *MP* gene was then isolated from the passaged virus by RT–PCR; the sequence of the gene was found to be identical to the original TAD 10 sequence.

Mutants TAD 1 to TAD 16, and TAD 23 did not induce symptoms when inoculated to non-transgenic tobacco plants. On Xanthi NN plants, no local lesions were found, and on Xanthi nn plants no symptoms of systemic infection were observed, and no infectivity was recovered from upper leaves (Figure 1). These mutants could, however, produce infections on transgenic MP(+) plants, showing that virus replication and long-distance spread were not affected.

Mutants TAD 19, TAD 21, TAD 22 and TAD 24 to TAD 26 produced local lesions on non-transgenic Xanthi NN plants, indicating that they retain the capacity to enable local movement. With the exception of TAD 26, these mutants also moved systemically on Xanthi nn plants. TAD 26 lies in the vicinity of the subgenomic promoter that directs production of the coat protein (CP) (Deom *et al.*, 1994). Western blot analysis showed that TAD 26 does not produce CP (not shown), which is required for efficient systemic infection by TMV in *N. tabacum* (Dawson, 1992).

Previously constructed mutant viruses carrying deletions of various lengths near the N-terminus of the MP indicated the importance of the N-terminus to the function of the protein (Gafny *et al.*, 1992). The results presented here show that the entire first half of the protein contains regions essential for function. Truncation of the C-terminal 73 amino acids (mutant CT-4) abolishes function, while truncation of 55 amino acids (mutant CT-3) does not (Berna *et al.*, 1991; Gafny *et al.*, 1992). Therefore it is not surprising that TAD mutations in the last third of the protein did not prevent virus movement on non-transgenic plants (with the exception of TAD 23). Deletions of residues 189–191 (TAD 19) and residues 209–211 (TAD 21) did not abolish function, showing that small deletions in the region just

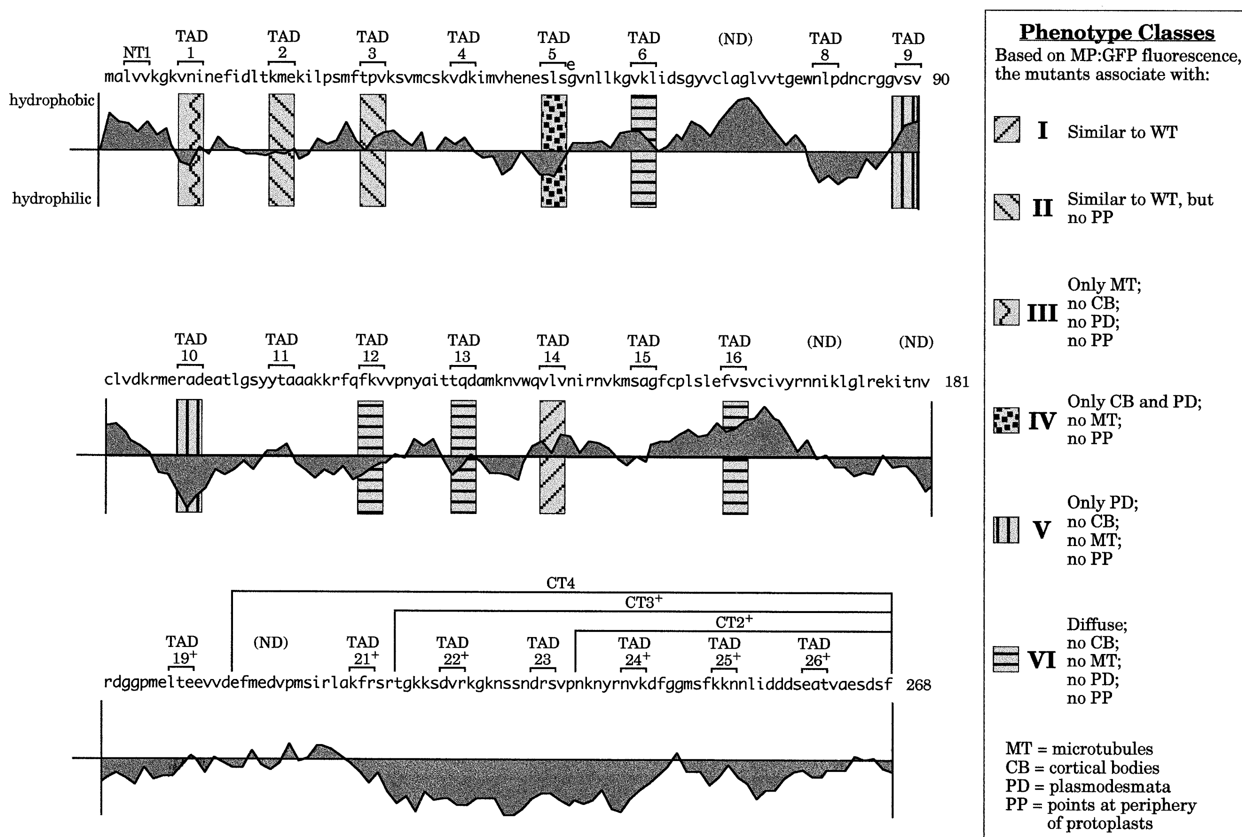


Figure 1. Amino acid sequence and hydropathy profile of the TMV MP.
The position of each TAD mutation is indicated, as are the positions of NT1 (Lapidot *et al.*, 1993), CT2, CT3 and CT4 (Berna *et al.*, 1991; Gafny *et al.*, 1992). Mutant TAD 5 also contains a glycine to glutamic acid mutation at position 52. A '+' sign is used to indicate those mutants that are functional. The vertical boxes indicate the classes of subcellular distribution for TAD mutants that were fused to GFP. 'ND' indicates mutants that were not constructed.

before the dispensable part of the C-terminus do not affect any critical functions. Surprisingly, deletion of amino acids 229–231 (TAD 23) abolished movement. This deletion may alter the structure of that region of the protein in a way that leads to misfolding of another region, instability of the protein, or an improper interaction with another protein.

While TAD 24 and 26 produced local lesions that expanded at the same rate as lesions produced by wt TMV, TAD 25 produced lesions that expanded at about 60% of the wt rate (Figure 2). Although by this measure TAD 25 has a reduced rate of local spread, it nevertheless retains the ability to systemically infect Xanthi nn plants. This mutation may have altered the structure of the protein in a way that is less severe than in the case of TAD 23.

As a test of whether non-functional mutant MPs could be complemented by a partially functional MP, hypersensitive (Xanthi NN) transgenic plants expressing a mutant in which the C-terminal 55 amino acids are truncated (mutant CT-3, Berna *et al.*, 1991; Gafny *et al.*, 1992) were inoculated with non-functional viruses TAD 1 to TAD 16, with virus carrying a deletion of residues 3–5 (mutant NT1; Lapidot *et al.*, 1993), and with virus carrying a mutant that lacks the C-terminal 73 amino acids (mutant CT4, Berna *et al.*, 1991; Gafny *et al.*, 1992). All of the viruses were complemented, showing that the last 55 amino acids need not be present in order for complementation to occur.

Fusion of GFP with TAD mutants

In order to directly observe the subcellular localization of the TAD mutant MP molecules in plants and protoplasts, infectious clones of TMV were prepared that encoded mutants TAD 1, 2, 3, 5, 6, 9, 10, 12, 13, 14 and 16, each fused to the gene encoding the S65T (Heim *et al.*, 1995) mutant of GFP. The resulting TAD:GFP constructs do not encode the CP, and can therefore only be used to study local movement of infection in *N. tabacum*. The TAD:GFP constructs were inoculated to transgenic MP(+) plants to complement the non-functional TAD mutants. Infection sites were observed microscopically in live tissue five or six days after inoculation. When the wt MP:GFP construct was inoculated to the transgenic plants, the subcellular distribution of fluorescence appeared identical to that seen in non-transgenic plants, suggesting that the production of MP by the transgenic plants does not alter the distribution of MP produced by the virus during infection. In

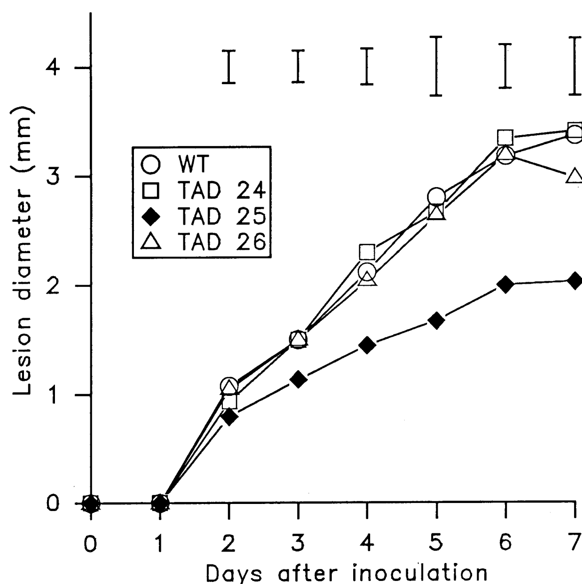


Figure 2. Time course of necrotic local lesion development caused by wt TMV and mutant TMV carrying TAD 24, 25 and 26.
RNA transcripts of each mutant were inoculated to half leaves of *N. tabacum* Xanthi NN, and transcripts of wt TMV were inoculated to the opposite half leaves. The diameters of 20–30 lesions were measured for each time point. Bars at the top of the graph indicate the least significant difference for each time point based on one-way analysis of variance. Filled symbols for TAD 25 indicate a statistically significant difference from the other viruses at a 95% confidence level.

addition, the TAD:GFP constructs were inoculated by electroporation to BY-2 non-transgenic tobacco protoplasts, which were fixed 18 and 22 h after inoculation for subsequent microscopic observation.

In plants, TMV producing wt MP:GFP gave essentially the same results that have been described for the fusion of GFP to the MP of the tobamovirus Ob (Padgett *et al.*, 1996). To summarize (Figure 3), all cells in an infection site show punctate localization in the cell wall, indicating association of the MP with plasmodesmata (Oparka *et al.*, 1997). Cells near the leading edge of infection show small cortical flecks of fluorescent material, probably associated with the ER (Heinlein *et al.*, 1998; C. Reichel, unpublished results), and faint fluorescent fibers indicating MP co-alignment with microtubules (Heinlein *et al.*, 1995). Moving inward from the leading edge, the cortical flecks become larger fluorescent bodies, and the fibers become brighter. In the brightest part of an infection site, the cortical bodies can be up to 10 μm long. Farther inward the bodies are less numerous while the filaments are at their brightest. In the center, near the origin of infection, only the association with plasmodesmata remains. Throughout the infection site, but especially near the brightest part of the ring, diffuse fluorescence can be seen within the cytoplasmic strands.

The appearance of wt MP:GFP in protoplasts essentially parallels the sequence seen in plants (Figure 3; Heinlein *et al.*, 1998). Protoplasts do not contain plasmodesmata, but fluorescence first accumulates in numerous points around the periphery of the cells, tentatively identified as putative cell-wall adhesion sites (Pickard, 1994), and persists in these sites throughout the infection process. Because MP accumulation in these peripheral points parallels the accumulation in plasmodesmata in infected plants, it is tempting to speculate that peripheral points may represent precursors of plasmodesmata. Later, fluorescence accumulates in small bodies that appear to be associated with the cortical ER, and that also contain TMV replicase (Heinlein *et al.*, 1998; C. Reichel, unpublished results). These bodies become larger, and cortical filaments representing MP association with microtubules become visible. Still later, the cortical bodies disappear, leaving smooth cortical filaments. Finally the filaments disappear, leaving only fluorescent points at the periphery of the protoplasts.

Figure 3. Fluorescence micrographs of plant leaves and protoplasts infected with TMV producing the S65T mutant of GFP fused to wt and mutant MP.
A Nikon B-2A filter set was used; as a result, chlorophyll fluorescence produces a red background, which causes GFP fluorescence to appear yellow. In some leaf photographs, the microscope field aperture was partially closed in order to increase contrast and resolution. This results in a circular region of illumination. A bar representing 10 μm is shown in each image.
(a) The subcellular localization of the wt MP:GFP protein is shown. The protein localizes to bodies associated with cortical endoplasmic reticulum (ER) and to microtubules in plants and protoplasts, as well as to plasmodesmata (PD) in plants and to peripheral points (periph. points) in protoplasts.
(b) Members of class III associated only with microtubules.
(c) Members of class IV associated only with ER in plants and protoplasts, and with PD in plants.
(d) Members of class VI showed only diffuse fluorescence in the cytoplasm.

Figure 5. Fluorescence micrographs of plants co-infected by two mutants of TMV.
A Chroma 31022 long-pass filter set was used for both photographs, largely eliminating red fluorescence from chlorophyll. The microscope field aperture was partially closed, resulting in a circular region of illumination. A bar representing 10 μm is shown in each image.
(a) *N. benthamiana* leaf co-infected with TMV-M:Bfus, producing the MP fused to a blue mutant of GFP, and TMVΔC-GFP, producing unfused wt GFP. The blue MP:BFP fusion protein is localized to a cortical body (probably associated with ER), while the green GFP fills the cytoplasmic strands and the area around the nucleus with diffuse fluorescence.
(b) *N. tabacum* leaf co-infected with the same viruses. The blue MP:BFP fusion protein is localized to plasmodesmata, some of which are indicated by arrows, while the green GFP produces diffuse fluorescence.

Fig. 3

cortical bodies | microtubules | PD, periph. points

(a) wt — plant / protoplast

10 μm

(b) Class III — plant / protoplast

no association | | no association

no association | | no association

(c) Class IV — plant / protoplast

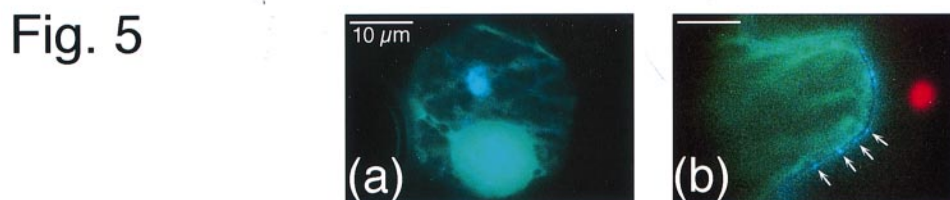
| no association |

| no association | no association

(d) Class VI

plant (no association) | protoplast (no association) | plant (no association)

Fig. 5



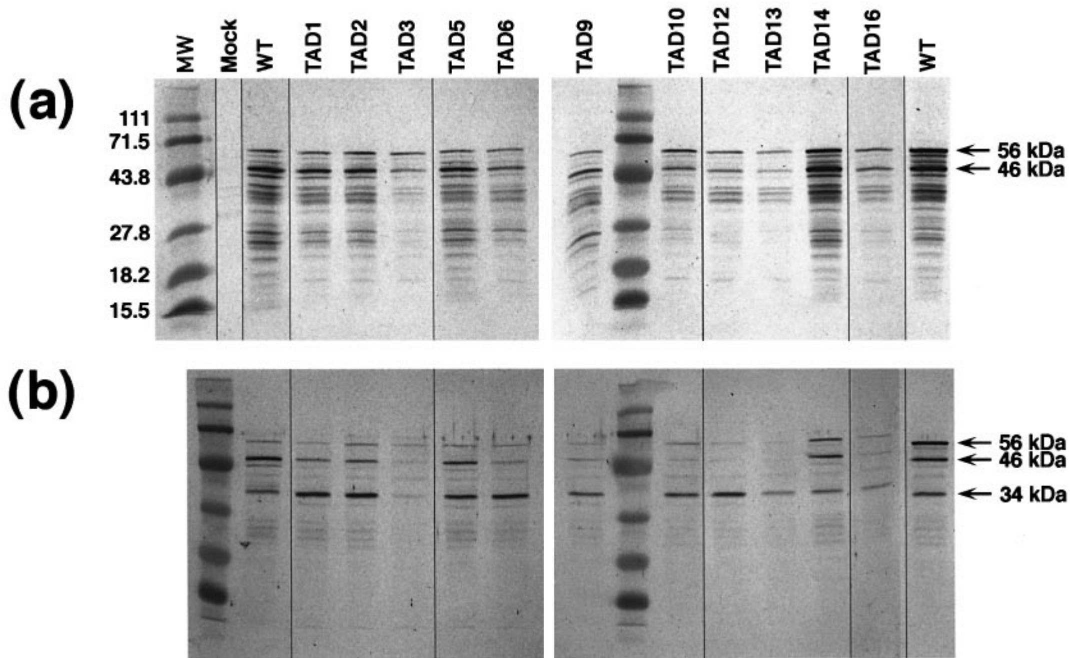


Figure 4. Western blots of extracts from protoplasts at 18 h after infection.
Protoplasts were infected by electroporation with RNA transcripts; mock-infected protoplasts were treated identically, but were electroporated with buffer only. Blots were developed with an antibody against the MP (a) or against GFP (b). The positions of the full-length MP:GFP fusion protein (56 kDa) and of two major degradation products are indicated.

The fluorescence produced by the TAD mutants can be grouped into six classes (Figures 1 and 3). Each class contains a subset of the subcellular localization sites seen with the wt protein. No new sites of localization resulted from the mutations. Class I, containing TAD 14, appeared identical to wt MP in both plants and protoplasts. Class II, containing TAD 2 and 3, appeared similar to wt MP in plants, but failed to associate with peripheral points in protoplasts. Class III, containing TAD 1, showed a strong association with microtubules in plants and protoplasts, but none of the other accumulations produced by the wt protein. Class IV, containing TAD 5, was found only in cortical bodies (probably ER-associated) and plasmodesmata in plants, and only in cortical bodies in protoplasts. Class V, containing TAD 9 and 10, associated only with plasmodesmata in plants, and was completely diffuse in protoplasts. Class VI, containing TAD 6, 12, 13 and 16, formed only diffuse fluorescence in plants and protoplasts, and did not accumulate in any of the sites characteristic of the wt protein.

To ascertain whether the mutations affected the stability of the MP, protoplast extracts prepared 18 h after infection were analyzed by Western blotting (Figure 4). The blots show the presence of full-length MP:GFP fusion protein as well as a series of smaller peptides that presumably correspond to degradation products. Both anti-MP antibody and anti-GFP antibody detected the full-length fusion protein (56 kDa) and a product with a molecular mass of about 46 kDa. In addition, the anti-GFP antibody detected a band with a molecular mass of about 34 kDa, presumably corresponding to a breakdown product containing intact GFP plus a small C-terminal fragment of the MP. The presence of this latter product may explain the appearance of diffuse fluorescence in the cytoplasm of cells infected with all of the constructs. The wt MP:GFP fusion protein and all of the TAD:GFP fusion proteins show the same pattern of bands, suggesting that the mutations did not result in significant changes in the mechanism of degradation of the MP. Some of the mutants, particularly some in class VI, which produce diffuse fluorescence, show a relative increase in the intensity of the 34 kDa band, suggesting that they are not as stable as the wt protein.

Mutants that retained the ability to associate with one or more of the subcellular sites must retain a significant portion of the native structure of the protein. Thus, the loss of function is not the result of complete unfolding of the protein. The fact that the Western blot degradation patterns of the wt protein and the mutants do not differ also supports the idea that the mutations did not radically alter the overall structure of the protein. Mutants in class II are located close to each other in the protein sequence, as are mutants in class V, showing that those phenotype classes result from disruption of relatively large domains of the protein, and not from deletion of a particular amino acid.

Mutants in all of the categories except class I (TAD 14) lost the ability to accumulate in peripheral points in protoplasts. This suggests that accumulation in those sites represents a fundamental aspect of MP function, which requires the entire structure of the protein. The loss of targeting to any other sites also leads to an inability to accumulate in peripheral points. Only TAD 14 accumulated in peripheral points, and it also accumulated in all of the other sites targeted by the wt protein. The fact that TAD 14 is non-functional, however, shows that correct targeting is not sufficient for MP function. Thus, there is at least one characteristic of the MP that is critical to function, yet cannot be observed by fluorescence microscopy. Studies are underway to identify other characteristics of the protein that may have been altered by the mutations, including susceptibility to phosphorylation.

When plants are infected with TMV carrying the wt MP fused to GFP, the earliest site of accumulation that can be observed is in the plasmodesmata (Oparka *et al.*, 1997; Padgett *et al.*, 1996). Mutants in class V associated only with plasmodesmata, and not with any of the other subcellular sites seen with the wt protein. Thus, the MP need not be capable of associating with any of the other sites in order to reach the plasmodesmata. Class III and class VI mutants failed to associate with plasmodesmata. The fact that class VI mutants do not associate with any subcellular structures suggests that they are completely unfolded. Class III (TAD 1), however, retains the ability to associate with microtubules despite the fact that it cannot associate with plasmodesmata. Therefore, deletion of amino acids 9–11 may have disrupted a domain of the protein involved in association with plasmodesmata.

Mutants in several classes (II, IV and V) accumulated in plasmodesmata in plants despite the fact that they did not accumulate in points at the periphery of protoplasts. This suggests that although MP accumulation at peripheral points seems to correspond with accumulation in plasmodesmata, these may in fact be non-equivalent sites. Alternatively, it is possible that mutants that did not associate with peripheral points in protoplasts were able to reach plasmodesmata in transgenic plants by interacting with wt MP. However, in all other ways, the phenotype observed for each mutant was the same in protoplasts and transgenic plants. Therefore, it seems unlikely that the wt MP influenced the localization of the mutants.

TAD 1 (class III) associated only with microtubules, showing that this association occurs independently of accumulation of MP in other subcellular sites. Thus, the wt protein does not reach microtubules after first passing through other structures. Similarly, the association with microtubules is not the result of saturation of a downstream site that prevents the protein from leaving the microtubules.

Accumulation of MP in cortical bodies can occur in the absence of association with all structures except plasmodesmata (TAD 5, class IV). This could imply that accumulation in cortical bodies follows accumulation in plasmodesmata in a targeting pathway, but is independent of accumulation in other sites.

Based on the results obtained with the TAD mutants fused to GFP, different regions of the MP sequence can be assigned roles in targeting to different sites. The region around amino acids 9–11 may be involved in targeting to the cortical bodies and plasmodesmata. The region around amino acids 49–51 may be involved in association with microtubules. The region around amino acids 88–101 may be involved in association with the cortical bodies and microtubules. The fact that a mutation can disrupt one or more targeting functions of the MP, while leaving other functions intact, suggests that the MP contains independent functional domains.

Lack of complementation between viruses carrying non-functional MP mutants

Fluorescence microscopy of cells infected with TAD:GFP fusions showed that small mutations can abolish individual targeting activities of the MP without affecting other targeting activities. For this reason, three different approaches were taken to test whether non-functional TAD mutants, when co-expressed in the same leaf cell, could complement each other to restore function. In the first approach, Xanthi NN plants were co-infected with mixtures of virions of non-functional mutants TAD 1 to TAD 16. The viruses were mixed in groups of two, five and ten mutants before inoculation, such that all possible combinations could occur. Surprisingly, no necrotic lesions were observed, showing that complementation did not occur.

This unexpected result prompted us to use a second approach, which increased the likelihood that multiple viruses would enter the same cell. Grafted plants were prepared in which the lower part of the plant (the root stock) was transgenic for wt MP, and the upper part (the scion) was non-transgenic. Mixtures of non-functional viruses were co-infected in a leaf of the root stock, where the wt MP produced from the transgene would allow the viruses to spread throughout that part of the plant, increasing the likelihood that individual cells would become co-infected. Mutants capable of complementing each other would be co-transported from the root stock into the non-transgenic scion, resulting in infection of the scion. No evidence of infection of the scion was found in these experiments even after several months, showing that complementation between non-functional mutants did not occur. By contrast, when transgenic scions were used, or when wt TMV was inoculated to root stocks, the scions showed symptoms within one month.

The third approach to test for complementation con-

sisted of inoculating mutant viruses to transgenic plants expressing mutant *MP* genes. This approach assures that two mutants of the MP will accumulate in the same cell. Non-functional TAD mutant viruses were inoculated to two different hypersensitive plant lines accumulating the non-functional MP mutant NT1, from which amino acids 3–5 are deleted (Lapidot *et al.*, 1993). Systemic host (Xanthi nn) transgenic plants accumulating a non-functional MP mutant from which the C-terminal 73 amino acids are truncated (mutant CT-4, Berna *et al.*, 1991; Gafny *et al.*, 1992) were inoculated with the TAD mutant viruses and the NT1 virus, individually and in groups of three. Virus carrying the CT-4 mutant was inoculated to hypersensitive transgenic plants accumulating the NT1 mutant. No complementation was observed in any of these experiments.

The lack of complementation in the co-infection experiments conceivably could have been the result of an inherent inability of individual leaf cells to support replication of two mutants of the same virus. To address this question, *N. benthamiana*, *N. tabacum* Xanthi nn, and transgenic MP(+) *N. tabacum* Xanthi nn plants were co-inoculated with TMV-M:Bfus, a mutant of TMV that produces MP fused to a blue mutant of GFP (BFP), and TMVΔC-GFP, which produces free GFP instead of the CP. Leaf tissue containing overlapping blue- and green-fluorescent infection sites was examined by fluorescence microscopy. The results were the same in all plant types. As shown in Figure 5, both GFP (diffuse) and BFP (localized to subcellular sites targeted by the MP) could be seen in the same cell. The overlapping regions in which co-infection was found were at least three cells wide. The overlap is indicative of co-infection, and not of movement of the MP out of infected cells. Although injection of MP into cells suggests that the MP can mediate its own intercellular transport (Waigmann and Zambryski, 1995), this does not appear to be the case in infected plants, for the following reasons. In an infection site, the MP is most abundant in a narrow macroscopic ring of cells that expands in diameter as the virus spreads outward from the site of inoculation, consistent with the fact that the MP is produced during a brief period in each cell and is then degraded (Epel *et al.*, 1996; Padgett *et al.*, 1996). The MP does not diffuse outward from the narrow ring. Furthermore, the size exclusion limit of the plasmodesmata is increased transiently in cells with the highest level of MP, while cells ahead of or behind the narrow ring show a normal limit (Oparka *et al.*, 1997). Thus, the results presented here show that infection from two TMV mutants can spread into the same cell within a plant.

The fact that no complementation was observed between non-functional MP mutants suggests that within the first 160 amino acids reside essential activities that require this entire region of the protein to be intact. Each molecule of the MP needs to be independently competent to carry out these activities.

Test for altered host range, and search for intramolecular complementation

A mutant MP that is not functional in *N. tabacum* could conceivably have retained, or perhaps gained, the ability to function in a different host. To test for this possibility, all non-functional TAD viruses were inoculated alone and in groups to *N. benthamiana*; mutant viruses TAD 1–12 were inoculated individually to *Chenopodium amaranticolor*, a hypersensitive host for many viruses; mutant virus NT1 was inoculated to *Arabidopsis thaliana* (normally a poor host for TMV); mutant viruses NT1 (non-functional), CT3 (partially functional) and CT2 (functional) were inoculated to green bunching onion (normally not a host for TMV); and wt TMV and mutant viruses CT3 and CT2 were inoculated to shallot (normally not a host for TMV). In each case, no symptoms were observed, and no infectivity was recovered from new leaves.

No non-functional TAD mutant reverted to functionality even several weeks after inoculation to plants. Chemical mutagenesis was carried out in an attempt to induce second-site mutations that could restore function to TAD mutants. TAD mutants 1–12 were transcribed, and the RNA was subjected to nitrous acid mutagenesis and then inoculated to plants, as described in Experimental procedures. No functional mutants were recovered. These results suggest that new point mutations cannot intramolecularly restore activities that were lost as a result of the disruption of a domain by a TAD mutation.

Final conclusions

The direct observation of the *in vivo* effects of small deletions reported here allows specific targeting activities to be assigned to domains of the TMV MP. Biochemical assays on the MP performed *in vitro*, such as nucleic acid binding (Citovsky *et al.*, 1990), nucleotide binding (Li and Palukaitis, 1996), and phosphorylation, will reveal whether the effects of the mutations on these activities correlate with the altered subcellular distributions of the MP reported here. Certain non-functional TAD mutants may also lead to the development of transgenic plants that are resistant to viral infection, as was the case for a non-functional mutant in which amino acids 3, 4 and 5 were deleted, leading to resistance against TMV and unrelated viruses (Cooper *et al.*, 1995; Lapidot *et al.*, 1993). TAD 14 is particularly interesting in this regard because it localizes to all of the sites targeted by the wt protein, yet is non-functional. This mutant might therefore interfere with the ability of

the wt protein to carry out its activities at one or more locations.

Based on *in vitro* studies of MP mutants expressed in and purified from *E. coli*, amino acids 112–185 (region A) and 185–268 (region B) were assigned the ability to bind RNA independent of each other (Citovsky *et al.*, 1992). Region B, which can bind RNA when dissected from the protein, corresponds to the region in which most of the TAD mutants were functional (TADs 19, 21, 22, 24, 25, 26). This could indicate that most of the TAD mutations in this region did not disrupt RNA binding. Alternatively, RNA binding by region A alone may be sufficient, or RNA binding may not be necessary for *in vivo* function. The one TAD mutant in this region that was not functional (TAD 23) lies within the C-terminal portion that can be truncated without abolishing function, as discussed above, and therefore presumably does not abolish function by disrupting RNA binding. Region A, which does not bind RNA when dissected from the protein, contains TAD 12, 13 and 16, which when fused to GFP produce only diffuse fluorescence, suggesting that these mutations disrupted the entire structure of the protein. TAD 14, which also lies in region A, is non-functional yet accumulates at all the subcellular sites seen with the wt protein. Thus, TAD 14 may interfere with the ability of the protein to bind RNA; this hypothesis remains to be tested.

Based on experiments in which purified MP mutants were co-injected with fluorescently labeled dextrans into plant cells, the region of the protein from amino acids 126–224 has been assigned the ability to modify the size exclusion limit of plasmodesmata (Waigmann *et al.*, 1994). A protein fragment comprising amino acids 116–268 could modify plasmodesmata, although in a way that was different from the wt protein. This region corresponds to the sum of the two RNA-binding regions described above. Therefore, the same arguments given in the case of RNA binding lead to the conclusion that TAD 14 may render the MP non-functional by interfering with the ability of the protein to modify plasmodesmata. This mutant has not lost the ability to associate with plasmodesmata; whether it can modify the size exclusion limit remains to be tested.

The mutations in the MP appear to exert their effects by disrupting functional domains of the protein. The structures of those domains, rather than specific amino acids within the domains, are important for the function of the protein. This is consistent with the fact that tobamovirus MPs share relatively little sequence similarity, yet perform the same function. The shared function most likely arises from similar structures, in which homologous domains perform the same activities. The information obtained from the TAD mutants will be critical to assigning activities to regions of the protein as structural information becomes available.

Experimental procedures

Plants

N. tabacum cv. Xanthi nn and *N. tabacum* cv. Xanthi NN were used as systemic and hypersensitive hosts, respectively, for testing the functionality of mutants. Transgenic MP(+) tobacco plants accumulating the TMV MP were Xanthi nn line 277 (Deom *et al.*, 1987) and Xanthi NN line 2005 (Deom *et al.*, 1991). Mutants were tested for changes in host range on *N. benthamiana*, *Chenopodium amaranticolor*, *Arabidopsis thaliana* (ecotype Columbia), green bunching onion (*Allium* sp.), and shallot (*Allium* sp.). Plants were grown and maintained under fluorescent lights or in a greenhouse.

Grafted plants were produced with line 277 as the root stock and non-transgenic Xanthi NN or Xanthi nn as the scion, or with line 2005 or line 277 as the scion for control experiments, following the protocol described by Arce-Johnson *et al.* (1997).

Construction of mutant TMV cDNA clones

Deletions of nine nucleotides (encoding three amino acids) at intervals of 30 nucleotides were constructed by site-directed mutagenesis using the Sculptor *in vitro* mutagenesis system (Amersham). The *MP* gene was excised from pU3/12 (Holt and Beachy, 1991) using *Kpn*I and *Bam*HI, and was cloned into pBluescript KS+ (Stratagene). Synthetic oligonucleotides extending 16–20 nucleotides in each direction from the deletion site were used to mutate the *MP* gene. Each mutant was sequenced, and was then excised with *Aat*II and *Acc*I and ligated into the TMV cDNA clone pU3/12DM-RV (Nejidat *et al.*, 1991) to generate full-length TMV containing the TAD mutations.

The S65T mutant of GFP (Heim *et al.*, 1995) was fused to the TAD mutants by excising a *Bam*HI–*Bsm*BI fragment from each TAD TMV construct and ligating it into the same sites in pTMV-M:Gfus (Heinlein *et al.*, 1995). The *MP* gene of each construct was sequenced to confirm its identity.

To demonstrate co-infection by two TMV mutants, plants were co-inoculated with TMVΔC-GFP, in which the CP is replaced with wt GFP, and TMV-M:Bfus, in which a blue fluorescent mutant of GFP is fused to the MP. To construct TMV-M:Bfus, a plasmid containing the Y66H blue mutant of GFP (Reichel *et al.*, 1996) was modified to incorporate mutations F99S, M153T and V163A to increase the solubility and fluorescence of the protein (C. Reichel, G. Jach and J. Schell, unpublished results). In addition, silent changes that remove a cryptic splice site and improve codon usage were included (Reichel *et al.*, 1996), and the gene was then substituted for the *GFP* gene in pTMV-M:Gfus.

In vitro *transcription from TMV clones, and infection of plants and protoplasts*

TMV clones were transcribed *in vitro* from the T7 promoter as described by Holt and Beachy (1991) or using MEGAscript T7 kits (Ambion). Plants were mechanically inoculated (Holt and Beachy, 1991), and protoplasts were transfected by electroporation (Watanabe *et al.*, 1987). For those constructs that encode the CP, virions were purified from infected plant tissue (Asselin and Zaitlin, 1978). On hypersensitive plants, infectivity was assayed by counting and measuring the diameter of local lesions. On systemic hosts, symptoms were observed in upper leaves, and samples taken from upper leaves were homogenized in 20 mM potassium phosphate pH 7.2 and mechanically inoculated to hypersensitive plants.

RT–PCR of TAD 10 was performed using a Perkin-Elmer Gene-Amp RNA PCR kit in order to determine whether repeated passaging on transgenic MP(+) plants introduced new mutations in the gene.

Chemical mutagenesis

Transcripts of TMV containing TAD mutants 1–12 were subjected to nitrous acid mutagenesis under conditions that alter approximately two nucleotides per RNA molecule (Gierer and Mundry, 1958). One-half volume of 4 M $NaNO_2$ and one-half volume of 1 M $NaC_2H_3O_2$ pH 4.8 were added to each transcript. After incubation at room temperature for 18.5 min, the pH was neutralized by addition of KH_2PO_4 at pH 7.2, and the transcripts were inoculated to *N. tabacum* Xanthi NN. After 10 days, the inoculated leaves were homogenized and used to inoculate new plants. No local lesions developed, indicating that intramolecular complementation did not occur.

Western blots

Infected protoplasts were pelleted by brief, low-speed centrifugation, and were immediately resuspended in SDS–PAGE sample buffer and boiled. The samples were subjected to SDS–PAGE and blotted to nitrocellulose. The blots were developed using an affinity-purified antibody against residues 209–222 of the MP, or a polyclonal antibody against GFP (Clontech), followed by an alkaline phosphatase-conjugated secondary antibody (Southern Biotechnology Associates).

Microscopy

Infected protoplasts were fixed with 3% paraformaldehyde in the presence of 2.5 mM EGTA, deposited onto polylysine-coated slides in a Cytospin centrifuge (Shandon), and mounted with Mowiol containing DABCO. Plant infection sites were excised, mounted with 0.4 M mannitol, and viewed and photographed within 1 h of excision. Microscopy was performed with a Nikon Optiphot2-UD equipped with a Nikon PlanApo 100X oil-immersion lens with a numerical aperture of 1.40. The S65T mutant of GFP fused to the MP was visualized with a Nikon B-2A filter set consisting of a 450–490 excitation filter, a 505 dichroic mirror, and a 520 barrier filter. BFP and wt GFP were visualized together using a Chroma 31022 long-pass filter set consisting of a 378–400 excitation filter, a 420 dichroic mirror, and a 430 long-pass filter. Images were recorded on Kodak Ektachrome 320T, Elite 400 or P1600 film.

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