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Accumulation of Mild and Severe Strains of Tobacco Mosaic Virus in Minor Veins of Tobacco

Xin Shun Ding,[1] Michael H. Shintaku,[2] Shelly A. Arnold,[1] Richard S. Nelson[1]

[1]Plant Biology Division, Samuel Roberts Noble Foundation, P. O. Box 2180, Ardmore, OK 73402 U.S.A.; [2]College of Agriculture, University of Hawaii at Hilo, 200 W. Kawili, Hilo, HI 96720 U.S.A.
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The Masked (M) and more severe common (U1) strains of tobacco mosaic virus (TMV) accumulate at similar rates in protoplasts or inoculated leaves of *Nicotiana tabacum* 'Xanthi nn' (R. S. Nelson, G. Li, R. A. J. Hodgson, R. N. Beachy and M. H. Shintaku, 1993, Mol. Plant-Microbe Interact 6:45-54). We now have compared accumulation of progeny virus from a transcript of an M-TMV cDNA clone (M^{IC}-TMV) with U1-TMV in chlorotic lesions of inoculated, mature leaves, and determined that M^{IC}-TMV accumulated in fewer vascular parenchymal (VP) and companion (C) cells within the predominant class of veins in these leaves. Independent of virus strain, the proportion of infected VP cells to infected C cells was approximately 5 to 1 early after inoculation. In systemically infected leaves, the proportion of infected VP cells to infected C cells was approximately 1 to 1 due mainly to an increase in the number of C cells infected. The difference in number of VP cells infected by M^{IC}-TMV compared with U1-TMV in inoculated leaves reflects a cell type-specific delay, not predicted from results of protoplast or leaf extract analyses. The presence of the 126-kDa protein of M^{IC}-TMV in those VP cells where M^{IC}-TMV accumulated indicated that the factors necessary for translation of viral RNA were present. Overall, fewer C cells were infected in the inoculated leaves compared with systemically infected leaves, which suggests that TMV does not enter many of the C cells of inoculated leaves or that viral replication is inhibited in these specific cells.

Additional keywords: companion cells, long-distance virus movement, phloem, symptom determinants, vascular parenchymal cells, tobamovirus.

The number of studies on the mechanisms of cell-to-cell movement and long-distance movement, sometimes called systemic or leaf-to-leaf movement, of plant viruses have increased in the last few years, advancing our understanding of both of these phenomena (Robards and Lucas 1990; Hull 1991; Maule 1991; Jackson *et al.* 1991; De Jong and Ahlquist 1991; Deom *et al.* 1992; Dawson and Hilf 1992; Meshi *et al.* 1992; Leisner and Turgeon 1993; Citovsky 1993). However, our understanding of long-distance movement lags behind our understanding of cell-to-cell movement. It is known that both host and viral factors are necessary for long-distance movement. Host factors influencing long-distance movement have been demonstrated for cowpea chlorotic mottle virus (CCMV) in soybean (Kuhn *et al.* 1981; Goodrick *et al.* 1991), potato leafroll virus (PLRV) in potato (Barker 1987), cauliflower mosaic virus (CaMV), and turnip crinkle virus (TCV) in *Arabidopsis thaliana* (Leisner *et al.* 1993; Simon *et al.* 1992), maize dwarf mosaic virus in maize (Lei and Agrios 1986; Law *et al.* 1989), and cucumber mosaic virus (CMV) in pepper (Dufour *et al.* 1989). Although these host factors have not been characterized, in some cases the locations within the movement-restrictive plant where the effect was manifest have been identified. Goodrick *et al.* (1991) determined that CCMV was restricted from accumulating in minor vein tissue of a resistant host. Thus, the bundle sheath (BS) cell-vascular parenchymal (VP) cell or BS cell-companion (C) cell boundary appeared to be the site of this movement restriction. Derrick and Barker (1992) determined that in some PLRV-resistant potato lines grown from PLRV-infected tubers, cells in external phloem bundles were generally not infected.

Several viral factors have been implicated as determinants of long-distance movement. These include the RNA encoding the coat protein (CP), or the CP itself, of tobacco mosaic virus (TMV; Siegel *et al.* 1962; Takamatsu *et al.* 1987; Dawson *et al.* 1988; Hilf and Dawson 1993), CaMV under certain conditions (Qiu and Schoelz 1992), beet necrotic yellow vein virus (Quillet *et al.* 1989), and tobacco rattle virus (Hamilton and Baulcombe 1989). In addition, the RNA encoding the movement protein (MP), or the MP itself, can affect the long-distance movement of TMV and CaMV (Saito *et al.* 1990; Qiu and Schoelz 1992). CaMV genes II, V, and VI and the large intergenic region of CaMV (Schoelz *et al.* 1991; Qiu and Schoelz 1992; Wintermantel *et al.* 1993); the 5′ leader of barley stripe mosaic virus RNA γ (Petty *et al.* 1990); and RNA 1 or its protein product of CMV (Lakshman and Gonsalves 1985) and CCMV (Wyatt and Kuhn 1980) have also been implicated in affecting long-distance movement. However, the site or sites within the plant where these viral factors impact long-distance movement have not been determined.

We have been studying the phenotypes of symptom formation and ability to accumulate in systemically infected leaves displayed by the M strain of TMV on *Nicotiana tabacum* 'Xanthi nn' or 'Xanthi NN' in comparison with the more severe U1 strain of TMV. The M strain produces, at most, a mild chlorosis on systemically infected leaves, whereas the U1 strain produces a severe, light-green, dark-green mosaic (Holmes 1934; Siegel and Wildman 1954). By exchanging

Corresponding author: R. S. Nelson.

cDNA fragments between clones of M-TMV and U1-TMV, Holt *et al* (1990) determined that the mild phenotype genetically mapped to the open reading frame encoding the 126- and 183-kDa proteins in or between the domains that code for a putative methyltransferase and helicase (see Dolja and Carrington 1992 for a review of sequence comparisons between RNA helicases and further references). Expression of these proteins has been shown to be important for viral replication (Ishikawa *et al.* 1986), and thus it was surprising that Nelson *et al.* (1993) found in comparing the mild strain with the severe strain that similar amounts of infectious virus, and viral proteins and RNAs were present in extracts from inoculated tobacco leaves or protoplasts and that they accumulated at similar rates. They also determined, however, that there was a slight delay in virus accumulation in the upper leaves and in the vascular traces of the petioles of inoculated leaves infected with the mild strain compared with the severe strain. The lack of accumulation of M^{IC}-TMV in tissue infected following vascular transport thus was positively correlated with the mild symptom phenotype. These results also suggested that the delay in accumulation in the vascular traces of petioles and in upper leaves may be due to less virus exiting the inoculated leaves, although the possibility of inhibited virus replication in systemically infected tissue could not be excluded.

Identifying barriers to systemic accumulation of plant viruses is important both to further characterize the ontogeny of

Table 1. Accumulation of M^{IC}-TMV and U1-TMV over time in chlorotic lesions of mature inoculated leaves and in tissue from systemically infected leaves[a]

Tissue	Virus	Day post inoculation		
		3	4	5
Chlorotic lesion[b]				
	U1	1.6 ± 0.3	3.0 ± 0.2	–
	M^{IC}	1.5 ± 0.2	2.7 ± 0.2	–
Systemic leaf[c]				
	U1	–	4.3 ± 0.4	6.3 ± 0.5
	M^{IC}	–	1.6 ± 0.4	2.7± 0.3

[a] Plants at similar developmental stages were paired, inoculated, and harvested as described in the materials and methods. TMV accumulation was measured by double sandwich direct enzyme-linked immunosorbent assay.

[b] Values for extracts from chlorotic lesions from inoculated leaves represent the mean ± S.E. (µg/lesion) for 25 lesions from five leaves on five plants per virus inoculum. No statistically significant difference was found between the mean values as determined by the Mann-Whitney U test.

[c] Values for extracts from systemic leaves (second leaf above the inoculated leaf) of plants inoculated with U1- or M^{IC}-TMV represent the mean ± S.E. (µg/leaf disk) for values from five plants (five disks per plant). Virus accumulation in M^{IC}-TMV infected leaf tissue was less than that in U1-TMV infected leaf tissue; $P < 0.001$ for equality by Mann-Whitney U Test.

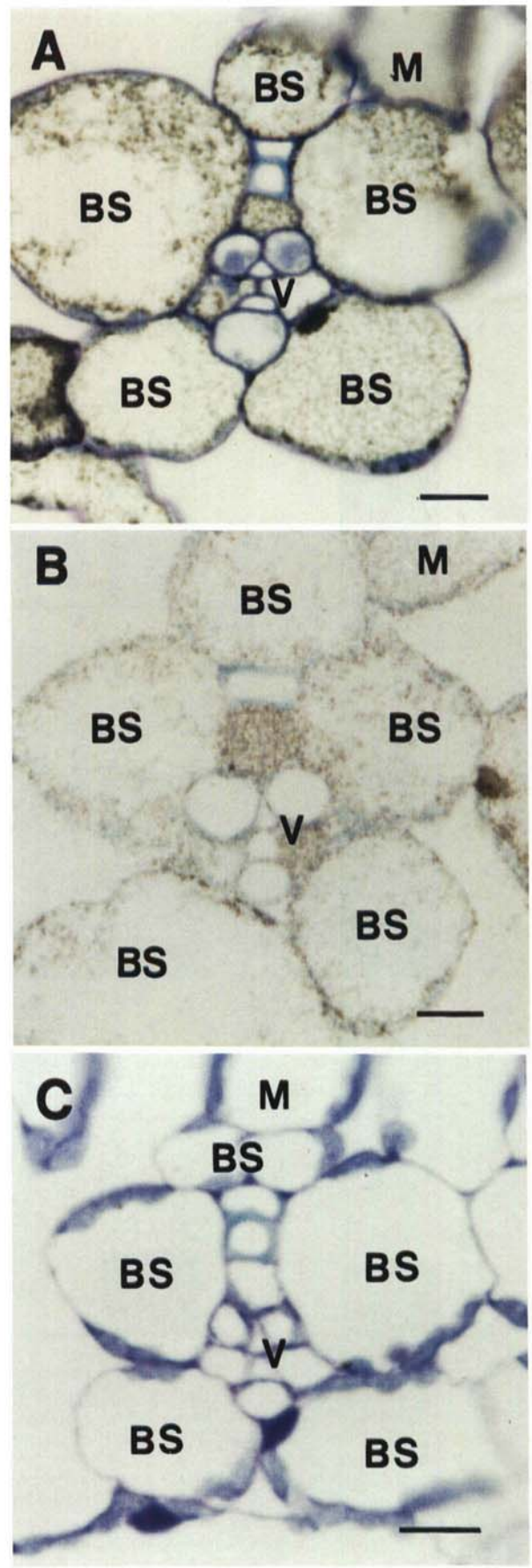

Fig. 1. Accumulation of virus in mesophyll (M), bundle sheath (BS), and minor vein (V) cells in M^{IC}- or U1-TMV infected chlorotic lesions at 3 days post inoculation. Analysis for virus was by immunocytochemistry using polyclonal antibody against TMV virions and goat anti-rabbit IgG gold (5 nm) conjugate followed by silver enhancement. Cells were stained with toluidine blue. **A**, A section prepared from a M^{IC}-TMV infected chlorotic lesion. **B**, A section prepared from a U1-TMV infected chlorotic lesion. **C**, A section prepared from a healthy Xanthi nn leaf. Bar = 12 µm.

virus infection in the plant host and as a source of information for the development of plants resistant to systemic infection. Previous cytological studies have not analyzed TMV accumulation in vein cells (e.g., Reinke and deZoeten 1991) or veins during the period of rapid systemic movement from inoculated leaves (e.g., Esau 1968). In this report we show that there are barriers to the accumulation of TMV in the VP and C cells of the minor veins of inoculated mature leaves of 'Xanthi nn' just prior to or during systemic disease appearance. VP and C cells of the minor veins are infected more slowly by M^{IC}-TMV than by U1-TMV. Regardless of virus strain, fewer C cells were infected than VP cells in inoculated leaves but not in systemically infected leaves, indicating that there is an impediment to infection of C cells in inoculated leaves.

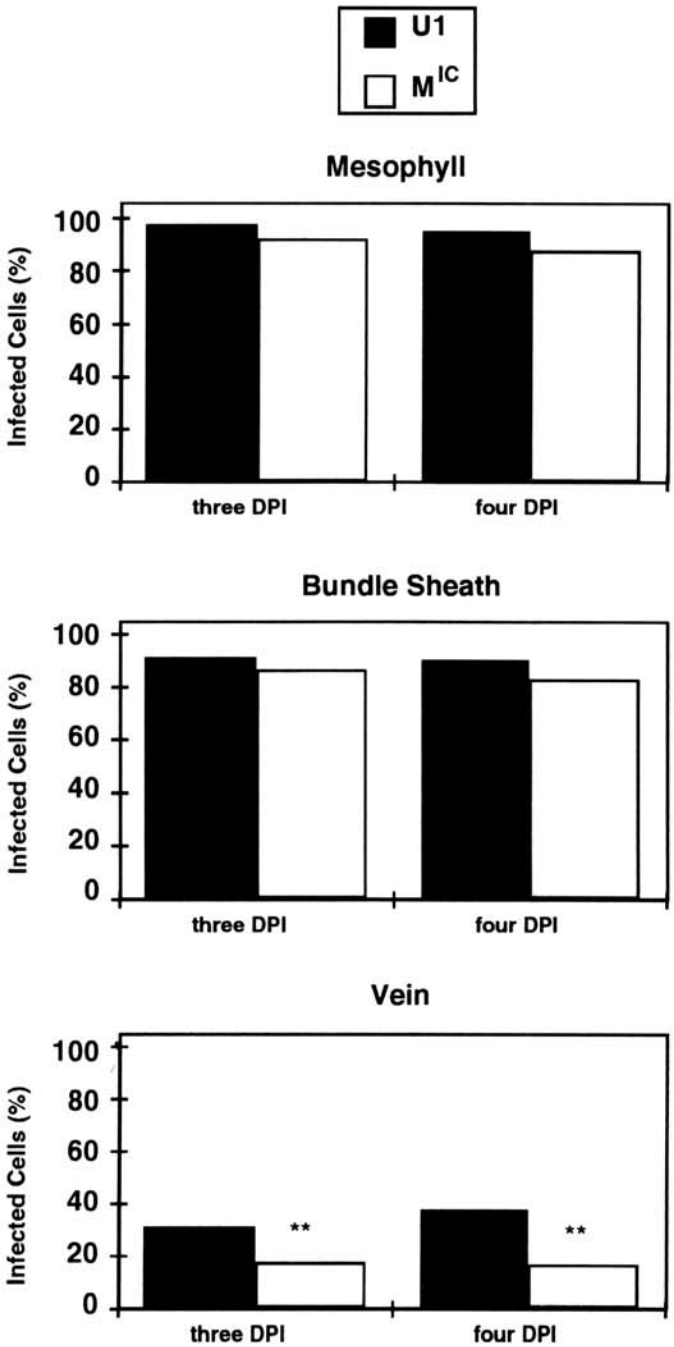

Fig. 2. Percentage of cells within a particular cell type infected with M^{IC}-TMV or U1-TMV over time from chlorotic lesions of inoculated leaves of *Nicotiana tabacum* 'Xanthi nn.' Tissue was analyzed as described in Figure 1. Values for mean percentage of cells infected were obtained from analysis of six to eight sections, two minor veins per section, and one section per chlorotic lesion, from M^{IC}-TMV or U1-TMV inoculated leaves at each sampling date. **, probability of equality < 0.01 by Mann-Whitney U test. DPI, days post inoculation.

RESULTS

Symptom phenotype and virus accumulation in inoculated and systemically infected tissue.

N. tabacum 'Xanthi nn' was inoculated with progeny virus from an infectious transcript of M-TMV cDNA (M^{IC}-TMV) or U1-TMV, and symptom development was observed. Mature leaves (leaf 5, numbering progresses toward shoot apex) inoculated with either virus showed chlorotic lesions (2–3 mm in diameter) by 3 days postinoculation (DPI) under growth chamber conditions. Leaf 7 of all U1-TMV inoculated plants showed mild vein-clearing symptoms at 4 DPI. However, the corresponding leaves of M^{IC}-TMV inoculated plants did not show vein-clearing symptoms until 5 DPI. At 9 DPI, the M^{IC}-TMV and U1-TMV inoculated plants showed, respectively, mild and severe mosaic symptoms, as described previously (Holmes 1934; Nelson *et al.* 1993).

At 3, 4, and 5 DPI, tissue from individual chlorotic lesions on inoculated leaves or tissue from systemically infected leaves were sampled at random and ELISA was used to determine virus concentrations in tissues analogous to those analyzed by immunocytological methods. The amount of virus that accumulated in M^{IC}-TMV infected lesions of inoculated leaves at 3 and 4 DPI was similar to that in U1-TMV infected lesions (Table 1). However, in systemically infected tissue at 4 and 5 DPI, the accumulation of M^{IC}-TMV was significantly less than that of U1-TMV (Table 1).

Immunocytological localization of virus proteins in inoculated leaves.

For immunocytological studies of virus accumulation, 92 chlorotic lesions were sampled from the inoculated leaves of 28 plants from three experiments and within these lesions approximately 90% of the minor veins were composed of between 9 and 12 cells in transverse section. A small percentage of veins were composed of 14–18 cells. Veins having 9–12 cells and 14–18 cells are defined as class V or class IV veins, respectively, by Ding *et al.* (1988). Silver-enhanced, gold-conjugated antibody immunocytochemical localization of TMV in fixed, plastic-embedded tissue sections resulted in a brown to gray/black signal within the cell interior in virus-infected tissue (Fig. 1A and B). The dispersion of signal into the vacuolar regions is likely due to the fixation conditions. No labeling was seen in cells of virus-free tissue (Fig. 1C).

In an attempt to identify the site of barriers that reduce systemic movement of M^{IC}-TMV, we initially recorded virus-infected cells as a percentage of all cells for mesophyll cells, bundle sheath (BS) cells, and vein cells within the mestome sheath. In tissue obtained from chlorotic lesions at 3 and 4 DPI, the proportion of infected cells in M^{IC}-TMV infected class IV or V veins was significantly less than that observed

for veins infected with U1-TMV (Fig. 2). Results from a second experiment gave similar results for BS or vein cells from chlorotic lesions at 4 DPI, U_1-TMV; BS, 85.5 ± 6.1; vein, 24.1 ± 5.8; M^{IC}-TMV; BS, 87.0 ± 5.2; vein, 13.8 ± 3.2; $P > 0.1$ for equal accumulations of M^{IC}-TMV and U1-TMV in BS cells, $P < 0.1$ for equal accumulation of M^{IC}-TMV and U1-TMV in vein cells. In systemically infected leaves the percentage of infected mesophyll, BS, and vein cells in M^{IC}-TMV infected samples were all lower than that for U1-TMV infected samples at 4 DPI (data not shown).

Classification of cells and distribution of virus within archetypical class V veins.

It was clear from the examination of minor veins that antibody against TMV virions was preferentially localized in specific cells (Fig. 1B) and that we needed to identify these cells. Previous reports (Turgeon and Helper 1989; Beebe and Evert 1992) indicated that vascular parenchymal (VP) cells and companion (C) cells within veins could be identified through differential plasmolysis because C cells and their associated sieve elements have much higher solute potentials than the neighboring parenchymal cells. We utilized this technique to identify VP and C cells within archetypical class V veins of tobacco. Without sorbitol treatment, all cells within a class V vein were fully turgid when examined by electron microscopy (Fig. 3A). However, after tissues were treated with 1 M sorbitol, particular cells within the vein became plasmolyzed (Fig. 3B). These cells were numbered 4, 6, and 8 and correspond to VP cells (Fig. 3A). This pattern was observed in seven veins by electron microscopy and 38 veins by light microscopy (data not shown). On occasion, cell number 4 was a xylem tracheary element and not a VP cell and could be identified by electron microscopy because of the presence of lignified cell walls with pits or by light microscopy after staining sections with toluidine blue. Thus, from our studies and published information regarding minor vein structures in tobacco (Ding *et al.* 1988) we determined that an archetypical class V vein contained a file of xylem tracheary elements (cell nos. 2 and 3, Fig. 3A) and two sieve elements (cell nos. 10 and 11, Fig. 3A) surrounded by a single alternating set of VP and C cells, three of each.

To determine which cell type accumulated TMV, a subsample of sections which had been probed with antiserum against TMV virions and which contained veins having the archetypical pattern of a class V vein (e.g., Fig. 3B) was reexamined by light microscopy. In class V veins within chlorotic lesions from leaves inoculated with either virus the proportion of infected VP cells was always much greater than the proportion of infected C cells, ranging from a mean ratio of 5.5:1 at 3 DPI to 2.9:1 at 7 DPI (Table 2). In class V veins from systemically infected leaf tissue more similar percentages of VP and C cells were infected, ranging from a mean of 1.4:1 at 4 DPI to 1.1:1 at 5 DPI (Table 2). A representative example of a systemically infected vein is shown (Fig. 4).

Using the same immunocytological procedures described above for virion detection, it was determined that the same cells that were labeled using antiserum against TMV virions were labeled with antiserum against the 126-kDa protein of TMV (Fig. 5A and B), and the same result was observed in larger fields of view (data not shown). No labeling was seen in healthy cells (Fig. 5C) or infected cells probed with preimmune serum (data not shown). Results from the examination of sections for TMV accumulation enhanced by immunocytochemistry under the electron microscope supported light microscopy results, indicating that TMV could be present in large amounts in VP cells but absent in adjacent C cells (compare Fig. 5D and B).

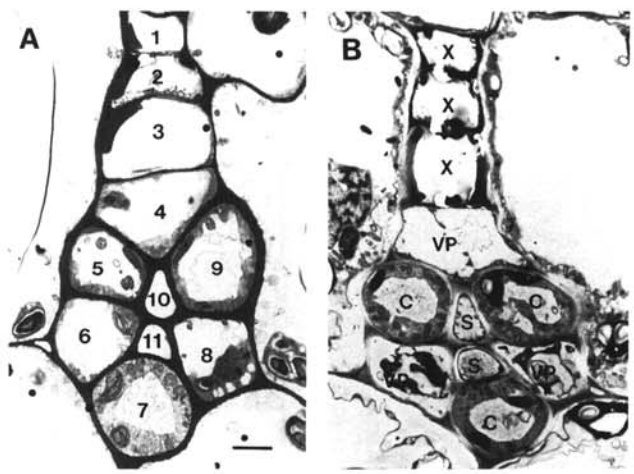

Fig. 3. Electron micrographs of archetypical class V veins from mature leaf sections of *Nicotiana tabacum* 'Xanthi nn' with or without 1.0 M sorbitol treatment before fixation. **A**, A vein not treated with 1.0 M sorbitol prior to fixation. Cells are numbered to allow text discussion. Note that cellular constituents for cells 4–9 are flush with the cell wall, indicating no plasmolysis. **B**, A vein treated with 1.0 M sorbitol prior to fixation. Note that the plasmalemma of cells 4, 6, and 8 (see Fig. 3A for numbering nomenclature) are completely separated from their cell walls and thus are plasmolyzed while cells 5, 7, and 9 are not. By this analysis, cells 4, 6, and 8 are considered to be vascular parenchymal (VP) cells while cells 5, 7, and 9 are considered to be companion (C) cells. Cells 2 and 3 are xylem tracheary elements (X). Cells 10 and 11 are sieve elements (S). Magnification 2,500×; Bar = 4 µm.

DISCUSSION

We previously determined that M-TMV was delayed in accumulation in the vascular trace of petioles of inoculated

Table 2. Percentage of infected VP and C cells within archetypical class V veins of M^{IC}- or U1-TMV infected Xanthi nn leaves

Tissue	DPI	Virus	No. of veins examined	Cell type	
				VP[a]	C
Chlorotic lesion					
	3[b]	U1	16 (12)[e]	89 ± 5[f]	25 ± 8
		M^{IC}	18 (15)	55 ± 9	7 ± 4
	7[c]	U1	10 (8)	85 ± 8	30 ± 14
		M^{IC}	8 (5)	60 ± 10	21 ± 11
Systemic leaf					
	4[d]	U1	14 (8)	80 ± 7	50 ± 10
		M^{IC}	6 (4)	61 ± 10	50 ± 14
	5[d]	U1	9 (6)	64 ± 12	52 ± 14
		M^{IC}	8 (6)	56 ± 14	50 ± 17

[a] VP, vascular parenchymal cell; C, companion cell (see Fig. 4 for vein structure and cell type designation).
[b] Samples from second and third experiments.
[c] Samples from third experiment.
[d] Samples from second experiment.
[e] Numbers in parenthesis indicate the number of sections from which the archetypical veins were selected and examined.
[f] Values are means ± standard errors.

leaves (Nelson *et al.* 1993), suggesting the possibility that the delay in virus accumulation in systemic tissue may be due to an effect manifested in the inoculated leaves. Our previous results showed that replication of M^{IC}-TMV in protoplasts and inoculated leaves over time was similar to U1-TMV (Nelson *et al.* 1993) and our current results indicate the mild and severe strains accumulated similarly in chlorotic lesions (Table 1). In contrast to estimating gross accumulation from homogenized tissue we proceeded to study virus accumulation in specific cells by immunogold labeling and silver enhancement. We determined that M^{IC}-TMV accumulated more slowly or to lower levels than U1-TMV in vascular parenchymal (VP) and companion (C) cells of minor veins of inoculated leaves at times just preceding or during systemic symptom appearance (Figs. 1 and 2 and Table 2). The minor veins we examined were mainly class V veins with some class IV veins as defined by Ding *et al.* (1988). Class V veins represent approximately 90% of the veins seen in sections from mature tobacco leaves (X. S. Ding and R. S. Nelson, unpublished results). They are the major site of photoassimilate loading and export (Turgeon 1989) and are the first veins virus would contact following cell-to-cell radial spread. Since these veins represent the majority of the venal network of a mature leaf, resistance to vascular invasion of the class V veins could lead to a significant decrease in inoculum for long-distance invasion. Also, since the cells within these veins account for a small minority of the total number of cells present in a leaf, this decrease in mild strain accumulation would not easily be detected in total extracts from chlorotic lesions (Table 1) or from whole leaves (Nelson *et al.* 1993).

Since fewer VP and C cells in chlorotic lesions accumulated M^{IC}-TMV than U1-TMV, both cell types could be considered potential barriers for systemic infection by M^{IC}-TMV. The possibility exists that the mild strain may not replicate efficiently in the VP cells of inoculated leaves; however, the determination that the VP cells which accumulated M^{IC}-TMV CP also accumulated 126-kDa protein (Fig. 5) suggests that M^{IC}-TMV is not simply accumulating in these cells during transport but is being translated and therefore likely replicating. Thus the cellular machinery is likely present to allow M^{IC}-TMV replication in these cells. This, along with evidence that M^{IC}-TMV accumulates in VP and C cells of systemically infected leaves (Fig. 4) suggests that the delay in accumulation of M^{IC}-TMV in VP or C cells in mature leaves may be due to impeded movement into these cells and not an inability to replicate. There is evidence that a barrier to movement exists between BS and VP cells from other systems. Ding *et al.* (1992) found that MP from TMV expressed in transgenic plants increased the size exclusion limit of plasmodesmata between mesophyll cells and BS cells but not of plasmodesmata connecting BS cells with phloem parenchymal cells. Goodrick *et al.* (1991) identified the BS-VP or -C boundary as the site of effect of a host resistance gene. Our work suggests that crossing this cell boundary may be critical for vascular accumulation of M^{IC}-TMV. Ding *et al.* (1992) suggested that the CP may be the additional factor necessary to allow passage across this barrier based on the literature showing that the CP of TMV is necessary for phloem-mediated transport (Siegel *et al.* 1962; Takamatsu *et al.* 1987; Dawson *et al.* 1988). However, this cannot explain the observations for M^{IC}-TMV, since the CPs for M^{IC}-TMV and U1-TMV are identical and accumulate to high levels (Holt *et al.* 1990; Nelson *et al.* 1993).

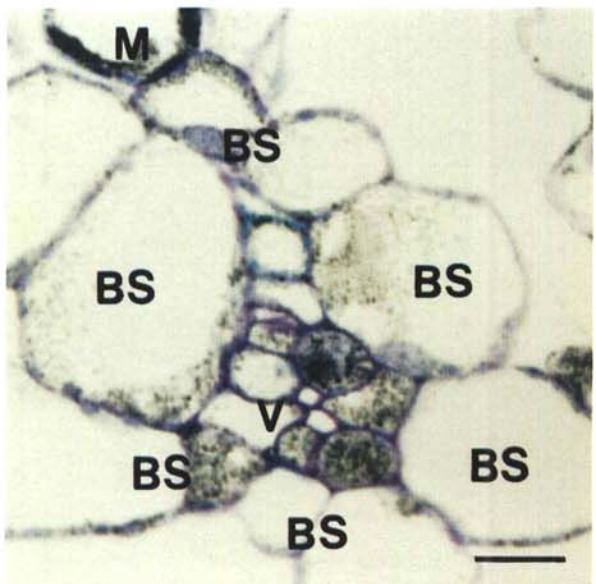

Fig. 4. Accumulation of virus in mesophyll (M), bundle sheath (BS), and minor vein (V) cells in U1-TMV infected systemic tissue at 5 days post inoculation. Analysis was performed as described in Figure 1. Bar = 12 µm.

Both viruses accumulated in fewer C cells than VP cells in inoculated leaves (Fig. 1 and Table 2). McCauley and Evert (1989) noted that particles of an unidentified virus were present in phloem parenchymal and mesophyll cells of minor veins of mature leaves from potato but rarely in C cells and never in sieve elements. Our results support the idea that the connections between the VP cells and the C cell-sieve element complex are modified for virus entry and, at a minimum, delay virus infection of C cells in mature leaves. Assuming C cells are in the direct route for systemic TMV movement, as they are thought to be for photoassimilate transport, it is possible that the reduction in the number of infected C cells compared with the number of VP cells limits systemic infection for both viruses and more severely for M^{IC}-TMV. Previously, Cohen *et al.* (1957) suggested that small numbers of infectious units give rise to a systemic infection and the VP-C cell boundary should be considered as a possible location where virus accumulation could be limited. However, since a small number of plasmodesmata exist between VP cells and sieve elements in class III veins of tobacco (Ding *et al.* 1988), it is possible that vascular movement proceeds by this route in class V veins and the limited infection of these cells by M^{IC}-TMV may result in limited systemic infection. However, McCauley and Evert (1989) observed no plasmodesmal connections between phloem parenchyma and sieve elements within seventh-order veins in potato. These veins are analogous in structure and cell number to class V veins of tobacco which we studied. Thus, further studies are necessary to characterize the plasmodesmata between the C cell-sieve element complex and the VP cells and BS cells in class V veins of *N. tabacum* and to investigate the form in which TMV moves through each cell type.

In contrast to the inoculated leaves where both strains of

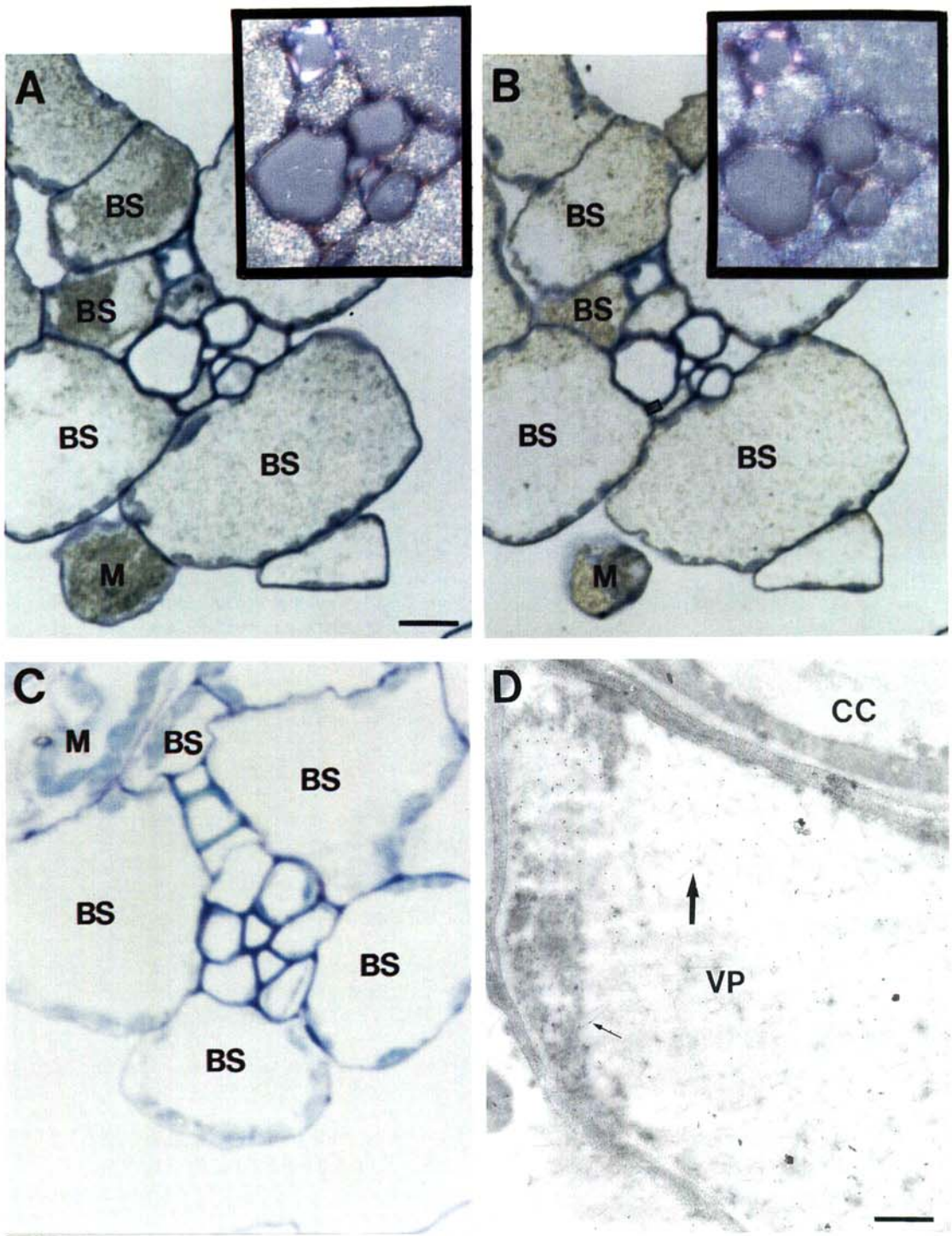

Fig. 5. Accumulation of viral coat protein (CP) and viral 126-kDa protein in bundle sheath and minor vein cells in serial sections prepared from a M^{IC}-TMV infected chlorotic lesion at 4 days post inoculation. **A**, A section probed with antibody against the U1-TMV 126-kDa protein. **B**, A section probed with antibody against TMV virions. Bar = 12 μm. Insets in **A** and **B** show signal in vein cells under polarized light. Signal intensity in C cells is similar to that observed in intercellular spaces. **C**, A section from healthy tissue probed with antibody against the U1-TMV 126-kDa protein. **D**, Electron microscopic visualization of the boxed area in **B** showing a strong deposition of gold particles over a vascular parenchymal (VP) cell that contains virus rods but not in a companion cell (CC) that contains no virus rods. Large arrow indicates virus rod and small arrow indicates gold particles. (Magnification, 32,000×; Bar = 620 nm). Cells were stained with toluidine blue in panels A, B, and C.

TMV accumulated in only a small number of C cells compared with VP cells, the results from the analysis of systemically infected tissue indicate that both strains of TMV exited sieve elements into similar numbers of VP and C cells (Table 2, Fig. 4). Free movement of virus into C cells from sieve elements might be expected since plasmodesmata between these cells are present in abundance (Ding *et al.* 1988; McCauley and Evert 1989; Beebe and Evert 1992), and the size exclusion limits of the plasmodesmata are greater than at other cell-to-cell interfaces (Kempers *et al.* 1993). However, movement from the C cell-sieve element complex to VP cells in systemic leaves is more difficult to explain and should be discussed in context with what was observed in inoculated mature leaves. In mature (i.e., source) leaves of tobacco or potato, the plasmodesmal connections between the C cell-sieve element complex and the VP cells are few in number (Ding *et al.* 1988; McCauley and Evert 1989). If the importing (i.e., sink) leaves have a similar lack of connection between the C cell-sieve element complex and the VP cells it is difficult to understand how similar numbers of C cells and VP cells become infected if symplastic transport is involved. However, in sink leaves, class V veins are immature (Turgeon 1987; Ding *et al.* 1988) and movement of virus between the C cell-sieve element complex and VP cell might be freer than in mature leaves. Developmental loss of symplasmic connections is known to occur between the C cell-sieve element complexes and VP cells in class III veins in tobacco leaf sink tissue (Ding *et al.* 1988), and in maturing stem phloem of *Lupinus luteus* (van Bel and van Rijen 1994). Turgeon (1989) has suggested that the diameter of plasmodesmata in veins of importing leaves decreases as a leaf matures and the finding that accumulation of cauliflower mosaic virus, representing a large molecule or molecular complex, decreases earlier than that observed for the smaller photoassimilates in maturing leaves (Leisner *et al.* 1992) supports such a hypothesis. Thus both the higher number and larger size of the plasmodesmata between VP cells and C cells of sink leaves may explain the accumulation of TMV in the VP cells of this tissue. We are in the process of determining whether tobamoviruses accumulate in the same cell types in other hosts and whether other viruses accumulate in the same cells as TMV in *N. tabacum* 'Xanthi nn.'

MATERIALS AND METHODS

Host cultivars, virus strains, and antisera.

Nicotiana tabacum L. 'Xanthi nn' (systemic host) or *N. tabacum* L. 'Xanthi NN' (hypersensitive host) were used. Plants were routinely raised in a greenhouse in 12-cm pots in an artificial soil medium (Metro-Mix 350, Grace). M and U1 strains of TMV were obtained from previously described sources (Holt *et al.* 1990). U1-TMV from experiments of Holt *et al.* (1990) was inoculated onto Xanthi NN followed by extraction of a single lesion and inoculation onto a systemic host (Xanthi nn). Leaves from plants showing the expected symptom phenotype were harvested and virus purified as described by Asselin and Zaitlin (1978). M^{IC}-TMV refers to progeny of the M strain infectious cDNA clone (Holt *et al.* 1990) and was purified as described (Holt *et al.* 1990; Nelson *et al.* 1993). Rabbit polyclonal antiserum against purified TMV particles was purified by DEAE-cellulose chromatography (Clark and Bar-Joseph 1984). Polyclonal antiserum against TMV 126-kDa protein was supplied by Hal Padgett (Washington University, St Louis, MO; current address; Scripps Research Institute, La Jolla, CA) and purified as described by Nelson *et al.* (1993).

Virus inoculation, tissue sampling, and analysis of virus accumulation.

For immunocytochemical studies, three experiments were conducted utilizing either four plants (experiment I) or five plants (experiments II and III) for each virus strain inoculated. In each experiment, two mock-inoculated plants were used as controls. At 38 days post-planting (DPP), the leaves 4 and 5 of individual plants which were paired according to their developmental status, were inoculated with M^{IC}-TMV or U1-TMV (50 µg/ml, 50 µl/leaf). The inoculated plants were then grown in a growth chamber at 23–24° C with 16 hr of light (~152 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and 8 hr of dark. At various times after inoculation, two single chlorotic lesions were randomly sampled from leaf 5 of each virus-inoculated plant. Two similarly sized and positioned leaf pieces to those harvested from virus-inoculated plants were harvested from mock-inoculated plants. At 5 DPI (experiment I) or 4 DPI (experiment II), two leaf pieces were arbitrarily sampled from third-order veins (for a description of vein designations see Turgeon 1987 and Ding *et al.* 1988) approximately 5 cm from the base of the systemically infected leaf 7 of each plant. At 7 DPI (experiment I) or 5 DPI (experiment II), tissues were sampled again as above but from third-order veins about 4 cm from the base of leaf 7 of each plant. Tissues obtained were used for immunocytochemical analyses. At various DPI during the second experiment, leaves 5 and 7 were also sampled as described above and assessed for virus CP accumulation by ELISA (Clark and Bar-Joseph 1984).

Tissue fixation and embedding.

The fixation procedure was essentially as described by Westcot *et al.* (1993) with the following specifics and modifications. Tissues sampled as described in the previous section were immediately submerged individually in 1 ml of 4% (w/v) paraformaldehyde and 2.5% (w/v) sucrose in a 0.1 M phosphate buffer (PB), pH 7.3, in small glass tubes. Glutaraldehyde was omitted during fixation since it is known to decrease the antigenicity of various proteins and decreases the penetration of antibodies (Westcot *et al.* 1993; Wick 1993). The tubes were placed in a microwave oven (Kenmore, model no. 5658804180, 1450W, 60 Hz) and treated for 10 sec at high power. The fixative was discarded and the samples were washed 3 times in 0.06 M PB, pH 6.5 (15 min per wash). The fixed samples were dehydrated by placing them in a series of ethanol solutions (i.e., 30% ethanol, 30 min; 50% ethanol, 30 min; 70% ethanol, 30 min; 90% ethanol, 30 min; 100% ethanol, 20 min) all at room temperature (RT). The samples were then embedded for 20 hr at 52° C in LR White resin (London Resin Company, Hampshire, UK). Semi-thin (2 µm) and ultrathin (80–90 nm) sections were cut using glass knives, on a Reichert-Jung 2050 microtome or MT 6000 ultramicrotome, for examination of light or electron microscopy, respectively.

Immunogold labeling.

Procedures for immunogold labeling were as described by VandenBosch (1991) with the following specifics and modifi-

cations. For light microscopic studies, semi-thin sections were mounted on glass slides precoated with 2% gelatin or 0.1% poly-L-lysine. The sections were first incubated in 20 µl of 2% bovine serum albumin (BSA) in 0.01 M PB, pH 7.0, for 1 hr at RT. After three washes (5 min each) in 0.01 M PB containing 0.1% Tween 20, the sections were incubated overnight at 4° C in 10 µl of antibody solution (1:100 dilution of rabbit polyclonal antiserum against TMV in 1% BSA in 0.01 M PB, pH 7.0) per section. The sections were washed three times as above, incubated in 2% BSA for 15 min, and then 30 min in 5-µl drops per section of goat anti-rabbit IgG gold (5 nm) conjugate diluted 1:100 in 1% BSA solution in 0.01 M PB, pH 7.0 (BioCell Research Laboratories, Cardiff, UK). Silver enhancement of gold labeling was done by adding 5 µl of silver enhancing solution (BioCell Research Laboratories, Cardiff, UK) to each section followed by incubation for 10–15 min in the dark on ice. The sections were counterstained with 0.01% toluidine blue.

For electron microscopy, thin sections were mounted on slot (2 × 1 mm) nickel grids precoated with 0.5% Formvar. The blocking and washing buffers were the same as that used in light microscopy. Polyclonal antibody against TMV and goat anti-rabbit IgG gold (10 nm) conjugate were used, respectively, at 1:100 and 1:50 (v/v) dilutions in 1% BSA in 0.01 M PB, pH 7.0. Poststaining was done by floating grids, section side down, on drops of 2% uranyl acetate for 8 min and then Reynold's lead citrate for 3 min at RT.

Examination of leaf tissue sections.

In each semi-thin section, minor veins (class IV or V) with third-order veins at one side, and their neighboring mesophyll cells (cells in a circular area of appoximately 0.7 mm diameter) were examined and photographed with a Nikon Microphot-FX microscope and dedicated camera. Cells which showed silver-enhanced labeling (usually light brown to black spots) were considered to be infected cells. Differences between the proportion of infected cells for various cell types in M^{IC}-TMV or U1-TMV infected sections were analyzed for statistical significance using the Mann-Whitney U Test (Siegel 1956). All ultrathin sections were viewed and photographed in a Zeiss 10A electron microscope operating at 80 kV.

Differentiation of companion cells and vascular parenchymal cells by plasmolysis.

Procedures were as described by Beebe and Evert (1992) with following specifics and modifications. Five leaf pieces were randomly sampled from leaf 7 (about 17 cm in midrib length) of each of six greenhouse-grown Xanthi nn plants at 42 days after planting. The leaf pieces were immersed for 30 min in 1 M sorbitol at RT. Four leaf pieces collected as above were immersed in distilled water only and used as controls. After three washes for 10 min each in 0.1 M PB, pH 7.0, the tissues were fixed overnight in 2% glutaraldehyde in 0.1 M PB, pH 7.0 at 4° C followed by 40 min in 2% osmium tetroxide in 0.1 M PB, pH 7.0 at RT. Dehydration, embedding and sectioning and staining were the same as described above. A cell was considered to be plasmolyzed if its plasmalemma was found to be separated from the cell wall when observed by light or electron microscopy.

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