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Molecular characterization and biological function of the movement protein of tobacco mosaic virus in transgenic plants

(virus movement/plasmodesmata function/plant cell wall)

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ABSTRACT **We previously demonstrated, in transgenic tobacco plants, that the role of the movement protein (MP) of tobacco mosaic virus is to facilitate the cell-to-cell spread of viral progeny during infection. An analysis of different tissues of these transgenic plants indicated that the MP accumulated in leaf, stem, and root tissue. The highest levels were detected in older leaves. The relative levels of MP in leaf tissue from transgenic plants were equivalent to, or higher than, the levels of MP in tobacco mosaic virus-infected leaf tissue. Results of subcellular fractionation of homogenates of transgenic leaf tissue showed that the MP was most abundant in the cell wall fraction of older leaves and that the protein remained at high levels in the cell wall fraction as the leaves continued to age. Significant levels of the MP were detected in a crude membrane/organelle fraction and a soluble fraction in younger leaves but decreased to low levels in older leaves. These results suggest that the MP accumulates and is stable in cell walls. We have previously shown that the MP modifies the molecular exclusion limit of plasmodesmata, which is consistent with the hypothesis that plant viruses move from cell to cell through altered plasmodesmata. We show here that the ability of the tobacco mosaic virus MP to modify the molecular exclusion limit of plasmodesmata in tobacco depends on the developmental stage of the leaf. The implications of these findings on understanding virus movement and how plasmodesmata function are discussed.**

The movement of a plant virus from the initial site of infection into adjacent healthy cells is essential for establishing a productive virus infection. In some virus–host interactions, pathogenesis includes systemic movement of virus by means of the vascular tissue. Although little is known about the virus–host interactions necessary for virus spread, recent studies have provided direct evidence that a protein encoded by tobacco mosaic virus (TMV) is required for cell-to-cell movement of the virus. When expressed in transgenic plants, this protein, the movement protein (MP; 32 kDa), complemented the temperature-sensitive defect in movement of the Ls1 mutant of the L strain of TMV (1). Using a different approach, Meshi *et al.* (2) showed that when the single amino acid change that distinguishes the MP gene of the Ls1 virus from that of the parental L strain was introduced into the MP gene of the L virus, the mutated L strain showed the same phenotype as the Ls1 virus.

Plant viruses (or nucleic acids) move from cell to cell through plasmodesmata, channels that extend through cell walls and provide cytoplasmic continuity between adjacent cells. However, on the basis of present knowledge of plasmodesmata architecture (3, 4) and the molecular size exclusion limits of plasmodesmata (5–7), it is generally assumed that the structure must be modified during virus infection for virus progeny to move from cell to cell. In the case of TMV in tobacco, the MP may be responsible for altering plasmodesmata. At present three lines of evidence support this hypothesis. (*i*) The TMV MP alters the function of plasmodesmata in mature tobacco leaves. The molecular size exclusion limits of plasmodesmata were significantly altered in transgenic plants expressing the MP gene (7). In these studies, it was shown that movement of fluorescein isothiocyanate-conjugated dextran (FITC-dextran), which has a molecular mass of 9.4 kDa, was detected between leaf mesophyll cells of plants expressing the MP gene, whereas the size exclusion limit for control plants was 0.7–0.8 kDa. (*ii*) Immunogold labeling of thin sections from TMV-infected leaf tissue has localized the MP in cell walls to plasmodesmata (8). (*iii*) The TMV MP is associated with cell wall fractions from leaf tissue of transgenic plants expressing the MP gene (1) as well as virus-infected plants (1, 9).

The TMV MP is an important component in determining pathogenicity (1), virulence (1, 2), and host range (10, 11). Because little is known about the MP and how it functions in facilitating virus movement, it is important that more information be obtained about the protein and the protein–host interaction. In this report, we describe molecular and biological characteristics of the TMV MP in transgenic plants.

MATERIALS AND METHODS

Plants. Transgenic plant lines 274 and 277 express the TMV MP gene and complement the defect in movement of the Ls1 mutant of TMV (1). Plant line 274H (R2 generation) is homozygous (H) for expression of the MP gene. Transgenic control line 306H was used as a control in all experiments. Plants were grown in a greenhouse under natural light conditions, supplemented in winter months with sodium halide lamps [75 $\mu Em^{-2} \cdot sec^{-1}$; 1 einstein (E) = 1 mol of photons; 14-hr photoperiod], or in a growth chamber (14-hr light/10-hr dark periods at 22°C).

Virus. The U1 (common) strain of TMV was used throughout. The virus was propagated in *Nicotiana tabacum* cv. Xanthi and purified according to Bruening *et al.* (12).

Subcellular Fractionation, Extraction, and Quantitation of the MP. Tissues were powdered subsequent to freezing in liquid nitrogen and lysed by grinding with a mortar and pestal in two volumes of ice-cold grinding buffer (GB; 100 mM Tris·HCl, pH 8/10 mM EDTA/5 mM dithiothreitol). The tissues were further triturated in a ground-glass homogenizer. The homogenates were centrifuged at 1000 × *g* for 10 min to give a crude cell wall pellet and supernatant (S1). The crude cell wall pellet was washed once with two volumes of GB and twice with two volumes of GB containing 2% Triton X-100. The first wash with GB plus 2% Triton X-100 was accompa-

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Abbreviations: MP, movement protein; TMV, tobacco mosaic virus; FITC-dextran, fluorescein isothiocyanate-conjugated dextran.

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nied by grinding of the suspended tissue in a ground-glass homogenizer. After the final wash, the crude cell wall fraction was suspended in two volumes of extraction buffer (EB; 75 mM Tris·HCl, pH 6.8/6 M guanidine hydrochloride), heated in a boiling water bath for 10 min, and centrifuged at 1000 × g for 20 min. An aliquot was dialyzed against TE (10 mM Tris·HCl, pH 6.8/1 mM EDTA) and stored at −80°C. This extract is referred to as the P1 or cell wall fraction. The supernatant (S1) was further fractionated to give the 30,000 × g pellet (P30) and a supernatant fraction (S30). The P30 was resuspended in EB, heated in a boiling water bath for 5 min, and centrifuged at 12,000 × g for 10 min. The supernatant was dialyzed against TE and stored at −80°C. This extract represents the P30 fraction. In some experiments, a mixture of protease inhibitors was used during subcellular fractionation (13). Protein concentrations of extracts were determined by the bicinchoninic acid (BCA) protein assay (Pierce).

Proteins were separated by SDS/PAGE (14) in 12.5% gels and blotted onto nitrocellulose (1). The MP was detected by reacting the nitrocellulose with a rabbit antibody to TMV MP and then with ^{125}I-labeled donkey anti-rabbit serum (Amersham) (1). Immunoreactive bands were excised from the nitrocellulose blots, and the amounts of ^{125}I bound were quantitated in a γ counter.

Northern Blot Analysis. Total RNA was isolated from leaf tissue and analyzed for RNA that encodes the MP (1). Radioactive bands representing the MP mRNA were excised from the nitrocellulose blots and quantitated.

Fluorescent Probes and Microinjection. Lucifer yellow CH (0.45 kDa) and FITC-dextran with a molecular mass of 9.4 kDa were used as fluorescent probes. The FITC-dextran and liposome encapsulation of the probes were prepared as described (7). Liposome suspensions were microinjected into the vacuoles of spongy mesophyll cells where liposomes fused to the tonoplasts. Movement between cells was monitored by using a Hamamatsu analytical photon-counting system (Photonic Microscopy, Oakbrook, IL) as described (7).

RESULTS

Accumulation of MP in Different Tissues of Transgenic Plants. The TMV MP was detected in previous studies in subcellular fractions of young leaf tissue from transgenic plants expressing the MP gene under the regulatory control of the 35S promoter of cauliflower mosaic virus (1). However, relative amounts of the protein in different subcellular fractions and in different tissues were not assessed. To determine the relative levels of MP in various tissues, we analyzed young and old leaves, stems, and roots from transgenic plant line 274H. MP was detected in subcellular fractions from all tissues analyzed from line 274H (Fig. 1). No MP was detected in extracts from control line 306H (1). These results are consistent with previous findings that genes under the control of the 35S promoter are expressed in all plant tissues (15, 16). The electrophoretic mobilities of MP obtained from the subcellular fractions of each tissue were identical. A lower molecular mass component, presumably caused by limited proteolysis of the MP, was detected. The antibody recognized a polypeptide of 27 kDa, which was found in all subcellular fractions from root tissue and occasionally in the P30 fraction of older leaves. The presence of protease inhibitors during subcellular fractionation had no effect on the levels of the 27-kDa polypeptide. In contrast, a minor band of 52 kDa was consistently detected in the P1 fraction from older leaves. The 52-kDa component may be a dimer of the MP that migrates anomalously or may result from a stable interaction of the MP with a specific cellular component.

The relative levels of MP in each subcellular fraction, normalized per gram of fresh weight of leaf tissue, are presented in Table 1. The highest levels of total MP were consistently detected in older leaves. MP was more abundant in the cell wall fraction of older leaves and roots than in the P30 or S30 fractions. In contrast, MP was most abundant in the S30 fraction from young leaves. Similar levels of MP were consistently detected in the P1 and S30 fractions of stem tissue.

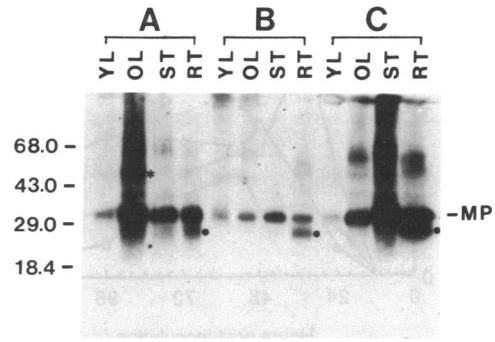

FIG. 1. Immunoblot analysis of MP in different tissues of transgenic plant line 274H. Extracts were obtained from three subcellular fractions: P1 (cell wall), P30, and S30. Tissues analyzed were young leaves (YL, ≤7 cm), old leaves (OL, ≥12 cm), stems (ST), and roots (RT). (*A*) Detection of MP in P1 extracts (20 μg of protein per lane). (*B*) Detection of MP in S30 extracts (200 μg of protein per lane). (*C*) Detection of MP in P30 extracts (100 μg of protein per lane). The asterisk indicates the 52-kDa protein. The filled circles indicate the 27-kDa protein. Protein molecular mass markers (in kDa) are indicated (Bethesda Research Laboratories).

Levels of MP in Transgenic and TMV-Infected Nontransgenic Plants. To compare the level of MP in transgenic plants to MP levels in TMV-infected plants, it was necessary to determine when (i.e., hours postinoculation) the MP was most abundant in infected leaf tissue. A time course of MP accumulation in TMV-infected plants was followed for 120 hr postinoculation, and the levels of MP in subcellular fractions were quantitated. Results of a typical experiment are shown in Fig. 2. MP reached a maximum level in the P1 fraction at ≈48 hr postinoculation and decreased only slightly through 120 hr postinoculation. The approximate time at which MP was most abundant in the S30 fraction varied in three experiments from 34 to 54 hr postinoculation, whereas in the P30 fraction the protein consistently reached maximum levels at ≈54 hr postinoculation. The levels of MP decreased rapidly in the S30 fraction, but more gradually in the P30 fraction, after maximum levels were detected.

A time point of 48 hr postinoculation was chosen for the comparison of MP levels in infected tissue with those in transgenic plants. As seen in Table 2, the amount of total MP extracted from leaves of plant line 274H was similar or higher than that extracted from TMV-infected Xanthi plants. The relative amounts of MP detected in the P30 and S30 fractions were similar in transgenic and infected tissue. However, the P1 fraction from line 274H contained significantly higher levels of

Table 1. Accumulation of MP in different tissues of transgenic plants

Plant organ	Levels of MP, cpm × 10^{-3}			
	P1	P30	S30	Total
Young leaf (≤7 cm)	123	43	205	370
Mature leaf (≥12 cm)	580	204	329	1112
Stem	146	53	195	394
Root	267	121	81	469

The values given are cpm × 10^{-3} of ^{125}I-labeled secondary antibody excised from the immunoblot analyzed in Fig. 1 and represent the relative amounts of MP per gram of fresh weight leaf tissue. Additional experiments gave similar results. Plants with 12–14 leaves were analyzed.

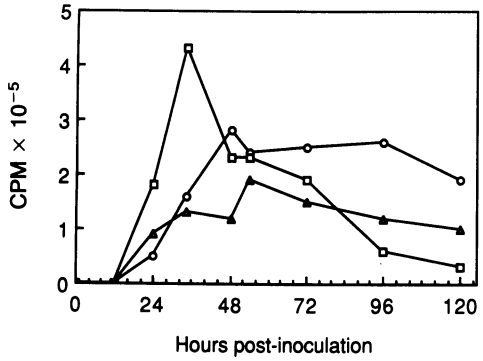

FIG. 2. Time course of MP accumulation in subcellular fractions prepared from TMV-infected *N. tabacum* cv. Xanthi. The second leaf from the top of seedlings at the six-leaf stage was inoculated with TMV (0.5 mg/ml). Inoculated leaves were harvested at the indicated times, and subcellular fractions were analyzed for relative levels of MP. Samples were analyzed and quantitated as described in *Materials and Methods*. ○, P1 fraction; △, P30 fraction; □, S30 fraction.

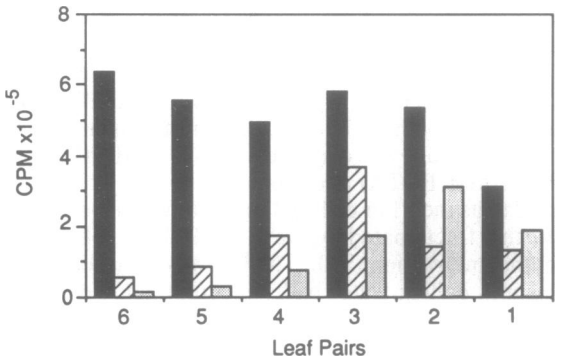

FIG. 3. Relative levels of MP in subcellular fractions as a function of leaf position in plant line 277. The amounts of MP in subcellular fractions were determined by quantitative immunoblot analysis. The relative levels of MP per gram of fresh weight leaf tissue are represented by the cpm of ^{125}I-labeled secondary antibody bound to MP. Leaf pairs were numbered from the top to the bottom of the plants; the youngest leaves analyzed were ≥5 cm in length. P1, ■; P30, ▨; S30, ▥.

MP than the equivalent fraction from infected tobacco plants. These results indicate that the MP is abundant in leaf tissue expressing the MP gene relative to infected tissue. Indeed, as the leaves of transgenic plants age, MP becomes increasingly predominant in the cell wall fraction (see below).

Accumulation of MP in Leaves of Transgenic Plants as a Function of Leaf Age. The difference in relative amounts and localization of the MP in young vs. old leaves (Table 1) suggested that MP accumulated in the cell wall (P1) fraction of older leaves. To further examine this accumulation, we determined the levels of MP in subcellular fractions from leaves of plant line 277 as a function of leaf position. The MP reached maximum levels in leaves 5 and 6, representing leaf pair 3 (Fig. 3). Subcellular fractionation analysis showed that the MP was most abundant in the cell wall fractions, irrespective of leaf position (Fig. 3). Whereas significant levels of MP were detected in the P30 and S30 fractions from younger leaf tissue, the MP was predominantly cell wall associated in the older leaves (leaf pairs 2–6). The presence of high levels of MP in older leaves was accompanied by a decrease in the amount of total protein extracted from the cell wall. The levels of cell wall-extracted protein in leaf pairs 3–6 were similar and represented only 20% of the level detected in leaf pair 1 (data not shown). The concomitant decline of MP in the P30 and S30 fractions from older leaves relative to the high levels of cell wall-associated MP suggests that the protein is relatively stable in cell walls. Similar results were obtained when 274H plants were analyzed (data not shown). In all experiments, the electrophoretic mobility of MP extracted from leaf tissue of different ages was identical.

Table 2. Levels of MP in transformed plants vs. nontransformed TMV-infected plants

Plant	Levels of MP, cpm × 10^{-3}			
	P1	P30	S30	Total
	Experiment 1			
274H	535	86	75	696
Xanthi-TMV	125	66	78	270
	Experiment 2			
274H	260	65	71	396
Xanthi-TMV	155	77	104	336

Four-week-old plants of *N. tabacum* cv. Xanthi were inoculated with TMV (Xanthi-TMV; 0.5 mg/ml). At 48 hr postinoculation, leaf 2 (inoculated leaf) of each seedling was analyzed. Leaf 2 from 4-week-old 274H plants was also analyzed. Four leaves were grouped per sample. Values given are cpm × 10^{-3} of ^{125}I-labeled secondary antibody and represent the relative amounts of MP per gram of leaf fresh weight.

The levels of MP mRNA in leaf tissue of line 274H were also analyzed. The highest levels of MP mRNA were detected in the youngest tissue and decreased in older leaf tissue. This decrease was concomitant with a decrease in total RNA from younger to older leaves (data not shown). The results are consistent with the decrease of MP in the P30 and S30 fractions from older leaves being due to a decrease in the mRNA levels.

In younger leaf tissues, a significant amount of the MP was found in the S30 fraction (Table 1 and Fig. 3). To determine if the MP in the S30 fraction was soluble or membrane associated, S30 fractions from young leaves of plant lines 274H and 277 were centrifuged at 140,000 × *g*. Approximately 90% of the MP in the S30 fractions remained in the supernatant under these conditions (data not shown).

Movement of a Fluorescent Probe Between Leaf Mesophyll Cells of Transgenic Plants as a Function of Leaf Age. The TMV MP has been shown to alter plasmodesmatal function in experiments in which fluorescent probes were microinjected into cells of leaves *in situ* (7). In these experiments, an FITC-dextran probe of 9.4 kDa moved between mesophyll cells in mature expanded tobacco leaves expressing the MP gene (plant lines 277 and 274H), whereas the molecular exclusion limit was less than 0.8 kDa in control cells (plant line 306H). To determine if leaf age or maturity has an influence on the modification of plasmodesmatal function in plants expressing the MP gene, liposomes containing the 9.4-kDa fluorescent probe were injected into mesophyll cells of leaves of plant line 277 at different levels of maturity. The effects of the MP on the molecular exclusion limit of plasmodesmata, as determined by movement of the 9.4-kDa probe between leaf mesophyll cells, depend upon leaf maturity (Fig. 4 and Table 3). In leaf 3 the 9.4-kDa probe was restricted to the injected mesophyll cell at 10 min after injection (Fig. 4*C*), whereas movement of the low molecular mass probe, Lucifer yellow CH (0.45 kDa), was unrestricted in mesophyll cells of leaf 3 (Fig. 4*B*). In contrast, movement of the 9.4-kDa probe in leaf 10 was detected a few seconds after injection; when movement of the probe occurred, it was readily detected 1 min after injection (Fig. 4*D*). In repeated experiments, movement of the 9.4-kDa probe was consistently detected in leaves 5 or 6 on plants that contained 14–18 leaves. Therefore, movement of the 9.4-kDa probe correlated with leaf position. Leaves 5 and 6 were fully, or nearly fully, expanded in these experiments.

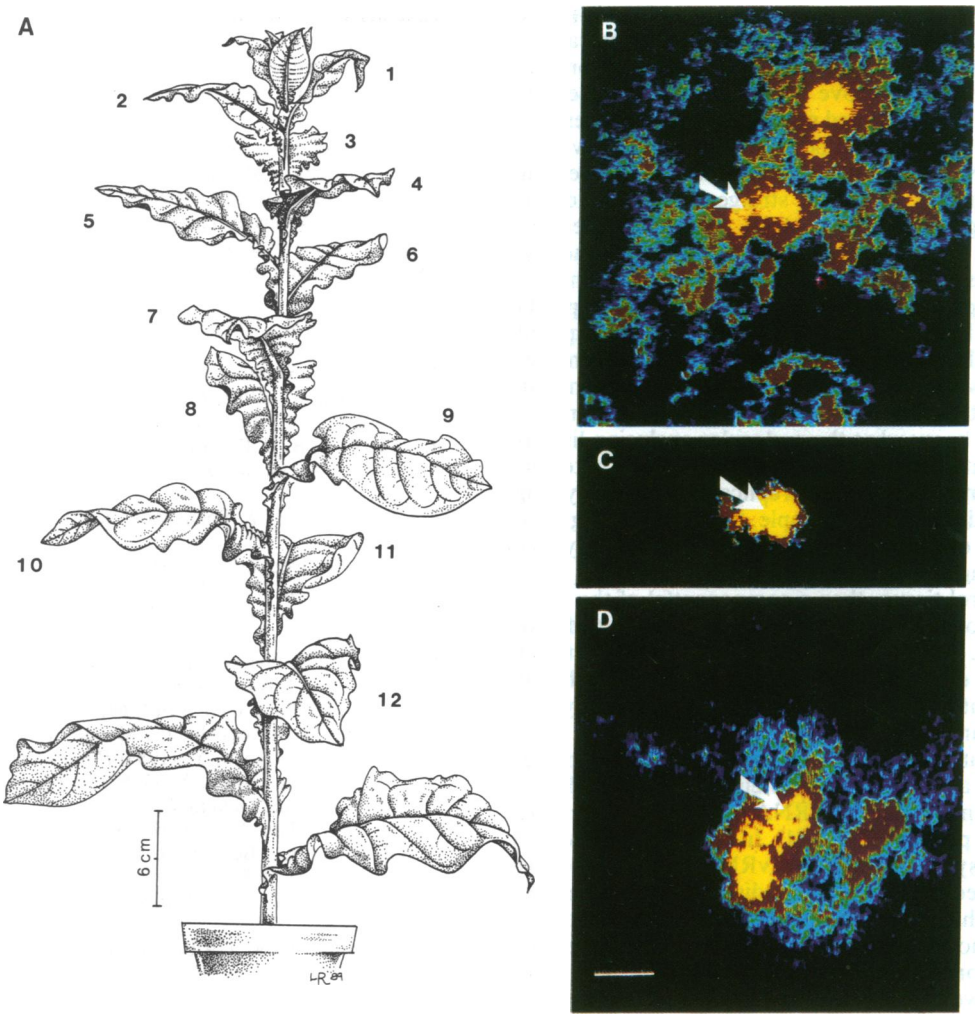

FIG. 4. Movement of fluorescent probes between leaf mesophyll cells. (*A*) Schematic representation of a transgenic tobacco plant in which cell-to-cell movement of microinjected FITC-dextran was studied. (*B–D*) Movement of the injected probes between spongy mesophyll cells of the different leaves, as indicated by false-color images analyzed by the Hamamatsu image analysis system (model C1966-20). Yellow represents the highest fluorescence intensity and the darker colors represent reduced fluorescence. (*B*) Movement of Lucifer yellow CH in leaf 3 at 1 min after injection. (*C*) Containment of 9.4-kDa FITC-dextran within the injected mesophyll cell of leaf 3 at 10 min after injection. (*D*) Movement of 9.4-kDa FITC-dextran in leaf 10 at 1 min after injection. (For *B–D*, bar = 50 μm.) Arrows indicate the cells in which the liposome-encapsulated dye was microinjected.

DISCUSSION

The expression of a functional TMV MP gene (under the regulatory control of the 35S promoter of cauliflower mosaic virus) in transgenic tobacco plants enables us to study the protein in the absence of the expression of other viral genes and to obtain information about the molecular details of the MP–host interaction. The MP accumulated in all plant tissues analyzed. The protein was most abundant in older leaves, where it was present at high levels in the cell wall fraction. The accumulation of MP in this fraction suggests that the protein has an affinity for, and/or is very stable in, this fraction. This conclusion is consistent with the finding that the MP can modify the function of the plasmodesmata (7) and is supported by two findings presented here. First, in older leaves the relative amounts of MP in the P30 and S30 fractions are low compared to younger leaves, while the levels of MP in the cell wall fractions are high (Fig. 3). Second, in TMV-infected leaves MP accumulates in cell walls, reaches a maximum level at approximately 48 hr postinoculation, and remains at nearly constant levels through 120 hr postinoculation. In contrast, the level of MP in the S30 fraction from infected tissue decreased after reaching maximum levels. Curiously, the level of MP in the P30 fraction appeared relatively stable and only decreased slightly after reaching maximum levels.

Recent results show that MP modifies the molecular exclusion limits of plasmodesmata in mesophyll cells of mature tobacco leaves (7). However, plasmodesmata in young leaf mesophyll cells expressing the MP gene were not functionally

Table 3. Representative data obtained on the movement of the 9.4-kDa fluorescent probe in leaves at different developmental stages

	Movement	
Leaf	Exp. 1	Exp. 2
2		−
3	−	−
4	−	
5	−	+
6	+	
7	+	
10		+
14	+	

Movement of the 9.4-kDa probe between mesophyll cells (see legend to Fig. 4) was determined in plants having 14–18 leaves.

altered, as judged by the restricted movement of the 9.4-kDa probe (Fig. 4). A number of intriguing explanations can be proposed to explain this finding based on the assumption that the FITC-dextran probes move by the same route used by viral progeny. (*i*) The restricted movement of the fluorescent probe in younger leaves could be a function of leaf development. Although we know of no documented evidence that plasmodesmata might be more stringently controlled in young, expanding leaves than in older, more mature leaves, it is possible that MP may only be able to modify plasmodesmata in older tissue. This interpretation could explain why there are no apparent effects on organogenesis in plants expressing the MP. (*ii*) A critical level of MP may be required in mesophyll cells before the fluorescent probe can move. The increased levels of MP in older tissue, predominantly in the cell wall fraction, lend support to this interpretation. We presently do not know what the molecular exclusion limit is for plasmodesmata in infected cells. However, it is noteworthy that TMV moves into, and replicates in, very young leaves of infected plants (for example, leaves 1–4 in Fig. 4*A*). If the molecular exclusion limit of plasmodesmata in young leaves expressing the MP gene is restricted to the limits detected in control tissue, then a component(s) of the infectious process would be required in addition to the MP for virus movement. Alternatively, movement of virus between mesophyll cells of young leaf tissue may require a highly localized concentration of MP that is not attained in young leaf tissue of plants expressing the MP gene.

It is likely that the TMV RNA genome (vRNA) spreads from cell to cell as a ribonucleoprotein (vRNP) particle (17, 18). Cell-to-cell movement of vRNA does not require functional TMV coat protein (17, 19–21). In contrast, TMV vRNA may also move systematically as a vRNP, although the coat protein is required for efficient long-distance movement (18). The possibility that long-distance movement is in the form of a TMV rod cannot be excluded (22). Unfortunately, because of the lack of conclusive data describing the form in which vRNA moves, we cannot, at present, predict the molecular size exclusion limits required for cell-to-cell movement of the TMV vRNP.

The TMV MP is the only nonstructural viral protein identified that is known to function in virus movement. Nonstructural proteins have also been localized in tissue infected with other viruses. The P1 protein (40 kDa) of cauliflower mosaic virus, a DNA virus, has been localized to areas around plasmodesmata (23). Localization of the putative MPs of two tripartite RNA viruses, albeit having different morphologies, offers conflicting results. The P3 protein (32 kDa) of alfalfa mosaic virus was localized to the middle lamella of the cell wall (24), whereas the 3A protein (35 kDa) of cucumber mosaic virus was localized to the nucleoli in infected tissue (25). Although the P3 and the 3A proteins may potentiate virus movement, they may function by very different mechanisms.

Further analysis of MP in transgenic plants will increase our knowledge of the MP–host interaction and provide details about the mechanism(s) by which the MP alters plasmodesmata and potentiates virus movement. Of equal importance, the MP may represent an invaluable tool in studying plasmodesmatal structure and function.

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