Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 1433–1437, February 1994
Cell Biology

Direct functional assay for tobacco mosaic virus cell-to-cell movement protein and identification of a domain involved in increasing plasmodesmal permeability

(microinjection/plasmodesmata/size exclusion limit)

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Communicated by George Bruening, November 1, 1993

ABSTRACT **Plasmodesmata are cytoplasmic bridges between plant cells thought to generally allow only the passage of small molecules and metabolites. However, large structures such as plant viruses also move from cell to cell via plasmodesmata. In tobacco mosaic virus (TMV) infection a viral movement protein (TMV-MP) mediates viral spread. Here, a microinjection assay is used to monitor the dynamics of TMV-MP function directly in wild-type plants. The results indicate that TMV-MP interacts with an endogenous plant pathway increasing plasmodesmal size exclusion limit to permit passage of 20-kDa dextrans. Furthermore, TMV-MP influences plasmodesmal size exclusion limit several cells distant from the injection site, indicating either that TMV-MP itself crosses plasmodesmata or that TMV-MP induces a diffusable signal capable of dilating microchannels of plasmodesmata. The region of TMV-MP responsible for increasing plasmodesmal size exclusion limit was mapped to the carboxyl-terminal part of the 268-amino acid residue protein between amino acid residues 126 and 224.**

In plants, cell communication and molecular trafficking are largely dependent on cytoplasmic bridges between plant cells, the plasmodesmata (PD). These plasma membrane-lined pores link neighboring cells and thereby generate a three-dimensional cellular network (1). Each PD contains about 10 microchannels (diameter, 2.5 nm) thought to function as transport pathways (for more details see ref. 2). In mesophyll cells, only small molecules, such as metabolites or dextrans with a molecular mass up to 1 kDa (corresponding to a Stokes radius of 0.75 nm), can pass through these channels freely (3). Due to their structural complexity, it seems possible that PD might also function in transport of endogenous macromolecules (4). In fact, PD are the major route for plant viruses moving from infected to adjacent healthy cells. Compared to the size of PD microchannels, plant viral dimensions are enormous—e.g., tobacco mosaic virus (TMV) particles are 300 × 18 nm and even free-folded TMV RNA has an average diameter of 10 nm (5). How can these large structures fit through so small a passageway?

Several lines of evidence suggest that viral encoded proteins are involved in the movement process. For TMV, a 30-kDa protein, which is expressed only transiently during infection (6), has been implicated in movement (7). Clues about the function of TMV movement protein (TMV-MP) are derived from *in vitro* experiments with purified TMV-MP and from *in vivo* experiments with transgenic tobacco plants expressing TMV-MP. Two functions have been identified: (*i*) TMV-MP cooperatively binds single-stranded (ss) nucleic acids (8) and (*ii*) transgenic tobacco plants expressing TMV-MP show an elevated PD size exclusion limit (SEL) of >10 kDa for dextrans (Stokes radius of 2.4 nm to 3.1 nm) (3). These transgenic plants also facilitate intercellular movement of unencapsidated viral RNA, thereby indicating that formation of viral particles is not necessary for TMV-MP-mediated movement (9).

A model for TMV-MP-mediated viral movement has been proposed (8) whereby the MP binds viral nucleic acid to coat and unfold it completely. Electron microscopic observations indeed show that ss nucleic acid complexed with TMV-MP is extremely thin and elongated, with a diameter of <2 nm (10). The combined effects of unfolding ss nucleic acid and increasing SEL likely permit the TMV-MP-mediated passage of the viral nucleic acid through PD microchannels into adjoining cells.

Here, we describe an approach to study TMV-MP function in plants: direct microinjection of purified TMV-MP into plant mesophyll cells with subsequent monitoring of its influence on PD SEL. Thus, the dynamics of TMV-MP interaction with PD can be visualized in real time. The results show that TMV-MP is able to increase PD SEL within 3–5 min after injection, indicating that TMV-MP operates a pathway already present in the cell. Similar experiments using TMV-MP deletion mutants led to the identification of a domain at the C terminus of TMV-MP involved in increasing PD SEL.

MATERIALS AND METHODS

Cloning, Overexpression, and Purification of TMV-MP and TMV-MP Deletion Mutants. For DEL 15, an *Nde* I site including a start codon was introduced into the MP gene of TMV U1 strain between the codons for amino acids 110 and 111 by oligonucleotide-directed mutagenesis (11). A TMV-MP fragment containing amino acid residues 111–268 was then cloned into a pET3a expression vector (12). DEL 15 as well as TMV-MP and DEL 2, 3, 4, 7, and 10 proteins were overexpressed in *Escherichia coli* and purified as described (8) except that buffer L (7) was modified to contain 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 10% glycerol, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride (pH 8).

Microinjection and Image Analysis. Experiments were as described (13). Injection conditions: 0.05–0.1 μg of TMV-MP or deletion mutant proteins per μl; 0.5 mM 10-kDa Lucifer yellow-labeled dextran (Sigma) or 0.5 mM 20-kDa fluorescein isothiocyanate-labeled dextran (Sigma) in 2.5 mM $KHCO_3$. Injection pressure: 200–400 kPa. Estimated injection vol-

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Abbreviations: TMV, tobacco mosaic virus; TMV-MP, TMV movement protein; SEL, size exclusion limit; PD, plasmodesmata; ss, single-stranded.

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ume: 1 pl. Technically successful injections were defined by smooth introduction of sample, clear highlighting of cellular outline, and withdrawal of the needle without loss of dye from the injected cell. Movement of dye was monitored at video rate (30 frames per sec) using an intensified photonic camera system (Hamamatsu Photonic systems, model C1966-20) as described (3). Fluorescence images shown were either averaged over 64 frames (2 sec of real time) or a "trace" picture was generated, where signal intensities of two individual video frames were subtracted and the difference was stored. This process was repeated with 950 frames at 5 frame intervals and the accumulation of all differential pictures gave rise to an image termed trace.

RESULTS

Microinjected TMV-MP Increases PD SEL in Mesophyll Cells of Wild-Type Tobacco Plants. TMV-MP purified from *E. coli* was mixed with fluorescently labeled dextrans and microinjected (13) into spongy mesophyll cells of tobacco leaves. The fate of the fluorescently labeled dextrans—whether confined to the microinjected cell or able to move to other cells—was then monitored with a video image analysis system that allows the actual visualization of the dynamics of the movement process (3). Since in transgenic plants expressing TMV-MP the increased molecular SEL for dextrans was >10 kDa but <17 kDa, TMV-MP was first coinjected with a fluorescently labeled 10-kDa dextran (Fig. 1*A*). Within 3–5 min a substantial amount of the 10-kDa dextran left the microinjected cell to appear primarily in another cell not directly connected to the injected cell (Fig. 1 *B* and *C*). This result can be understood considering that mesophyll cells are part of a three-dimensional network. Each cell is connected not only to neighbors on the surface but also to cells beneath it (Fig. 1*F*), and under the light microscope only the surface layer of cells can be clearly monitored. In nearly all experiments the dye moved down into non-surface cells first (see also Fig. 2 *A* and *B*) and eventually reappeared in cells located in the surface layer within the field of view of the camera (Fig. 2*C*). Therefore, the fluorescent 10-kDa dextran in Fig. 1 *A–C* passed through at least one and possibly several cells before reappearing on the surface. In contrast, fluorescent 10-kDa dextran injected without TMV-MP was contained within the injected cell for at least 30 min (Fig. 1 *D* and *E*). That fluorescent 10-kDa dextrans can move into cells not directly connected to the injected cell shows that TMV-MP influences the SEL of PD in cells other than the injected cell. Thus, either TMV-MP itself crosses PD channels to then interact with PD in the adjoining cell(s) or TMV-MP triggers a signal capable of influencing SEL in cells distant from the injection site.

Microinjected TMV-MP Is Functionally Different from TMV-MP Expressed in Transgenic Plants. In transgenic plants, the maximal molecular SEL for dextrans is between 10 and 17 kDa. Here, coinjection of TMV-MP with fluorescent 20-kDa dextran resulted in extensive movement: fading of the fluorescence signal in the injected cell occurred after 10–15 min (Fig. 2 *A* and *B*) and by 30 min dye had distributed to at least 20–30 cells (Fig. 2*C*). Afterwards, no further changes were observed, indicating that a final distribution had been reached. In control experiments, fluorescent 20-kDa dextran injected without TMV-MP did not leave the injected cell (data not shown). Next, the ability of TMV-MP to promote movement of fluorescent 40-kDa dextran was tested; coinjection of this dextran with TMV-MP did not result in detectable movement within 1 hr (data not shown).

Thus, under the influence of injected TMV-MP, the PD molecular SEL for dextrans is >20 but <40 kDa. This corresponds to a Stokes radius of 3.2–4.3 nm (14) and exceeds the maximal SEL observed in TMV-MP transgenic plants (Table 1). Therefore, other differences may exist between the two systems. An interesting, but as yet unexplained, observation in TMV-MP transgenic plants is the lack of SEL increase in young leaves (5- to 7-cm length), although TMV-MP is expressed there (13, 15). When TMV-MP was coinjected with fluorescently labeled 10-kDa dextran into a leaf of 5-cm length from a wild-type plant, extensive movement occurred (Fig. 3), although the time course of dye distribution was slower than in mature leaves.

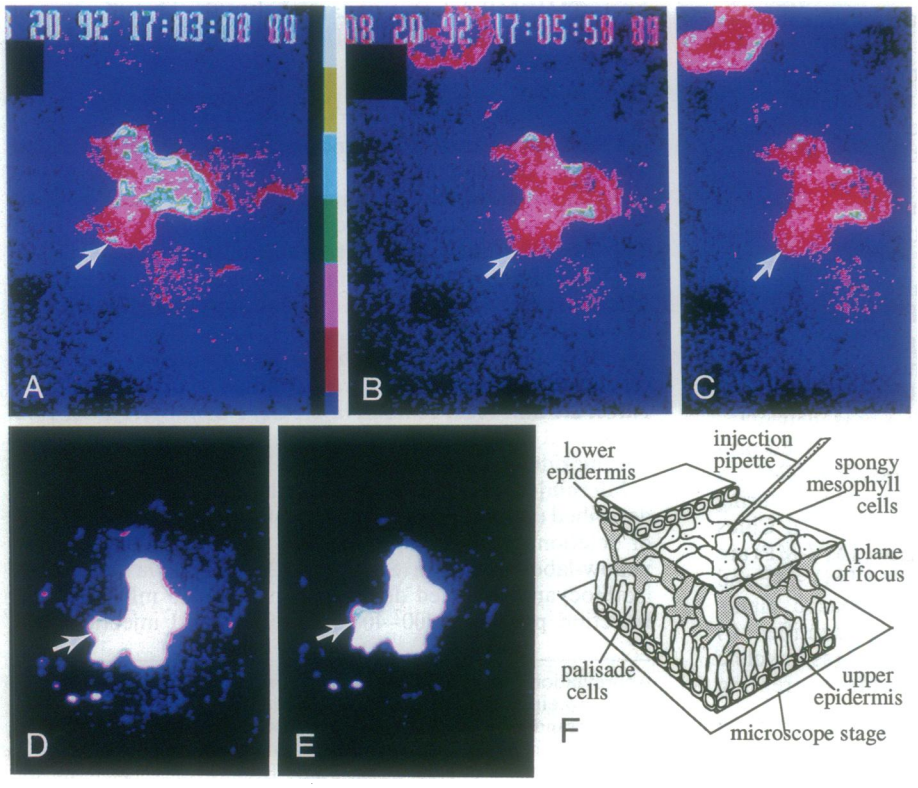

FIG. 1. Microinjection of fluorescently labeled 10-kDa Lucifer yellow dextran together with TMV-MP (*A–C*) and without TMV-MP (*D* and *E*) into spongy mesophyll cells of mature tobacco leaves. The time course of 10-kDa dextran movement is shown. Elapsed time after injection: (*A*) 1 min, (*B*) 4 min, (*C*) 5 min, (*D*) 1 min, (*E*) 30 min. Arrows point to the injected cell. Pictures are averaged; colors are derived by false color imaging and represent intensity of the fluorescent signal, ranging from blue (lowest intensity) to white (highest intensity) with black and blue representing background; see color bar at the right of fluorescent image in *A*. In the top part of the picture an automatic timer gives date and time of the experiment. The timer has been turned off in the image in *C* to allow a better view of the fluorescent cells. (*A–C*, ×230; *D* and *E*, ×130.) (*F*) Schematic view through a tobacco leaf. Spongy mesophyll cells form a three-dimensional interconnected network. To inject into spongy mesophyll cells, a small portion (5 mm^2) of the lower epidermis of the leaf is pealed off. The leaf is then placed, abaxial side up, on the microscope stage. Only surface cells in the plane of focus can be injected and subsequently monitored. These cells are indicated in a lighter pattern.

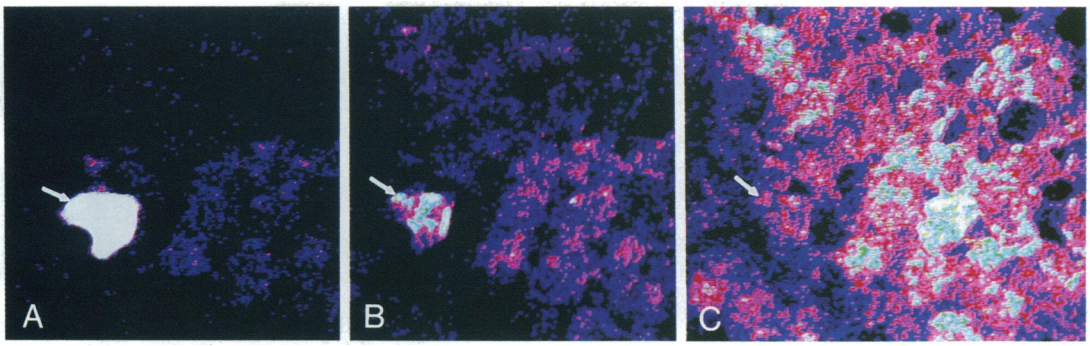

FIG. 2. Microinjection of a mixture of TMV-MP and 20-kDa fluorescein isothiocyanate-labeled dextran into spongy mesophyll cells of a mature tobacco leaf. The time course of 20-kDa dextran movement is shown. Elapsed time after injection: (*A*) 3 min, (*B*) 14 min, (*C*) 34 min. Arrows mark the microinjected cell. (×130.) Image analysis as in Fig. 1.

Deletion Analysis of TMV-MP Identifies a Domain Responsible for PD SEL Increase. Several deletion derivatives of TMV-MP were tested to characterize the region(s) involved in PD SEL increase. These proteins were microinjected into tobacco mesophyll cells together with a fluorescent 10-kDa dextran (Fig. 4). A protein was considered active if a significant amount of 10-kDa dextran left the injected cell during the observation time of 30 min; it was considered inactive if the injected cell showed no significant change of signal intensity during the observation time. Previous studies with transgenic plants expressing TMV-MP deletion mutants revealed that removal of 55 C-terminal amino acids did not impair the ability of the truncated TMV-MP to increase PD SEL, whereas removal of 73 C-terminal amino acids abolished it (16). Deletion mutants DEL 7 and DEL 10 analyzed here closely resemble the mutants used in the transgenic plant study. Indeed, DEL 7 with only 43 C-terminal amino acids deleted was functional (Fig. 4), although the time course of movement appeared slower (first signs of movement, 5–10 min after injection) compared to wild-type TMV-MP. In contrast, DEL 10, which lacks 83 C-terminal amino acids, was inactive (Fig. 4). For further analysis internal deletion mutants DEL 4, DEL 3, and DEL 2 were tested. DEL 4 was inactive as well, whereas DEL 3—although considered active according to our definition—was severely impaired. This reduced activity was manifested in very slow movement and inefficient release of fluorescent dyes from the injected cell. DEL 2 was active and its time course of movement was comparable to that of DEL 7. In summary, from five tested deletion mutants, two with adjacent deletions were found inactive (DEL 10 and DEL 4), one with a very small deletion next to DEL 4 was impaired (DEL 3), and two flanking mutants were active (DEL 7 and DEL 2). This pattern of activity suggested that a region involved in PD SEL increase is encompassed by amino acid residues 126–224 (domain E, Fig. 4).

Table 1. Comparison of TMV-MP influence on PD SEL in wild-type tobacco plants microinjected with TMV-MP and transgenic plants expressing TMV-MP

Parameter	Wild-type plants microinjected with TMV-MP	Transgenic plants expressing TMV-MP
Movement		
10-kDa dextran	+	+
20-kDa dextran	+	–
40-kDa dextran	–	–
Maximal Stokes radius, nm	3.2–4.3	2.4–3.1
Time course of movement		
10-kDa dextran	3–5 min*	3–5 sec*
20-kDa dextran	10–15 min*	–
Movement in young leaves (<7-cm length)		
10-kDa dextran	15–20 min*	–

*Times indicate major signs of movement.

The C-Terminal Part of TMV-MP Increases PD SEL. The above results suggested that the C terminus of TMV-MP is responsible for increasing SEL. This suggestion derives from negative results that may reflect an overall disturbance of protein conformation rather than deletion of a functional region. This is unlikely since the deletion mutants retained at least one TMV-MP function, binding to ss nucleic acid (10). However, we decided to produce a truncated protein lacking the N-terminal 110 amino acids, DEL 15, and to directly test it for its ability to increase SEL. When analyzed on an SDS/polyacrylamide gel, DEL 15 migrated according to its predicted molecular mass of 20 kDa (Fig. 5*A*). DEL 15 contained both ss nucleic acid binding domains and its ss nucleic acid binding activity was comparable to that of wild-type MP (Fig. 5*B*). Thus, the major structural characteristics of the C terminus of the protein appear to have been preserved.

DEL 15 was comicroinjected with a fluorescent 10-kDa dextran into tobacco mesophyll cells. Within 3–6 min a significant amount of dye left the injected cell, but no experiment resulted in a clear accumulation of dye in other cells within the field of view. This movement pattern contrasted sharply with that observed for TMV-MP and its other deletion mutants, where movement of dye was restricted to the vicinity of the injection site. To visualize DEL 15 movement an image analysis function called trace was employed.

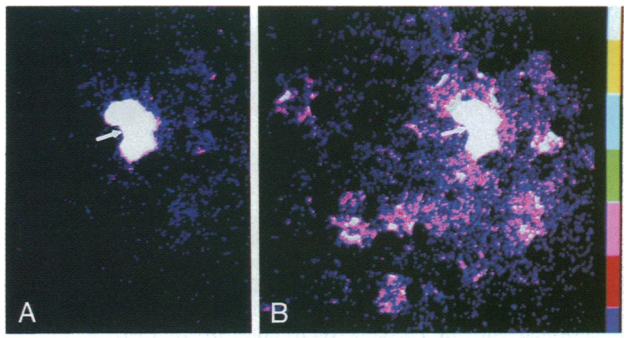

FIG. 3. Microinjection of a mixture of TMV-MP and 10-kDa Lucifer yellow dextran into spongy mesophyll cells of a young tobacco leaf (5-cm length). Injection site was in the leaf tip region. The arrow designates the injected cell. Time after injection: (*A*) 5 min, (*B*) 60 min. Note that first signs of movement are visible after 5 min (see pink spots in cells surrounding the injected cell in *A*), but major movement took place after 15–20 min. A final distribution was reached after 50–60 min (*B*). (×110.) Image analysis as in Fig. 1.

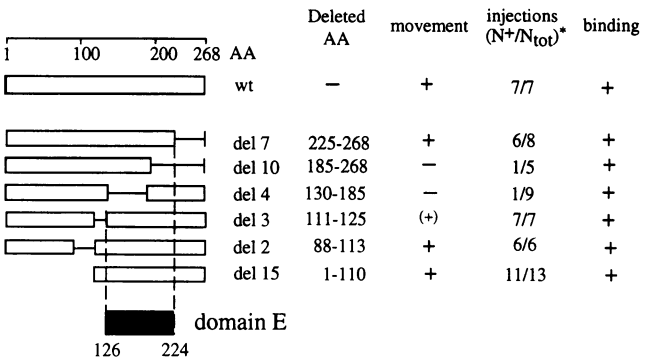

FIG. 4. Analysis of TMV-MP deletion mutants for their ability to increase SEL. Boxes indicate retained amino acids; lines indicate deleted amino acids. N^+, number of injections resulting in significant loss of signal (= fluorescent dextran) from the injected cell and/or accumulation of signal in other cells; N_{tot}, total number of technically successful injections as defined in the text.

A trace picture reflects a summary of signal changes within a time period; it does not constitute a steady-state picture as presented in, for example, Fig. 2*C*. As even small changes in signal distribution can be tracked, the pathway of low-intensity fluorescent signals can be visualized. The trace picture for DEL 15 was accumulated over a time span of 2 min 38 sec starting 3 min after injection (Fig. 6*B*). A significant amount of dye left the injected cell during that time, at least partially through an adjacent cell on the surface. If fluorescent 10-kDa dextran alone was injected, the fluorescent signal was confined to the injected cell and no change in signal intensity took place; therefore, the trace analysis shows the injected cell black with only a cellular outline due to light scattering at the edges of the cell (Fig. 6*A*). In summary, DEL 15 is capable of increasing PD SEL to allow movement of 10-kDa dextrans within 3–6 min after injection. As judged by the rapid and efficient loss of signal, DEL 15 seems to be more active than wild-type TMV-MP.

DISCUSSION

In the present study, we microinjected TMV-MP together with fluorescent dextrans into tobacco mesophyll cells. This

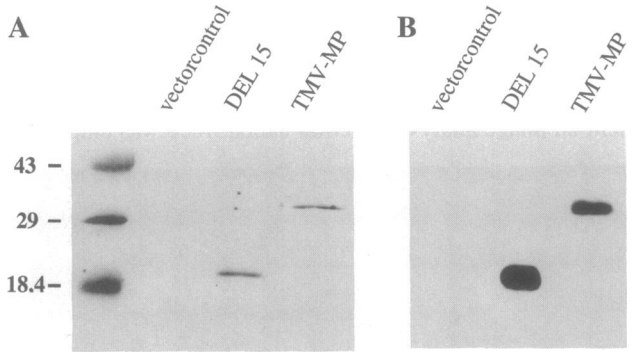

FIG. 5. Production and ss nucleic acid binding activity of DEL 15. (*A*) Proteins overexpressed and purified from *E. coli* BL21(DE3)pLysE strain were resolved on 15% SDS/polyacrylamide gel and stained with Coomassie brilliant blue R-250. Bacteria were transformed with pET3 (lane vectorcontrol), pET3-DEL 15 (lane DEL 15), and pET-TMV-MP (lane TMV-MP). Molecular masses of marker proteins are indicated in kDa. (*B*) RNA-protein binding as determined by UV crosslinking. Experiments were done as described (8). Briefly, 5–10 μg of protein (same protein solutions as shown in *A*) and 100,000 cpm of radioactively labeled RNA ([α-^{32}P]UTP label) were incubated (30 min, 4°C) and subsequently UV crosslinked. Unbound RNA was removed by RNase A digestion. The reaction mixture was resolved on a 15% SDS/polyacrylamide gel and autoradiographed.

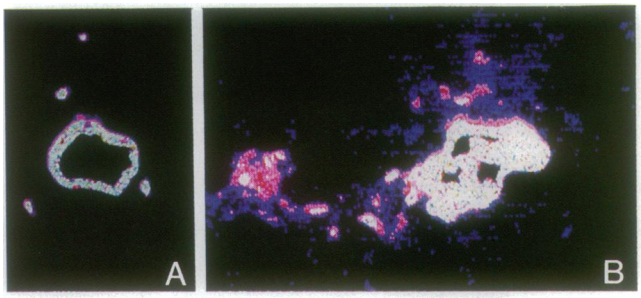

FIG. 6. (*A*) Microinjection of 10-kDa Lucifer yellow dextran: a trace performed 4–7 min after injection. False color imaging is used to represent different amounts of change in signal intensity (see color bar in Fig. 1); black indicates no change in signal intensity. (*B*) Microinjection of 10-kDa Lucifer yellow dextran together with DEL 15: trace analysis reflecting movement from 3 to 6 min after injection. (×130.)

experimental system allows the direct and immediate monitoring of TMV-MP function. TMV-MP was shown to increase PD SEL to permit passage of dextrans as large as 20 kDa but smaller than 40 kDa (this corresponds to a Stokes radius of 3.2–4.3 nm, Table 1). The increased SEL would require PD microchannels with a diameter of >6 nm. This greatly exceeds the 2.5-nm diameter of PD microchannels in unperturbed plant cells (2); thus, to support movement of large molecules, PD proteins likely undergo rearrangement.

As TMV-MP functions within 3–5 min after injection, it probably operates a pathway already present in plant cells rather than inducing formation of new structures. Thus, macromolecular trafficking via PD may not be restricted to virus-specific processes. Recently it was demonstrated that during viral infection, SEL increase occurs as late as 2–5 hr after the initial inoculation with virions (17). Compared to this, microinjected TMV-MP acts very quickly. The time course observed during viral infection likely is determined by other factors such as uncoating, viral replication, and protein synthesis.

Unexpectedly, fluorescent dextrans comicroinjected with TMV-MP can spread not only to neighboring cells but also to cells farther away from the originally injected cell. This implies that TMV-MP can influence PD SEL several cells distant from the injection site. The mechanism of this influence has not yet been elucidated, but two possibilities exist: (*i*) TMV-MP itself is able to cross PD microchannels to then affect PD in other than the injected cell or (*ii*) TMV-MP remains confined to the injected cell but triggers a signal, which operates PD microchannels and can spread through several cells. At present, we favor the first possibility: estimates of the molecular mass of proteins able to pass through PD microchannels can be derived from elution profiles of dextrans and globular proteins on size exclusion columns (18). Channels supporting movement of a 20-kDa dextran should, in principle, allow passage of globular proteins with a molecular mass up to 60 kDa (18). Therefore, TMV-MP with a lower molecular mass of 30 kDa and potentially elongated shape (10) should easily fit the increased SEL of the PD microchannels. Considering the large increase in SEL, other (even larger) proteins might be able to cross PD microchannels. So far, endogenous protein transport through PD has been proposed for one other plant system, the companion cell–sieve element complex of the phloem. The molecular mass of these endogenous proteins ranges from 8 to 70 kDa (19, 20), which is consistent with the increase in PD SEL observed here.

Direct microinjection of TMV-MP results in an increase in PD SEL significantly larger than that observed in TMV-MP transgenic plants (Table 1). Furthermore, developmental constraints on microinjected TMV-MP seem to be less pro-

nounced: while in transgenic plants young leaves do not support movement of 10-kDa dextrans, microinjected TMV-MP promotes movement of 10-kDa dextrans even in young leaves of wild-type plants. This latter result is more consistent with the development of symptoms during viral infection, which starts and is most pronounced in the tips of young leaves. However, dextran movement is slower when comicroinjected with TMV-MP than when microinjected into transgenic plants [major movement of 10-kDa dextran after several minutes compared to 3–5 sec in transgenic plants (ref. 3; Table 1)]. This delay may reflect the time TMV-MP needs to establish its function and/or the time course of movement of TMV-MP or a TMV-MP-induced diffusable signal across PD. Since in transgenic plants TMV-MP is already present in all mesophyll cells, it does not have these requirements. Differences in PD structure between wild-type and transgenic plants could also account for the change in time course of movement.

Results derived from deletion mutant analysis suggest that a domain, designated domain E and encompassed by amino acid residues 126–224, is required, increasing PD SEL (Fig. 4). Domain E overlaps with ss nucleic acid binding domains A and B (10) but not with phosphorylation domain D (ref. 21; Fig. 7). Increasing PD SEL is probably a complex process and several individual functions may reside in this large domain, such as interaction with a cytoplasmic shuttle protein and/or a PD receptor that could be involved in localizing the TMV-MP to PD (22). Domain E is also active in DEL 15, a truncated protein lacking amino acid residues 1–110; however, the distribution pattern of fluorescent 10-kDa dextran in DEL 15 injected tissue has been considerably changed compared to wild-type TMV-MP, as no clear accumulation of fluorescent signal within the vicinity of the injected cell took place (Fig. 6).

To account for the different movement pattern observed with DEL 15, it is crucial to explain why accumulation of dye can take place in TMV-MP-induced movement. In general, pictures as in Fig. 2*C* reflect the end stage of the movement process, as from then on no further changes in the distribution pattern were observed. There are two possible explanations. (*i*) In wild-type TMV-MP, amino acids 1–110 are the target of specific cellular factors involved in inactivation. DEL 15, lacking these amino acids, is less efficiently inactivated than TMV-MP and therefore capable of influencing PD SEL over a longer time and distance. Thus, the fluorescent signal could spread beyond the field of view and would be diluted below the detection limit. This idea would imply that the N-terminal region in wild-type TMV-MP has a regulatory function and is capable of modulating the movement activity located in the C-terminal part of the protein. Two other observations suggest functional importance of the N-terminal part of TMV-MP: viral transcripts containing various N-terminal deletions in TMV-MP were no longer infectious (23) and their expression in transgenic plants resulted in reduced increase in SEL (24). Furthermore, amino acid residues 56–96 are highly conserved within tobamoviruses (25). (*ii*) An alternative and simpler explanation assumes that MP itself moves out of the injected cell to interact with more distant PD: the smaller size of DEL 15 and the deletion of a hydrophobic region between amino acid residues 60 and 80 (see hydrophobicity plot in ref. 8) could lead to a higher diffusion rate resulting in wider spread of the protein. This could account for the dilution of the fluorescent signal below the detection limit.

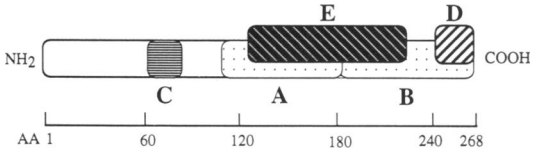

FIG. 7. Functional domains of TMV-MP. Domain A (112–185 amino acids) and domain B (185–268 amino acids), ss nucleic acid binding regions. Domain C (65–86 amino acids), required for proper protein folding. Domain D, phosphorylated region. Domain E (126–224 amino acids), region involved in SEL increase.

The following is a possible scenario for TMV-MP-induced movement: in a wild-type plant, specific plant proteins open PD microchannels and thereby facilitate transport of macromolecules. As shown here, TMV-MP mimics these plant proteins and operates the transport pathway, thereby facilitating movement of viral RNA. In a wild-type plant, the SEL eventually will be reduced to its normal level by inactivation of plant proteins responsible for its increase. The same might be true for TMV-MP and explain why the movement process is stopped after a certain time. Direct microinjection of TMV-MP into plant cells represents a powerful system to further characterize molecular requirements, mechanisms, and regulation of this critical cell-to-cell communication pathway.

We thank S. Gilroy, J. S. Haudenshield, and J. Zupan for help with initial experiments. E.W. was supported by the Austrian Fonds zur Foerderung der Wissenschaftlichen Forschung, Project JO695-MOB. This work was supported by National Science Foundation Grant DCB-90-05722 to W.J.L. and National Institutes of Health Grant GM 45244-01 to P.Z.

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