Impeded Phloem-Dependent Accumulation of the Masked Strain of Tobacco Mosaic Virus

Richard S. Nelson[1], Guoxuan Li[1], Richard A. J. Hodgson[2], Roger N. Beachy[2], and Michael H. Shintaku[1]

[1]Plant Biology Division, Samuel Roberts Noble Foundation, Ardmore, OK 73402, U.S.A., and [2]Department of Biology, Washington University, St. Louis, MO 63130, U.S.A.

Received 18 June 1992. Accepted 22 October 1992.

The Holmes' masked (M) strain of tobacco mosaic virus produces mild (attenuated) symptoms compared with those of the U1 strain. We compared the accumulation of M strain viral protein and RNA with that of the severe U1 strain in protoplasts and leaf tissue from *Nicotiana tabacum* 'Xanthi nn.' In protoplasts or inoculated leaves, the accumulation of coat protein, movement protein, 126- and 183-kDa proteins, and all species of viral RNA were similar for the M and U1 strains. In addition, the specific infectivity of extracts from M-inoculated tissue was similar to that of extracts from U1-inoculated tissue. However, in tissue infected as a result of phloem transport, accumulation of M strain coat protein and viral RNA was transiently less than that observed for the U1 strain. Results from tissue print and temperature shift experiments indicated that the lower level of M strain accumulation was apparent in the petioles of inoculated leaves from 2.5 to 4 days postinoculation and in the shoot apex at least through 11 days postinoculation. We conclude that, in comparison with the U1 strain, the M strain is capable of normal rates of replication and cell-to-cell movement in inoculated leaves, but accumulation (i.e., replication or movement) over long distances (i.e., by phloem movement) is inhibited. This inhibition is observed in both developed tissue (petioles of inoculated leaves) and in developing tissue (tissue near the shoot apex).

Additional keywords: attenuated viruses, pathogenicity, symptom determinants.

In recent years, nucleotide sequences that conditionally determine disease symptoms have been identified in plus-stranded RNA plant viruses (reviewed in De Jong and Ahlquist 1991; Hull 1991; Jackson *et al.* 1991; Jupin *et al.* 1991; Daubert 1992; Dawson 1992). In many cases, the nucleotide changes responsible for altered disease symptoms reside in either the open reading frame for the capsid protein or for the protein known or thought to be responsible for cell-to-cell movement of the virus. Recently, determinants of disease phenotypes have been identified within nucleotide sequences that regulate, or code for, proteins believed to be involved in viral replication. Besides simply affecting virus replication (Kroner *et al.* 1989, 1990; Petty *et al.* 1990a; Traynor *et al.* 1991; Hacker *et al.* 1992; Lewandowski and Dawson 1993), these regions have been shown to be ultimately involved in altering host range (Meshi *et al.* 1988; Yamafuji *et al.* 1991), cell-to-cell spread in the host (Watanabe *et al.* 1987; Traynor *et al.* 1991), temperature sensitivities (Kroner *et al.* 1989; Roossinck 1991), and ability to replicate satellite RNAs (Roossinck and Palukaitis 1991; Collmer *et al.* 1992).

Traynor *et al.* (1991) produced mutants in the 5′ and 3′ coding regions of RNA 2 of brome mosaic virus (BMV), the genomic RNA encoding a protein with a sequence motif (i.e., GDD) found in viral RNA-dependent RNA polymerases (RdRps). When some of these RNA 2 mutants were inoculated with infectious transcripts from wild-type cDNA of RNAs 1 and 3 of BMV, virus accumulated to high levels in barley protoplasts. In contrast, these RNAs replicated poorly in both inoculated and uninoculated leaves of plants. This lack of accumulation in uninoculated leaves may have been due to a decrease in virus movement in the inoculated leaves, reduced leaf-to-leaf spread, decreased virus replication or movement in the systemically infected tissue, or to a combination of these factors. Recently, disease determinants have been identified in barley stripe mosaic virus that resulted in lack of systemic movement, yet near normal levels of virus accumulation in the inoculated leaves (Petty *et al.* 1990b). In *in vitro* studies, Petty *et al.* (1990b) determined that a short open reading frame 5′ of the AUG for the γa protein in the viral RNA decreased expression of the γa protein. The γa protein also contains the GDD motif. They further concluded that the lack of systemic infection in *N. benthamiana* may be due to the decreased expression of the γa protein.

The Holmes' masked (M) strain of tobacco mosaic virus (TMV) (Holmes 1934) is markedly attenuated in symptom severity compared with the U1 strain. This strain was first isolated from tomato stems inoculated with a distorting strain of TMV and placed at 34.6 °C for 15 days (Holmes 1934). The symptom induced by the M strain on systemically infected leaves of *Nicotiana tabacum* L. 'Xanthi nn' is at most a mild chlorosis. Holmes (1934) showed that necrotic and starch lesions in inoculated leaves of several *Nicotiana* species were similarly sized between the distorting strain and the M strain. However, the M strain produced fewer and smaller starch lesions in the tissue

Address correspondence to R. S. Nelson.
Present address of R. A. J. Hodgson: Department of Plant Pathology, Waite Agricultural Institute, Glen Osmond, SA 5064, Australia.
Present address of R. N. Beachy: Division of Plant Biology MRC7, Scripps Research Institute, La Jolla, CA 92037, U.S.A.

MPMI Vol. 6, No. 1, 1993, pp. 45-54

infected by vascular transport. In a previous study, utilizing recombinants of infectious cDNA clones of the M and U1 strains, we mapped the determinants of the attenuated symptoms to the nucleotides within the open reading frame encoding the 126- and 183-kDa proteins (Holt *et al.* 1990). Here we present an analysis of the accumulation of viral products in plant tissue after inoculation with either the M or the more virulent U1 strain of TMV. The results from this work increased our understanding of how changes in the nucleotide sequence of the TMV genome manifest themselves as measurable biochemical differences that may lead to attenuated symptoms.

RESULTS

In this paper we separated results involving local infection by spread of TMV within an inoculated leaf from those involving infection initiated solely by phloem transport of TMV (e.g., leaf to petiole, leaf to leaf), although both can include the systemic spread of infection via cell-to-cell movement. We refer to infections occurring through phloem transport as phloem-dependent infections and to infections occurring in inoculated leaves or protoplasts as local infections. Evidence for phloem transport of TMV has been described (Matthews 1991, and references therein).

Accumulation of viral protein and RNA products.

Local infections. The accumulation of TMV coat protein (CP) in Xanthi nn protoplasts inoculated with purified M, U1, or L19 strains was determined (Fig. 1). No differences in CP accumulation were observed among the three virus inocula up to 48 h postinoculation. Extracts from inoculated leaves of plants gave similar results up to 11 days postinoculation (DPI) (Fig. 2). At 11 DPI, the specific infectivities of extracts from leaves inoculated with M or U1 were similar (M, 0.70 ± 0.30; U1, 0.99 ± 0.04; units defined in Materials and Methods) as were CP accumulation results (M, 159 ± 9 ng; U1, 160 ± 56 ng). Results from additional experiments with extracts from plants harvested at 3 DPI indicated that the specific infectivity of M was essentially identical to U1 on a fresh weight or nanograms of CP basis (data not shown).

The accumulation of movement protein (MP) was determined in cell wall extracts from inoculated leaves and compared with CP accumulation. MP accumulation during the critical 3- to 5-DPI period, when cell-to-cell movement was expected to be evident, was similar for tissues inoculated with the M and U1 strains (Fig. 3). Both MP and CP accumulation continued through the period.

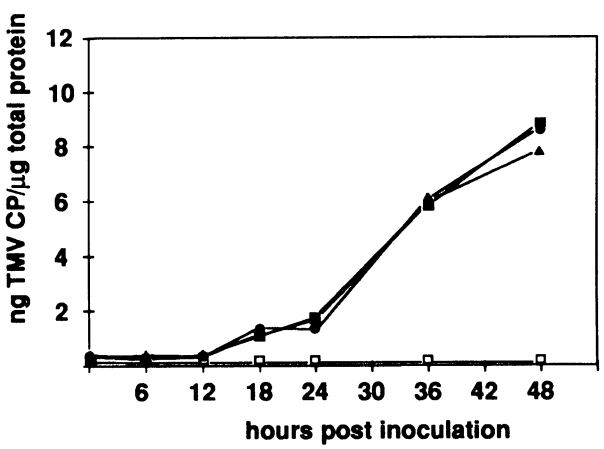

Fig. 1. Accumulation of TMV-coat protein (CP) over time in protoplasts infected with M-TMV, U1-TMV, or L19-TMV. TMV concentrations in all inocula were 5 µg/ml, and specific infectivities were determined to be equivalent by necrotic lesion assay on *Nicotiana tabacum* 'Xanthi NN'. CP accumulation was measured by ELISA. ● = M-TMV; ■ = U1-TMV; ▲ = L19-TMV; □ = mock inoculated.

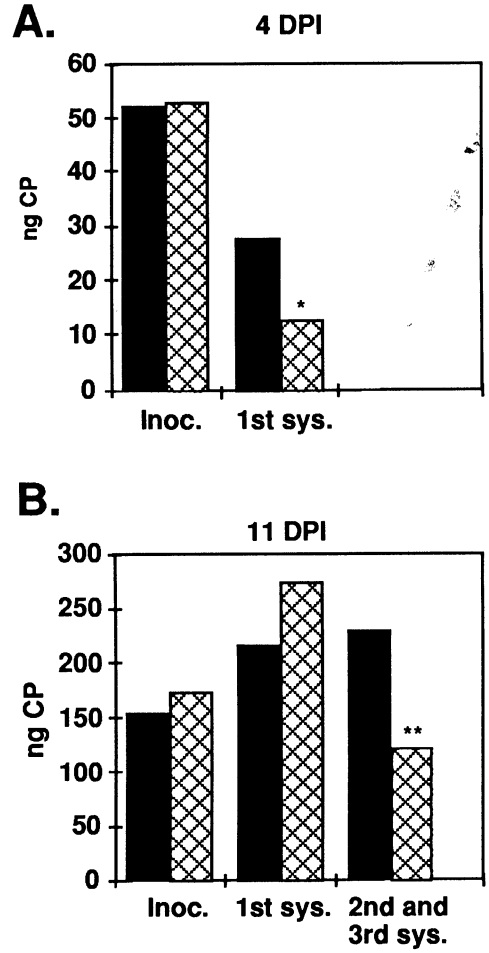

Fig. 2. Accumulation of TMV-coat protein (CP) over time in leaves of *Nicotiana tabacum* 'Xanthi nn' plants infected with M- or U1-TMV. Two consecutive leaves were inoculated with 0.1 µg/ml U1-TMV (solid bar) or 0.3 µg/ml of M-TMV (crosshatched bar). Infectivities of M- and U1-TMV inocula, as measured by the mean lesion numbers produced (± standard deviation) on eight leaves of *N. tabacum* 'Xanthi NN,' were 29 (±18) and 37 (±18) for 0.1 µg/ml of U1-TMV and 0.3 µg/ml of M-TMV, respectively. CP accumulation was measured by enzyme-linked immunosorbent assay. Equivalent amounts of leaf extract (on a gram fresh weight basis) were loaded per sample, and the values are given as nanograms of CP accumulated. An asterisk above a bar indicates a significant difference between this value and the companion U1 value at the 0.1 (4 DPI, *) and 0.05 (11 DPI, **) probability levels as determined by analysis of variance followed by least significant difference analysis. There were five replicates per mean value. **A,** Values for inoculated (Inoc.) and first systemically infected (1st sys.) leaves at 4 days postinoculation (DPI). **B,** Values for inoculated, first systemically infected, and second and third systemically infected (2nd and 3rd sys.) leaves at 11 DPI.

The accumulation of 126- and 183-kDa proteins was determined in protoplasts and compared with CP accumulation (Fig. 4). Accumulation of 126- or 183-kDa protein or of CP in M-strain-infected protoplasts was as great or greater than that determined for U1-strain-infected protoplasts. Accumulation of similar amounts of 126- and 183-kDa protein at 4 DPI in leaves of Xanthi nn inoculated with strains M or U1 was also observed (data not shown).

Viral RNA accumulation (genomic plus-strand and minus-strand and subgenomic plus-strand) in inoculated leaves of tissues infected with M or U1 strains was determined (Fig. 5). In this experiment (the same as that described in Fig. 6A), plants were inoculated with a greater amount of M strain than U1 strain with respect to infectivity. The apparent increase in viral RNA accumulation in the inoculated leaves of the plants infected with the M strain

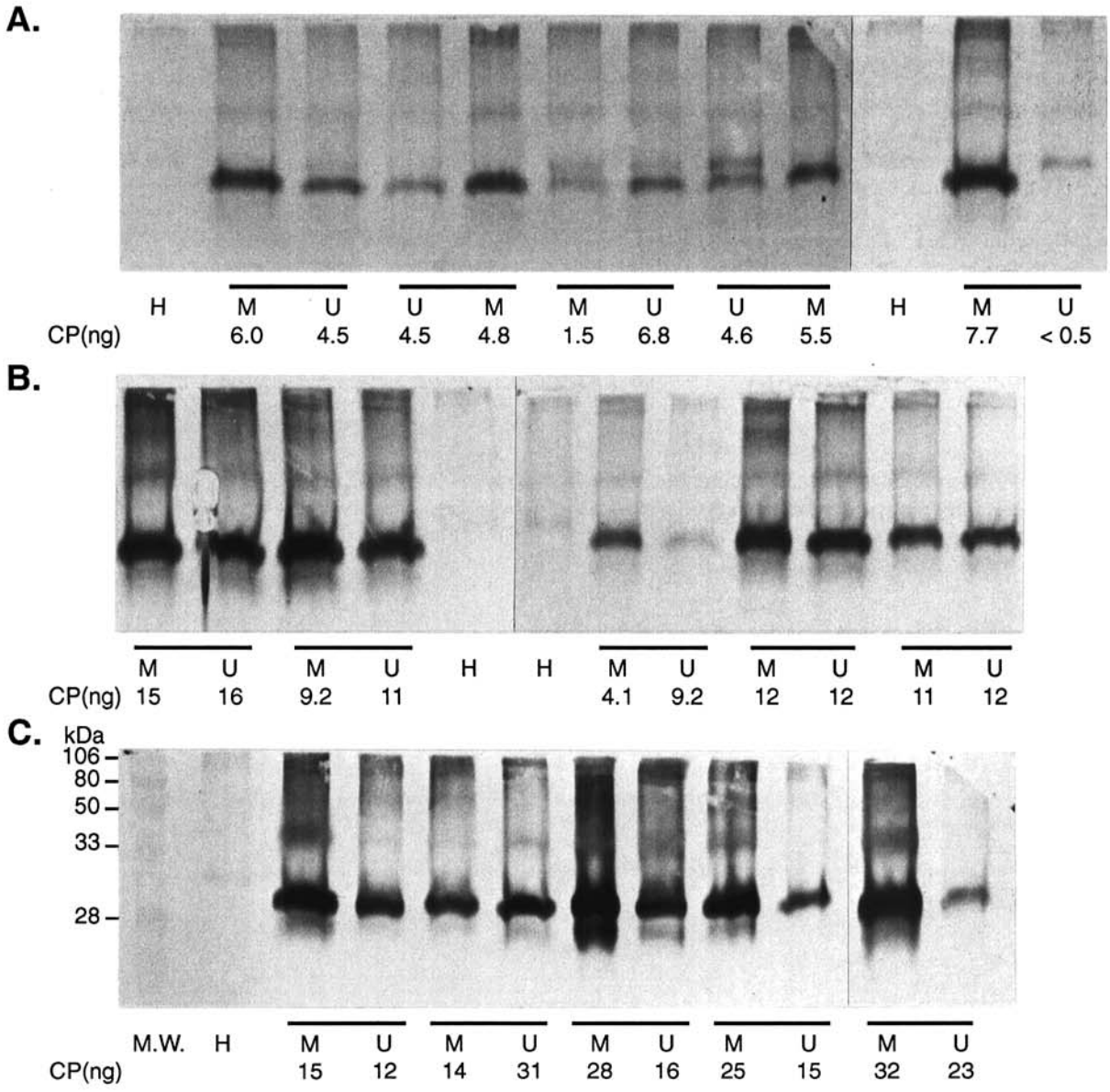

Fig. 3. Accumulation of TMV-movement protein (MP) and coat protein (CP) in leaves of *Nicotiana tabacum* 'Xanthi nn' inoculated with M- or U1-TMV. Respective infectivities of M- and U1-TMV inocula, as measured by the mean lesion number produced (± standard error), on 11 leaves of *N. tabacum* 'Xanthi NN' were 23 (±4) and 24 (±7) lesions per half leaf. The youngest inoculated leaf on Xanthi nn plants was >5 cm in midrib length, and two consecutive leaves were inoculated. There were five replicates per time point per inoculum. MP accumulation was measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot. In each lane, a 7.5-μg sample of total protein for each cell wall extract was loaded. CP accumulation was measured by enzyme-linked immunosorbent assay. Equivalent amounts of leaf extract (on a gram fresh weight basis) were loaded per sample, and the values are given as nanograms of CP accumulated. **A,** Values at 3 days postinoculation (DPI); **B,** 4 DPI; **C,** 5 DPI. H = Tissue from mock-inoculated plants; U = tissue from plants inoculated with U1-TMV; M = tissue from plants inoculated with M-TMV. Bars under M and U designations indicate samples from plants that were paired by approximation of their plastochron indices. Note that the results for the last three samples in **A,** the last seven samples in **B,** and the last two samples in **C** came from separate blots. Positions and sizes (kilodaltons) of molecular weight markers are shown to the left of the first lane in **C**.

reflects this difference in inoculum. Results from Northern blot analysis of extracts from the inoculated leaves supported the slot blot RNA analysis by showing that the CP-subgenomic RNA accumulation was similar in leaves inoculated with M and U1 (data not shown). Thus, in local infections the accumulation of CP, MP, 126- and 183-kDa proteins, and viral RNA species was similar for the M strain and the virulent U1 strain.

Phloem-dependent infections. In petioles of inoculated leaves and in stem and leaf tissue above the inoculated leaves, the accumulation of the M strain CP was delayed with respect to U1-infected tissue (Figs. 2 and 6). In addition, viral RNA accumulation at 4 and 6 DPI in systemically infected leaf tissue was less for the M strain than the U1 strain (Fig. 5). In a separate experiment, a reduction in M strain RNA compared with U1 strain RNA was determined for systemically infected leaves sampled at 11 DPI (data not shown).

At all sampling times, the shoot apices of plants inoculated with the M strain accumulated less CP than the apices of plants inoculated with the U1 strain (Fig. 6, and at 6 DPI, data not shown). However, as the infected tissue (initially accessed by phloem transport) aged, the accumulation of M strain was similar to that observed for U1 (Fig. 2B, first systemic leaf; Fig. 6A, 11 DPI, petiole tissue). Thus, while viral proteins for both strains accumulated in local infections at similar rates and levels, during phloem-dependent infections the M strain CP accumulated in a delayed manner compared with the U1 strain.

Extracts from leaf tissue infected with M or U1 by phloem-dependent movement and harvested at 11 DPI were analyzed for specific infectivities. The specific infectivity of the M and U1 extracts again roughly agreed with the amount of CP present in these extracts (specific infectivity: M, 0.30 ± 0.10; U1, 0.77 ± 0.15. CP accumulation: M, 143 ± 46 ng of CP; U1, 277 ± 59 ng of CP).

Detection of virus spread in hypersensitive hosts after temperature shift.

At 32° C the normal necrotic N-gene response is suppressed, and TMV spreads systemically in plants containing the N gene (Takahashi 1975; Weststeijn 1981). Xanthi NN plants inoculated and kept at 28° C, or kept at 32 °C for 2 days and then transferred to 28° C, yielded lesions of virtually identical size and appearance for the M and U1 strains (Fig. 7A and B). A similar result was previously observed for inoculated leaves of *N. langsdorffii, N. glutinosa*, and other local lesion hosts (Holmes 1934). Inoculated plants that were shifted from 32 to 26–28° C at 5 DPI became necrotic in areas where virus had accumulated during high-temperature movement and replication. Inoculated leaves from the plants inoculated with M and U1 strains collapsed and became totally necrotic within 2 days of the temperature shift. However, only plants inoculated with the U1 strain showed substantial necrosis of the systemically infected upper leaves and shoot apex (compare C and D in Fig. 7). These data support the conclusions drawn from the tissue print experiments suggesting that the M strain accumulates more slowly than the U1 strain in phloem-accessed tissue.

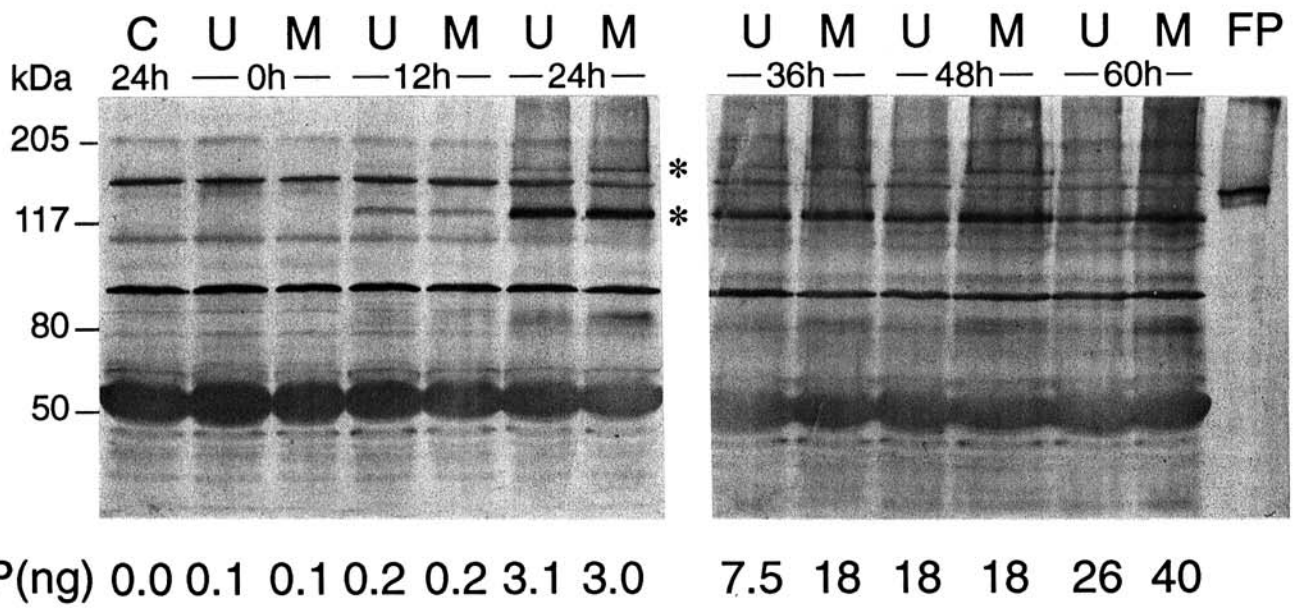

Fig. 4. Accumulation of 126- and 183-kDa protein and coat protein (CP) over time in protoplasts infected with M- or U1-TMV. Inoculum concentrations for M and U1 were 5 μg/ml, with equivalent specific infectivities as determined by necrotic lesion assay on *Nicotiana tabacum* 'Xanthi NN.' Analysis of 126- and 183-kDa proteins was by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot. For protoplast extracts, 52 μg of total protein was loaded per lane. Antiserum was not preadsorbed with uninfected plant tissue. Positions and sizes (kilodaltons) of molecular weight markers are noted in the left column. CP accumulation (in nanograms) was measured by enzyme-linked immunosorbent assay. Equivalent amounts of extract per sample (on a total protein basis) were loaded. C = Sample from uninoculated protoplasts; U = sample from protoplasts inoculated with U1-TMV; M = sample from protoplasts inoculated with M-TMV; and FP = sample of a partially purified *Escherichia coli* extract containing a β-galactosidase-TMV 126-kDa protein translational fusion with an expected molecular mass of 144 kDa. Asterisks indicate the positions of the 126- and 183-kDa proteins.

DISCUSSION

The accumulation of M and U1 strain viral products at various times postinoculation in local and phloem-dependent infections are compared (Table 1). Holmes (1934) determined that the M strain produced necrotic and starch lesions similar in size to those produced by the parental distorting strain on inoculated leaves. Thus, for this attenuated strain, lesion size positively correlates with virus product accumulation in the inoculated leaves and the specific infectivity of virus from these leaves. Lewandowski *et al.* (1993) characterized an attenuated strain of U1-TMV (V-36) where the determinant is a single nucleotide substitution that results in a single amino acid substitution in the 126-kDa protein. Inoculation of *N. tabacum* 'Xanthi-nc' with this virus results in a normal necrotic lesion phenotype but a specific infectivity of only about 20% of the parental virus. Thus, lesion phenotype does not always correlate with infectious virus and viral RNA accumulation.

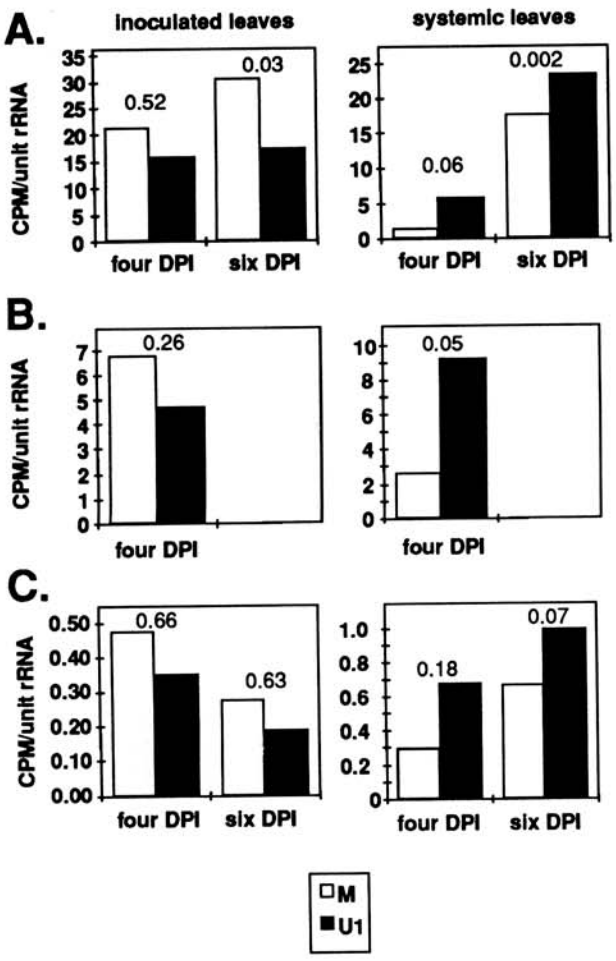

Fig. 5. Accumulation of TMV plus-strand genomic, subgenomic, and minus-strand genomic RNA over time in inoculated and systemically infected leaves of *Nicotiana tabacum* 'Xanthi nn' plants, after inoculation with M- or U1-TMV. Analyses were conducted on tissue from plants described in Fig. 6A. Values are given as counts per minute (cpm) of TMV RNA probe per unit of ribosomal RNA (rRNA) $\times 10^{-3}$ (**A** and **B**) and $\times 10^{-1}$ (**C**) and represent the mean of three replicates (4 days postinoculation [DPI]) or four replicates (6 DPI). Mean values for uninoculated plant tissue were subtracted from those of virus-infected tissue. Numbers above the bars indicate the probability that the means are from the same population as determined by paired *t* tests. **A,** Plus-strand genomic RNA accumulation; **B,** plus-strand genomic and subgenomic RNA accumulation; **C,** minus-strand genomic RNA accumulation.

Both the M strain and the V-36 strain differ in lesion phenotype from that determined for a third attenuated strain of TMV, $L_{11}A$, which has been cloned and sequenced (Nishiguchi *et al.* 1985). $L_{11}A$ produces small lesions on inoculated leaves (Nishiguchi and Oshima 1977). Although a mutation in the 126- and 183-kDa proteins of $L_{11}A$ is believed to be the initial cause of attenuated symptoms, the lack of MP accumulation appears to be the direct cause of the phenotype (Watanabe *et al.* 1987). These authors concluded that the lack of MP accumulation resulted in decreased cell-to-cell spread and consequently the small lesion phenotype. This explanation for the attenuated symptoms observed after $L_{11}A$ infection does not apply to M-strain infections, since accumulation of MP in the cell wall fraction of inoculated leaves was similar to that determined for the severe U1 strain (Fig. 3).

In a separate experiment, the M-strain virus was inoculated onto transgenic plants that expressed the MP gene of TMV-U1; these plants displayed no increase in the severity of disease symptoms (C. M. Deom and R. S. Nelson, unpublished). Thus although all three attenuated TMV strains (M, V-36, and $L_{11}A$) were isolated under high-temperature selection regimes (Holmes 1934; Oshima *et al.* 1965; Lewandowski *et al.* 1993), and the determinant of the mild symptoms has been shown to be in the nucleotide sequence encoding the 126-kDa protein, a difference is apparent in the mechanism by which attenuated symptoms are produced by these strains.

In phloem-dependent infections, a delay in virus accumulation was apparent for the M strain in both developed and developing tissue (Figs. 2, 5–7). Holmes (1934) noted that starch lesions were smaller in number and size in such tissue. The mechanism by which the M strain is prevented from accumulating in such tissue is unknown. It is possible that the mechanism involves a change in the 126-kDa protein, leading to either a decreased ability to recognize a viral subgenomic promoter or a decreased ability to interact with a host or viral factor(s). It is also possible that the secondary structure of the viral RNA in this domain has been altered in its ability to interact with a host or viral factor(s).

Unlike the mechanism of attenuation, the site of inhibition of M-strain accumulation can be discussed in more detail. Cohen *et al.* (1957) determined that the U2 strain of TMV does not accumulate well in young tissue (i.e., leaves less than 2 cm in midrib length), whether directly inoculated or accessed by phloem-dependent transport, when compared with the U1 strain. The delayed accumulation of the M strain in petioles of inoculated leaves indicated that for the M strain the delay in phloem-dependent accumulation can be observed in developed as well as in young tissue. In addition, after mechanical inoculation the M strain is capable of infecting young leaves (midrib less than 2 cm) of Xanthi nn or Xanthi NN, producing similar amounts of CP and necrotic lesions of similar sizes and number to that produced after U1 inoculation (R. S. Nelson

and M. Shintaku, unpublished). If further work supports these findings, a blockage in virus entry into, replication in, or exit from the phloem-associated tissue would be the most likely explanation for the lack of accumulation. Holmes (1934) noted such a possibility in his seminal work.

Regardless of the outcome of future work, the results presented in this paper and by Holmes (1934) comparing the M and U1 strains indicate that phloem-dependent infection is controlled by different factors from those that control local infection. As an example, a similar phenomenon was observed when sunn-hemp mosaic virus was coinoculated with the cowpea strain of southern bean mosaic virus (SBMV-C) in bean, a nonpermissive host for SBMV-C (Fuentes and Hamilton 1991). These researchers found that coinfection with sunn-hemp mosaic virus allowed cell-to-cell spread of SBMV-C in bean but not accumulation at long distances. Ding *et al.* (1992) have shown that the MP of TMV does not increase the size exclusion limit of phloem parenchyma cells as it does for mesophyll and bundle sheath cells in tobacco plants. In addition, Atabekov and colleagues (Atabekov and Dorokhov 1984) have identified a transport form of the virus that aids in leaf-to-leaf movement. This transport form contains the viral CP and other uncharacterized proteins. The identification of the proteins in this transport form and studies on their potential ability to modify vascular cells for leaf-to-leaf movement may aid in elucidating the relationships between the transport form, mutation(s) in the 126-kDa protein gene, and phloem-dependent infections.

In conclusion, the M strain of TMV is capable of accumulating normal amounts of viral products and infectious virus in inoculated leaves. However, it is impeded in accumulating viral products and infectious virus over long distances. This phenotype is in contrast to those of other attenuated TMV strains (V-36 and $L_{11}A$) that have been sequenced and whose ultimate cause of attenuation maps to the 126- and 183-kDA open reading frames. Whether

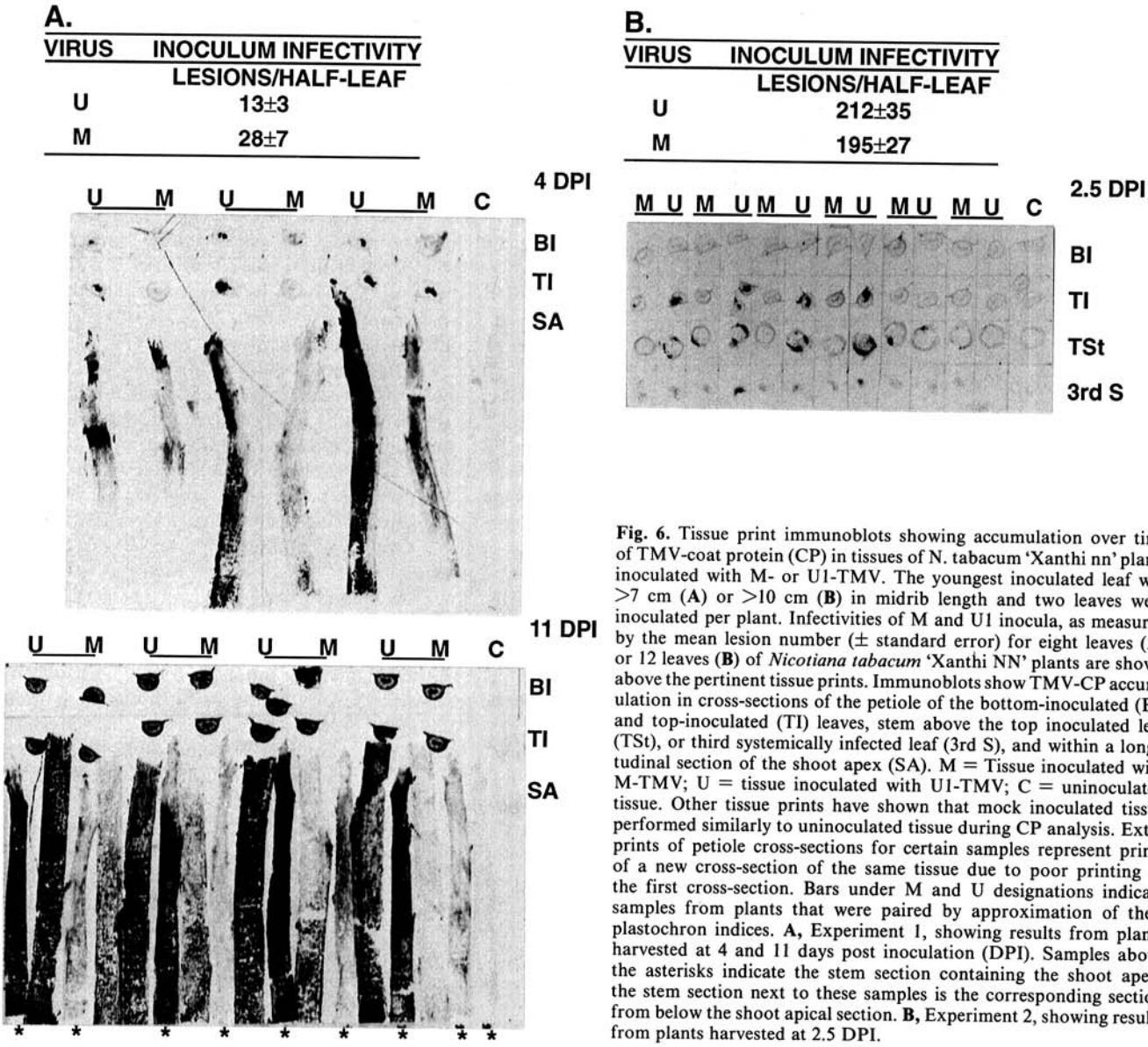

Fig. 6. Tissue print immunoblots showing accumulation over time of TMV-coat protein (CP) in tissues of N. tabacum 'Xanthi nn' plants inoculated with M- or U1-TMV. The youngest inoculated leaf was >7 cm (**A**) or >10 cm (**B**) in midrib length and two leaves were inoculated per plant. Infectivities of M and U1 inocula, as measured by the mean lesion number (± standard error) for eight leaves (**A**) or 12 leaves (**B**) of *Nicotiana tabacum* 'Xanthi NN' plants are shown above the pertinent tissue prints. Immunoblots show TMV-CP accumulation in cross-sections of the petiole of the bottom-inoculated (BI) and top-inoculated (TI) leaves, stem above the top inoculated leaf (TSt), or third systemically infected leaf (3rd S), and within a longitudinal section of the shoot apex (SA). M = Tissue inoculated with M-TMV; U = tissue inoculated with U1-TMV; C = uninoculated tissue. Other tissue prints have shown that mock inoculated tissue performed similarly to uninoculated tissue during CP analysis. Extra prints of petiole cross-sections for certain samples represent prints of a new cross-section of the same tissue due to poor printing of the first cross-section. Bars under M and U designations indicate samples from plants that were paired by approximation of their plastochron indices. **A,** Experiment 1, showing results from plants harvested at 4 and 11 days post inoculation (DPI). Samples above the asterisks indicate the stem section containing the shoot apex; the stem section next to these samples is the corresponding section from below the shoot apical section. **B,** Experiment 2, showing results from plants harvested at 2.5 DPI.

the determinant(s) for this attenuation is the nucleotide sequence itself or the protein encoded by that sequence remains to be determined for the M strain.

MATERIALS AND METHODS

Virus strains and host varieties.

The M and U1 strains of TMV were obtained from previously described sources (Holt *et al.* 1990). L19-TMV refers to progeny of the M-strain-infectious cDNA clone (Holt *et al.* 1990), which was purified from systemically infected leaves of *N. tabacum* 'Xanthi nn' inoculated with the infectious transcript. The purification procedure for M, U1, and L19 was as described by Holt *et al.* (1990). *N. tabacum* 'Xanthi nn' and 'Xanthi NN' were the systemic and local lesion hosts, respectively.

Protoplast experiments.

Protoplasts were isolated from fully expanded leaves of 7- to 9-wk-old Xanthi nn grown in a growth chamber programmed for 16 h of light (23–24° C, ~152 $\mu E \cdot s^{-1} \cdot m^{-2}$) and 8 h of dark (23–24° C). The protoplast isolation procedure was as described by Hills *et al.* (1987). Equal numbers of protoplasts were treated with each virus (5 μg) for uptake as previously described (Loesch-Fries and Hall 1980). Protoplasts were incubated at 23–24° C under continuous illumination after virus inoculation. For CP determinations, protoplasts were sonicated in buffer (0.14 M NaCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl, and 0.05% [v/v] Tween 20), and the total protein concentrations were determined by Bradford assay (Bio-Rad, Richmond, CA). Extracts were then analyzed by double-sandwich enzyme-linked immunosorbent assay (ELISA; Clark and Bar-Joseph 1984) to determine CP content. Polyclonal antibody against TMV-CP was produced by injecting rabbits with purified TMV. The antibody was purified by DEAE-cellulose chromatography (Clark and Bar-Joseph 1984).

For analysis of 126- and 183-kDa proteins, 5×10^4 protoplasts (100 μl) were pelleted and frozen in liquid N_2 after the incubation buffer was removed; then 100 μl of high-salt buffer (0.03 M potassium phosphate, 0.4 M NaCl,

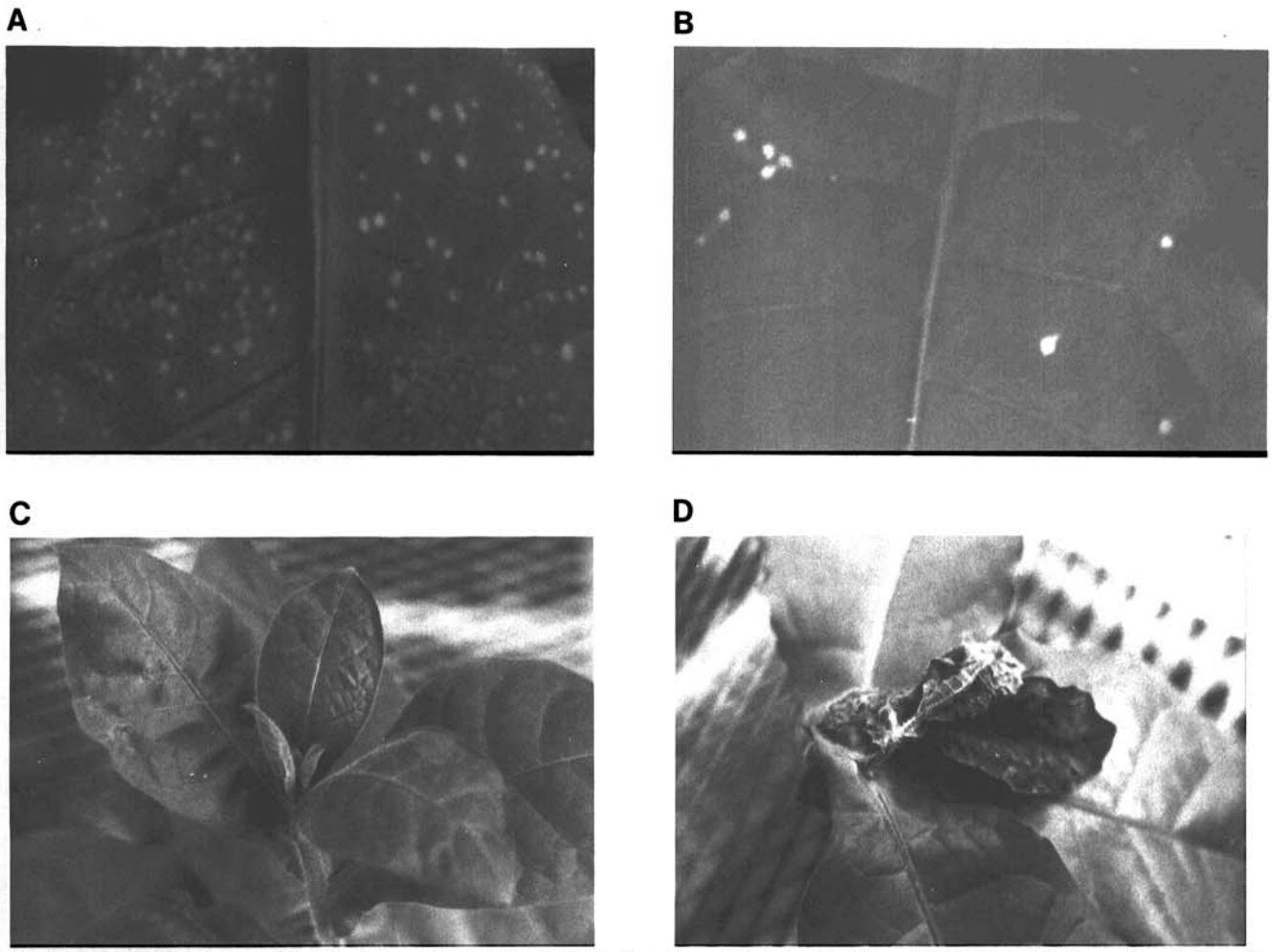

Fig. 7. Symptom development in *Nicotiana tabacum* 'Xanthi NN' plant tissue infected with M- or U1-TMV at 28° C or after shifting the growth temperature from 32 to 28° C. **A,** Plant inoculated and grown at 28° C. Left half of leaf inoculated with M-TMV, and right half of leaf inoculated with U1-TMV. **B,** Plant inoculated and grown at 32° C for 2 days postinoculation (DPI) and 28° C for 1.5 additional days. Left half of leaf inoculated with M-TMV, and right half of leaf inoculated with U1-TMV. **C and D,** Plants grown at 32° C for 5 DPI and 28° C for 5 DPI. **C,** Plant inoculated with M-TMV; **D,** Plant inoculated with U1-TMV.

10 mM β-mercaptoethanol, pH 7.5) was added. The protoplasts were then sonicated after thawing, centrifuged at 10,000 × *g* for 5 min, and the supernatant was saved. Protein concentrations were determined as described above. Glycerol was then added to the samples to a final concentration of 5% (w/v) together with 1 μl of bromophenol blue, and the samples were immediately analyzed by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5% [w/v] polyacrylamide). Following electrophoresis, proteins were transferred to nitrocellulose (Towbin *et al.* 1979), and the blotted proteins were probed with a 1:100 dilution of a polyclonal antibody (Ab) (IgG fraction) against an in-frame translational fusion of seven amino acids from the gene 10 protein of bacteriophage T7 and a portion of the TMV 126-kDa protein (amino acids 63–423; antiserum supplied by Hal Padgett, Washington University, St. Louis, MO). The anti-126-kDa protein serum had been purified by passage over a protein A/G column according to the manufacturer's specifications (Pierce, Rockford, IL). The anti-126-kDa protein polyclonal IgG was unable to immunoprecipitate BMV translation products but capable of binding to a β-galactosidase fusion protein containing most of the N-terminal sequence of the TMV 126-kDa protein in Western blot analysis (the region including amino acids 63–310 of the 126-kDa protein; G. Li and R. Nelson, data not shown). The β-galactosidase fusion construct was supplied by W. O. Dawson (University of Florida, Lake Alfred, FL), and the fusion protein was affinity purified over *p*-aminobenzyl 1-thio-β-D-galactopyranoside agarose by J. Sherwood (Oklahoma State University, Stillwater, OK). Immunoblots were probed according to the Bio-Rad protocol modified as follows; the blots were allowed to incubate in primary Ab (anti-126-kDa protein IgG) overnight at room temperature and in a 1:7,500 dilution of secondary polyclonal Ab (goat anti-rabbit IgG, Promega, Madison, WI) for 4 h at room temperature.

Whole plant experiments.

Xanthi nn plants served as a systemic host for studies of virus accumulation in inoculated and systemically infected tissue. Biological activity of the virus inocula was standardized by inoculating Xanthi NN plants (hypersensitive hosts) and counting necrotic lesions. Xanthi nn plants at the same physiological stage of growth were paired by approximating their plastochron indices (Erickson and Michelini 1957). Procedures for inoculating plants were as described by Nelson *et al.* (1987) except that the pH of the inoculum buffer was 7.8.

Table 1. Accumulation of viral products after infection by the M strain of TMV relative to the U1 strain in various plant tissues

Viral product	Leaves	
	Inoculated	Systemic
Protein accumulation		
126- and 183-kDa proteins	M ≅ U1[a]	ND[b]
MP[c]	M ≅ U1	ND
CP[d]	M ≅ U1[a]	M < U1
CP in petioles (tissue prints)	M < U1	M < U1
RNA accumulation		
(+) Strand genomic	M ≅ U1	M < U1
(+) Strand genomic and subgenomic	M ≅ U1	M < U1
(−) Strand genomic	M ≅ U1	M < U1
Infectious virus		
Specific infectivity	M ≅ U1	M ≅ U1

[a] Also observed for protoplasts infected with virus.
[b] Not determined.
[c] Movement protein.
[d] Coat protein.

Accumulation of CP and specific infectivity assay.

Xanthi nn plants were inoculated with 0.1 μg/ml of U1-TMV or 0.3 μg/ml of M-TMV at 33 days postplanting (DPP). After inoculation the plants were kept in the greenhouse. At 4 and 11 days postinoculation (DPI) the two inoculated leaves and systemically infected leaves from each plant were removed, combined as necessary, and stored at −70° C. Tissue was later extracted in buffer approximating phosphate-buffered saline (0.14 M NaCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, and 2.7 mM KCl, pH 7.4). The extract was centrifuged (10 min at 10,000 × *g*), and the supernatant was assayed for CP by ELISA. For three of the five replicates for each virus, the extracted tissue was also inoculated onto half leaves of a local lesion host (Xanthi NN). The opposite half leaf was inoculated with a suspension of diluted, purified U1 virus. Lesion counts were taken at 3 DPI, and the ratio of lesions produced for each leaf was determined for two leaves per replicate. Values are given as means ± standard deviations for the three replicates.

CP and MP accumulation.

Xanthi nn plants were inoculated with virus 43 DPP. Virus inoculum concentrations were 0.25 μg/ml for M and 0.125 μg/ml for U1. After inoculation, the plants were kept in a greenhouse. At various DPI the two inoculated leaves from each plant were removed, combined, and stored at −70° C. The samples were extracted for CP and MP determinations according to the procedure of Deom *et al.* (1990) with the following modifications. The tissue was not further triturated in a ground-glass homogenizer after extraction in grinding buffer. The supernatant (S1) was analyzed for the presence of CP by ELISA. The pellet containing the crude cell wall fraction was washed as described but not further extracted. The final cell wall fraction (P1) was analyzed for the presence of MP by Western blot analysis with a primary rabbit polyclonal Ab IgG produced against a 19 amino acid peptide corresponding to residues 205–223 of the MP sequence (Deom *et al.* 1987; antiserum supplied by C. M. Deom) and a secondary alkaline phosphatase-conjugated polyclonal Ab IgG to rabbit IgG (heavy-chain specific; Promega). Total protein concentrations for P1 fractions were determined by bicinchoninic acid protein assay (Pierce, Rockford, IL).

CP (tissue print) and viral RNA analyses.

Xanthi nn plants were inoculated with virus 43 DPP. Virus inoculum concentrations were 1.0 μg/ml for M and 0.25 μg/ml for U1. After inoculation, plants were kept in a greenhouse. At various DPI, the two inoculated leaves from each plant were excised, tissue prints made of cross-sections of the petioles (Cassab and Varner 1987; Ye and

Varner 1991), and half leaves from each combined and stored at −70° C. The second through fourth (for the 4 and 6 DPI sampling times) and second through sixth (for the 11 DPI sampling time) systemically infected leaves were excised, and half leaves of each leaf were combined and frozen at −70° C for further analysis. For tissue print analysis, freehand longitudinal sections of the stem were made from approximately the first node above the top inoculated leaf to the shoot apex. Tissue prints were probed for TMV-CP accumulation with a rabbit polyclonal Ab against TMV purified over a DEAE-cellulose column followed with an alkaline phosphatase-conjugated polyclonal Ab IgG to rabbit IgG (heavy-chain specific; Promega). Blots were probed according to the manufacturer's protocol (Promega 1989/1990).

Total RNA was extracted from tissue sampled at 4 DPI according to the protocol of Logemann *et al.* (1987) and from 6 DPI tissue according to the protocol described by Palukaitis (1984). The protocol described by Logemann *et al.* gave more intact RNA as determined by denaturing formaldehyde gel electrophoresis (data not shown). RNA was quantitated spectrophotometrically, and the RNA and nitrocellulose sheets were prepared for slot blot analysis according to Sambrook *et al.* (1989). Samples were loaded onto the nitrocellulose using a Minifold II slot blot device (Schleicher & Schuell, Keene, NH). Complementary DNA clones of M-TMV were used as templates to produce ^{32}P-labeled transcripts according to a standard procedure (Promega 1989/1990). Blots were probed with transcripts specific for the 5′ end of the plus strand of M-TMV (nt 1–528) or its complement or the 3′ end of the plus strand of M-TMV (nt 3332–6395) or its complement. Standard protocols were used during prehybridization, hybridization, and washing steps (Sambrook *et al.* 1989). Radiation from blots was quantitated using an Ambis radioanalytic imaging system (Ambis Systems, San Diego, CA). The amounts of viral RNA were standardized by probing separate blots with a cloned soybean 18S ribosomal cDNA (Eckenrode *et al.* 1985), which had been labeled with ^{32}P by random priming (supplied by M. Rincon).

In a separate experiment, Xanthi nn plants were inoculated with virus 41 DPP. Virus inoculum concentrations were 0.75 μg/ml for M and 0.25 μg/ml for U1. After inoculation, plants were kept in a greenhouse. At 2.5 DPI, tissue prints were made from cross-sections of the petioles from the inoculated leaves, the stems above the top inoculated leaves, and the petioles from the third leaf above the top inoculated leaf. Tissue prints were analyzed as described above.

Virus accumulation after temperature shift.

Xanthi NN plants were inoculated with a virus inoculum titer that was standardized by inoculating M and U1 strains on separate halves of individual leaves and comparing necrotic lesion numbers. Fully expanded leaves were inoculated, and plants were placed in growth chambers maintained at 32° C (16 h light and 8 h dark). At various times, plants were moved from the growth chamber to the greenhouse, where the maximum temperature was approximately 28° C. The time of appearance and location of necrotic areas were recorded.

ACKNOWLEDGMENTS

We thank Hal Padgett for supplying antiserum against the 126- and 183-kDa TMV proteins, C. Michael Deom for supplying antiserum against the movement protein of TMV, Magaly Rincon for the labeled 18S ribosomal RNA probe, and Frank Coker for technical assistance and maintenance of plants in the greenhouse. Cuc Ly and Glenn Elmore are thanked for production of many of the figures and Allyson Wilkins for typing and preparation of the manuscript.

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