

Tobacco Mosaic Virus Movement Protein-Mediated Protein Transport between Trichome Cells

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Tobacco mosaic virus movement protein (TMV MP) is required to mediate viral spread between plant cells via plasmodesmata. Plasmodesmata are cytoplasmic bridges that connect individual plant cells and ordinarily limit molecular diffusion to small molecules and metabolites with a molecular mass up to 1 kD. Here, we characterize functional properties of *Nicotiana clevelandii* trichome plasmodesmata and analyze their interaction with TMV MP. Trichomes constitute a linear cellular system and provide a predictable pathway of movement. Their plasmodesmata are functionally distinct from plasmodesmata in other plant cell types; they allow cell-to-cell diffusion of dextrans with a molecular mass up to 7 kD, and TMV MP does not increase this size exclusion limit for dextrans. In contrast, the 30-kD TMV MP itself moves between trichome cells and specifically mediates the translocation of a 90-kD β -glucuronidase (GUS) reporter protein as a GUS::TMV MP fusion. Neither GUS by itself nor GUS in the presence of TMV MP moves between cells. These data imply that a plasmodesmal transport signal resides within TMV MP and is essential for movement. This signal confers selectivity to the translocated protein and cannot function in *trans* to support movement of other molecules.

INTRODUCTION

Cell–cell communication and molecular traffic between plant cells are largely dependent on plasmodesmata, cytoplasmic bridges that transverse the plant cell wall and link individual cells into a three-dimensional network (Beebe and Turgeon, 1991; Lucas et al., 1993). Plasmodesmata are membrane-lined pores that consist of several microchannels thought to function as transport pathways between plant cells (Ding et al., 1992b). Effective channel size is typically monitored by cell-to-cell movement of differently sized, fluorescently labeled dextrans that serve as inert tracer molecules and define the size exclusion limit (SEL) of plasmodesmata. In many types of plant cells, plasmodesmal microchannels are thought to limit molecular traffic to small molecules and metabolites with a molecular mass up to 1 kD (Tucker, 1982; Goodwin, 1983; Terry and Robards, 1987; Wolf et al., 1989; Derrick et al., 1990). In a wild-type plant, only plasmodesmata of the companion cell–sieve element system of the phloem have been shown to display a higher basal SEL and mediate transport of dextrans at least 3 kD in size (Kempers et al., 1993). Despite these severe restrictions on endogenous molecular traffic, large structures, such as plant viruses, most likely use plasmodesmata to spread from an initially infected cell to neighboring cells. This cell-to-cell movement is essential to establish systemic infection throughout the plant. Because the size of viral particles and even that of free viral nucleic acid exceed plasmodesmal channel dimensions, how do viruses exploit plasmodesmata for movement?

Several lines of evidence suggest that virus-encoded proteins are involved in the movement process. For tobacco mosaic virus (TMV), a 30-kD protein has been implicated in movement through plasmodesmata (Deom et al., 1987; Meshi et al., 1987). Two functions for TMV movement protein (TMV MP) have been identified: (1) purified TMV MP cooperatively binds single-stranded nucleic acids *in vitro* (Citovsky et al., 1990); and (2) TMV MP expressed in transgenic tobacco plants opens (“gates”) plasmodesmata to permit passage of 10-kD dextrans (Wolf et al., 1989) and even 20-kD dextrans when purified TMV MP is comicroinjected into leaf mesophyll cells of wild-type tobacco plants (Waigmann et al., 1994). Transgenic plants expressing TMV MP also facilitate movement of unencapsidated tobacco rattle virus RNA, thereby indicating that TMV MP can interact with and promote cell-to-cell movement of viral RNA in the absence of virion formation (Ziegler-Graff et al., 1991). Based on these TMV MP functions, a model for viral movement has been proposed (Citovsky and Zambryski, 1991): MP binds viral nucleic acid to coat and unfold it. The resulting thin, elongated nucleic acid–protein complex (Citovsky et al., 1992) targets plasmodesmata, opens plasmodesmal channels, and permits the passage of viral nucleic acid into the adjoining cell. This model may also describe movement of other plant viruses, because several MPs bind single-stranded nucleic acids *in vitro* (Citovsky et al., 1991; Osman et al., 1992; Pascal et al., 1994; Rouleau et al., 1994; Schoumacher et al., 1994). Some MPs also gate plasmodesmata with respect to dextran movement (Fujiwara et al., 1993; Noueir et al., 1994; Waigmann et al., 1994) as well as move themselves between adjacent cells (Fujiwara et al., 1993; Noueir et al., 1994).

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Electron microscopic studies of infected plants have led to a second model for viral cell-to-cell movement. Plants infected with, for example, cowpea mosaic virus show striking tubular structures that extend through cell walls into neighboring cells. These tubules are associated with viral MP and contain viral particles. Based on these observations, it was suggested that virions or subviral nucleocapsid may move cell to cell through these tubular extensions (van Lent et al., 1990; Weiczorek and Sanfacion, 1993; Kormelink et al., 1994).

Gating plasmodesmal channels is unlikely to be a unique function of viral MPs. That gating occurs within minutes of microinjection of MPs suggests that MPs operate an endogenous plant pathway designed for intercellular transport of macromolecules (Waigmann and Zambryski, 1994). To date, only one endogenous plant protein transport system through plasmodesmata has been observed, the companion cell–sieve element complex of the phloem. Proteins with a molecular mass up to 70 kD are most likely synthesized in companion cells and subsequently transported into the enucleate sieve elements (Fisher et al., 1992; Nakamura et al., 1993). In support of this hypothesis, the mRNA of one abundant phloem protein, PP2, has been found to be exclusively localized to companion cells (Bostwick et al., 1992), although the protein is found in both cell types (Smith et al., 1987). Because endogenous plant proteins responsible for regulating plasmodesmata transport have yet to be identified, viral MPs remain the best probes to study plasmodesmal function.

Earlier studies have used mesophyll cells for analyzing MP function by observing an MP-mediated change in movement patterns of various fluorescently labeled molecules. However, there is a major drawback: mesophyll cells are highly interconnected and form a complex three-dimensional cellular network such that only the surface cells can be clearly monitored under the light microscope. It is therefore not possible to observe or predict the exact pathway of a fluorescently labeled molecule. Studies of plasmodesmal transport would be greatly facilitated if the injected cell were part of a linear array of cells. Leaf trichomes of *Nicotiana clevelandii* consisting of four to eight cells arranged in single file provide such a system and therefore were used in this study. Moreover, trichomes are known to support movement of plant viruses (Derrick et al., 1992; Angell and Baulcombe, 1995) and thus represent a biologically relevant system for our microinjection studies.

Here, we show that mesophyll and trichome plasmodesmata are functionally distinct from each other. Trichome plasmodesmata have a considerably higher basal SEL for dextrans than do mesophyll plasmodesmata, and microinjected TMV MP does not further increase this SEL for dextrans. Despite the lack of gating with respect to dextrans, TMV MP itself moves between trichome cells. Furthermore, TMV MP specifically mediates the movement of a β -glucuronidase (GUS):TMV MP fusion protein (90 kD) between trichome cells; however, GUS (60 kD) by itself or coinjected with TMV MP does not move. These results, discussed below, suggest several criteria for macromolecular transport through plasmodesmata.

RESULTS

Trichome Plasmodesmata Have an Unusually High Basal SEL for Dextrans

To characterize *N. clevelandii* trichome plasmodesmata initially, we attempted to establish their basal SEL and kinetics of movement for various tracer molecules. Low molecular mass molecules such as Lucifer Yellow (521 D) and commercially available fluorescently labeled dextrans with average molecular masses of 4.4 and 10 kD were microinjected into a trichome cell. Figure 1 shows a diagram of a trichome; usually, cell 2 or 3 of a large trichome was used for injection. Lucifer Yellow was seen within the next cell after 1 to 2 min and had spread throughout the entire trichome in ~ 5 min (Oparka and Prior, 1992; data not shown). Surprisingly, commercially available 4.4- and 10-kD fluorescein isothiocyanate (FITC)-dextran preparations also moved between trichome cells, although at a slower rate. This indicated that the basal SEL of trichome plasmodesmata was significantly >1 kD.

To assess trichome SEL more accurately, commercially available 4.4- and 10-kD FITC-dextran preparations were fractionated on a Sephadex G-50 column (Figure 2A). The separation profile revealed a considerable molecular mass diversity of dextrans, with peak fractions corresponding to the average molecular mass given by the supplier. This was confirmed by light-scattering experiments to determine the molecular mass of two individual dextran fractions (Figure 2A, 13 and 9 kD). These two fractions as well as the peak fractions from the 4.4-kD dextran separation (Figure 2A, 4 to 5 kD)

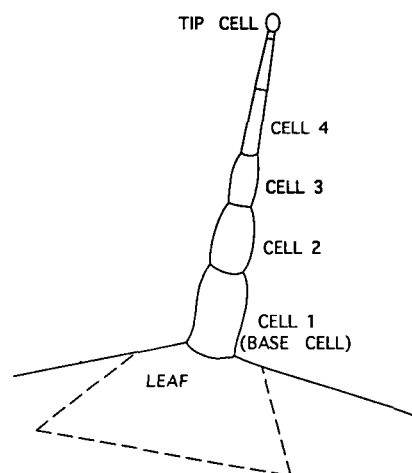


Figure 1. Schematic Drawing of an *N. clevelandii* Trichome Illustrating the Numbering System for Cells.

Microinjected trichomes were isolated for tissue fixation and immunolocalization with a piece of leaf (indicated by the dotted lines) attached to them.

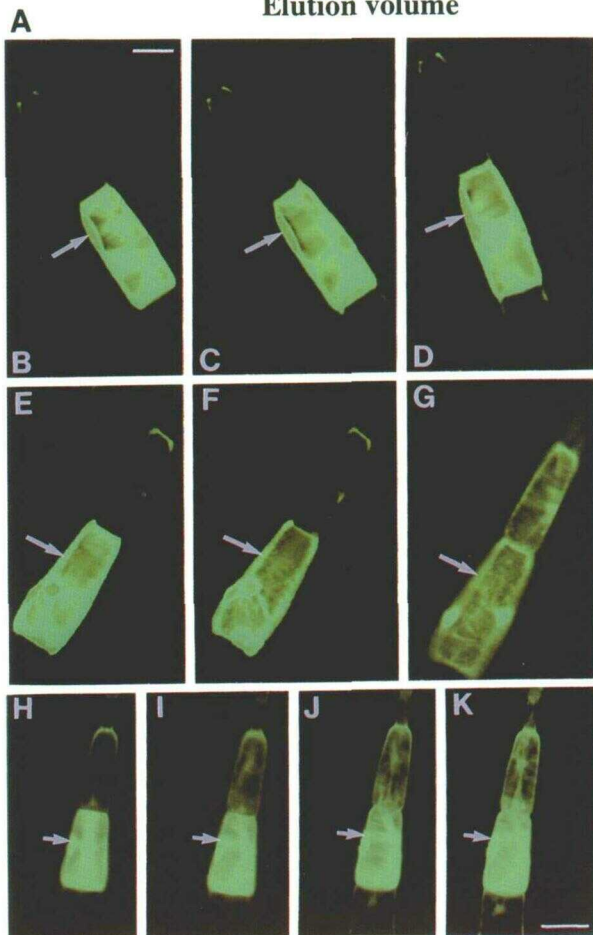
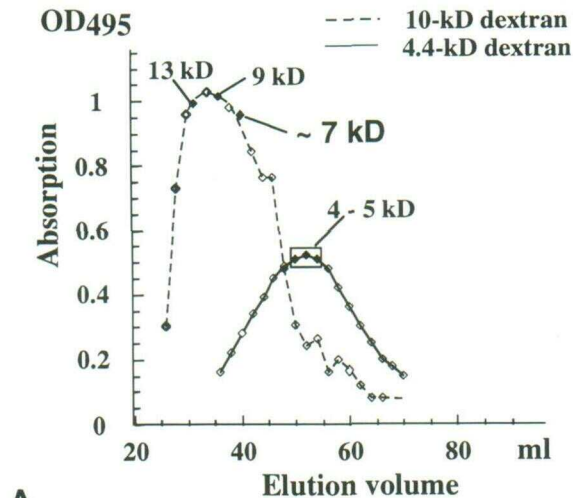


Figure 2. Determination of SEL for Dextran in Trichomes.

(A) Separation of commercially available 4.4- and 10-kD FITC-dextrans on Sephadex G-50.

(B) to (D) Microinjection of purified 9-kD FITC-dextran. Time course of movement is shown at 5 min (B), 25 min (C), and 2 hr (D) after microinjection.

Table 1. Dextran Movement in Trichomes in the Presence and Absence of TMV MP

Injected Molecule	Time Course of Movement ^a	Total Injections/Positive ^b Injections
Unpurified		
20-kD dextran	— ^c	5/1
10-kD dextran	20 min–1 hr	9/9
4.4-kD dextran	10–20 min	2/2
Purified		
13-kD dextran	—	2/0
9-kD dextran	—	3/0
7-kD dextran	1–2 hr	3/3
4- to 5-kD dextran	10–30 min	5/5
9-kD dextran + TMV MP	—	4/0
7-kD dextran + TMV MP	1–2 hr	2/2

^a Time when major movement was observed.

^b Injections that resulted in movement.

^c No movement observed.

and one intermediate fraction with an estimated molecular mass of 7 kD (Figure 2A) were tested in microinjection experiments. Dextran in the 13-kD fraction (Table 1) and 9-kD fraction (Figures 2B to 2D and Table 1) did not move between trichome cells within the observation time of 2.5 hr, whereas 7-kD dextrans moved slowly (Figures 2E to 2G and Table 1), with major movement requiring 1 to 2 hr. The 4- to 5-kD dextrans moved more rapidly, with major movement observed after 10 to 30 min (Figures 2H to 2K and Table 1). Based on our experiments, we conclude that the basal SEL of trichome plasmodesmata for dextrans is >7 kD but <9 kD.

In principle, movement of low molecular weight dyes and dextrans occurs bidirectionally (Figures 2H to 2K). However, movement from the injected cell into cells nearer to the leaf is often slower than movement into upper cells (Figures 2H to 2K). In some injections, even unidirectional movement from the injected cell toward the tip cell has been observed (data not shown). This preference in directionality of movement might reflect inherent properties of the trichome, such as the glandular function of the tip cell. To support its synthetic and secretory

(E) to (G) Microinjection of purified 7-kD FITC-dextran. Time course of movement is shown at 5 min (E), 25 min (F), and 2 hr (G) after microinjection.

(H) to (K) Microinjection of purified 4- to 5-kD FITC-dextran into a trichome cell. Time course of movement is shown at 3 min (H), 17 min (I), 30 min (J), and 41 min (K) after microinjection.

White arrows point to injected cells. Bar for (B) to (G) at top right of (B) = 50 μ m; bar for (H) to (K) at bottom right of (K) = 100 μ m.

activity, biochemical compounds might be transported routinely from the leaf into the tip cell, thus establishing a directional flow.

Although, in mesophyll cells, dye movement occurs rapidly to drain the injected cell of fluorescence (Wolf et al., 1989), in trichomes substantial fluorescence remains in the injected cell. This difference can be attributed most easily to the number of cells available for intercellular transport in the two tissue types: mesophyll cells are highly interconnected into a large network, whereas trichomes consist of only four to eight cells that we observe to be isolated from significant communication with the rest of the leaf.

TMV MP Does Not Increase Basal SEL for Dextrans Although TMV MP Itself Moves between Trichome Cells

In mesophyll cells of wild-type tobacco, coinjection of purified TMV MP with fluorescently labeled dextrans results in a dramatic increase in SEL from 1 to 20 kD (Waigmann et al., 1994). We therefore expected a similar effect on the SEL of *N. clevelandii* trichome plasmodesmata. Surprisingly, when TMV MP was coinjected with dextran fractions of 9 kD, exceeding the basal SEL, we observed no TMV MP-induced movement (Table 1). In addition, coinjection of TMV MP with dextran fractions of

7 kD, smaller than the SEL, resulted in no significant change in the time course of movement (Table 1). Therefore, TMV MP does not gate trichome plasmodesmata to increase their SEL for dextrans.

That viruses are capable of movement through trichomes (Derrick et al., 1992; Angell and Baulcombe, 1995) strongly suggests an interaction between MP and trichome plasmodesmata. A direct way of testing the interaction between TMV MP and trichome plasmodesmata is to determine whether TMV MP itself can move between trichome cells. It was not possible to monitor movement of fluorescently labeled TMV MP directly, because TMV MP solubility is low and fluorescent labeling of the protein results in a signal too dilute to detect by fluorescence microscopy. In addition, fluorescently labeled TMV MP no longer binds single-stranded nucleic acids, which suggests inactivation of the protein by the labeling procedure (data not shown).

We therefore used a combination of whole-mount fixation and immunolocalization to detect TMV MP movement. A mixture of purified TMV MP and the fluorescent low molecular mass dye Lucifer Yellow was injected into an *N. clevelandii* trichome cell; Lucifer Yellow served as a quality control for microinjection and was expected to move between cells. As a negative control, trichomes were injected with Lucifer Yellow only. After 20 to 30 min, trichomes were fixed and subjected

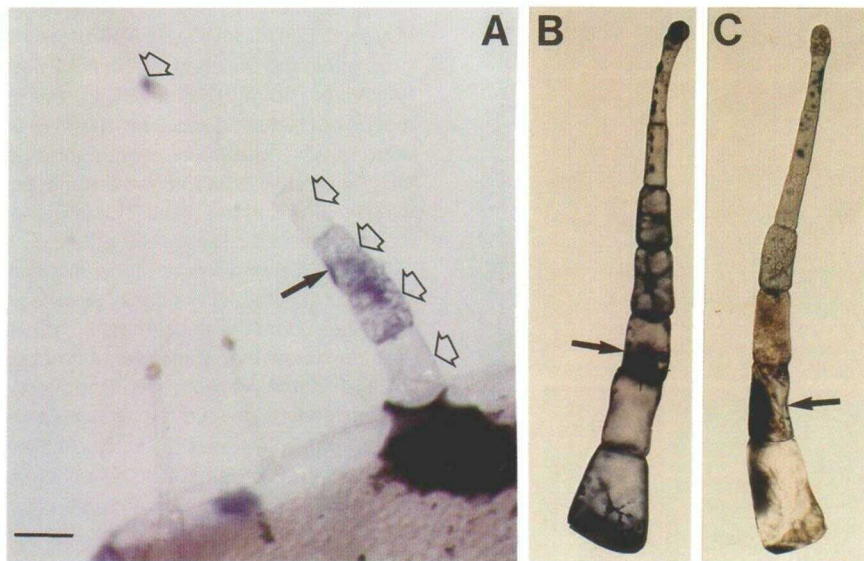


Figure 3. Immunolocalization of Microinjected TMV MP within a Trichome.

(A) and (B) Trichomes injected with purified TMV MP and Lucifer Yellow. The primary antibody dilution in (A) is 1:50 and in (B) it is 1:5. (C) Trichome injected with Lucifer Yellow only. The primary antibody dilution is 1:5.

The solid black arrows point to microinjected cells. Open black arrows indicate individual trichome cells, including tip cells, that show localization of TMV MP. Photographs were taken with either a Nikon dissecting microscope, as in (A), or a Zeiss Axiophot, as in (B) and (C). Due to different illumination techniques, trichomes appear almost translucent under the fiber optic illumination of the dissecting microscope, as shown in (A), whereas the trichomes have a brownish background under the Zeiss Axiophot compound microscope, as shown in (B) and (C). The black spot at the base of the trichome in (A) and on the left side of the two bottom cells of the trichome in (C) is derived from marker pen ink (see Methods). Bar for (A) to (C) at bottom left of (A) = 100 μ m.

Table 2. Results from the Fluorescent GUS Assay in Trichomes

Injected into		Substrate Concentration in Needle (μM)	Total Injections/Positive ^a Injections
First Cell	Second Cell		
GUS, S	— ^b	10	1/1 ^d
	—	100	5/3
	—	1000	1/1
S ^c	—	100	4/0
GUS	S	100	4/1 ^d
GUS::TMV MP	S	100	5/4
GUS, TMV MP	S	100	4/1 ^d

^a Positive injections were injections in which a fluorescent signal developed in the cell injected with substrate within 2 hr.

^b Second cell was not injected.

^c Substrate.

^d Extremely weak signal.

to immunocytochemistry. TMV MP localization was detected by binding to an anti-TMV MP antibody/alkaline phosphatase-coupled secondary antibody, with subsequent visualization by a chromogenic reaction creating a blue precipitate. Trichomes microinjected with TMV MP showed reaction with anti-TMV MP antibody (denoted by the blue color) throughout, indicating that TMV MP moved in both directions, up into the tip cell and down into the base cell (Figure 3B). Essentially the same movement pattern was observed when trichomes were incubated with 10 times less concentrated primary antibody, although a gradient in signal intensity was observed in which the microinjected cell, immediately surrounding cells, and the tip cell showed the most intense coloring (Figure 3A). In contrast, control trichomes injected with dye alone showed only light brown background color (Figure 3C). These experiments indicate that microinjected TMV MP moves between *N. clevelandii* trichome cells.

TMV MP Specifically Mediates Movement of a GUS::TMV MP Fusion Protein

The studies discussed above suggest that trichome plasmodesmata are selective in supporting molecular transport because proteins (TMV MP) can move but dextrans >7 kD cannot. A selection based purely on size is unlikely, because the hydrodynamic radius of TMV MP is probably greater than that of 7-kD dextrans (le Maire et al., 1989). One possible basis for this selection is that a plasmodesmal transport signal is required for efficient translocation. Only molecules designed for movement between cells, like TMV MP, harbor such a plasmodesmal transport signal, whereas dextrans and presumably other unrelated proteins do not. To address this question, we tested whether GUS, a bacterial enzyme, was capable of moving between cells. We also asked what would be the effect

of TMV MP on such movement, either during comicroinjection of TMV MP and GUS or in the context of a GUS::TMV MP fusion protein. The GUS reporter protein has two advantages: it can be detected by a highly sensitive enzymatic assay to determine its localization within the trichome, and detection requires that GUS be functional following translocation through plasmodesmata.

The GUS assay involves cleavage of the nonfluorescent substrate Imagen Green into a highly fluorescent yellow greenish product. Both substrate and product are lipophilic because of the presence of a hydrocarbon chain, and they localize to the plasma membrane. Neither substrate nor product moves between cells, because lipids incorporated into the plasma

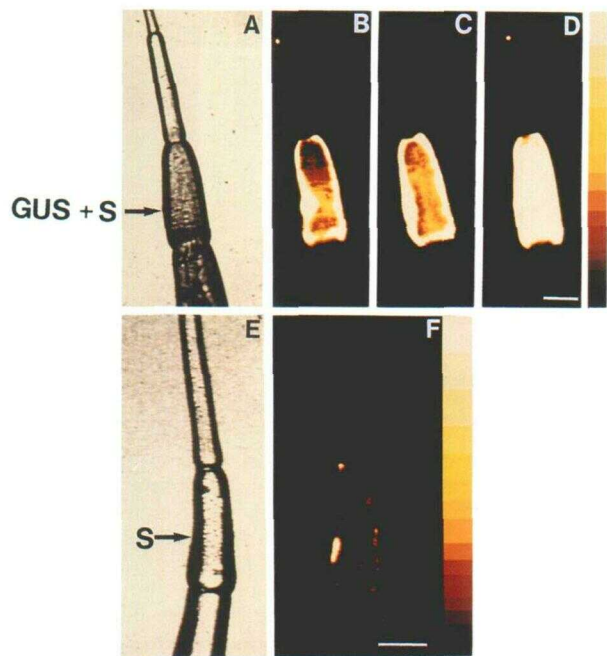


Figure 4. Time Course and Specificity of Microinjected GUS Activity in Trichomes.

(A) to (D) Time course of GUS activity in trichomes, assayed by coinjection of GUS enzyme and substrate Imagen Green. (A) shows bright-field microscopy of a trichome 4.5 hr after microinjection. (B) to (D) False color representations of fluorescence images of the same trichome at 35 min (B), 1 hr (C), and 4.5 hr (D) after microinjection. Note that according to the supplier, Imagen Green localizes to the plasma membrane, does not move between cells, and is therefore confined to the injected cell.

(E) and (F) Test for background GUS activity in trichomes. (E) shows bright-field microscopy and (F) is a false color representation of a fluorescence image of the same trichome 2.5 hr after microinjection of the substrate Imagen Green.

Color bar to the right shows signal intensity in color code. Black and red indicate background; white denotes the highest signal intensity. Arrows point to injected cells. S, substrate. Bar for (A) to (D) at bottom right of (D) = 50 μm ; bar for (E) and (F) at bottom right of (F) = 50 μm .

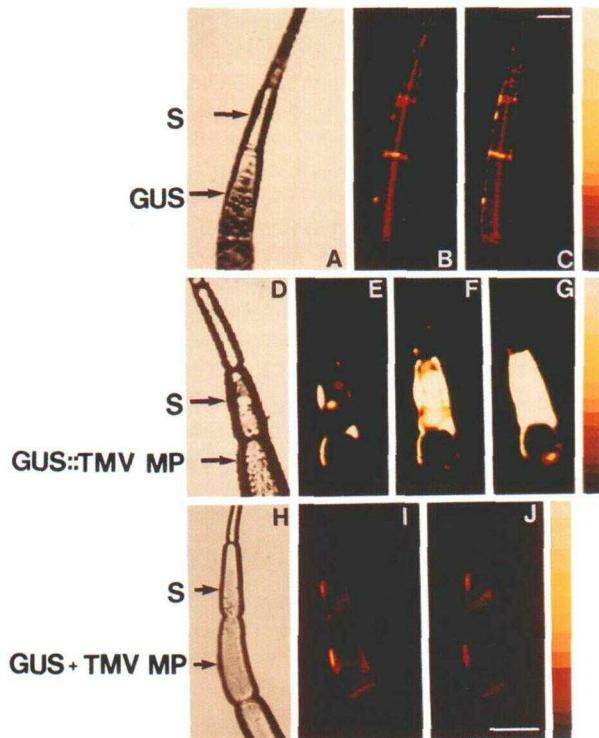


Figure 5. Analysis of Protein Movement between Trichome Cells.

(A) to (C) Microinjection of GUS enzyme and substrate into separate trichome cells. (A) shows bright-field microscopy of a microinjected trichome 1.5 hr after substrate injection. (B) and (C) are false color representations of fluorescence images of the same trichome 5 min (B) and 1.5 hr (C) after substrate injection. The camera was set to be sensitive enough to detect background fluorescence (red color); this ensures that even slight increases in fluorescence can be monitored. (D) to (G) Microinjection of GUS::TMV MP fusion protein and substrate into separate trichome cells. (D) shows bright-field microscopy of a microinjected trichome 2.5 hr after substrate injection. (E) to (G) are false color representations of fluorescence images of the same trichome 5 min (E), 25 min (F), and 2.5 hr (G) after substrate injection.

(H) to (J) Comicroinjection of GUS and TMV MP as two separate proteins into one trichome cell and substrate into a neighboring cell. (H) shows bright-field microscopy of a microinjected trichome 1 hr and 40 min after substrate injection. (I) and (J) are false color representations of fluorescence images of the same trichome 5 min (I) and 1 hr and 40 min (J) after substrate injection.

S, substrate. Bar for (A) to (C) at top right of (C) = 100 μ m. Bar for (D) to (J) in bottom right of (J) = 100 μ m. Color bar and arrows are as given in Figure 4.

membrane do not move between cells (Grabski et al., 1993). To ensure that these features existed in our system, GUS was premixed with substrate at different concentrations (10 μ M, 100 μ M, and 1 mM) and immediately injected into the same trichome cell (Table 2). As a quality control for microinjection in this and all following experiments, the low molecular mass dye Cascade Blue was used to ensure cell viability and potential

for movement. This dye can be monitored in a separate channel, and its signal does not interfere with the signal generated by cleavage of Imagen Green. Upon coinjection of GUS and the substrate, an intense fluorescent signal developed at substrate concentrations of 100 μ M (Figures 4A to 4D and Table 2) and 1 mM (Table 2) and was confined to the injected cell. With a substrate concentration of 10 μ M, only a very weak signal developed (Table 2). Based on these results, a substrate concentration of 100 μ M was used in all subsequent experiments. In contrast, no fluorescent signal developed after substrate injection alone (Figures 4E and 4F, and Table 2). These experiments show that product and substrate do not move between cells and that the fluorescent signal is generated by microinjected GUS and not by endogenous cellular activity.

To test whether GUS moves between cells, GUS was injected into one cell; 30 min later the substrate was injected separately into an adjacent cell (Figure 5A). No signal developed in the substrate-injected cell after 1.5 hr (Figures 5B and 5C, and Table 2) or after overnight incubation (data not shown). Thus, GUS is unable to move between cells.

Next, we examined whether TMV MP could mediate GUS movement. We constructed a fusion protein by linking the TMV MP coding sequence to the C terminus of the GUS coding sequence. The GUS::TMV MP fusion protein was overexpressed and purified from *Escherichia coli*, as described by Waigmann et al. (1994). Purified GUS::TMV MP fusion protein was injected into a trichome cell, followed 30 min later by substrate injection into an adjacent cell (Figure 5D). Within the first 25 min after substrate injection, a significant amount of fluorescent signal developed in the substrate-injected cell and continued to increase during the next hours (Figures 5E to 5G, and Table 2). Thus, the GUS::TMV MP fusion protein moves between trichome cells.

To determine whether a physical link between the two proteins is necessary for movement or whether TMV MP could also support GUS movement in *trans*, we injected the separate GUS and TMV MP proteins into one trichome cell. Again, the substrate was injected 30 min later into an adjacent cell (Figure 5H). There was no development of fluorescent signal in the substrate-injected cell after 1 hr and 40 min (Figures 5I and 5J, and Table 2). Based on these observations, TMV MP cannot facilitate GUS movement in *trans*. Taken together, these results suggest that TMV MP most likely contains functional information to promote its own movement through plasmodesmata.

DISCUSSION

We used direct microinjection of various fluorescently labeled molecules to analyze transport characteristics of *N. cleavelandii* trichome plasmodesmata. In contrast with the highly interconnected mesophyll cells used in previous studies, trichomes constitute a linear cellular system and thus simplify movement analysis. Also, trichomes are known to support

movement of plant viruses (Derrick et al., 1992; Angell and Baulcombe, 1995), which suggests a biologically relevant interaction between trichome plasmodesmata and viral MPs. TMV MP has been used as a probe to study protein transport through plasmodesmata, to analyze possible implications on viral movement, and to characterize trichome plasmodesmata functionally.

The results presented indicate that *N. clelandii* trichome plasmodesmata are functionally distinct from mesophyll plasmodesmata, the only other functionally and structurally well-characterized plasmodesmal system, because their SEL for dextrans is 7 kD (versus 1 kD in mesophyll cells), and this SEL cannot be increased further by coinjection with TMV MP. The presence of TMV MP had no effect on either the time course of dextran movement or the SEL of *N. clelandii* trichome plasmodesmata (Table 1), whereas coinjection of TMV

MP into tobacco mesophyll cells dramatically increases SEL for dextrans from 1 to 20 kD (Waigmann et al., 1994). Thus, the basal functional properties of these two plasmodesmata types as well as their interaction with TMV MP are different. In addition, movement through trichomes is generally slower than movement through mesophyll cells: even low molecular mass dyes, such as Lucifer Yellow or Cascade Blue, require 1 to 2 min to move between adjacent trichome cells (Oparka and Prior, 1992; this work), whereas they move within a few seconds in mesophyll cells (Wolf et al., 1989). Taken together, the results indicate that plasmodesmata from different cell types might have a spectrum of functional properties, depending on the specific transport requirements of a particular tissue type or on the developmental state of the plant.

Microinjection of MPs into mesophyll cells of wild-type plants results in a dramatic increase in SEL for dextrans within minutes; moreover, MPs themselves move with similar kinetics between cells (Fujiwara et al., 1993; Noueir et al., 1994; Waigmann et al., 1994). In our experiments, TMV MP movement was not monitored directly but analyzed immunocytochemically 30 min after microinjection; during that time period, but possibly much sooner, TMV MP had moved throughout the whole trichome. In contrast with TMV MP movement in mesophyll cells, TMV MP movement in trichomes was not accompanied by an increase in SEL for dextrans. Therefore, interaction between plasmodesmata and TMV MP can occur independent of an increase in effective channel size as monitored by dextrans.

Earlier published studies concluded that the basal SEL for dextrans in trichome plasmodesmata is <4.4 kD (Derrick et al., 1992; Vaquero et al., 1994). In both cases, however, unfractionated, polydisperse fluorescently labeled dextrans were used, which most likely complicated assessment of the SEL. Also, the differences in observation time (20 min in the study by Derrick et al. [1992] and 4 min in the study by Vaquero et al. [1994] versus 2.5 hr in our work) might account for this discrepancy. However, even during their short observation time, Vaquero et al. (1994) reported movement of 4.4-kD dextrans in 12% of their experiments; this supports our studies that score major movement of 4.4-kD dextrans between 10 and 30 min. Both of these earlier studies also analyzed the potential influence of viral MPs on the SEL of trichome plasmodesmata. Again, polydisperse dextrans were used for assessment of SEL; in addition, different viruses and completely different experimental strategies were employed. We therefore cannot directly compare their data with ours.

The current model for TMV movement (Citovsky and Zambryski, 1991) proposes that TMV MP binding to viral nucleic acid and gating of plasmodesmata are essential features of the movement process. However, the proposed requirement for gating was based on studies conducted in mesophyll cells (Wolf et al., 1989). Here, we report that TMV MP movement between trichome cells is not accompanied by an increase in SEL as monitored by dextrans. This latter observation apparently contradicts the model's requirement for TMV MP-mediated gating of plasmodesmata.

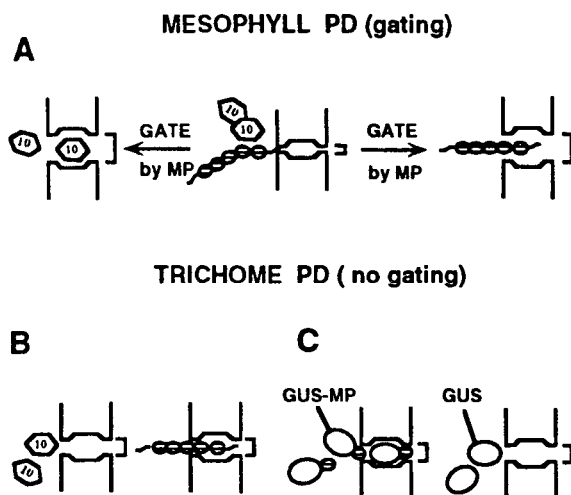


Figure 6. Model for Mesophyll versus Trichome Plasmodesmal Transport.

The following different molecules for transport are shown: a hexagonal-shaped molecule indicates 10-kD dextran, a large unshaded oval indicates GUS, and a small shaded oval indicates TMV MP. TMV MP is shown either as a complex with single-stranded nucleic acid (see wavy tails at ends of complex) or as a fusion with GUS. Plasmodesmata are depicted as open passageways; square brackets to the right of each plasmodesma indicate estimates of their size. Plasmodesmal transport is indicated by the presence of the molecules within the plasmodesmal channel.

(A) Transport through mesophyll plasmodesmata. Plasmodesmata size is small (center) before gating by TMV MP (at left and right). Movement of both 10-kD dextrans and TMV MP–single-stranded nucleic acid complex occurs after MP gating.

(B) and **(C)** Transport through trichome plasmodesmata. The size of the plasmodesmal channel is the same in all panels and is larger than the ungated mesophyll channel but smaller than the gated mesophyll channel. Neither 10-kD dextrans nor GUS can move on their own, but a GUS:TMV MP fusion protein can (see text).

PD, plasmodesmata.

The present results modify our perception of viral movement in several ways. One is that, depending on the plasmodesmata type, viral movement may show different characteristics. For example, in mesophyll cells, the basal molecular SEL for diffusing molecules is 1 kD. This indicates that transport channels are small in size. In this system, even molecules like MP or viral nucleic acid–MP complexes, which are presumably actively translocated, require an increase in channel size (Figure 6A). This increase in channel size can be monitored by movement of larger dextrans, for example, 10-kD dextrans (Figure 6A). In contrast, in trichomes the already high basal SEL of plasmodesmata, together with the elongated shape of the nucleic acid–MP complex (Citovsky et al., 1992), might be sufficient for viral movement to proceed without additional gating (Figure 6B).

Alternatively, in trichomes, dextran movement might not reflect an MP-mediated increase in channel size, even though this increase takes place. Possibly, MP moves fast and only transiently increases plasmodesmal channel size, for example, only while MP moves through plasmodesmata. Plasmodesmal channels could be opened rapidly and, as soon as TMV MP has entered the passageway, reduced to normal size again, thus effectively preventing dextrans exceeding the basal SEL from entering the transport channel. In such a case, no TMV MP-mediated increase in SEL for dextrans would be observed.

Or, MP-induced increase in effective channel size for dextrans, as observed in mesophyll cells, may not be directly related to the viral movement process. Instead, viral movement and increase of SEL for dextrans could be independent events that take place individually at specific types of plasmodesmata. For example, in mesophyll cells, two structurally distinct plasmodesmata types have been identified: primary and secondary. During leaf development within a plant, the relative amounts of these two plasmodesmata types in mesophyll cells change with leaf age such that very young leaves contain predominantly primary plasmodesmata, whereas older, fully expanded leaves contain predominantly secondary plasmodesmata (Ding et al., 1992a).

Taking these observations into account, the following scenario seems possible: MP might be sequestered into secondary mesophyll plasmodesmata upon phosphorylation by a cell wall-associated kinase (Citovsky et al., 1993), thereby structurally modifying these plasmodesmata, as has been demonstrated in transgenic plants expressing TMV MP (Ding et al., 1992a). This results in a larger SEL for the modified secondary plasmodesmata, and consequently, larger dextrans can move. Viral movement, on the other hand, proceeds through primary plasmodesmata, and the movement process is not accompanied by an increase in effective channel size for dextrans. If this scenario were the case, increase in effective channel size may not be an essential prerequisite for viral movement. *N. clelandii* trichome plasmodesmata could reflect a type of plasmodesmata that supports TMV movement but whose interaction with TMV MP cannot be monitored by an increase in SEL for dextrans.

We also attempted to elucidate whether TMV MP could mediate movement of single-stranded nucleic acids between trichome cells. Complexes between TMV MP and covalently labeled fluorescent RNA were formed in vitro and microinjected into a trichome cell. Generally, we observed only a low level of fluorescent signal in the neighboring cells; this did not indicate substantial movement (E. Waigmann and P. Zambryski, unpublished data). However, we cannot rule out technical reasons for the lack of substantial movement: even in dilute solutions, complexes tend to form aggregates with each other that are readily observed under the microscope as small, highly fluorescent particles. These complex aggregates not only complicate microinjection itself but also may represent a biologically inactive form. Alternatively, the lack of efficient complex movement observed in trichomes could indicate the requirement for additional viral factors.

N. clelandii trichome plasmodesmata constitute an efficient transport system with respect to protein movement. TMV MP itself not only moves between trichome cells, but it specifically mediates the movement of a GUS:TMV MP fusion protein with a molecular mass of 90 kD, whereas GUS alone with a smaller molecular mass (60 kD) cannot move between cells. Clearly, in trichomes, protein movement is not a simple diffusion process, with size as the major selection criterion for movement as assumed for dextrans. Instead, the data suggest that a plasmodesmal transport signal resides within MP and is essential for an active transport mechanism. As summarized in Figure 6, the present results imply a complex situation with at least three general criteria for transport through plasmodesmata: (1) size of plasmodesmal channels (Figure 6A), (2) size and shape of transported molecules (Figure 6B), and (3) the presence or absence of a plasmodesmal targeting/transport signal sequence (Figure 6C). In the case of TMV MP-mediated protein movement, this latter requirement might be of paramount importance. The proposed signal is functional not only within TMV MP but also in the more general context of a GUS:TMV MP fusion protein. In contrast, it is not present in GUS alone (Figure 6C). That a plasmodesmal transport signal is primarily responsible for protein movement through trichomes is supported by the observation that TMV MP cannot promote GUS movement in *trans*; instead, the physical connection of the two proteins (as a fusion) is required for GUS movement.

Possibly, the transport process is a chaperoned event that involves unfolding/refolding of the translocated polypeptide chain; this could explain why protein size per se does not appear to be a selection criterion. Because several viral MPs have been shown to move to cells considerably distant from the injection site (Fujiwara et al., 1993; Noueiry et al., 1994; this work; Figure 3) and thus are capable of crossing multiple plasmodesmata, proteolytic cleavage of a putative plasmodesmal transport signal is probably not involved in translocation. So far, it is unclear whether different viral MPs might have different transport signals, with each interacting with a specific plasmodesmal receptor, or whether they might share the same plasmodes-

mal transport signal and receptor. In the latter case, the putative signal would probably be characterized by structural rather than sequence information because computer-based comparisons of available viral MP sequences do not point to any highly conserved sequences (Koonin et al., 1991; Mushegian and Koonin, 1993).

Endogenous plant proteins might also contain such a plasmodesmal transport signal and be capable of interaction with, and movement through, plasmodesmata. A candidate for such a protein is the maize homeodomain protein *KNOTTED1*, which is implicated in the initiation of leaf and flower organ development. Whereas the protein is detected in all cell layers of the inflorescence meristem, including the outer tunica layer, the mRNA is confined to the inner meristem layers (Smith et al., 1992; Jackson et al., 1994). To explain this observation, Jackson et al. (1994) suggested that the *KNOTTED1* protein itself might move from the inner tissue layers to the outer layer. Although not yet proven, this movement might occur through plasmodesmata. Thus, movement of plant proteins through plasmodesmata might be of importance in the regulation and coordination of numerous events within a plant, such as development and signal response. Elucidation of a plasmodesmal transport signal using viral MP paradigms might provide a tool for identifying endogenous plant proteins that traffic the plasmodesmal network.

METHODS

Plant Material

Six-week-old *Nicotiana clevelandii* plants grown from seed were used for all microinjection experiments. Plants were grown in the greenhouse under long-day conditions (16 hr light/8 hr dark).

Cloning and Expression of Proteins Used for Microinjection

For GUS::TMV MP, the β -glucuronidase (*GUS*) gene was derived from plasmid pRTL2SG-N1a/ Δ B+K (Restrepo et al., 1990), and the tobacco mosaic virus movement protein (TMV MP) gene was derived from plasmid petP30 (Citovsky et al., 1990). petP30 was cut with *Nde*I, and the recessed end was filled in with the Klenow fragment of DNA polymerase I. The TMV MP gene was subsequently released from the petP30 plasmid by digestion with *Bam*HI. This fragment was ligated to pRTL2SG-N1a/ Δ B+K previously cut with *Bgl*II (filled in with the Klenow fragment) and *Bam*HI, thereby joining the 3' end of the *GUS* gene to the 5' end of the TMV MP gene and creating plasmid pRTL2SG-N1a/ Δ B+K::TMV MP. As a result of the cloning strategy, two additional codons were generated at the junction site between the *GUS* gene and the TMV MP gene. To put the fusion gene under the control of a bacterial promoter, plasmid pRTL2SG-N1a/ Δ B+K::TMV MP was then cut with *Nco*I and *Bam*HI and ligated into the bacterial expression vector pet3D previously cut with the same enzymes to generate plasmid pet3-GUS::TMV MP. Proteins GUS::TMV MP and TMV MP were overexpressed in *Escherichia coli* BL21(DE3)pLysE and purified to homogeneity as originally described by Citovsky et al. (1990) with modifications by Waigmann et al. (1994).

Dextran Separation on Sephadex G-50

Two hundred microliters of 10 mM fluorescein isothiocyanate-dextran (FITC-dextran; average molecular mass either 10 or 4.4 kD; Sigma) in 5 mM KHCO_3 , pH 7.6, was loaded onto a size exclusion column (dimensions 2.5 \times 50 cm) filled with Sephadex G-50 preswollen in 5 mM KHCO_3 . The column was run with 5 mM KHCO_3 buffer, and 1-mL fractions were collected. FITC-dextran concentration in individual fractions was detected by optical density measurements at a wavelength of 495 nm. Selected dextran fractions were concentrated by precipitation in 80% ethanol for 1 hr at -20°C and redissolved in 5 mM KHCO_3 at a concentration of 0.1 to 0.5 mM. The hydrodynamic radius and molecular mass of individual fractions were determined by light scattering (model No. Dynapro/801S; Protein Solutions Inc., Charlottesville, VA).

Microinjection Conditions

Microinjection and fluorescence microscopy were performed on an Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a silicon intensified target videocamera (Dage-MTI, Inc., Michigan City, IN) and a cooled charge coupled device camera (Princeton Instruments, Trenton, NJ). Microinjection of trichome cells was performed as described by Oparka et al. (1991). Briefly, leaves were cut off at the stem with a razor blade and immediately placed into an Eppendorf tube containing water. The Eppendorf tube was sealed with Parafilm (American National Can, Greenwich, CT) to secure the leaf. The severed leaf was propped up on a small piece of foam under the microscope to prevent damage of trichomes. Only trichomes at the leaf edge were used for injection. Injection was performed while supporting the trichome with the microforged tip of a glass needle to avoid damage to the cell. Support and injection needles were exactly aligned on opposite sides of the cell to be injected. Injection was carried out at a pressure of 50 to 60 psi. Withdrawal of the injection needle from the cell was done slowly over the course of several minutes to facilitate sealing of the injection wound. The concentration of injected substances in the needle was as follows: FITC-labeled dextrans (Sigma), 0.1 to 0.2 mM; Imagen Green (Molecular Probes, Inc., Eugene, OR), 0.1 mM; Cascade Blue (Molecular Probes), 0.05 mM; Lucifer Yellow (Sigma), 0.1 mM; TMV MP and GUS::TMV MP, 0.1 to 0.2 $\mu\text{g}/\mu\text{L}$; GUS (Boehringer Mannheim), 0.02 units per μL .

Whole-Mount Fixation and Immunolocalization

Trichomes were marked under the microscope with black marker pen at or near their base cell. A small piece of leaf with the injected trichome at its edge (Figure 1) was cut out; the piece of leaf served as a "grip" for all subsequent operations because the trichome itself was too fragile to be handled. Fixation and cell wall permeabilization by proteinase K digestion were performed essentially as described by Tautz and Pfeifle (1989). Briefly, trichomes were fixed for 30 min in 3% formaldehyde, rinsed several times with methanol and ethanol, and stored overnight at -20°C in ethanol. They were postfixing in 5% formaldehyde in PBST (0.137 M NaCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , 2.7 mM KCl, 0.1% Tween 20, pH 8) for 25 min, rinsed several times in PBST, and digested with proteinase K (40 $\mu\text{g}/\text{mL}$ in PBST) for 10 min. Postfixing and washing were repeated once as described above. Trichomes then were blocked for 40 min in 1% BSA in PBST and incubated overnight with affinity-purified anti-TMV MP antibody (purified from

polyclonal rabbit serum as described by Smith and Fisher [1984]). Before incubation, affinity-purified anti-TMV MP antibody was diluted either five- or 50-fold in PBST. Samples were washed in PBST and subsequently incubated for 2 hr with alkaline phosphatase-coupled goat anti-rabbit antibody (1:1000 diluted in 1% BSA in PBST). After several washes in PBST, alkaline phosphatase activity was detected by incubation with the chromogenic substrates 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium, which are converted in situ into a dense blue compound. Developing time was 30 min.

Photographic images were taken with Kodak Ektar 100 color film; samples were viewed either on a dissecting microscope (Figure 3A) or on a Zeiss Axiophot microscope (Figures 3B and 3C). In the latter case, groups of trichome cells were photographed (due to the size of trichome cells, only two or three cells fit into the field of view), and a picture of the whole trichome was reconstructed from individual prints. Whole-mount fixation of microinjected trichomes and immunolocalization of TMV MP were carried out three times: an alkaline phosphatase-coupled secondary antibody was used twice, and an FITC-coupled secondary antibody was used once. All three experiments gave positive results, indicating TMV MP movement; however, the signal with the fluorescently labeled antibody was considerably weaker than the signal with the alkaline phosphatase-coupled secondary antibody.

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