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The Amount of Movement Protein Produced in Transgenic Plants Influences the Establishment, Local Movement, and Systemic Spread of Infection by Movement Protein-Deficient Tobacco Mosaic Virus

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The movement protein (MP) of tobacco mosaic virus (TMV) is required for the virus to spread from cell to cell in *Nicotiana tabacum*. To explore the role of the MP in the initiation of infection and in local and systemic spread of virus, transgenic plants that accumulate varying levels of MP, and that accumulate MP only in selected leaf tissues, were inoculated with TMV mutants that lack a functional *MP* gene. Expression of *MP* is not required in most epidermal cells for successful infection by mutant TMV, although we cannot rule out the possibility that a small amount of MP may have diffused into the epidermis from other tissues. Wild-type TMV produces substantially more MP than is required for each stage of a successful infection. Establishment of multicellular infection sites by mutant TMV on an inoculated leaf at maximum efficiency requires about 30% of the amount of MP produced by wild-type TMV in a systemic infection. The subsequent cell-to-cell spread of the mutant virus through the inoculated leaf requires no more than 2% of this level of MP in order to occur at the maximum rate. Systemic spread of the mutant virus throughout the plant occurs at the maximum rate when 4% of this level of MP is present.

Additional keywords: cauliflower mosaic virus 35S promoter; chlorophyll A/B promoter; complementation; phenylalanine ammonia lyase promoter; tissue-specific gene expression.

Infection of a tobacco plant by tobacco mosaic virus (TMV) can be divided into several steps: (i) infection of the first cell in a leaf; (ii) establishment of a multicellular infection site (movement of the virus out of the first cell); (iii) short-distance (cell-to-cell) spread through the leaf; (iv) long-distance spread, which can be subdivided into entry into, travel through, and exit from the vascular system; and (v) further cell-to-cell spread once the virus has left the vascular system. The movement protein (MP) is known to be required for at least steps (iii) and (v), but the mechanism by which it functions has not been fully determined.

Several studies have led to some understanding of the mode of action of the TMV MP. The protein has a direct effect on the function of plasmodesmata. The molecular size exclusion limit of plasmodesmata in transgenic Xanthi tobacco plants expressing the *MP* gene from the cauliflower mosaic virus (CaMV) 35S promoter (Deom et al. 1987) is at least 10-fold greater than that in control plants (Wolf et al. 1989). These transgenic plants also exhibit changes in photosynthate partitioning when compared with control plants (Lucas et al. 1993). In addition, electron microscopy employing immunogold labeling has shown that the MP is localized in plasmodesmata in tobacco leaf tissue from TMV-infected plants (Tomenius et al. 1987) and transgenic plants (Atkins et al. 1991; Ding et al. 1992; Moore et al. 1992). MP produced in and purified from *Escherichia coli* binds single-stranded nucleic acids in vitro in a cooperative but nonspecific manner and forms a thin extended structure (Citovsky et al. 1990, 1992). These results suggest that in vivo, MP and TMV RNA may form a thin protein–RNA complex that passes through plasmodesmata that have been modified by MP (Citovsky et al. 1990, 1992; Deom et al. 1992). *E. coli*-produced MP, when injected into a tobacco mesophyll cell, can increase the plasmodesmal size exclusion limit of that cell and of adjacent mesophyll cells, suggesting that the MP may be able to move from cell to cell in the absence of virus (Waigmann et al. 1994).

The tissues through which TMV must spread in order to successfully infect a plant are not known. In addition, it is not known in which tissues or in what quantities MP must accumulate in order for local or long-distance spread of the virus to occur. To explore these questions, we produced transgenic plants that express the *MP* gene from two tissue-specific promoters: the chlorophyll A/B (cAB) promoter of pea (Cashmore 1984) and the phenylalanine ammonia lyase gene 2 (pal2) promoter of bean (Cramer et al. 1989). In contrast to the CaMV 35S promoter, which yields high-level expression in most tissues (Teeri et al. 1989), the cAB

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Table 1. Description of the transgenic plant lines used

Plant line	Tobacco cultivar	Promoter	Coding sequence	Descriptive name used in text
Sx-O	Xanthi nn	cAB	none	control
Esc4-7	Xanthi nn	cAB	*uidA*	nn-cAB-*uidA*
cAB-nn E, F, I, 6	Xanthi nn	cAB	TMV *MP*	nn-cAB-*MP*
cAB-NN F, J, O, 4	Xanthi NN	cAB	TMV *MP*	NN-cAB-*MP*
G 6-1	Xanthi NN	CaMV 35 S	*uidA*	NN-35S-*uidA*
277	Xanthi nn	CaMV 35 S	TMV *MP*	nn-35S-*MP*
2004, 2005	Xanthi NN	CaMV 35 S	TMV *MP*	NN-35S-*MP*
PGN	Xanthi nn	pal2	*uidA*	nn-pal2-*uidA*
PMN	Xanthi nn	pal2	TMV *MP*	nn-pal2-*MP*

promoter is active in chloroplast-containing tissues, but is not active in the leaf epidermis, except in guard cells. The pal2 promoter is active primarily in the leaf upper epidermis and developing xylem. Plants that produce varying levels of MP under the control of the cAB, 35S, or pal2 promoter were inoculated with TMV and with mutants of TMV that lack a functional *MP* gene. The results of these experiments allow us to draw conclusions about the role of the MP at several stages during a virus infection.

RESULTS

Characterization of transgenic plants.

cAB promoters drive the nuclear expression of chlorophyll A/B binding protein genes (Apel and Kloppstech 1978). The expression is light inducible, phytochrome mediated, and limited to green tissue (Mitra et al. 1989; Simpson et al. 1986; Teeri et al. 1989). We transformed tobacco (*N. tabacum*) leaf disks with the *MP* gene under the control of the cAB promoter *AB80*, and recovered more than 10 transgenic lines each of *N. tabacum* cv. Xanthi nn (a systemic host for TMV) and cv. Xanthi NN (hypersensitive to TMV). We also transformed Xanthi nn with the *MP* gene or the *uidA* gene (the gene for β-D-glucuronidase [GUS]) under the control of the pal2 promoter and recovered six pal2-*MP* lines and five pal2-*uidA* lines. In addition, transgenic plants that express the *MP* gene or the *uidA* gene under the control of the cAB or the CaMV 35S promoter were used (described in Materials and Methods). Tobacco transformed with the cAB promoter with no MP coding sequence was used as a negative control. The plant lines used in this study are described in Table 1. Heterozygous R_1 progeny plants that contained the *MP* gene were used in the virus infection studies. In all plants, gene integration was confirmed by Southern blot analysis (data not shown), and in the case of plants expressing the *MP* gene, MP accumulation was confirmed by Western blot analysis (not shown).

Tissue specificity of the cAB and pal2 promoters.

To determine in which tissues expression occurs in transgenic *N. tabacum* plants, plants expressing the *uidA* gene from the cAB, 35S, or pal2 promoter were analyzed by histological GUS assays. Figure 1 shows leaf sections of R_1 progeny of transgenic plants after incubation with the substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclo-ammonium salt (X-Gluc). As has been previously demonstrated (Jefferson 1988), plants that express the 35S-*uidA* gene construct showed high levels of GUS activity in all tissues (Fig. 1A). In contrast, plants that express the cAB-*uidA* gene construct showed no activity in epidermal cells (except for guard cells and leaf hairs), low activity in vascular cells (not shown), and high activity in mesophyll cells, guard cells, and leaf hair cells (Fig. 1B). It is assumed that plants expressing the *MP* gene follow the same pattern of gene expression. Plants expressing the pal2-*uidA* gene construct showed GUS activity primarily in the leaf upper epidermis (Fig. 1C), as previously reported (Reimann-Philipp and Beachy 1993).

Plants expressing the pal2-*MP* gene construct were also analyzed by SDS-PAGE followed by Western blotting (Fig. 2A). Total protein was extracted from leaf upper epidermis, whole leaf tissue, and stem tissue. MP accumulation was found to occur predominantly in the leaf upper epidermis in these plants.

Samples of upper epidermis were collected from Xanthi nn plants, both uninfected and infected with TMV, and from a nn-35S-*MP* plant line, a nn-pal2-*MP* plant line, and two different nn-cAB-*MP* plant lines. Equal amounts of total protein extracted from these tissues were subjected to SDS-PAGE followed by Western blot analysis (Fig. 2B). The epidermis of the nn-pal2-MP plant accumulated the most MP, followed by the TMV-infected Xanthi nn plant, the nn-35S-MP plant, and the nn-cAB-MP plants, in that order. We cannot determine if the presence of a low level of MP in the epidermis of the nn-cAB-MP plants is due to movement of the MP from the mesophyll into the epidermis, or is simply the result of some mesophyll cells adhering to the epidermis as it was peeled from the leaf. The histological GUS assays (described above) indicated that there was little or no expression from the cAB promoter in most epidermal cells.

Quantitation of MP in transgenic plants.

The amount of MP that accumulates in each cAB-*MP* and 35S-*MP* transgenic plant line was determined and compared to the amount produced by TMV infection in nontransgenic plants at 5 days after inoculation. MP was extracted from leaves 3 and 4 (from the top) of plants 6 weeks after planting, and was quantitated by ELISA (see Table 2 for Xanthi nn plants and Table 3 for NN plants). TMV infection produced the highest level of MP, and was assigned the value of 100% for comparative purposes. The various transgenic lines showed a wide range of MP levels, from as little as about 1% to as much as about 60% of the level found in TMV-infected plants.

Complementation of TMV mutants by transgenic plants.

Plants were inoculated with RNA transcripts produced in vitro from cDNA clones of TMV lacking the *MP* gene

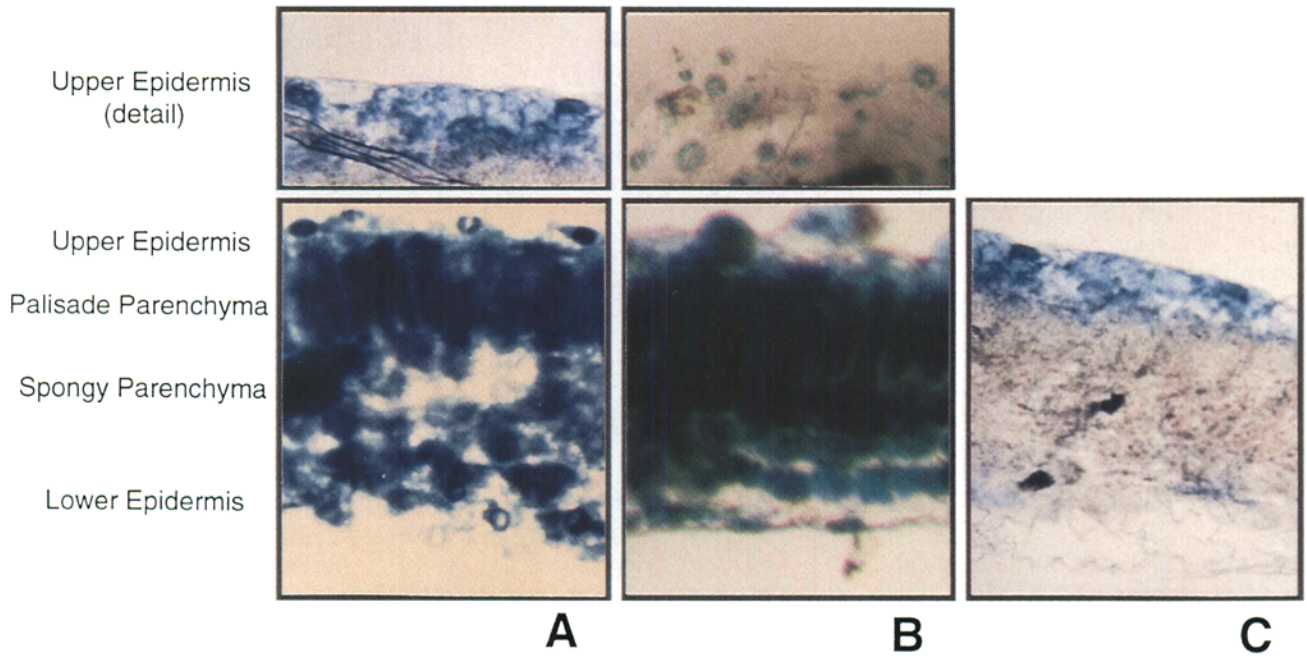

Fig. 1. Pattern of GUS activity in leaves of transgenic plants that contain the **(A)** 35S-*uidA*, **(B)** cAB-*uidA*, and **(C)** pal2-*uidA* gene constructs. Upper photographs show GUS activity in isolated epidermis. Lower photographs show transverse sections through leaf tissues.

(TMVΔMP; Holt and Beachy 1991) or in which the *MP* gene was replaced by the *uidA* gene (TMVΔMP-GUS; Lapidot et al. 1993). All of the plant lines that express the *MP* gene from the cAB or 35S promoter were able to functionally complement both TMV mutants, although the severity of symptoms caused by infection was dependent on the level of MP accumulation (Tables 2 and 3). Histochemical detection of GUS activity following inoculation with TMVΔMP-GUS showed that in both the NN-cAB-*MP* and NN-35S-*MP* plant lines, virus replication (as revealed by GUS activity) is restricted to the vicinity of local lesions. Characteristic results of GUS activity for lines cAB-NN F and 2005 infected with TMVΔMP-GUS are shown in Figure 3A and B, respectively. In both nn-cAB-*MP* and nn-35S-*MP* plants inoculated with TMVΔMP-GUS, GUS activity was found in large irregularly shaped areas (not shown).

When nn-pal2-*MP* plants, which accumulate MP primarily in the epidermis, were inoculated with TMVΔMP, no symptoms of virus infection were seen. Twenty-four plants were inoculated with TMVΔMP-GUS, and GUS activity was found only in isolated epidermal cells (Fig. 3C). No sign of virus replication was found in mesophyll cells.

The visual appearance of the local lesions produced by TMVΔMP in plant lines that express the *MP* sequence from the cAB promoter (e. g., line cAB-NN F; Fig. 3D) is different from that produced in plants that harbor the 35S-*MP* gene construct (e. g., line 2005; Fig. 3E). The lesions produced on NN-35S-*MP* plants are similar in appearance to those produced by TMV on Xanthi NN plants, i.e., the lesions have a light-brown central area surrounded by a ring of dark pigment. By contrast, the lesions produced in cAB-NN F plants are white and do not have a dark ring, and are also significantly smaller in size (see below). This unusual morphology was seen in all the NN-cAB-*MP* plant lines when infected with TMVΔMP, regardless of the amount of MP produced by the plant. Infection of these plant lines with wild-type TMV produced normal local lesions; two examples are shown in Figure 3F and G.

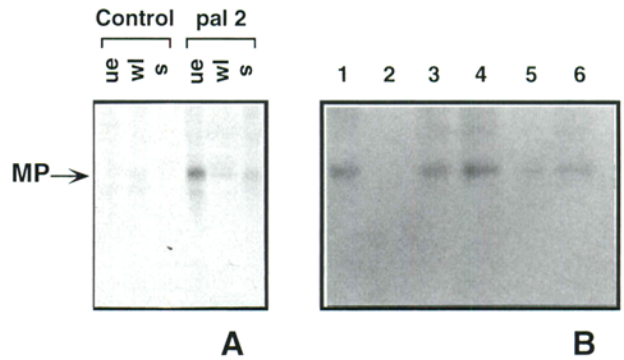

Fig. 2. (A) Western blot detection of movement protein (MP) in extracts of tissues from Sx-0 (control) and nn-pal2-MP plants. ue: upper epidermis; wl: whole leaf; s: stem. Equal quantities of total protein (as determined by BCA assay) were loaded in each lane. **(B)** Western blot detection of MP in extracts from leaf upper epidermis from: 1. Upper leaf of a nontransgenic Xanthi nn plant infected with tobacco mosaic virus in a lower leaf, 1 week after inoculation; 2. Uninfected nontransgenic Xanthi nn plant; 3. nn-35S-*MP* plant; 4. nn-pal2-*MP* plant; 5. cAB-nn I (nn-cAB-*MP*) plant; 6. cAB-nn E (nn-cAB-*MP*) plant. Equal quantities of total protein (as determined by BCA assay) were loaded in each lane.

Effect of amount of MP on symptom severity and virus accumulation.

Transgenic Xanthi nn plants carrying the cAB-*MP* gene construct that accumulated widely varying levels of MP were inoculated with equal amounts of TMVΔMP to determine the impact of the amount of accumulated MP on systemic infection. Ten days after inoculation disease symptoms were recorded, and the level of virus accumulation in upper leaves was determined by quantitating CP accumulation and by inoculating leaf homogenates onto plant line 2005 (Table 2).

The level of virus accumulation and the severity of symptoms in line 277 (nn-35S-*MP*) infected with TMVΔMP were similar to those in nontransgenic plants infected with TMV, even though the amount of transgenically produced MP was only 60% as great as that produced by TMV infection. Lines cAB-nn E and cAB-nn F (nn-cAB-*MP*), with much lower levels of MP (about 4% of TMV infection), showed strong disease symptoms and significant amounts of virus accumulation in upper leaves. Lines cAB-nn I and cAB-nn 6 (nn-cAB-*MP*), with even lower amounts of MP, showed moderate or no symptoms and significantly lower levels of virus accumulation. Thus, the severity of disease symptoms and the level of virus accumulation in these plant lines was correlated with the amount of MP in the plants. However, a small amount of MP (about 4% of the level found in TMV infection) is sufficient to produce essentially full development of symptoms and virus accumulation.

Effect of amount of MP on number and size of local lesions.

Transgenic Xanthi NN plants carrying the cAB-MP and the 35S-*MP* gene constructs were inoculated with TMV or TMVΔMP, and the number and size of the resulting local lesions were determined (Table 3; Fig. 3F and G). The number of local lesions produced by TMV was similar in all plant lines tested. In contrast, the number of lesions produced by TMVΔMP depended on the level of MP in the plants. On lines cAB-NN O and cAB-NN 4 (NN-cAB-*MP*), which have very low levels of MP (about 2% of the amount in TMV-infected Xanthi nn plants), very few lesions were produced. On line 2004 (NN-35S-*MP*), with 20% as much MP as in systemic TMV infection, the number of lesions was higher, and in the three lines with 27% or more of the MP level found in systemic TMV infection, the number of lesions reached a maximum level.

The size of the lesions induced by TMVΔMP did not depend on the amount of transgenically produced MP or on the promoter that was driving *MP* gene expression, except on line 2005 (NN-35S-*MP*). Likewise, the size of lesions induced by TMV, while larger than those induced by TMVΔMP, was invariant regardless of the amount of MP produced by the plant (including nontransgenic plants), except on line 2005. The anomalous size of lesions on line 2005 may be due to the exceptionally high level of MP or to some other characteristic of this line.

To better characterize the effect of the amount of MP on the

Table 2. Severity of symptoms and amount of virus detected in upper leaves of transgenic Xanthi nn tobacco plants inoculated with TMVΔMP

Plant line	Relative amount of MP (%)[a]	Symptoms[b]	Relative CP level in upper leaves (%)	Infectious units in upper leaves (no.)[c]
Sx-0	0	None	0	0
Nontransgenic Xanthi nn infected with TMV	100	Severe mosaic in upper and middle leaves, rugosity in middle leaves	100	186 ± 35
277	61	Severe mosaic in upper leaves, rugosity and chlorosis in middle leaves	100	182 ± 31
cAB-nn E	4.2	Mosaic in upper leaves and rugosity in middle leaves	88	144 ± 26
cAB-nn F	3.8	Attenuated mosaic in upper leaves	84	112 ± 21
cAB-nn I	2.4	Rugosity in upper leaves, chlorosis in middle leaves	66	68 ± 11
cAB-nn 6	1.3	None	12	11 ± 4

[a] The values are averages of three disks taken from leaves 3 and 4 from each of three plants. The percentages are relative to the amount of MP in extracts of Xanthi nn tobacco plants 5 days after infection with TMV (25 μg MP per g of frozen leaf tissue).

[b] Three-week-old plants were inoculated with 50 μl of TMVΔMP (at a concentration that would form 150 lesions on a plant line 2005 leaf) on each of two lower leaves. The symptoms were recorded in four plants 10 days after inoculation.

[c] Two leaf disks 5.9 mm in diameter (0.4 g) were collected from each plant and ground in 400 μl of phosphate buffer pH 7.2. 50 μl of each extract was inoculated onto a leaf of plant line 2005 and local lesions were counted. Results are the averages from six leaves of three 2005 plants, 4 days after inoculation.

Table 3. Number and size of local lesions in transgenic NN tobacco plants inoculated with TMV or TMVΔMP

Plant line	Relative Amount of MP (%)[a]	TMV[c]		TMVΔMP[c]	
		Number	Diameter (mm)	Number	Diameter (mm)
Xanthi NN	0	24.0 ± 16	3.0 ± 1.8	0	N/A
Nontransgenic Xanthi nn infected with TMV	100	N/A[d]	N/A	N/A	N/A
2005	58	32.0 ± 18	6.0 ± 2.4	35.1 ± 17	4.0 ± 1.8
cAB-NN F	33	21.1 ± 19	3.6 ± 1.4	48.8 ± 23	1.8 ± 1.5
cAB-NN J	27	30.2 ± 14	3.4 ± 1.6	58.7 ± 32	1.5 ± 1.4
2004	20[b]	27.6 ± 13	3.5 ± 1.1	18.5 ± 10	1.4 ± 0.4
cAB-NN O	2.3	28.3 ± 21	3.5 ± 1.4	0.3 ± 0.8	1.1 ± 0.5
cAB-NN 4	1.6	26.6 ± 17	3.4 ± 1.2	4.2 ± 1.8	1.2 ± 0.8

[a] See Table 2 for explanation of values.

[b] Determined from values described by Deam et al. (1991).

[c] Plants were inoculated with 50 μl per leaf of a standard virion inoculum (TMVΔMP) solution that would produce approximately 50 local lesions on a plant line 2005 leaf. The number and size of local lesions are the average of two inoculated leaves on each of three plants 4 DPI.

[d] N/A = not applicable.

number of lesions formed, several lines were inoculated with a range of concentrations of TMVΔMP or TMV (Fig. 4). Over this range, the number of local lesions produced varied approximately linearly with the inoculum concentration. At higher virus concentrations, the differences between the lines become more apparent than in Table 3. With TMVΔMP, the slopes of the dose/response lines for cAB-NN F and 2005, which contain high levels of MP, were approximately equal, and were significantly greater than for cAB-NN 4, which contains a very low level of MP, showing that the level of MP affects the likelihood of a multicellular infection being established. With TMV, the slopes of the dose/response lines for cAB-NN F and cAB-NN 4 were roughly equal to the slope for nontransgenic plants. The slope for plant line 2005 was greater than for the other three plant lines. In the case of infection with TMVΔMP, 33% of the level of MP found in nontransgenic plants systemically infected with TMV is sufficient to produce the maximum number of lesions. In the case of TMV, the level of MP in the plant does not affect the number of lesions except in line 2005, which contains the highest amount of MP.

DISCUSSION

Transgenic plants that contain the cAB-*MP* gene construct, which is not expressed in most epidermal leaf cells, complemented two mutants of TMV that lack a functional *MP* gene, TMVΔMP and TMVΔMP-GUS. This demonstrates that mutant TMV infection does not require MP expression in most epidermal cells. These results suggest that expression of MP in the epidermis may not be needed for the mutant virus to move from an epidermal cell to a mesophyll cell. This can be explained if: (i) virus can move from epidermal cells to mesophyll cells without the aid of MP; (ii) the plasmodesmata connecting epidermal cells to mesophyll cells can be modified by MP produced solely in mesophyll cells, thereby allowing virus movement; or (iii) MP can move from mesophyll cells into epidermal cells, where it might interact with and promote movement of the virus; Waigmann et al. (1994) have shown that MP purified from *E. coli* and injected into tobacco leaf mesophyll cells can move into adjacent mesophyll cells.

Inoculation of nn-pal2-*MP* plants with TMVΔMP-GUS resulted only in infection of individual epidermal cells (Fig. 3C). This suggests that the presence of *MP* in epidermal cells may not be sufficient to allow virus to spread laterally through the epidermis or downward into the mesophyll cells (and, for that matter, that virus cannot spread in either of these ways without the aid of MP). The inability of nn-pal2-MP plants to complement TMVΔMP or TMVΔMP-GUS may simply be due to an insufficient amount of MP in the epidermis. Although immunoblot analysis (Fig. 2B) shows a greater accumulation of MP in the leaf upper epidermis of a nn-pal2-*MP* plant than in a TMV-infected nontransgenic plant, TMV may transiently produce an even higher level of MP during an early stage of infection in order to cause spread out of the initially infected cell. Fannin and Shaw (1987) found that TMV infection begins in isolated epidermal cells and remains in these cells for some time before beginning rapid spread to mesophyll cells and other epidermal cells.

The unusual appearance of lesions induced by TMVΔMP on NN-cAB-*MP* plants compared with those induced by TMV (Fig. 3D–G) may be due to the absence of MP in most epidermal cells in NN-cAB-*MP* plants, which could affect how the hypersensitive response occurs. The difference is not the result of a low level of MP, since line 2004 (NN-35S-*MP*), with a level of MP comparable to that of cAB-NN J, shows small lesions with normal appearance. Other factors can also affect lesion morphology: Some TMV mutants (in most cases known to contain mutations in the MP gene) form lesions without rings (Jockusch 1968; Zimmern and Hunter 1983; T. W. Kahn and R. N. Beachy, unpublished results).

The availability of transgenic plants with varying amounts of MP allowed us to study the effect of MP level on the initiation, local spread, and systemic spread of virus infection. The effect of the amount of MP on the establishment of infection sites was determined by counting the number of local lesions formed on Xanthi NN plants. The effect of the amount of MP on the rate of local spread was determined by measuring the diameters of local lesions at a given time after inoculation. The effect of MP level on systemic spread of virus was determined by measuring the amount of TMV in upper leaves of plants at a given time after inoculation of lower leaves. The rate of systemic spread will be a function of the efficiency with which infection is established and the rates of local and long-distance movement.

The efficiency with which TMVΔMP established infections on transgenic Xanthi NN plants reached a maximum when the amount of transgenically produced MP was about 30% of the amount produced by systemic TMV infection of nontransgenic Xanthi nn plants (Table 3; Fig. 4). The efficiency of TMV infection of transgenic plants did not depend on the amount of MP produced by the plant, except perhaps in the case of line 2005 (NN-35S-*MP*), which accumulates the greatest amount of MP. A possible explanation for these results is that the level of MP in a transgenic leaf may vary from one area of the leaf to another. At low total levels of MP, some areas of the leaf may have less MP than is required to allow an infection to spread out of the initially infected epidermal cells and cause the formation of local lesions, while other areas may exceed the threshold level. As the total amount of MP is increased, a point will be reached at which all areas are above the threshold; further increases in the MP level will not lead to a greater efficiency of establishment of infection sites.

In infection by TMVΔMP, the size of local lesions was not statistically different regardless of the amount of MP in the plant line, except in the case of line 2005 (Table 3). In TMV infections, the lesions were larger than in TMVΔMP infections, possibly because of the production by TMV of locally high MP levels, but were comparable in size to each other and to lesions formed by TMV on nontransgenic plants. Again, the exception was line 2005, on which lesions were larger. The level of MP in line 2005 may be so high that the virus is able to escape the hypersensitive response to some extent (lesions on this line continue to grow for a longer time than lesions on other plant lines). Alternatively, there may be some other characteristic of this line that causes TMV and TMVΔMP to spread faster, since Dawson (1990) and Culver et al. (1993) found that TMV mutants that produce 10 to 50 times more MP than wild-type TMV do not spread faster than TMV. Our results differ from those of Lehto and Dawson

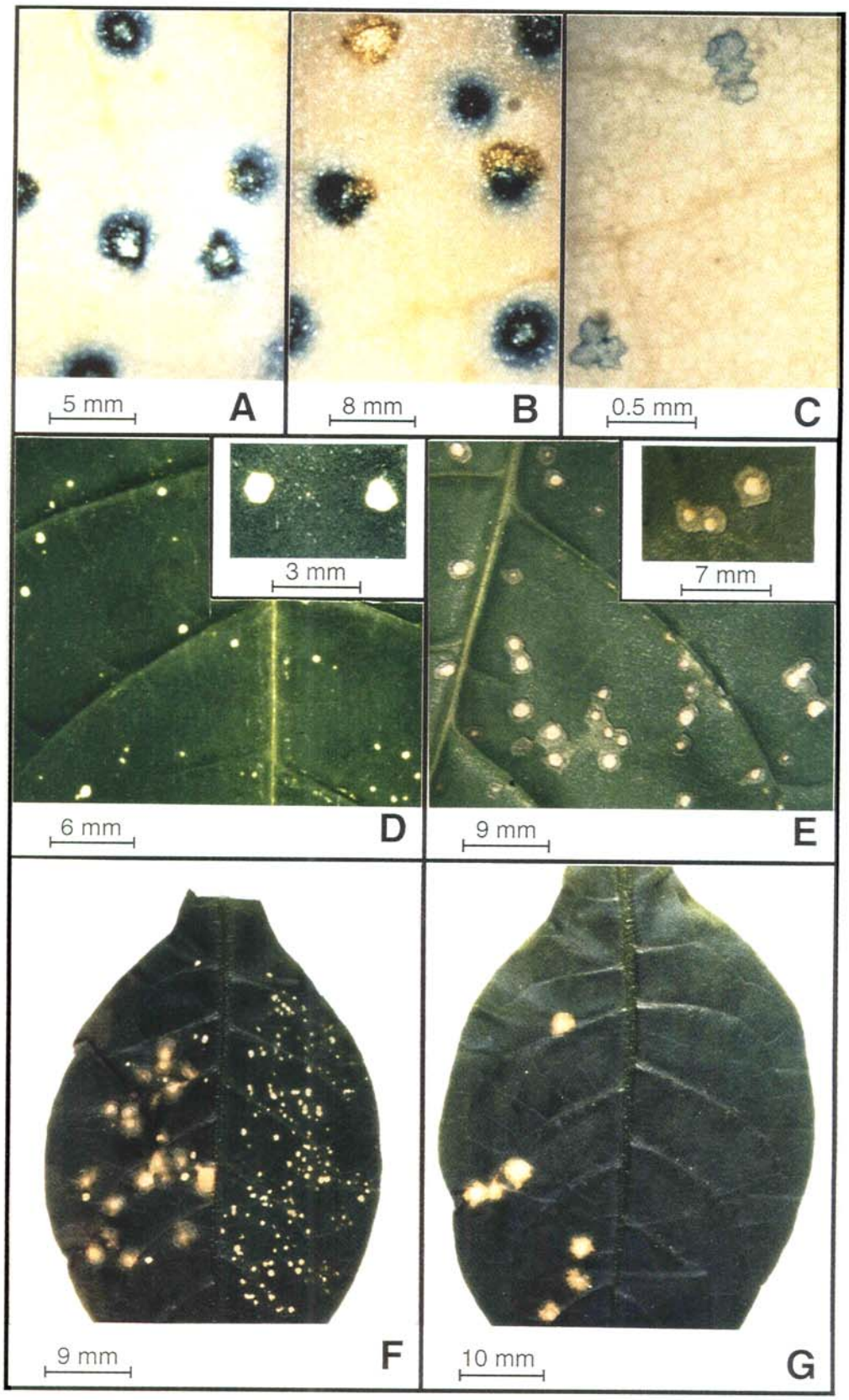

Fig. 3. Functional complementation of viruses lacking the movement protein (MP) gene by transgenic tobacco plants that express the *MP* gene. GUS activity in leaves of lines cAB-NN F **(A),** 2005 **(B),** and PMN **(C)** is shown 3 days after inoculation with RNA transcripts of TMVΔMP-GUS. Morphology of local lesions in lines cAB-NN F **(D)** and 2005 **(E)** plants is shown 3 days after inoculation with transcripts of TMVΔMP. Insets in (D) and (E) show lesions at higher magnification. **(F)** cAB-NN J leaf inoculated with TMVΔMP virions (right side of leaf) at a concentration that would produce approximately 75 lesions on a plant line 2005 leaf, and with TMV virions (left side of the leaf) at a concentration that would produce about one quarter as many lesions. **(G)** cAB-NN O line inoculated with TMVΔMP virions (right side of leaf) and TMV virions (left side of leaf) using the same inocula as in (F).

(1990a, 1990b) who found, by using TMV mutants that produce reduced amounts of MP, that when the MP level is below about one-tenth the wild-type level, the rate of cell-to-cell spread is reduced.

Our data suggest that the establishment of a multicellular infection is distinct from the subsequent cell-to-cell spread of virus through a leaf, since a relatively high level of MP is required to achieve the maximum number of local lesions, while a very low level is required for the maximum rate of growth of lesions. The presence of MP is not required for the establishment of a primary infection, since single epidermal cells in a nontransgenic plant can be infected by TMVΔMP-GUS (not shown). Therefore, the level of MP appears to influence the likelihood that an infection will be converted from the single-cell stage to the multicellular (two or more cells) stage. Once this conversion occurs, the rate of cell-to-cell spread through a leaf does not depend on the level of MP available over the range tested in this study. Fannin and Shaw (1987) found that a much longer period of time is required for TMV to spread from an initially infected epidermal cell to its immediate neighbors than for subsequent spreading to further cells, again suggesting two distinct stages of infection.

When transgenic Xanthi nn plants were infected with a high concentration of TMVΔMP, the severity of systemic disease symptoms and the level of virus accumulation in upper leaves depended on the amount of MP in the plants (Table 2). However, above a rather low level of MP (about 4% of the level seen in TMV infection of nontransgenic plants), an increase in the amount of MP did not lead to an increase in virus accumulation. Although this may suggest that a process or function that is involved in systemic infection has been saturated by even a low level of MP, a more likely explanation is that the upper leaves have reached the point at which no more virus can be produced. These results indicate that TMV produces substantially more MP than is required to achieve full systemic infection of a tobacco plant.

In summary, the establishment of a multicellular infection by TMVΔMP does not depend on the expression of an *MP* gene in the majority of epidermal cells, and indeed, appears not to benefit from expression. This step does, however, require a moderate level of MP in the mesophyll cells in order to achieve maximum efficiency. Once a multicellular infection has been established, even a very low level of MP is sufficient for the virus to spread locally at its maximum rate. The maximum rate of systemic spread of TMVΔMP infection requires less transgenically produced MP than is produced during a wild-type TMV infection.

MATERIALS AND METHODS

Plasmid construction and plant transformation

A. Plasmids containing the cAB promoter.

A DNA fragment corresponding to nucleotides 4903 to 5709 of TMV U_1 was inserted into the *Xho*I and *Bam*HI sites of pUC18. This fragment was excised and ligated into the *Sal*I and *Bam*HI sites of the plasmid pGV1511 (kindly provided by M. Van Montagu) downstream of the cAB promoter *AB80* isolated from *Pisum sativum* (Cashmore 1984), to form the plasmid pGV1511-30K. The construct is referred to as the cAB-*MP* gene construct.

Triparental mating (Matzke and Matzke 1986) was used to mobilize pGV1511-30K into the rifampicin-resistant C58C1 strain of *Agrobacterium tumefaciens* (Van Larebeke et al. 1974) carrying the disarmed Ti plasmid pGV3850 (Zambryski et al. 1983). The resulting chimeric construct was used to transform *Nicotiana tabacum* cv. Xanthi NN and Xanthi nn by the leaf disk procedure (Horsch et al. 1985). *N. tabacum* cv. Xanthi nn was also transformed with pGV1511 lacking the *MP* gene sequence to produce control plants. R_1 plants were used for all experiments. Plants were grown at 25 to 30°C under artificial light with a 14-h light/10-h dark photoperiod.

B. Plasmids containing the pal2 promoter.

The pal2 promoter was derived from the gPAL 2 gene of *Phaseolus vulgaris* (Cramer et al. 1989). A *Dra*I fragment containing a 1,157-bp fragment was ligated into the *Xho*I site

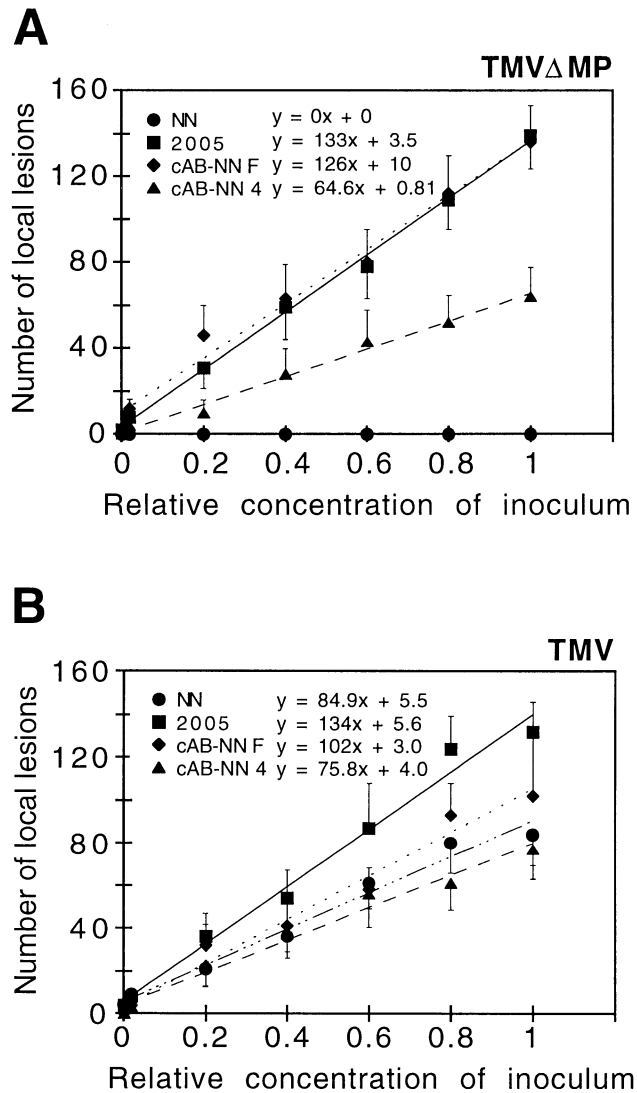

Fig. 4. Number of local lesions produced on tobacco cv. Xanthi NN plants by different concentrations of TMVΔMP (**A**) and TMV (**B**). The values are the averages of numbers of lesions on two leaves on each of three plants, 3 days after inoculation with 50 µl per leaf of a standard virion inoculum solution (TMVΔMP or TMV) that would produce approximately 150 local lesions on a plant line 2005 leaf (defined as a relative concentration of 1), and with dilutions of this solution. Lines of best fit were determined by linear regression.

(made blunt by a fill-in reaction) of pUC18-MN, which contains the *MP* sequence of TMV isolated from pTM-934 (comprising TMV nucleotides 4855 to 5868; Oliver et al. 1986) ligated with the nos 3′ end. A fragment containing the pal2 promoter, the TMV *MP* sequence, and the nos termination sequence was isolated by partial digestion with *Hin*dIII and complete digestion with *Eco*RI. The isolated gene was ligated with the binary plant transformation vector pMON-505 (Rogers et al. 1987) that had been previously digested with *Eco*RI and *Hin*dIII. The plasmid pPGN was constructed by first ligating the *E. coli uidA* gene, derived from the plasmid pRAJ-260 (Jefferson et al. 1986) with the nos termination signal in pUC19. The pal2 promoter (described above) was ligated into the *Sma*I site of this plasmid resulting in the plasmid pUC19-PGN. This gene was excised as an *Eco*RI/*Hin*dIII fragment and ligated with pMON-505 to give pMON-505-PGN. Transformation of tobacco was carried out as described by Reimann-Philipp and Beachy (1993). R_1 plants were used for all experiments. Plants were grown as described above.

Other plant lines.

R_3 progeny of plant lines 277, 2004, and 2005 (Deom et al. 1987, 1991) were used. These lines express the *MP* open reading frame under the control of the 35S promoter of cauliflower mosaic virus (referred to as the 35S-*MP* gene construct). Plants were grown from R_1 seeds of G 6-I, a transgenic plant line that expresses the *uidA* gene from the 35S promoter, and which was previously developed in this laboratory (L. Farrell and R. N. Beachy, unpublished). R_1 seeds of line Esc4-7, which contains a cAB-*uidA* gene construct, were kindly provided by J. Simpson, CINVESTAV, Irapuato, Mexico. Plants were grown as described above.

In vitro transcription and plant inoculation.

Full-length clones of TMV U_1 (Holt and Beachy 1991), TMVΔMP (Holt and Beachy 1991), and TMVΔMP-GUS (Lapidot et al. 1993), were transcribed in vitro, and the transcripts were inoculated onto plants using the procedure of Holt and Beachy (1991). TMVΔMP lacks the first two-thirds of the MP sequence, and retains the last third, which contains the viral origin of assembly and the coat protein subgenomic promoter, in an untranslated form. TMVΔMP-GUS contains the *uidA* coding sequence in place of the first two-thirds of the *MP* gene, retaining the last third of the gene as in TMVΔMP.

Histochemical detection of GUS activity.

Leaf tissue from transgenic plants that express the *uidA* gene or from plants infected with TMVΔMP-GUS was vacuum infiltrated with GUS assay buffer (50 mM sodium phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.5 mM potassium ferrocyanide/ferricyanide [Jefferson et al. 1986]) containing 0.5 mg/ml X-Gluc. Tissues were incubated overnight at 37°C and then transferred to 70% ethanol for clearing and examination.

Detection and quantification of MP.

Fresh leaf tissue (excluding the midribs) from transgenic plants or plants infected with TMV was homogenized in extraction buffer (62.5 mM Tris-HCl, pH 6.8, 1% [w/v] SDS and 20% [v/v] glycerol) followed by heating for 5 min in a boiling water bath. Proteins were precipitated by addition of 4 volumes of ice-cold acetone followed by centrifugation at 14,000 × *g* for 5 min, and the pellet was redissolved in extraction buffer. Total protein concentration was determined using the BCA assay (Pierce, Rockford, Ill.). MP was detected by SDS-PAGE (Laemmli 1970) followed by Western blotting (Sambrook et al. 1989; Towbin et al. 1979) using an anti-MP antibody (Deom et al. 1987) as the primary antibody and an alkaline phosphatase conjugate (Southern Biotechnology Associates) as the secondary antibody. MP was quantified by ELISA in microtiter plates using the same antibodies.

Quantification of TMV CP in plants.

Two 5.9-mm-diameter leaf disks were ground in 100 µl of 35 mM potassium phosphate, pH 7.5, 10 mM β-mercaptoethanol, 400 mM NaCl. Insoluble material was removed by a 5-min centrifugation at 14,000 × *g*. Ten micrograms of protein (as determined by BCA assay) was used in an ELISA reaction in microtiter plates using an anti-CP antibody as the primary antibody, and an alkaline phosphatase conjugate as the secondary antibody.

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