Requirement of the Movement Protein for Long Distance Spread of Tobacco Mosaic Virus in Grafted Plants

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Systemic spread of tobacco mosaic virus (TMV) that lacks a functional movement protein (TMV Δ MP) was investigated in grafted tobacco (*Nicotiana tabacum*) plants. Transgenic plants that express the 30-kDa movement protein (MP) gene (*MP*) under the control of the *rolC* (phloem-specific) or *pal2* (xylem-specific) promoters were unable to support systemic infection by the mutant virus, while plants that express the *MP* gene from the cauliflower mosaic virus 35S promoter (35S:*MP*) led to systemic infection. Doubly grafted plants were constructed in which plants containing the 35S:*MP* gene were used as root stock and plants carrying various *MP* constructs constituted the middle scion. The upper scion contained the 35S:*MP* gene in plants that produce a hypersensitive response when systemically infected by TMV. TMV Δ MP moved systemically and produced complete necrosis in the upper scion when expression of *MP* in the middle scion was under the control of the *rolC* or 35S promoter, but not when the *pal2* promoter was used. When plants expressing a gene encoding a defective MP were used as the middle scion, there was no systemic infection by TMV Δ MP, and a delay in systemic infection by wild-type TMV. In grafted plants with middle scions that expressed the TMV 54 kDa gene sequence there was no apparent systemic infection by TMV Δ MP in the upper scion. The results obtained indicate that the MP has a role in long distance movement, and support the suggestion that replication is necessary for systemic infection of these grafted plants.

its ability to move both locally and long distance. The relatively slow, local movement that occurs on the order of micrometers per hour (Rappaport and Wildman 1957) involves cell-to-cell movement through plasmodesmata; long-distance spread involves relatively rapid movement that occurs at centimeters per hour (Helms and Wardlaw 1978). In long-distance spread, virus apparently moves throughout the plant via the vascular tissue.

Two proteins have been identified as important for movement of tobacco mosaic tobamovirus (TMV). The 30-kDa movement protein (MP) is dispensable for virus replication but is essential for cell-to-cell spread of the infection (Deom et al. 1987; Meshi et al. 1987); the coat protein (CP) is dispensable for cell-to-cell movement but is essential for virion assembly and rapid, long-distance movement (Hull 1989; Maule 1991; Saito et al. 1990). However, this requirement is not absolute and may vary from host to host. For example, mutants of TMV that lack CP spread systemically in *Nicotiana sylvestris*, but are incapable of infecting other *Nicotiana* spp. (Hilf and Dawson 1993). Other viruses, such as barley stripe mosaic virus and bipartite geminiviruses, appear not to require the CP for long-distance movement, although it has been demonstrated that geminiviruses require the products of BL1 and BR1 for this process (Brough et al. 1988; Gardiner et al. 1988; von Arnim and Stanley 1992).

Many viruses, especially those restricted to phloem, such as some geminiviruses or luteoviruses, display limited cell-to-cell movement but efficient long-distance movement. It is also possible that some types of host resistance to systemic infection involve the blockage of viral transport into the vascular tissue (Goodrick et al. 1991; Sanger et al. 1994). Most viruses, on the other hand, rely on both cell-to-cell and rapid, long-distance movement during systemic infection.

Recent evidence implicates the MP of some viruses in long-distance movement. Geisman-Cookmeyer and Lommel (1993) created two mutants in the MP of red clover necrotic mosaic dianthovirus that enable the virus to move from cell to cell in inoculated leaves but leave the virus unable to move systemically. Lehto and Dawson (1990) described a mutant TMV in which a delay in production of MP caused a delay in long-distance movement. Here we report our studies of the role of

The ability of a plant virus to cause a systemic infection depends on its capacity to replicate in initially infected cells and

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the TMV MP in long-distance movement; these studies made use of transgenic tobacco plants that express the *MP* gene under the transcriptional control of constitutive or vascular tissue-specific promoters. For these studies, we evaluated the ability of a TMV mutant that lacks a functional MP to cause systemic infection in grafted plants. These studies demonstrated that, under the conditions of this experiment, systemic spread of TMV requires MP in or near the vascular tissues.

RESULTS

Characterization of plant lines.

Transgenic tobacco plants were produced that express the TMV *MP* or *uidA* gene under the control of the following promoters (summarized in Table 1): the cauliflower mosaic virus 35S promoter; the *rolC* promoter from the T-DNA of the Ri plasmid from *Agrobacterium rhizogenes* (Oono et al. 1987); or the promoter derived from the *pal2* gene from *Phaseolus vulgaris* (Cramer et al. 1989). Transverse sections of stems from plants transgenic for the 35S:*uidA* gene showed, as expected, β -glucuronidase (GUS) activity in all tissues, with greater activity in phloem-associated cells than in other cells. Plants that contain the *pal2:uidA* gene exhibited GUS activity preferentially in primary xylem, while GUS activity in plants that contain the *rolC:uidA* gene was restricted to phloem tissues and the tips of leaf hairs (data not shown). The pattern of accumulation of MP as detected by tissue print immunoblot analyses was consistent with the pattern of GUS expression. Transgenic plants that contain either the *pal2:MP* gene or *rolC:MP* gene accumulated MP in primary xylem, and primary phloem and phloem-associated cells, respectively, while the 35S:*MP* gene led to MP accumulation in all tissues (Fig. 1).

Transgenic *Nicotiana tabacum* lines that contained the fragment of the polymerase gene encoding the 54-kDa protein of TMV (similar to those described by Golemboski et al. (1990)) were produced as described in Materials and Methods. Presence of the 35S:*54 kDa* gene was confirmed by Southern blot hybridization reactions (data not shown). Analysis of resistance in four lines of *N. tabacum* Xanthi NN that contain the gene was carried out by inoculating plants with TMV. One of the plant lines, 54505NN-4, displayed strong resistance to infection. No local lesions were observed on line 54505NN-4 inoculated with TMV at a concentration of 2 μ g/ml, and only a few lesions were produced when 10 to 100 μ g of TMV per ml was used.

Table 1. Description of the transgenic tobacco (*Nicotiana tabacum*) plant lines used

Plant line	Xanthi cultivar	Promoter	Coding sequence
RoGN-nn	nn	<i>rolC</i>	<i>Gus A</i>
RMN-Sx-3/2	nn	<i>rolC</i>	TMV- <i>MP</i>
RMN-NN-12	NN	<i>rolC</i>	TMV- <i>MP</i>
PGN-nn	nn	<i>pal2</i>	<i>Gus A</i>
PMN-Sx-7/2	nn	<i>pal2</i>	TMV- <i>MP</i>
PMN-NN-9	NN	<i>pal2</i>	TMV- <i>MP</i>
306	nn	CaMV 35S	None
G 6-1	nn	CaMV 35S	<i>Gus A</i>
277	nn	CaMV 35S	TMV- <i>MP</i>
2005	NN	CaMV 35S	TMV- <i>MP</i>
3 Δ 5-Sx-11	nn	CaMV 35S	TMV- <i>dMP</i>
3 Δ 5-NN-7B	NN	CaMV 35S	TMV- <i>dMP</i>
54505NN-4	NN	CaMV 35S	TMV- <i>54 kDa</i>

Assays for complementation of TMV Δ MP in transgenic plants.

All transgenic and nontransgenic plant lines used for this study were evaluated for the capacity to facilitate the local or systemic spread of TMV Δ MP, a TMV mutant lacking a functional MP (Holt and Beachy 1991). Local movement was evaluated on transgenic lines of Xanthi NN by the presence or absence of local lesions, while in Xanthi nn plants systemic infection and virus accumulation were evaluated. Only plants that express the *MP* under the control of the 35S promoter were able to support local or systemic virus infection following inoculation with infectious transcripts of the TMV Δ MP (Table 2). Plant lines that express a gene encoding a defective MP (*dMP*) (Lapidot et al. 1993) did not complement TMV Δ MP.

Plant lines that were nontransgenic or contained the 35S:*MP* gene were susceptible to infection and systemic spread by wild-type TMV. However, plant lines that expressed the 35S:*dMP* gene showed reduced local and systemic infection by TMV, as reported by Lapidot et al. (1993). Plant line 54505NN-4, which contains the 35S:*54 kDa* gene, was resistant to infection by TMV (Table 2) and no local lesions were produced.

Systemic spread of TMV Δ MP in singly grafted plants.

To determine the role of the MP in long-distance spread of TMV, we inoculated TMV Δ MP to grafted plants. In these studies, the root stocks were obtained from plant line 277 and are capable of supporting local and systemic spread of TMV Δ MP (Table 2). A slash-type approach graft was used to attach scion tissues of various Xanthi NN lines, as described in Table 1. Systemic infection by TMV Δ MP was considered to be successful when a strong, necrotic reaction was observed in the scion.

At 14 days after infection with TMV Δ MP, necrosis was observed only in plants that expressed the 35S:*MP* gene (plant line 2005; Table 2) in the scion (Fig. 2A). The relatively long time required for systemic infection suggests that transport of virus across the union was inefficient. Longitudinal sections through the scion stem revealed necrotic tissues (Fig. 3C) prior to complete necrosis of the tissues (Fig. 3D). Scions that did not develop external necrosis, as in the case of tissues that had no transgenes, or contained the *pal2:MP* gene or the *rolC:MP* gene, did not develop internal necrosis. Tissues in the root stock were free of necrosis. These studies demonstrated that TMV Δ MP was unable to cause infection of the scion in grafted plants unless the *MP* was expressed from the 35S promoter.

To confirm that the virus that caused apical necrosis had not gained a functional MP, apical tissues were collected and homogenized, and the extract was applied to Xanthi NN tobacco plants. The infectivity recovered from these tissues produced local lesions on plant lines that contained the 35S:*MP* gene, but not on nontransgenic control plants. It was therefore concluded that the virus recovered from the apex was similar or identical to the virus inoculated to the root stock.

Spread of TMV Δ MP through doubly grafted plants.

To determine whether or not MP was required for the passage of TMV Δ MP through the stem, doubly grafted plants were developed. In these studies, the root stock was transgenic Xanthi nn harboring the 35S:*MP* gene (plant line 277). In all

plants the apical scion was transgenic Xanthi NN harboring the 35S:MP gene (plant line 2005), and was separated from the root stock by a middle scion of different Xanthi nn and Xanthi NN plant lines. After the double graft plants were established, one or two leaves on the rootstock were inoculated and the middle and upper scions were observed for necrosis and virus accumulation.

Apical necrosis following inoculation with TMVΔMP occurred within 23 days when the middle scion was derived from plant line 277 (Fig. 2B), and 30 days when the line 2005 was the middle scion (Fig. 4A). This is the same length of time required for wild-type TMV to cause systemic necrosis on similar grafted plants (Fig. 4B). Although systemic movement is slower than TMV infection in nongrafted plants, when the middle scion was derived from transgenic plants containing the rolC:MP gene, apical necrosis occurred within 23 days after inoculation with TMVΔMP, the same time interval as

when the middle scions were nn plants that contained the 35S:MP gene (Fig. 2B). There was no apical necrosis when the middle scion contained the pal2:MP gene or the empty vector (Fig. 2B). These results indicated that apical necrosis caused by TMVΔMP infection occurs only when the MP accumulates in phloem and phloem-associated cells.

To determine whether or not there was internal necrosis in the middle scion, a number of Xanthi NN stems were cut longitudinally and examined at 14 and 21 days after inoculation of the root stock; we considered that necrosis would indicate virus replication. However, no necrosis was observed. We therefore concluded that either no replication had occurred in this tissue, or that if replication occurred, it did not induce a necrotic reaction.

To examine in greater detail whether or not virus replication was required for long-distance spread of TMVΔMP, we used grafted plants in which the middle scion was resistant or sus-

Fig. 1. Tissue print immunoblot detection of the 30-kDa movement protein (MP) in cross sections of stem of transgenic plants expressing the (A) 35S-MP, (B) rolC-MP, and (C) pal2-MP gene constructs, and (D) nontransgenic plant. The MP was detected with a polyclonal antiserum against a synthetic peptide of the MP (Deom et al. 1987).

Table 2. Capacity of tobacco mosaic virus (TMV) and TMV lacking the 30-kDa movement protein (TMVΔMP) to cause local or systemic infection of the transgenic plant lines^a

Plant line	Gene construct	Host genotype	TMV	TMVΔMP
306 (Sx)	...	nn	+	–
RMN-Sx-3/2	rolC:MP	nn	+	–
RMN-NN-12	rolC:MP	NN	+	–
PMN-Sx-7/2	pal2:MP	nn	+	–
PMN-NN-9	pal2:MP	NN	+	–
277	35S:MP	nn	+	+
2005	35S:MP	NN	+	+
3Δ5-Sx-11	35S:dMP	nn	+	–
3Δ5-NN-7B	35S:dMP	NN	+	–
54505NN-4	35S:54 kDa	NN	–	–

^a Six-week-old plants were inoculated with 100 μl of TMV or RNA transcripts of TMVΔMP per leaf. + = development of systemic mosaic in Xanthi nn plants or local lesions in Xanthi NN plants. – = no symptoms developed.

ceptible to infection. For this study, doubly grafted plants consisting of root stocks of plant line 277, various Xanthi nn and NN lines as middle scion, and plant line 2005 (Xanthi NN with the 35S:MP gene) as upper scion were constructed. In addition to middle scion tissues that contained the vector only (no MP; Fig. 4A) and lines with the 35S:MP gene, lines that contained either the 35S:dMP gene or the 35S:54 kDa gene were used. The dMP (defective MP, due to deletion of amino acids 3, 4, 5; Lapidot et al. 1993) reduces the local and long-distance spread of TMV (Lapidot et al. 1993). The 35S:54 kDa gene is similar to that reported by Carr and Zaitlin (1991) and confers a high level of resistance to TMV.

Following inoculation of TMVΔMP to the rootstock, apical necrosis occurred only on those plants that contained a middle scion from plants that contain the 35S:MP gene (Fig. 4A). There was no apical necrosis in plants in which the middle scion contained the 35S:dMP gene or the 35S:54 kDa gene in either the Xanthi nn or Xanthi NN genotypes. The lack of systemic infection in these grafted plants further supports the conclusion that movement of TMVΔMP requires functional MP in the middle scion.

NN No MP –	NN pal2-MP –	NN rolC-MP –	NN 35S-MP +
nn 35S-MP			

A

NN 35S-MP			
–	–	+ _{23 d}	+ _{23 d}
nn No MP	nn pal2-MP	nn rolC-MP	nn 35S-MP
nn 35S-MP			

B

Fig. 2. Development of apical necrosis in grafted plants inoculated with tobacco mosaic virus that lacks a functional movement protein (TMVΔMP). Apical necrosis indicated by (+) and absence of necrosis by (–). Five-week-old movement protein (MP) “+” plants were used for the (A) single or (B) double grafts. All grafted plants were inoculated on two leaves of the rootstock with 100 μl per leaf of TMVΔMP virions. Number of days after inoculation at which a significant amount of necrosis was observed is indicated next to (+).

To further address the question of whether or not virus replication occurs during systemic infection in the grafted plants, doubly grafted plants were constructed with root stocks of nontransgenic Xanthi nn and middle and upper scion plants, as above. Following infection with wild-type TMV, apical necro-

sis occurred in approximately the same period of time when the middle scion was transgenic Xanthi nn carrying the empty vector or the 35S:*MP* gene (Fig. 4B). Apical necrosis was delayed when Xanthi NN was used as middle scion, and when the middle scion contained the 35S:*dMP* gene there was fur-

Fig. 3. Appearance of symptoms in grafted plants inoculated with tobacco mosaic virus that lacks a functional movement protein (TMVΔMP). **A**, Non-inoculated control graft showing the junction between Xanthi NN apical and Xanthi nn basal segments. **B**, Mosaic and systemic chlorosis in a scion of the genotype Xanthi nn (line 277). **C**, Necrosis in a longitudinal section of a scion of genotype Xanthi NN (line 2005). **D**, Apical necrosis in a scion of Xanthi NN (line 2005) and chlorosis in the rootstock genotype Xanthi nn (line 277).

ther delay in systemic necrosis (Fig. 4B). Since all plants containing the dMP eventually showed necrosis, it was concluded that the dMP did not provide sufficient levels of resistance to prevent spread from cells in the scion in which replication occurred.

When the middle scion was line 54505 NN-4 (35S:54 *kDa* gene in Xanthi NN; see Table 1), there was no systemic necrosis in any of the plants. However, upon analysis of longitudinal sections, we observed a limited number of necrotic areas in the inner tissues of the middle scions that contained the 35S:54 *kDa* gene. Necrosis was limited to small areas and was insufficient to cause collapse or visible external necrosis on the scion tissues (not shown). These results suggest that virus replication occurs during passage of TMV through the middle scion of these grafted plants and that, in the absence of sufficient amounts of replication, systemic infection does not occur.

DISCUSSION

The long-distance movement of virus through the vascular system is essential for the rapid and efficient establishment of

systemic infection by TMV. Previous studies with TMV have identified the CP as necessary for rapid systemic movement in most species studied (Saito et al. 1989, 1990; Takamatsu et al. 1987). It has been suggested that slow, systemic spread of a TMV mutant that does not produce CP results from cell-to-cell spread through the stem (Dawson et al. 1988). We have examined the role of the TMV MP in long-distance movement of TMV and a TMV mutant that lacks an MP (TMVΔMP) with grafted plants that express the *MP* gene in specific types of tissues. In preliminary studies, we determined that expression of the *MP* gene in primary xylem (with the *pal2:MP* gene) or in phloem (*rolC:MP*) did not enable TMVΔMP to establish a systemic infection (Table 2). In contrast, plants that express the 35S:*MP* gene enable TMVΔMP to move systemically and develop mosaic symptoms, or cause local lesions characteristic of TMV infection (Table 2; also Holt and Beachy 1991)

Since TMVΔMP lacks the capacity for cell-to-cell movement, the absence of systemic symptoms in plants that carry the *rolC:MP* and *pal2:MP* genes (Table 2) could reflect the inability of this mutant to reach vascular tissues from the cells

Fig. 4. Development of necrosis in grafted plants inoculated with tobacco mosaic virus (TMV) that lacks a functional movement protein (TMVΔMP) or TMV. Tobacco plants that express genes encoding the *dMP* or 54 *kDa* open reading frame were inoculated with (A) 100 μl per leaf of TMVΔMP virions or (B) 100 μl per leaf of TMV (1 μg/ml). Number of days after inoculation at which necrosis was observed is indicated next to (+).

that are infected following inoculation of the leaves. To assure infection in all cells, TMVΔMP was inoculated to transgenic plants that contained the 35S:MP gene and that served as root stocks of grafted plants with scion tissues that express the different MP genes. Consistent with the results shown in Table 2, TMVΔMP caused systemic infection (i.e., necrosis) only when the scion contained the 35S:MP gene (Fig. 2A); we concluded from these studies that expression of MP only in xylem or phloem tissues was insufficient to facilitate systemic spread and/or sufficient level of replication of mutant virus to induce necrosis. In doubly grafted plants (Fig. 2B), expression of the MP gene from the rolC or 35S promoter in the middle scion, but not from the pal promoter, allowed TMVΔMP to systemically infect these plants (Fig. 2B). These results led us to conclude that expression of MP in phloem is required for spread of TMVΔMP in the stem. The fact that the mutant virus is unable to cause apical necrosis in singly grafted plants in which the scion contained the rolC:MP gene (Fig. 2A) suggests that necrosis requires that the virus replicate not only in cells in which the rolC:MP gene is expressed, but also in additional cells.

Based on the determined rate of translocation of photoassimilates (43 to 92 cm/h), and that of long-distance spread of TMV (0.11 to 23.3 cm/h), it has been proposed that virus moves long distances in the phloem together with photoassimilates (Helms and Wardlaw 1978; Schneider 1965). Such a rate of translocation makes it unlikely that TMV replication is required for long-distance spread in nonresistant, nongrafted plants.

To determine whether or not systemic infection requires replication of wild-type TMV in grafted plants, we carried out studies with plant lines that contain genes encoding the dMP (Lapidot et al. 1993) and 54 kDa genes of TMV (Golemboski et al. 1990), each of which confers a degree of resistance to TMV. Doubly grafted plants that included as middle scion tissue from plants expressing the dMP gene showed a delay in apical necrosis after inoculation with TMV (Fig. 4B). We conclude that this reflects a requirement that the infection move into and out of cells in the stem in which replication takes place. Similarly, the inability of TMV to move systemically in grafted plants that express the 54 kDa gene in the middle scion (Fig. 4B) supports the conclusion that replication is required for long-distance spread of TMV. Carr and Zaitlin (1991) demonstrated that TMV cannot replicate efficiently in cells that express the 54 kDa gene. Furthermore, systemic necrosis occurred more slowly when the middle scion was the “NN” resistant genotype rather than the “nn” susceptible genotype. These results support the suggestion that replication is required for movement of infection through the middle scion since NN plant lines would be less likely to become infected than would the nn lines.

In our study, long-distance spread of infection is substantially slower in the grafted plants than in nongrafted plants. It is possible that physical or physiological barriers that may occur in grafted plants restrict *trans*-union transport of virus and necessitate that virus replication take place in order to achieve sufficient levels for infection to reach the scion. An alternative possibility is that in these grafted plants infection occurs by cell-to-cell spread. Virus could move by this route through the graft union region, unloading from the sieve elements to the associated companion cells and then re-entering the sieve

elements in the scion. We consider this unlikely as we did not observe significant necrosis that would reflect infection in the Xanthi NN scions at the graft union.

Our data contrast with those recently reported by Gera et al. (1995), who carried out similar types of studies with doubly grafted plants and a mutant of TMV that lacks a functional MP gene. In contrast to our results, these workers, who used transgenic and nontransgenic tobacco plants with nn genotype, reported that mutant virus moved through the middle scion to cause systemic disease symptoms in nearly the same time as did wild-type TMV. They therefore concluded that virus replication was not required for phloem-mediated, long-distance spread of mutant virus. Although certain experimental details were not described by Gera et al. (1995), apparent differences between their studies and ours include plant genotype, length of time between completion of the graft and inoculation with virus, the conditions under which plants were held following inoculation, and the nature of the grafting procedure itself. It remains to be determined which of these or other differences account for the contrasting results of these two independent sets of studies.

MATERIALS AND METHODS

Plant material.

Tobacco (*Nicotiana tabacum*) plants used are described in Table 1. Transgenic Xanthi NN and Xanthi nn plants that express the MP sequence under the control of the cauliflower mosaic virus 35S promoter (Deom et al. 1987; 1991), transformed with the plasmid only, or the uidA coding sequence under the control of the 35S promoter (Reimann-Philipp and Beachy 1993) were used. Plants that express the dMP and the MP or uidA genes under the control of the pal2 promoter were included in this study (Arce-Johnson et al. 1995). Plants expressing the MP or uidA open reading frame (ORF) controlled by the rolC promoter, or expressing the 54 kDa ORF from TMV, are described below.

Construction of the rolC:MP gene.

For the construction of plasmid pMON 505-RMN, the TMV MP coding sequence was excised from plasmid B12 by digestion with *EcoRI*/*Bam*HI, and blunt-end ligated into the *EcoRI* site of pMON 316 (Rogers et al. 1987) to give pMON 316MP. Plasmid B12 was constructed by ligating the *EcoRI*/*Bam*HI insert from the plasmid pTM934 (Oliver et al. 1986) into Bluescript KS(+). The 1,289-bp *SmaI*/*Bam*HI insert taken from pMON 316MP contained the TMV MP coding sequence, and the nopaline synthase (nos) gene termination sequence was ligated into the *SmaI*/*Bam*HI sites of pUC18, resulting in plasmid pUC18 MN. The *Agrobacterium rhizogenes* rolC promoter sequence was contained in an 870-bp *HpaI*/*Hind*III fragment of pBR ORF12 (Oono et al. 1987; Sugaya et al. 1989). The fragment was blunt-end ligated into the *SmaI* site of pUC18, resulting in the plasmid pUC18 rolC, then excised from pUC18 rolC as an *EcoRI*/*Bam*HI fragment and blunt-end ligated into the *SmaI* site of pUC18 MN to give the plasmid pUC 18 PMN. The correct orientation of the rolC promoter sequence was confirmed by sequencing. The chimeric PMN gene containing the rolC promoter, the TMV MP coding sequence, and the nos termination sequence were then isolated by partial digestion with *Hind*III and complete digestion with

EcoRI and inserted into the *EcoRI/HindIII* sites of the binary plant transformation vector pMON 505 (Rogers et al. 1987) resulting in plasmid pMON 505-RMN.

The *Escherichia coli uidA* gene was derived from the plasmid pRAJ 260 (Jefferson et al. 1987). The *rolC:GUS* gene was constructed in the plasmid pGN100. This plasmid contains the *uidA* sequence followed by the *nos* termination sequence and an upstream *SmaI* site in pUC 19. The *rolC* promoter sequence was obtained as described above and ligated into the *SmaI* site, resulting in plasmid pUC 19 PGN. The chimeric gene was excised as an *EcoRI/HindIII* fragment and inserted into the *EcoRI/HindIII* sites of pMON 505 to generate pMON 505-RGN.

Cloning the 54-kDa sequence from TMV RNA.

The 54 kDa ORF of TMV was excised from the TMV cDNA clone pU3/12ΔM-RV (Nejdat et al. 1991) by digestion with *HaeII* and *EcoRV* followed by making blunt the *HaeII* 3' overhangs with T4 DNA polymerase and deoxynucleoside triphosphates (dNTPs). The appropriate fragment was recovered and ligated into the *StuI* site of an intermediate cloning vector that contains the 35S promoter, a polylinker, and the E9 3' end that had been treated with calf intestinal alkaline phosphatase (CIAP) following restriction. The resulting plasmid, pB4.23, was digested with *NotI* to release the insert and treated with the Klenow fragment of DNA polymerase plus dNTPs to fill in the 5' overhangs. The insert was then ligated into pMON 505 (Horsch and Klee 1986) that had been digested with *EcoRV* and treated with CIAP. The resulting construct, pB46.24, was introduced into *A. tumefaciens* GV311SE (Fraley et al. 1985) by triparental mating with pRK2013 (Figurski and Helinski 1979) as helper.

Plant transformation.

Transformation of *N. tabacum* cvs. Xanthi NN and Xanthi nn by the leaf disk procedure (Horsch et al. 1985) with pMON 505-RMN and pMON 505-RGN was carried out as described by Reimann-Philipp and Beachy (1993). Plants derived from tissue culture were verified with regard to tissue-specific accumulation of MP, or GUS activity; positive plants were self pollinated and R₂ progeny were used for all experiments.

The transgenic line 54505NN-4 that expresses the 54 kDa ORF was generated from *N. tabacum* cv. Xanthi NN via *A. tumefaciens* that harbors pB46.24. The resulting plants were like those reported by Golemboski et al. (1990) except that our plants were cv. Xanthi NN. Also, whereas the 54 kDa ORF described by Golemboski et al. contained a 5-amino-acid, C-terminal extension derived from m13MP18, the ORF used here was designed to terminate at the native stop codon of the replicase gene.

Plants derived from tissue culture were self pollinated and progeny from four lines were challenged with TMV (1 µg/ml). The number of local lesions produced on three of the lines was approximately the same as on control plants. Plants from line 54505NN-4 produced either small lesions or no lesions at all. One of the 54505NN-4 plants with no local lesions was allowed to self pollinate. Progeny of this plant were challenged with TMV, and one of the plants that showed no local lesions was self pollinated and harvested for seed. All experiments were performed with plants derived from this seed.

Tissue printing for MP.

Four- to six-week-old tobacco plants were analyzed by tissue print immunoblot reactions as described by Cassab and Varner (1987). Immunostaining was carried out with a polyclonal antiserum against a synthetic peptide representing amino acids 205 to 223 of the MP (Deom et al. 1987) as first antibody and anti-rabbit/alkaline phosphatase conjugate (Promega, Madison, WI) as second antiserum.

GUS assays in plant tissues.

Whole seedlings, stems, and young leaves were vacuum infiltrated with a solution containing 0.5 mg of X-Gluc per ml in 50 mM sodium phosphate buffer, pH 7.0, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, and 0.5% (vol/vol) Triton X-100 (Jefferson et al. 1987), and incubated overnight at 37°C. The samples were then transferred to 70% ethanol for destaining and storage.

Grafted plants.

Chisel-type approach grafts were carried out on 5-week-old tobacco plants approximately 41 cm in height with four pairs of extended leaves, at the time of carrying out the first graft. For double grafts, the second of which was carried out 3 weeks after the first, the middle scion contained a single auxiliary shoot. Apical scions were approximately 10 cm in length and included two to three leaves. The junctions between graft and receptor were sealed with Parafilm. Plants were kept in a transparent chamber with 100% relative humidity and external light for 4 days or until the graft wound healed. Plants were then transferred to a growth room (22 to 26°C; 330 µE · s⁻¹ · m⁻²; 14-h days; 60% relative humidity, approximately).

In vitro transcription and plant inoculation.

Full-length cDNA clones of TMV U₁ and TMVΔMP constructed downstream of the bacterial T7 promoter (Holt and Beachy 1991) were transcribed in vitro, and the transcripts were inoculated onto plants. TMVΔMP lacks the first two-thirds of the MP sequence, and retains the viral origin of assembly and the CP subgenomic promoter. In the experiments in which virions were used as inoculum, TMVΔMP was amplified in transgenic plants that express the MP gene (line 277), from which virus was subsequently isolated. Grafted plants were inoculated 8 days after completing the final graft.

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