

Mutational Analysis of the Movement Protein of Odontoglossum Ringspot Virus to Identify a Host-Range Determinant

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Tobacco mosaic virus (TMV) which contains the movement protein (MP) of odontoglossum ringspot tobamovirus (ORSV) in place of the TMV MP systemically infects orchids but causes local infection in tobacco unless the carboxy-terminal 48 amino acids of the MP are deleted (C. A. Holt, C. A. Fenczik, S. J. Casper, and R. N. Beachy; Virology, in press, 1995). Frameshift mutations were created within the 3' end of the MP gene that led to truncations of the ORSV MP by 11, 19, 28, 37, and 48 amino acids; each of the mutant MP genes was inserted into the cloned cDNA of TMV in place of the TMV MP and infectious transcripts were produced. Virus containing mutant MPs were used to infect vanilla orchids, a systemic host of ORSV, and tobacco plants. Removal of 11 amino acids from the ORSV MP prevented spread of the chimeric virus in orchids while restoring the ability to cause a systemic infection on tobacco. Further deletions of the MP affected the size of virus-induced necrotic local lesions on tobacco cv. Xanthi NN and the systemic spread and accumulation of virus in cv. Xanthi nn, a systemic host of TMV. However, each virus replicated to equivalent levels in protoplasts. A mechanism by which the ORSV MP limits the spread of the chimeric virus is proposed.

The members of the tobamovirus family, of which tobacco mosaic virus (TMV) is the type member, have specific natural host ranges. For example, TMV is an efficient pathogen of many members of the Solanaceae, whereas odontoglossum ringspot virus (ORSV) primarily infects plants of the Orchidaceae. However, the factors that influence the host range of these and other tobamoviruses is not fully understood.

Each of the proteins encoded by most viruses has the potential to impact host range. The TMV genome encodes four and possibly five proteins: the 183- and 126-kDa proteins are required for replication (Ishikawa et al. 1986), the 30-kDa movement protein (MP) is essential for cell-to-cell spread (Meshi et al. 1987), and the 17.5-kDa coat protein (Hunter et al. 1976) is dispensable for both replication and cell-to-cell

movement but is essential for long-distance movement and for virion assembly (Siegal et al. 1962; Takamatsu et al. 1987; Dawson et al. 1988; Saito et al. 1990). A possible fifth protein of 54 kDa has not been found in infected plants and its function is not known (Palukaitis and Zaitlin 1986). Other tobamoviruses encode proteins with similar activities.

Evidence for the role of the viral MP in host-range determination was initially reported in mixed-infection studies. Several studies have shown that certain viruses can systemically infect a nonhost plant when coinoculated with a second virus that normally infects that plant (Allison et al. 1990; Atabekov and Dorokhov 1984; Dodds and Hamilton 1976; Malysenko et al. 1988). In one series of studies, it was shown that a tobamovirus isolated from orchids could provide a helper function to another tobamovirus, cucumber green mottle mosaic virus (CGMMV) (Malysenko et al. 1989). It was suggested from these studies that the movement function of the compatible virus (the orchid virus) allowed the incompatible virus (CGMMV) to move in a nonhost plant (orchid).

The TMV MP is involved in the cell-to-cell movement of virus infection (Deom et al. 1987; Meshi et al. 1987). The MP alters the permeability of plasmodesmata in transgenic plants that express the MP gene (Wolf et al. 1989) or when MP is directly injected into plant cells (Waigmann et al. 1994). The MP localizes to plasmodesmata in TMV-infected plants (Tomenius et al. 1987), as well as in transgenic plants that contain the MP (Atkins et al. 1991; Moore et al. 1992). Data from in vitro experiments indicated that the MP binds to single-stranded nucleic acids and led to the hypothesis that the MP acts as a molecular chaperone for TMV RNA, escorting the RNA to and perhaps through the plasmodesmata (Citovsky et al. 1992).

The role of the MP in determining the host range of tobamoviruses and bromoviruses has been studied by creating hybrid viruses in which the MP from one virus is exchanged for that of another. The MP of brome mosaic virus (BMV) and cowpea chlorotic mottle virus (CCMV) were exchanged in infectious cDNA clones of the respective viruses; in each instance the hybrid virus was no longer able to infect its natural host (Mise et al. 1993). When the MP of tobacco mild green mosaic tobamovirus (TMGMV) was exchanged for the MP in TMV, the exchange influenced the size of local lesions induced by infection of *N. tabacum* cv. Xanthi NN (Nejdat et al. 1991). Both the MP and the CP of odontoglossum ringspot

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tobamovirus (ORSV) were shown to contain host range determinants by studies in which each cistron was transferred to an infectious cDNA clone of TMV (Hilf and Dawson 1993). As shown by Holt et al. (1995), the replacement of the ORSV MP for that of TMV allowed the hybrid virus to move long distance in orchids, whereas it was limited to the inoculated leaf in tobacco plants. In these studies two mutants of the hybrid virus, M₂L and V-1, were recovered that allowed the phloem associated long-distance movement of the chimeric viruses in tobacco but not in orchid plants. One of these mutations, M₂L, resulted in a truncation of the ORSV MP by 48 amino acids.

In this study we further defined the host-range determinant contained within the carboxy-terminal amino acids of the ORSV MP. Nucleotides were inserted into the ORSV MP gene that caused frameshift mutations leading to premature termination of translation resulting in proteins that are truncated at approximately 10 amino acid intervals. Five mutant hybrid viruses were inoculated onto tobacco and orchid plants to determine their infectivity. We concluded that the terminal 11 amino acids, 267 to 278, of the ORSV MP contain a region that is necessary for host range specificity, possibly through interaction with a host component.

RESULTS

Construction of mutations in the ORSV MP.

Hilf and Dawson (1993) proposed that the ORSV movement protein contains determinants that influence the host range of the virus. Holt et al. (1995) showed that these determinants are within the carboxy-terminal 48 amino acids of the movement protein. To further characterize the location of this determinant, mutations were made in the 3' end of the ORSV MP sequence and the mutant MPs were tested in a chimeric virus that included the TMV replicase and coat protein genes.

The chimeric virus used in this study was constructed by precisely replacing the TMV MP with the ORSV MP in the TMV genome (Fig. 1). A fragment containing the genes encoding the ORSV MP and TMV CP was generated by PCR-mediated gene fusion and cloned into a Bluescript plasmid to create the intermediate plasmid BS/OMP-TCP. BS/OMP-TCP was sequenced through the genes encoding the ORSV MP and TMV CP to confirm that the fusion between the two genes was as predicted and no additional mutations were found. To create the full-length infectious cDNA of the chimeric virus, T/OMP, the ORSV MP and the TMV MP genes were subcloned on an *EcoRV*-*KpnI* fragment into the plasmid U3/12ΔM-RV which contains the gene encoding the TMV replicase. U3/12ΔM-RV is a modified clone of TMV in which the first 450 nucleotides of the TMV MP were removed and replaced with a unique *EcoRV* restriction site (Holt et al. 1995).

Mutations within the carboxy-terminal end of the ORSV MP were created to further define the host-range determinant. BS/OMP-TCP was subjected to PCR mutagenesis to insert one or two nucleotides within specific sequences near the 3' end of the MP gene. The inserted nucleotides caused frameshift mutations which result in premature translational termination of the ORSV MP while maintaining the RNA sequence with little change. This strategy was selected because

the subgenomic promoter for the coat protein gene and a portion of the origin of assembly (OAS) for the virus are present in close proximity to the mutagenized sequences. Following mutagenesis, the resultant PCR products were cloned into pBluescript KS+ and sequenced to confirm the presence of the mutation. Five mutations were made that resulted in the synthesis of proteins truncated at approximately 10 amino acid intervals from the carboxy-terminal end of the ORSV MP. Full-length clones were generated by subcloning the mutant ORSV MP gene and TMV CP gene into U3/12ΔM-RV. Each mutant is named based on the number of amino acids removed from the carboxy-terminal end of the MP, e.g., M-11, M-19, M-28, M-37, and M-48. The chimeric virus and the altered sequence of each of the five mutations is shown in Figure 1.

RNA transcripts produced from each of the clones were tested for infectivity on *Nicotiana tabacum* cv. Xanthi NN and Xanthi nn plants. Transgenic Xanthi NN plants that express the TMV MP gene (plant line 2005; Deom et al. 1991) were also inoculated with each transcript as a positive control for infectivity of the inoculum. Virus was isolated from inoculated leaves of Xanthi nn at 5 days postinoculation (d.p.i.) for subsequent studies, rather than repeatedly relying on transcription reactions for inoculum. RNA from each of the viruses was directly sequenced to confirm the presence of the mutation.

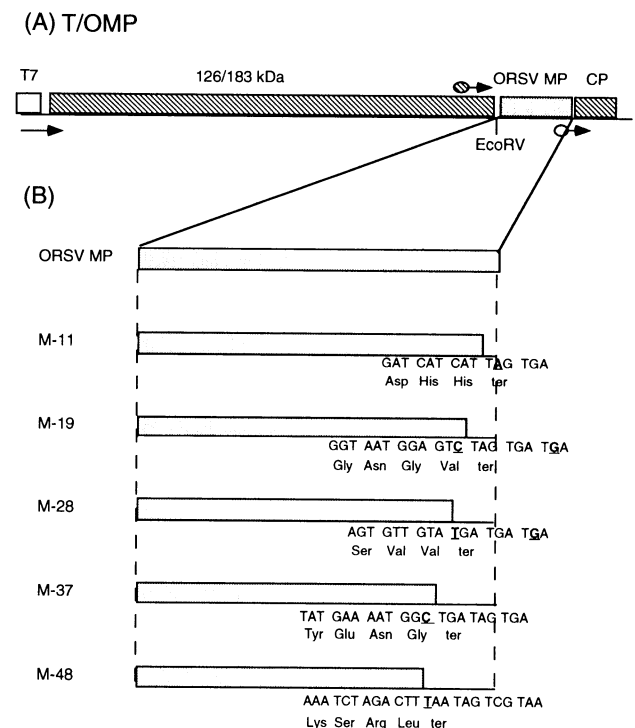


Fig. 1. Schematic representation of the hybrid virus T/OMP and mutants of odontoglossum ringspot virus (ORSV) movement protein (MP). **A**, T/OMP infectious cDNA clone under control of the T7 promoter. Subgenomic RNA promoter sequences are indicated by the ball and arrow figures. **B**, The five mutations made in the 3' end of the ORSV MP gene. The numbers to the left of the boxes indicate the number of amino acids truncated from the protein. The sequences below the boxes show the inserted nucleotides (underlined) and the resulting amino acids.

Infectivity of the chimeric viruses on *N. tabacum* cv. Xanthi NN.

Virus that was isolated from inoculated leaves of Xanthi nn plants infected with either T/OMP or any of the five mutants caused necrotic local lesions when inoculated to Xanthi NN plants. Necrotic local lesions appeared at 3 to 4 d.p.i. for each virus, compared with 2 to 3 d.p.i. for TMV. The size of local lesions induced by infection with T/OMP was about one third the size of lesions induced by TMV at 6 d.p.i. (Table 1).

Necrotic local lesions induced by T/OMP and each of the viruses with mutant ORSV MP sequences were compared by inoculating opposite half leaves of Xanthi NN tobacco plants (Table 1). The local lesions caused by M-11 infection were nearly double the size of local lesions caused by T/OMP. Lesions induced by M-19 were smaller in diameter than those caused by M-11, but were significantly larger (*t* test *P* value 0.0001) than those induced by T/OMP. The removal of 28 or more amino acids from the ORSV MP resulted in lesions that were smaller than those induced by either M-11 or M-19 (Table 1).

Differences in the sizes of local lesions are generally considered to reflect both the rate of cell-to-cell spread of virus infection and the rate of virus replication. To determine whether or not mutations in the MP gene affected the spread of infection in a plant line that produces the TMV MP, each virus was also inoculated onto plant line 2005 (Deom et al. 1991). This Xanthi NN plant line contains the TMV MP and facilitates the spread of TMV that lacks a functional MP (Holt and Beachy 1991). Each of the hybrid viruses, including T/OMP, caused similar sized lesions on this host (data not shown). The results suggest that the differences in the sizes of local lesions reported in Table 1 are due to the effect of the mutations in the MP on local spread of the virus rather than on virus replication, per se. Furthermore, the results indicate that there is a domain within the last 11 amino acids of the ORSV MP that affects the rate of cell-to-cell spread of T/OMP on tobacco plants.

The effect of the mutations of the ORSV MP on long-distance movement and symptom development on *N. tabacum* cv. Xanthi nn.

To ascertain whether the mutations in the MP affect the rate of rapid long-distance spread of virus infection, each mutant

Table 1. Comparison of lesion size caused by tobacco mosaic virus (TMV), T/OMP, and hybrid viruses that contain truncated ondontoglossum ringspot virus (ORSV) movement proteins (MPs)^a

Virus	Lesion sizes		Proportional size ^c
	(Truncated form : T/OMP) ^b		
M-11	1.80 ± 0.40 : 0.95 ± 0.28		1.90
M-19	1.44 ± 0.47 : 0.95 ± 0.33		1.52
M-28	1.31 ± 0.37 : 1.26 ± 0.39		1.04
M-37	0.71 ± 0.22 : 0.72 ± 0.25		0.99
M-48	0.82 ± 0.25 : 0.80 ± 0.21		1.02
TMV	2.72 ± 1.2 : 0.81 ± 0.28		3.36

^a Half leaves were inoculated with the viruses containing the truncated forms of the ORSV MP or TMV, while opposite half leaves were inoculated with T/OMP.

^b Averaged diameter (mm) at 6 days postinoculation of 80 or more individual local lesions on two different leaves of two individual plants ± the standard deviation.

^c Size of lesions as a proportion of the averaged size of T/OMP-induced necrotic lesions.

virus was inoculated to systemic hosts for TMV and ORSV, *N. tabacum* cv. Xanthi nn and *Vanilla planifolia*, respectively. Before inoculation, the infectivity of each virus preparation was determined by inoculation onto Xanthi NN and the concentration of each virus was adjusted to produce 150 to 200 lesions per leaf.

Five- to six-week-old tobacco plants which had five to six true leaves were inoculated with each virus and long-distance movement was followed for 14 days after inoculation by performing ELISAs with antibodies against the TMV coat protein using extracts prepared from noninoculated upper leaves. At least five plants were inoculated with each virus and samples were collected at 4, 7, 10, and 14 d.p.i. The topmost leaf that was sufficiently large that a sample could be taken without destroying the leaf was assayed at each time point. Three samples were taken from each of the five inoculated plants and samples were pooled, except when the leaf was very small. In such cases, one sample was taken per leaf and then pooled with the samples from the five other plants. ELISAs were performed as described in Materials and Methods. Each experiment was repeated twice with similar results; however, the data presented were collected from one experiment.

Under the conditions of our experiments, vein clearing and, on occasion, mosaic symptoms were observed in TMV infected plants at 4 d.p.i. By 14 d.p.i. plants exhibited severe symptoms in most of the leaves including leaf crinkling, severe vein clearing, and mosaic symptoms. The levels of TMV in these tissues at 4 d.p.i. were substantially greater (>10-fold) than any of the chimeric viruses at 7 d.p.i. The hybrid virus T/OMP was not detected in the upper leaves of inoculated plants until 7 d.p.i. and there were no symptoms on these plants at this time. At 14 d.p.i. small chlorotic spots appeared on the upper leaves and mild mosaic symptoms were seen on two or three out of five plants in each experiment.

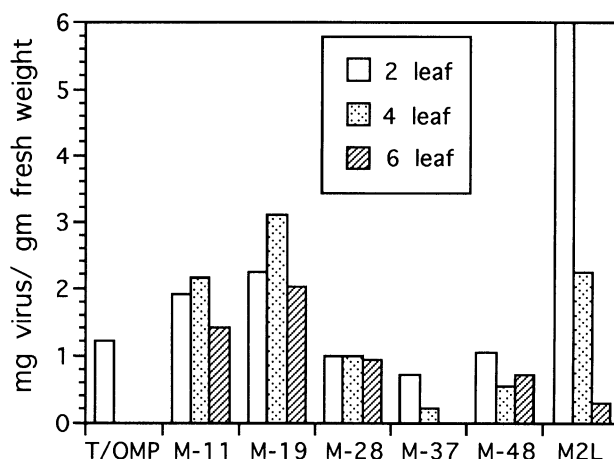


Fig. 2. Virus accumulation in upper leaves of *Nicotiana tabacum* cv. Xanthi nn at 10 days postinoculation. ELISAs were performed to quantitate the amounts of virus in upper leaves (second, fourth, or sixth leaf above the inoculated leaf) at various time points following inoculation. Three disks were taken from each leaf at 10 d.p.i. and pooled with samples taken from four other plants. ELISAs were performed as described in Materials and Methods. Tobacco mosaic virus (TMV) accumulation in similarly infected samples was 10-fold higher than those shown here at the earliest time point and so are not included. A standard curve using different amounts of purified TMV was included on each ELISA plate to quantitate the reactions. The values in the table represent mg of virus/gm fresh weight tissue.

T/OMP was only detected in the second leaf above the inoculated leaf until 14 d.p.i. when it was detected in the fourth leaf above the inoculated leaf (data not shown).

The M-11 mutant was detected in upper leaves by 7 d.p.i. and caused symptoms that were more severe than those caused by T/OMP. M-19 was detected in upper leaves by 7 d.p.i. and caused symptoms that were slightly more severe than those caused by either M-11 or T/OMP. M-28 moved more slowly than T/OMP and was not detected in systemic leaves until 10 d.p.i. The symptoms caused by this mutant were milder than M-11 and T/OMP. M-37 was detected at very low levels by 10 d.p.i. and only achieved high titers in the fourth leaf above the inoculated leaf (data not shown). The symptoms induced by this virus were very mild, causing only a small amount of vein clearing and, in some plants, small chlorotic spots by 14 d.p.i. Mutant M-48 was detected by 7 d.p.i. in leaf 4, but did not accumulate to high levels even by 14 d.p.i. and plants remained asymptomatic (data not shown). A comparison of the levels of virus accumulation in noninoculated leaves of plants infected with each of the hybrid viruses at 10 d.p.i. is shown in Figure 2.

To confirm that the mutations were maintained in the MP, virus was recovered from the noninoculated leaves and the viral RNA was subjected to direct sequence analysis. In each case, the mutations were maintained and no additional changes were found in the MP sequence.

Inoculation of T/OMP and mutant viruses on transgenic plants.

To determine whether the differences in systemic disease symptoms and virus accumulation caused by T/OMP and derived mutants were due to the MP, each virus was inoculated to transgenic Xanthi nn plants that express the TMV MP gene (plant line 277; Deom et al. 1987). The long-distance movement of T/OMP and each mutant on plant line 277 was evaluated by monitoring the appearance of disease symptoms and the systemic accumulation of virus. The accumulation of virus in noninoculated leaves of plants inoculated with hybrid virus was more rapid on the MP(+) transgenic plants than on nontransgenic plants, and was essentially the same for each virus (data not shown). The appearance of disease symptoms also occurred at a more rapid rate in transgenic plants, and the symptoms were more severe than on nontransgenic controls (data not shown). These results indicate that the differences in phloem-mediated long-distance spread of the hybrid viruses on nontransgenic plants are due to the expression and/or function of the movement protein rather than other characteristics of the different viruses.

Long-distance movement and symptom development of T/OMP and the five mutant viruses on *Vanilla planifolia*.

T/OMP, TMV, ORSV, and each of the mutant viruses were inoculated to the orchid, *Vanilla planifolia*. This species of orchid produces a new leaf every 1 to 2 weeks, in contrast to other orchids, which produce new leaves only once in several months. ORSV causes chlorotic spots on both inoculated and noninoculated leaves of *V. planifolia*; systemic infection results in shorter internode lengths by 3 months post-inoculation (Holt et al. 1995). ORSV was detected in non-inoculated leaves by 2 months postinoculation. Plants in-

fectured with TMV remained asymptomatic and, while virus was detected in inoculated leaves, none was detected in upper leaves during this period (data not shown). Plants infected with T/OMP also remained asymptomatic, but virus was recovered from noninoculated leaves by 2 months post-inoculation, although the amount of virus was small compared to infection by ORSV. None of the five mutant viruses moved beyond the inoculated leaf or caused symptoms on orchids during the 2 months of observation, although virus was detected in the inoculated leaf in each case (data not shown).

Determination of MP and CP levels in infected protoplasts.

Tobacco protoplasts isolated from cultured BY-2 cells were infected with RNA isolated from virions of TMV, T/OMP, M-48, and M-11. Infected protoplasts were labeled with ³⁵S Met/Cys for 1.5 h at 20 and 30 h postinoculation. Labeled proteins were separated by SDS-PAGE (Fig. 3) and the

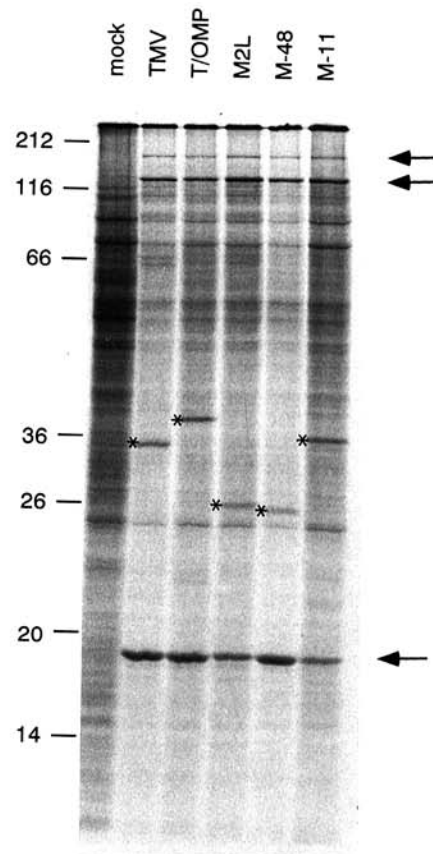


Fig. 3. Proteins labeled in protoplasts infected with tobacco mosaic virus (TMV), T/OMP, M-48, and M-11. Protoplasts were exposed to [³⁵S]-Met/Cys in the presence of actinomycin D (30 µg/ml) for 1.5 h at 20 h postinoculation prior to harvest and extraction of proteins. Proteins were separated by SDS-PAGE followed by autoradiography. Molecular weights of the standards are: 212 kDa, myosin; 158 kDa, MBP-β-galactosidase; 116 kDa, β-galactosidase; 97 kDa, phosphorylase B; 66 kDa, bovine serum albumin; 55 kDa, glutamic dehydrogenase; 42 kDa, maltose-binding protein; 36 kDa, lactate dehydrogenase M; 26 kDa, triosephosphate isomerase; 20 kDa, trypsin inhibitor; 14 kDa, lysozyme; 6.5 kDa, aprotinin. Arrows indicate the 183-kDa protein, 126-kDa protein, and the CP. Asterisks indicate the MP.

amount of each viral gene product was determined with a radioactive phosphoimaging system. The amount of CP and MP are expressed relative to the amount of the 126-kDa protein. The relative amounts of viral gene products at 30 h postinfection (data not shown) were similar to the amounts at 20 h postinfection (Table 2).

TMV, T/OMP, M-48, or M-11 each produced a MP of the predicted size in infected protoplasts (Fig. 3; indicated by *). The amount of MP produced by T/OMP compared with the amount of 126-kDa protein is not less than the amount produced by TMV; this result indicates that T/OMP probably does not produce smaller necrotic local lesions (Table 1) because the infection produces less MP. The amount of MP produced by each of the mutant viruses is not dramatically different, although relatively less MP was produced by M-48 than the other viruses (Table 2).

Because it was previously demonstrated that CP is essential for long-distance spread (Dawson et al. 1988), we considered the possibility that the viruses produced different amounts of CP. As shown by comparing the data presented in Table 2 and Figure 2, there was no direct correlation between the amount of CP produced during protoplast infection and systemic spread of the infection in tobacco plants. For example, the amount of CP (relative to the amount of 126-kDa protein) was not less for M-48 than for TMV, yet long-distance movement of M-48 was much less effective than the long-distance movement of TMV (Fig. 2). These results indicate, therefore, that the differences in rapid long-distance movement caused by T/OMP, TMV, and each of the five mutant chimeric viruses are apparently not due to differences in the production of CP.

DISCUSSION

The MP of ORSV was previously shown to play a role in determining the host range of this tobamovirus. Hilf and Dawson (1993) demonstrated that when the ORSV MP replaced the TMV MP in a hybrid virus, the resulting virus moved more slowly from cell to cell than did TMV and did not cause a "TMV-like" systemic infection. Likewise, replacement of the TMV CP with the ORSV CP rendered the hybrid virus unable to move systemically on tobacco plants. We confirmed that the ORSV MP limits spread of TMV and expanded the studies to show that a hybrid virus caused a systemic infection on orchids, the natural host for ORSV (Holt et al. 1995). The hybrid virus used by Holt et al. un-

derwent genetic changes that resulted in the selection of variants that caused systemic infection in tobacco plants but could not infect orchid plants. One of the variants, M₂L, resulted from deletion of 48 amino acids from the carboxy-terminus of the MP, implicating this region in determining the host range of ORSV.

In the current study, we analyzed the function of the ORSV MP by creating a series of frameshift mutations within the MP gene which resulted in premature translational termination. By examining both the cell-to-cell and long-distance movement of these viruses in tobacco and orchid plants, we identified a region of the ORSV MP between amino acids 267 and 278 (ORSV MP contains 278 amino acids) that is required for host-range specificity.

Because the chimeric viruses used in this study were less efficient in both local and long-distance spread than was TMV (Fig. 2, Table 1), they were inoculated to transgenic plants that express the TMV MP gene. All of the virus constructs tested in this study, T/OMP, M-11, M-19, M-28, M-37, and M-48 caused local lesions of equal size when inoculated onto transgenic MP(+) Xanthi NN plants, although the lesions were somewhat smaller than those induced by TMV infection on this plant line. Lesion size on Xanthi NN plants reflects the ability of the virus to move from cell to cell and by implication, the rate of virus replication. Therefore, we concluded that the differences in local spread of T/OMP, M-11, and M19 (Table 1) were due to an effect of the MP itself, rather than differences in the rate of replication of each virus.

The slow or reduced rate of cell-to-cell movement of the hybrid virus T/OMP is correlated with the inability of the virus to cause a successful systemic infection on tobacco plants. Infections by M-11 or M-19, which caused the formation of larger lesions than did T/OMP (Table 1), caused a higher accumulation of virus in noninoculated leaves in Xanthi nn than did T/OMP, M-28, M-37, or M-48 (Fig. 2).

One way in which MP may contribute to long-distance spread is by affecting the movement of virus into or out of cells in the stem during long-distance spread. Recent evidence from our laboratory has demonstrated that long-distance spread of a TMV mutant lacking a MP gene occurs only when stem tissue produces MP (P. Arce-Johnson, U. Reimann-Philipp, H. S. Padgett, R. Rivera-Bustamente, and R. N. Beachy, unpublished). Tissue print Western blot analysis of plants infected with T/OMP showed that the virus enters the vascular tissue in leaves during the same period of time as does TMV (Fenczik, Ph.D. thesis, Washington University), yet there is little or no systemic infection of T/OMP (Fig. 2). These results may indicate that for T/OMP to cause a rapid systemic infection it must, in addition to entering the phloem, enter other cells where virus replication occurs. The identity of such cells is discussed elsewhere (Arce-Johnson et al., manuscript in preparation).

The inability of T/OMP to cause rapid systemic infection in tobacco plants may result from suboptimal interactions between the MP and the coat protein, or the MP and a host component. Removal of the last 11 amino acids in mutant M-11 may then result in a better "fit" between the host factors and the MP allowing the protein to function more efficiently in tobacco but much less efficiently in orchids. Conversely, it is possible that the interaction is a negative one, and this region of the movement protein may interact with the product

Table 2. Relative amounts of movement protein (MP) and coat protein (CP) produced in tobacco protoplasts

Virus	MP/126-kDa ^b	CP/126-kDa ^b
TMV	0.60	1.66
T/OMP	0.91	1.84
M-48	0.42	1.87
M-11	0.61	0.63

^a Infected protoplasts were labeled with ³⁵S met/cys for 1.5 h at 20 h postinoculation. Proteins from 1.5 × 10⁴ protoplasts were subjected to SDS-PAGE. ³⁵S-Incorporation into proteins was quantified by the ImageQuant Software following phosphoimage analysis on a Molecular Dynamics Phosphorimager.

^b Results are expressed as a ratio of the amount of ³⁵S incorporated into MP or CP to the amount of ³⁵S in the 126-kDa protein.

of a host defense gene. The removal of the last 11 amino acids may result in an inability of the defense protein to interact with the ORSV MP, resulting in the virus escaping a host defense mechanism.

A comparison of the amino acid sequences of the known tobamovirus MPs revealed a high degree of identity in two areas in the amino-terminal portion of the MP, referred to as regions I and II (Saito et al. 1988). The carboxy-terminal region of the MP sequences, on the other hand, are highly variable and it was proposed that this region is involved in determining host specificity of the virus (Saito et al. 1988). The MP of a tobamovirus that infects shallots (a monocot) has 11 amino acid differences with the TMV MP (Kwon et al. 1994); eight of the changes are found within the last 80 amino acids of the carboxy-terminal end of the protein. Furthermore, mutations which result in changes of amino acids 238 and 244 of tomato mosaic tobamovirus (ToMV) enable ToMV to overcome resistance conferred by the locus *Tm-2²* (Weber et al. 1993). Studies described here demonstrate that sequences within the last 11 amino acids of the ORSV MP are involved in determining the host range of a TMV/ORSV hybrid virus. Amino acids in this region of the MP may directly interact with one or more host proteins or may be involved in protein conformation that allows an interaction between the MP and a host component.

MATERIALS AND METHODS

Plant material.

Transgenic *Nicotiana tabacum* cv. Xanthi nn plant line 277 (Deom et al. 1987) and Xanthi NN plant line 2005 (Deom et al. 1991) express the TMV MP gene under control of the cauliflower mosaic virus (CaMV) 35S promoter. Homozygous R₂ and R₃ progeny of transgenic plants lines were used in this study. Plants were grown in a growth room under artificial light (14 h light/10 h dark period) at 25 to 30°C.

Construction of TMV-ORSV hybrid viruses and mutations of the movement protein gene sequence.

T/OMP. The chimeric virus, *T/OMP*, was constructed by substituting the ORSV MP for the 3' half of the TMV MP in the infectious cDNA construct, U3/12ΔM-RV (Holt et al. 1995). Four primers were used to create the hybrid virus construct: F-OMPRV, 5'CCTAGGGATATCTTGTCTAC-AATGGCTCT3', which is complementary to the 5' end of the ORSV MP sequences (Isomura et al. 1990) and contains an *EcoRV* restriction site; T-end-Kpn, 5'ATAGGTACCTGGCCCCCTACCGGGGGTAAC3', which is complementary to nucleotides 6395 (the 3' end of TMV RNA) to 6375 (numbered according to Goelet et al. 1988) of the TMV genome flanked by a *KpnI* site; and a set of complementary primers, T/O, 5'CACAATCTGATTTCGTATTGAAT ATGTCTTACAGTATCACT3', which corresponds to the last 20 nucleotides of the ORSV MP and the first 18 nucleotides of the TMV CP and O/T, which is the complement to T/O.

Two PCR reactions were performed using the DNA complement of ΔM-OMP (Holt et al. 1995) which contains both the ORSV MP and the TMV CP genes, as template. PCR-mediated gene fusion was performed as outlined in Yon and Fried (1989). Two independent PCRs were performed with the same template DNA, the first used the forward primer T/O

and T-end-Kpn, and the second used the reverse primer O/T and F-OMPRV. The products of both PCR reactions were combined and gel purified to remove the primers (Gene Clean, Bio 101, La Jolla, Calif.). A second PCR was subsequently performed with the two outside primers (F-OMPRV and T-end-Kpn). The resultant PCR product contains the ORSV MP gene directly 5' of the TMV CP gene. This product was subcloned into Bluescript (Stratagene, La Jolla, Calif.) to create the intermediate plasmid BS/OMP-TCP. To create the full-length cDNA of the virus the genes for the ORSV MP and the TMV CP were released from Bluescript by digestion with *EcoRV* and *KpnI* and recloned into a full-length cloned cDNA of TMV RNA which was previously modified to contain an *EcoRV* site at the 3' end of the replicase gene (U3/12ΔM-RV; Holt et al. 1995). This step replaces the TMV MP and CP sequences of U3/12ΔM-RV with a DNA fragment containing the ORSV MP and TMV CP genes.

Truncated mutants of the ORSV MP.

Mutations of the ORSV MP were constructed using PCR-mediated gene fusion to insert one or two nucleotides within the 3' end of the ORSV MP gene. Forward and reverse primers were used to create each mutation based on the published sequence of the ORSV MP cistron (Isomura et al. 1990). The PCR was performed as described above, except that the set of internal complementary primers used was different. The sequences for the forward primers are as follows: F/M-11, 5'GATCATCATTAGTGAGTCATTTGA3'; F/M-19, 5'AA-TGAGTCTAGTGATGATTCGTA3' F/M-28, 5'GTGTTG-TATGATGATGATTGTGG3'; F/M-37, 5'ATGAAAA TGGCTGATAGTGATG3'; and F/M-48, 5'ATCTAGAC-TTTAATAGTCGTAAT3'. The nucleotides in bold type were introduced into the sequence of the ORSV MP in order to create frameshift mutations that would result in multiple translational termination codons and cause the synthesis of truncated MPs. BS/OMP-TCP was used as the template for the PCR reactions. The resultant PCR products were cloned into pBluescript KS+ (Stratagene) and were sequenced. Sequencing was by the dideoxy chain termination method (Sanger 1977) using the Sequenase Version 2.0 DNA Sequencing Kit (U.S. Biochemical, Cleveland, Ohio). Cloned cDNAs containing the predicted mutations were cut with *EcoRV* and *KpnI*, gel purified, and ligated with U3/12ΔM-RV previously restricted with *EcoRV* and *KpnI*. The resultant chimeric viruses containing mutant MP sequences are further described in Figure 1.

In vitro transcription, inoculation, and isolation of virus.

Plasmids containing *T/OMP* and derivatives fused with the T7 promoter sequence were linearized with *KpnI*, and transcribed with T7 RNA polymerase, as described by Holt and Beachy (1991). After incubation, 20 mM sodium phosphate buffer, pH 7.0, was added and the mixture was inoculated with Carborundum to tobacco plants 5 to 6 weeks after seeding; these plants had five to six true leaves. *N. tabacum* cv. Xanthi NN, Xanthi nn, and the transgenic plant lines described above were used as hosts. Immediately after inoculation, plants were rinsed with water and maintained in a growth room under artificial light at 25 to 30°C. Clones whose infectious RNAs caused equivalent numbers of lesions

on Xanthi NN and plant line 2005 were chosen for further study. Plants were observed daily for disease symptoms. Virus accumulation was monitored by ELISA at 4, 7, 10, and 14 d.p.i.

Virus was isolated at 5 d.p.i. from leaves inoculated with infectious RNAs as follows. One to two grams of leaf tissue was ground in 1 volume (1 ml/g fresh weight) 0.5 M phosphate buffer pH 7.0, 14.3 mM β -mercaptoethanol. Two volumes of water saturated chloroform/butanol (50:50) were added and mixed. Samples were centrifuged for 15 min in a clinical tabletop centrifuge at top speed. The aqueous phase was transferred to microfuge tubes and virus particles were precipitated with 4% PEG-8000 for 10 min on ice. Virus was collected by centrifugation in a microfuge for 10 min. Pellets were resuspended in 10 mM phosphate buffer, pH 7.0, and centrifuged again to remove insoluble material. Virus was again precipitated with 4% PEG (w/v) and 1% NaCl (w/v) and collected by centrifugation. Viral RNA was isolated (Bruening et al. 1976) and sequenced using reverse transcriptase from avian myeloblastosis virus (Seigagaku, Japan; Padgett, and Beachy 1993).

ELISA. Ninety-six well Maxisorb (Nunc-Immunoplate) ELISA plates were coated overnight at 4°C with rabbit anti-TMV antibodies in 0.05 M carbonate buffer, pH 9.6. Non-specific binding was reduced by treating plates with blocking buffer (1% [w/v] BSA in 50 mM Tris, 0.15 M NaCl pH 8.0 [TBS]) for 1 h at room temperature. Plant samples were ground in ELISA buffer (phosphate-buffered saline [0.01 M KH_2PO_4 , 0.1 M Na_2HPO_4 , 1.37 M NaCl, 0.027 M KCl, pH 7.0; PBS], 0.05% Tween-20 [w/v], 0.2% ovalbumin [w/v]), diluted, and incubated overnight at 4°C in the blocked ELISA plates. The plates were washed with TBS with 0.05% Tween-20, five times. The plates were then incubated with mouse anti-TMV antibody at 37°C for 1 h. Bound antibody was detected with goat anti-mouse antibodies conjugated to horseradish peroxidase (HRP). HRP was detected colorimetrically using *o*-phenyldiamine as a substrate (Harlow and Lane 1988). The A490 was determined using a BioRad multiplate reader.

Protoplast isolation, infection, and labeling in vivo.

BY-2 suspension culture cells were maintained and protoplasts were isolated essentially as described by Watanabe et al. (1982). Two $\times 10^6$ protoplasts were inoculated by electroporation with 4 μg of purified viral RNA as described by Watanabe et al. (1987) and cultured in 35-mm petri dishes at 27°C in the presence of 30 $\mu\text{g}/\text{ml}$ Actinomycin-D. Protoplasts ($2 \times 10^5/\text{ml}$) were pulse labeled with 5 $\mu\text{Ci}/\text{ml}$ [^{35}S] met plus cys (NEN, 1175 Ci/mmol) for 1.5 h before harvest. Cells were collected by gentle centrifugation and lysed in Laemmli sample buffer (Laemmli 1970). Samples containing the equivalent of 1.5×10^4 protoplasts were subjected to electrophoresis in gels of 12.5% polyacrylamide containing SDS. Gels were exposed overnight on a Molecular Dynamics phosphorimage screen and developed on the phosphorimager. For quantification of ^{35}S in specific proteins, the Molecular Dynamics ImageQuant software was used.

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