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Increase in Plasmodesmatal Permeability during Cell-to-Cell Spread of Tobacco Rattle Virus from Individually Inoculated Cells

Peter M. Derrick,[1] Hugh Barker, and Karl J. Oparka

Scottish Crop Research Institute, Invergowrie, Dundee, DD2 5DA, United Kingdom

A microinjection technique was devised for inoculation of single *Nicotiana clevelandii* leaf trichome cells with virus particles. By removing inoculated trichomes at various times after microinjection, it was shown that at least 4 hr were required for tobacco rattle virus (TRV; tobravirus group) to move out of primarily inoculated cells. Effects of the early stages of TRV infection on plasmodesmatal permeability were examined by microinjection of fluorochrome-labeled molecules. Fluorescein-labeled insulin A chain (M_r, 2921) and fluorescein-labeled dextran (M_r, 4400) were observed to pass out of individual *N. clevelandii* trichome cells that had been inoculated with TRV by microinjection 5 hr previously. By contrast, Lucifer Yellow CH-labeled dextran (M_r, 10,000) was restricted to the inoculated cell. None of these fluorescent probes were able to move out of uninoculated cells or out of cells that had been inoculated with TRV only 2 hr previously. The movement of macromolecules through plasmodesmata, therefore, coincided with and probably resulted from cell-to-cell movement of TRV. The results are discussed with reference to the interaction of viruses and plasmodesmata and mechanisms of intercellular virus movement.

INTRODUCTION

The spread of a plant virus from cell to cell involves symplasmic movement of viral genetic material across cell walls via plasmodesmata (for recent reviews, see Atabekov and Taliansky, 1990; Citovsky and Zambryski, 1991; Hull, 1991; Maule, 1991; Deom et al., 1992). Results of several studies have demonstrated that only molecules with a molecular mass of less than about 1 kD can move between cells via plasmodesmata (reviewed by Robards and Lucas, 1990). Virus particles and nucleic acids are much larger than these molecules and so virus-induced mechanisms that increase the permeability of plasmodesmata during intercellular virus movement must exist. Considerable evidence exists that implies that the genomes of many viruses encode movement proteins (MPs) that enable them to pass from cell to cell (reviewed by Atabekov and Taliansky, 1990; Maule, 1991).

Although little is known about how MPs enable intercellular transfer of virus particles or nucleic acid, recent work has revealed some of their properties. It has been demonstrated that in vitro, the MPs of tobacco mosaic virus (TMV; tobamovirus group), cauliflower mosaic virus (caulimovirus group), red clover necrotic mosaic virus (dianthovirus group), and alfalfa mosaic virus bind strongly and cooperatively to single-stranded nucleic acids in a sequence nonspecific manner (Citovsky et al., 1990, 1991; Osman et al., 1992; Schoumacher et al., 1992). Citovsky et al. (1991) proposed a model for the TMV MP as a bifunctional molecule that binds both to viral nucleic acid and to plasmodesmata (the latter property of the TMV MP had been previously reported by Tomenius et al., 1987). Other recent reports have implicated domains of the TMV MP amino acid sequence in locating the protein within plasmodesmata (Berna et al., 1991) and binding to nucleic acids (Citovsky et al., 1992). Wolf et al. (1989, 1991) introduced fluorescein isothiocyanate (FITC)–labeled dextrans by microinjection into individual mesophyll cells of tobacco transformed with constructs of the MP gene of TMV and observed a substantial increase in plasmodesmatal permeability compared with tests on control plants. This suggested that in transgenic tobacco the TMV MP is able to modify plasmodesmata.

We have attempted to examine changes in the size exclusion limit of plasmodesmata during the development of a true virus infection. Previous work (Derrick et al., 1990) indicated that for several viruses such changes are only short lived. Therefore, we investigated the early stages of tobacco rattle virus (TRV; tobravirus group) infection in *Nicotiana clevelandii* to observe any transient effects of infection on plasmodesmatal permeability. Using this approach, we were able to determine whether increases in permeability similar to those detected in plants transformed with the TMV MP gene would also be observable during interaction of a whole virus genome with its host plant. In this report, we describe a time scale for intercellular spread of TRV from cells individually inoculated with virus particles and show that, during this movement, the exclusion limit of plasmodesmata is increased to a size that may allow passage of viral ribonucleoprotein complexes but not intact virus particles.

[1] To whom correspondence should be addressed.

RESULTS

Inoculation of Single *N. clevelandii* Trichome Cells and Measurement of the Rate of Cell-to-Cell Movement of TRV

To measure the rate of cell-to-cell movement of TRV, purified virus particles were microinjected into the cytoplasm of single trichome cells, and the trichomes were subsequently detached from the leaf at various times following inoculation, thereby preventing (or allowing) virus movement from the trichome into the leaf lamina. Detachment of trichomes after various time intervals following inoculation of either cell 1, 2, or 3, as designated in Figure 1A, enabled estimation of the time required for TRV to move across one, two, or three cell boundaries.

Figure 1B illustrates microinjection of a trichome cell, with the spread of fluorescence within sharply defined cytoplasmic strands indicating an injection into the cytoplasm. Vacuolar injections were typified by a diffuse spread of fluorescence throughout the injected cell (see Figure 2 in Oparka et al., 1991). After inoculation, cytoplasmic streaming was unfortunately difficult to observe in many trichomes because of the highly refractive curved cell surface. However, in cases where it was observed, streaming was either undisturbed by microinjection or was temporarily arrested and recovered within 5 min.

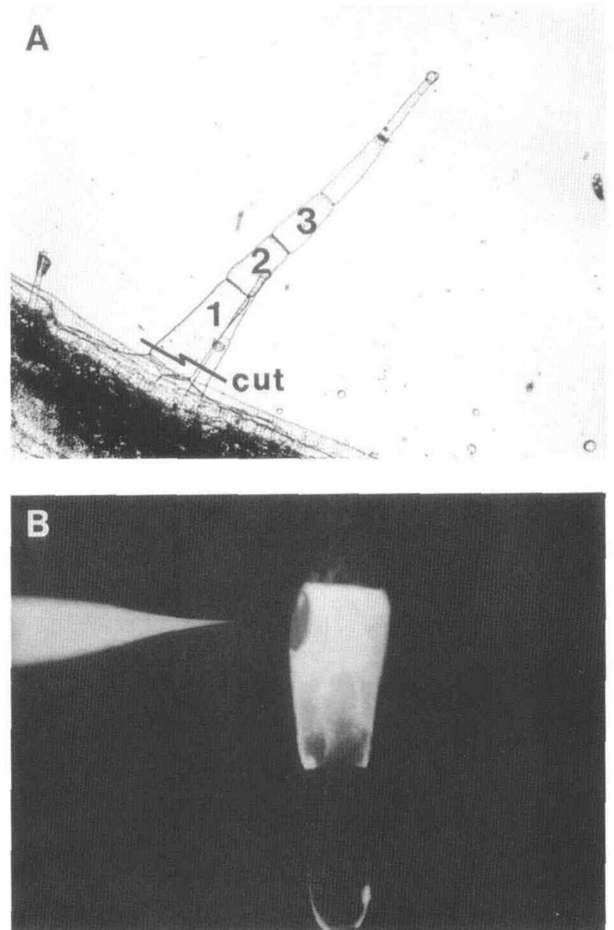

Figure 1. Microinjection of *N. clevelandii* Leaf Trichomes.

(A) Trichome showing the numbering system for cells 1, 2, and 3 and the position of cuts during detachment of trichomes.

(B) Microinjection of purified TRV and the symplasmically mobile fluorochrome sulforhodamine B into the cytoplasm of a trichome cell.

Development of TRV Infections and the Rate of Cell-to-Cell Virus Movement

Between 1 and 4 days after inoculation of a single trichome cell, a necrotic area developed on the leaf periphery of many injected leaves, as illustrated in Figure 2. Necroses developed adjacent to, and spread away from, the site of injection, indicating that TRV had moved from the trichome into the leaf lamina. In preliminary tests, when leaves bearing necrotic patches were tested by rub inoculation of leaf homogenates onto *Chenopodium amaranticolor* (a local lesion host of TRV used as a diagnostic indicator), the presence of virus in the necrotized leaf was invariably confirmed. Homogenates of TRV-microinjected *N. clevelandii* leaves that failed to develop the spreading necrotic symptoms did not elicit local lesion development in *C. amaranticolor.* The development of a necrotic lesion on *N. clevelandii* leaves inoculated by microinjection was, therefore, adopted as a reliable indicator of infection.

In initial trials, 17, 33, and 70% of leaves became infected after injection into the cytoplasm with 0.055, 1.0, and 2.5 mg/mL of TRV, respectively. Only 30% of vacuolar injections with 2.5 mg/mL of TRV resulted in infection. Injections of 2.5 mg/mL of TRV into the cytoplasm were therefore used as standard in this study, and leaves in which virus was accidentally injected into the vacuole were discarded.

Detachment of trichomes within 2 hr of microinjection of single trichome cells with TRV prevented the development of necrotic patches, presumably by removing the initial source of inoculum from the leaf. Figure 3 shows that the minimum time required for TRV to move across one cell boundary, i.e., from cell 1 into the leaf, was about 4 hr. The minimum times for TRV movement from cell 2 (across two cell boundaries) and cell 3 (across three cell boundaries) were about 5 and 7 hr, respectively. Because TRV had moved across one cell boundary in approximately 50% of successful inoculations within 5 hr (Figure 3), plasmodesmatal permeability during virus movement was examined at this time.

The Molecular Size Exclusion Limit of Plasmodesmata between Virus-Free and TRV-Infected Trichome Cells as Assessed by Microinjection of Membrane-Impermeant Fluorescent Probes

Lucifer Yellow CH and FITC-labeled glutamic acid (F-Glu) moved rapidly out of injected cells within the symplasm when

microinjected into the cytoplasm of individual trichome cells. With these probes, fluorescence was visible in immediately neighboring cells within, at most, 3 min of injection. When microinjected into virus-free trichome cells, FITC-labeled phenylalanyl-phenylalanyl-phenylalanine (F-Phe_3), FITC-labeled oxidized insulin A chain (F-insA), FITC-labeled dextran (M_r, 4400; F-dextran 4.4), and Lucifer Yellow CH-labeled dextran (M_r, 10,000; LYCH-dextran 10) failed to move out of the cells into which they were introduced, as shown in Table 1, at least for the duration of the 20-min observation period. By this time, fluorescence within injected cells had become diffuse owing to a more homogeneous distribution of dye throughout the transvacuolar and parietal cytoplasm, and probably sequestration of the dyes within the vacuole as reported by Tucker et al. (1989) and Goodwin et al. (1990) and as reviewed by Oparka (1991).

When F-insA or F-dextran 4.4 was microinjected into the cytoplasm of TRV-inoculated trichome cells, 5 hr after microinjection of TRV particles, fluorescence often moved into immediately neighboring cells within a few seconds, or at most within 3 min following injection of the dye, as shown in Figures 4C and 4D and detailed in Table 2. However, when F-insA, F-dextran 4.4, or LYCH-dextran 10 was microinjected into the cytoplasm of trichome cells that had been microinjected with TRV only 2 hr previously, fluorescence was restricted to the injected cell (illustrated in Figures 4A and 4B and detailed in Table 2). Similarly, F-insA did not move out of cells mock inoculated 5 hr previously with 5 mM sulforhodamine B in Hepes buffer, as shown in Table 2. LYCH-dextran 10 was retained in all cells inoculated 5 hr previously (Table 2).

The results presented here demonstrate an increase in plasmodesmatal permeability that is dependent on the early stages

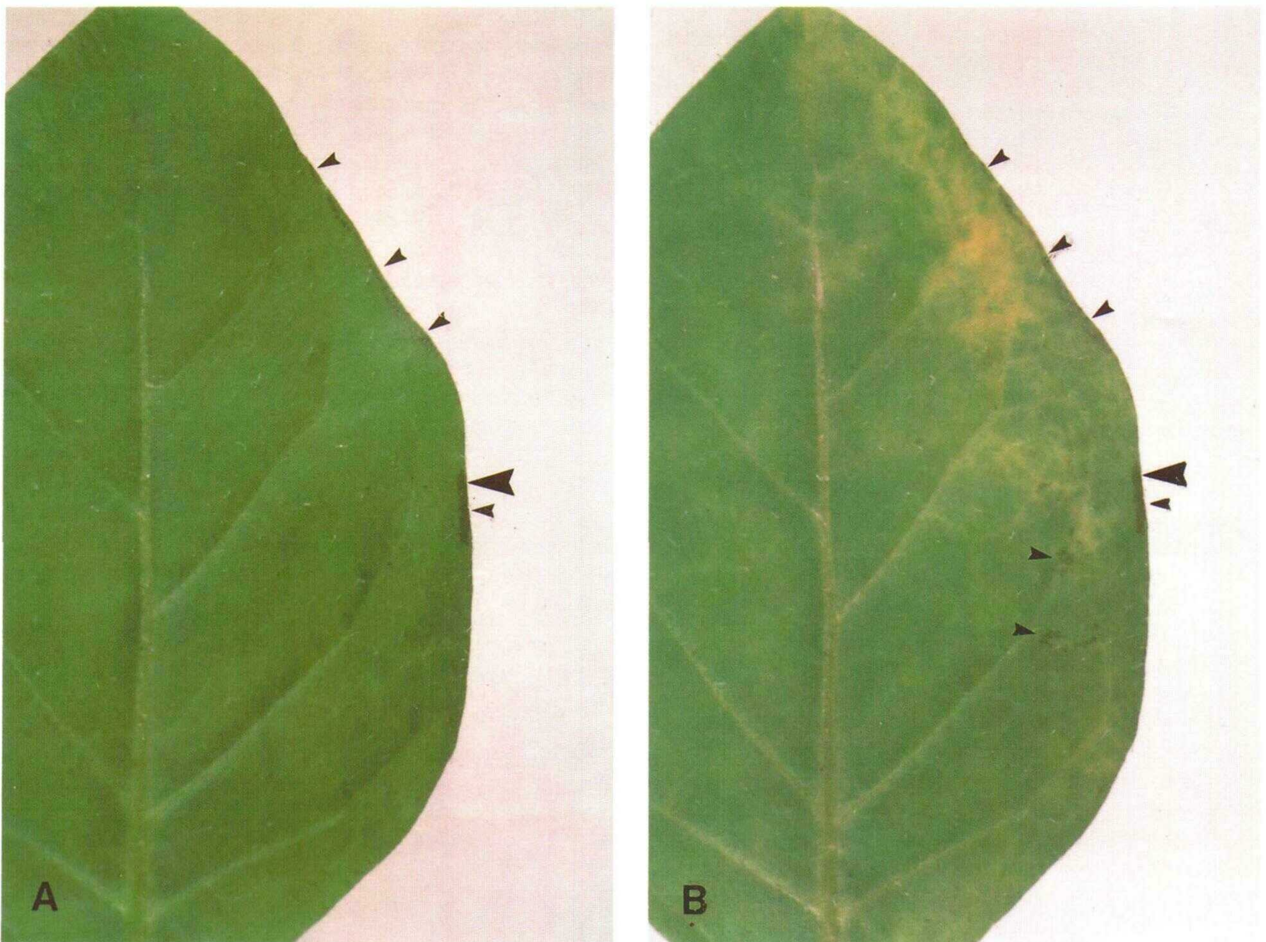

Figure 2. The Appearance of Necrotic Lesions on *N. clevelandii* Leaves after Microinjection of TRV into a Single Trichome Cell.

(A) Inoculated leaf at 3 days after microinjection.

(B) Inoculated leaf at 5 days after microinjection.

Large arrows indicate the site of the inoculated trichome. Small darts indicate necrotic areas along the leaf periphery in **(A)** and **(B)** and around veins in **(B)** caused by the spread of TRV away from the inoculated cell.

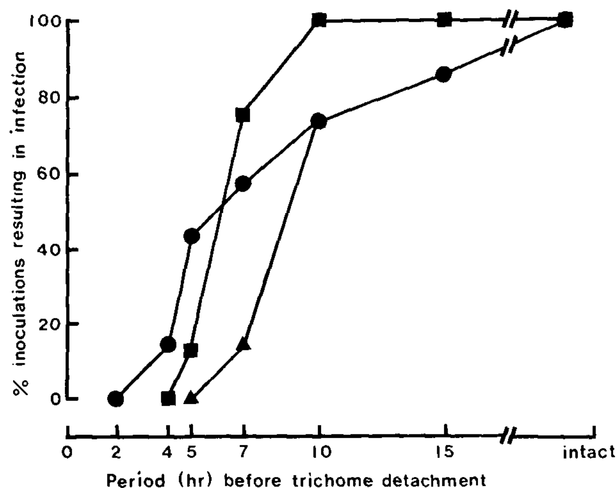

Figure 3. Time Course of Movement of TRV from Inoculated *N. clevelandii* Trichome Cells into the Leaf Lamina.

Shown is the percentage of inoculated leaves that became infected after removal of inoculated trichomes at various times following inoculation of cell 1 (●), cell 2 (■), or cell 3 (▲). Data are presented using a scale on which the proportion of leaves with intact inoculated trichomes that developed infection is taken as 100%. Intact indicates leaves from which inoculated trichomes were not detached.

of a TRV infection. The molecular exclusion limit of plasmodesmata in TRV-infected trichome cells increased from about 0.8 kD at 2 hr after inoculation to between 4.4 and 10.0 kD at 5 hr after inoculation. This increased exclusion limit is similar to, although slightly less than, that reported for plasmodesmata in tobacco transformed with the TMV MP gene (between 9.4 and 17.2 kD; Wolf et al., 1989, 1991). In terms of molecular size, which is more important than molecular weight in determining intercellular symplasmic mobility (Terry and Robards, 1987), the 4.4- and 10-kD dextrans used in this study would have Stokes radii of 1.48 and 2.31 nm, respectively (calculated as described by Squire, 1981). The exclusion limit of the modified plasmodesmata lies between these two values. Similarly, the exclusion limit of plasmodesmata in TMV MP–transformed tobacco (Wolf et al., 1989, 1991) was delimited by dextrans with Stokes radii of 2.23 and 3.10 nm.

It is difficult to estimate an exclusion limit for plasmodesmata in virus-free *N. clevelandii* trichomes in terms of Stokes radius using the fluorochromes listed in Table 1 because these do not have an approximately spherical conformation. However, Terry and Robards (1987) calculated that for plasmodesmata in *Abutilon striatum* nectary trichomes, a Stokes radius of about 0.9 nm approaches the exclusion limit. Because plasmodesmata in many plant species appear to have similar molecular weight exclusion limits (Robards and Lucas, 1990), including those of *N. clevelandii* epidermal cells (Derrick et al., 1990), it is reasonable to assume that this value is probably close to the exclusion limit for *N. clevelandii* trichome plasmodesmata.

DISCUSSION

In addition to its virological significance, research into viral modifications of plasmodesmatal function is likely to provide useful information relevant to the cell biology of plasmodesmatal function in normal virus-free plants. Inoculation of individual *N. clevelandii* trichome cells by microinjection of purified virus particles has provided a reliable system for studying the early stages of virus–plasmodesma interactions. Microinjection of inocula containing 2.5 mg/mL of virus particles resulted in consistently high levels of infection with both TRV (this study) and a nepovirus (tomato black ring virus; P. M. Derrick, unpublished results). In other studies, microinjection has been used to inoculate cultured plant cells (Nims et al., 1967; Halliwell and Gazaway, 1975; Toyoda et al., 1985) and protoplasts (Joshi and Vincentini, 1990) with viruses.

TRV moved out of inoculated *N. clevelandii* trichome cells about 4 hr after inoculation; this was the shortest time in which TRV moved from cell 1 into the leaf lamina (Figure 3). Similarly, in studies with cucumber mosaic virus in *Vigna sinensis* and TMV in *N. sylvestris* and *N. tabacum*, virus moved from epidermal cells into mesophyll cells after 3, 4, and 5 hr, respectively (Uppal, 1934; Welkie and Pound, 1958; Fannin and Shaw, 1987). This time interval coincides with the onset of viral replication in protoplasts inoculated with viruses. Aoki and Takebe (1975) detected viral RNA in TMV-infected tobacco protoplasts 4 hr after inoculation. The TMV MP is among the initial products of virus replication (Joshi et al., 1983) and has been detected at 4 and 6 hr after inoculation of tobacco protoplasts with TMV RNA or particles, respectively (Watanabe et al., 1984; Blum et al., 1989). The TRV genome encodes a 29-kD protein that is functionally homologous to the TMV MP (Ziegler-Graff et al., 1991). It seems reasonable to assume, therefore, that the TRV 29-kD protein was present in microinjected *N. clevelandii* trichome cells at about 4 hr following inoculation, enabling virus spread to neighboring cells.

It is interesting that although at least 4 hr were required for TRV to move across one cell boundary, the virus could cross

Table 1. Movement of Fluorescent Probes in Uninfected *N. clevelandii* Trichome Cells

Fluorescent Dye	M_r	Injections Resulting in Dye Movement[a]
LYCH	457	7 (7)[b]
F-Glu	537	5 (5)
F-Phe_3	850	0 (5)
F-insA	2,921	0 (7)
F-dextran 4.4	4,400	0 (5)
LYCH-dextran 10	10,000	0 (6)

[a] Data are presented as the number of injections of fluorescent dyes in which fluorescence moved out of the injected cell within 20 min.
[b] Values in parentheses are numbers of trichomes injected.

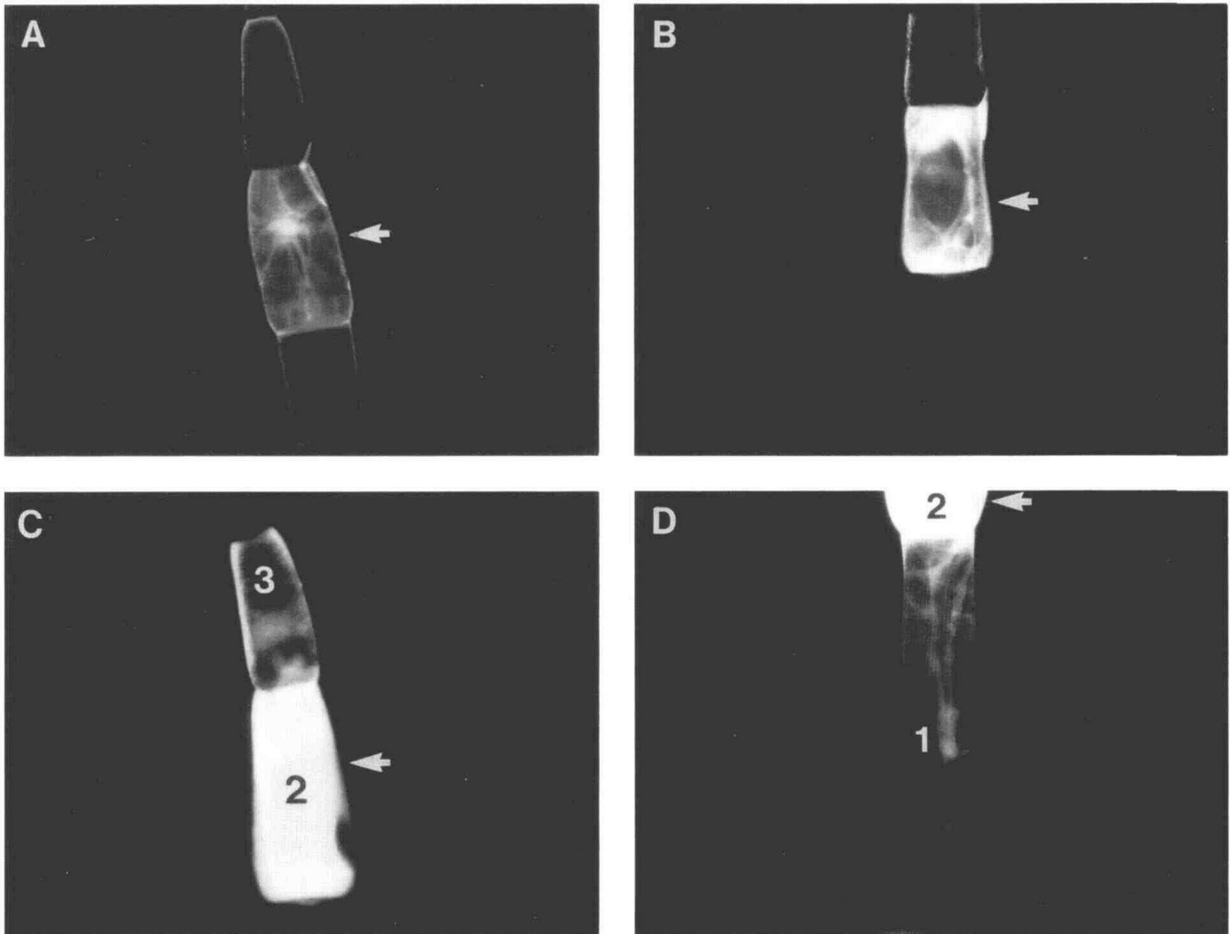

Figure 4. Movement of Fluorescent Probes out of TRV-Infected Trichome Cells.

(A) and **(B)** Trichomes inoculated 2 hr previously with TRV. Fluorescent probes are restricted to inoculated cells (arrowed). **(A)** shows a trichome 5 min after injection of F-insA, and **(B)** shows a trichome 15 min after injection of F-dextran 4.4.

(C) and **(D)** Movement of fluorescent probes out of cells inoculated 5 hr previously with TRV. In **(C)**, 5 min after injection of F-insA, and **(D)**, 5 min after injection of F-dextran 4.4, fluorescence has clearly moved symplasmically out of the inoculated cells (arrowed). Cell numbering (as described in Figure 1A) reveals that the fluorescent probes have moved acropetally in **(C)** and basipetally in **(D)**.

two cell boundaries within 5 hr and three cell boundaries within 7 hr. Because any injury to cells resulting from microinjection appeared to last only a few minutes, as determined by recovery of cytoplasmic streaming, we believe that damage caused by microinjection and subsequent recovery of the cell have only slight, if any, impact on the time required for TRV to cross the first cell boundary. The time differential between movement across one and two cell boundaries could be caused by several factors, including levels of the infectious entity moving into neighboring cells, the nature of the infectious entity (virus or nucleoprotein complexes that do not require uncoating), or possibly movement of replication complexes that circumvent time-consuming early events in the infection process.

The results of this study indicate that TRV moves from cell to cell via virus-modified plasmodesmata that have a functional channel radius of less than 2.3 nm. This finding supports the hypothesis that cell-to-cell movement of TRV must be in a form other than virus particles because the latter are cylindrical structures with a cross-sectional radius of about 9.0 nm for the SYM strain (Kurppa et al., 1981). Also supporting this theory is the existence of variants of TRV that do not form virus particles (so-called NM isolates) but can move from cell to cell at least as quickly as particle-forming strains (Frost et al., 1967). Therefore, it seems likely that TRV moves from cell to cell via modified plasmodesmata as a form of RNA or as a nonparticle ribonucleoprotein complex, perhaps similar to those described by Dorokhov et al. (1983, 1984).

Table 2. Movement of Fluorescent Macromolecular Probes in TRV-Infected and Mock-Inoculated *N. clevelandii* Trichome Cells

Fluorescent Dye	Time after Inoculation (hr)	Injections Resulting in Dye Movement[a]
F-insA	5	5 (7)[b]
F-dextran 4.4	5	3 (7)
LYCH-dextran 10	5	0 (7)
F-insA	2	0 (5)
F-dextran 4.4	2	0 (5)
F-insA[c]	5	0 (5)

[a] Data are presented as the number of injections of fluorescent dyes in which fluorescence moved out of TRV-inoculated cells within 20 min.
[b] Values in parentheses are numbers of trichomes injected.
[c] Mock inoculation. Cells were microinjected with 3.0 mM sulforhodamine B in Hepes buffer, pH 7.3.

We cannot exclude the possibility that the time interval required before cell-to-cell movement can occur could represent time needed to generate new plasmodesmata with increased permeability. It is also possible that during intercellular passage of the virus, a specific interaction of virus, MP, and plasmodesmata may transiently open plasmodesmata to a greater size than we have been able to measure. If, however, the infection-induced change in plasmodesmatal permeability detected in this study is sufficient in itself to allow intercellular transport of the movement form of TRV, then the steric conformation of the TRV RNA during movement must be very different from that of the protein-free, folded RNA molecule because these are too large to pass through the enlarged plasmodesmatal channels. In addition to the sequence nonspecific, cooperative binding of MPs to single-stranded nucleic acids reported for several viruses (see Introduction), Citovsky et al. (1992) demonstrated that binding of TMV MP to single-stranded nucleic acids produced ribonucleoprotein complexes that were much longer than the corresponding free nucleic acid (TMV MP–single-stranded RNA complexes were two- to threefold longer). These MP–nucleic acid complexes were also estimated to be much thinner than free nucleic acids, the TMV MP–single-stranded DNA complex having a width of about 1.5 to 2.0 nm. If TRV MP binding to viral RNA has a similar effect on the viral RNA conformation in vivo, the resulting nucleoprotein complex may well be capable of passing through enlarged plasmodesmata, as suggested by our experiments and those of Wolf et al. (1989, 1991).

METHODS

Plant Material

Virus-free *Nicotiana clevelandii* plants were grown in soil-less compost in a glasshouse with supplementary lighting during the winter months. Fully expanded, young leaves were removed from plants that were approximately 7 weeks old. The cut petiole was immediately submerged in water within a microcentrifuge tube sealed with Nescofilm (Nippon Shoji Kaisha Ltd., Osaka, Japan) and placed on the microscope stage for inoculation by microinjection. Trichomes for microinjection were uniseriate and composed of six to nine cells, discounting the clump of terminal secretory cells (Figure 1A).

Preparation of Virus Inoculum for Microinjection

The SYM strain of tobacco rattle virus (TRV) (Kurppa et al., 1981) was cultured in virus-free *N. clevelandii* plants grown in an aphid-proof glasshouse. Virus purification was essentially as described by Cooper and Mayo (1972), except that final purification was by sucrose density gradient centrifugation (Kurppa et al., 1981). Purified virus was resuspended in 10 mM Hepes adjusted to pH 7.3 with NaOH and the virus concentration estimated spectrophotometrically using an extinction coefficient of $A^{0.1\%}_{260\,nm,1\,cm}$ = 3.0 (Lister and Bracker, 1969). Inoculum for microinjection contained 2.5 mg/mL of TRV in Hepes buffer, pH 7.3, and either 5 mM sodium fluorescein (Sigma) or 5 mM sulforhodamine B (Aldrich). The addition of a fluorochrome made it possible to observe the progress of microinjection under blue (for fluorescein) or green (for sulforhodamine B) epifluorescence illumination. The proportion of infections resulting from microinjection of TRV was the same whether the inoculum contained either fluorescein or sulforhodamine B (data not shown).

Fluorescent Compounds

Fluorescein isothiocyanate isomer 1 (FITC)–labeled peptides (FITC-labeled glutamic acid [F-Glu], FITC-labeled phenylalanyl-phenylalanyl-phenylalanine [F-Phe_3], and FITC-labeled oxidized insulin A chain [F-insA]) were prepared and purified as described by Simpson (1978), and their purity was assessed as described by Goodwin (1983). Peptides for FITC conjugation, Lucifer Yellow CH, and FITC-labeled dextran with an M_r of 4400 (F-dextran 4.4) were purchased from Sigma. F-insA and F-dextran 4.4 were further purified by gel chromatography through a Sephadex G-25 column (Pharmacia, Sweden). LYCH-labeled dextran with an M_r of 10,000 (LYCH-dextran 10) (Molecular Probes Inc., Eugene, OR) was purified by twice precipitating the dye in 80% ethanol. Lucifer Yellow CH, F-Glu, and F-Phe_3 were microinjected as 30 mM solutions in water, and F-insA, F-dextran 4.4, and LYCH-dextran 10 as 3 mM solutions in water.

Microinjection

The micromanipulation equipment and micropipettes were as described in Derrick et al. (1990) and attached to a Nikon Optiphot epifluorescence microscope equipped with Nikon B2-E (blue) and G-2A (green) filter cassettes. A pressure injector was constructed, based on the pressure-probe design of Murphy and Smith (1989). This included a pressure transducer and a digital pressure indicator enabling pressure within the micropipette to be monitored.

During injection, trichomes were supported by pushing a micropipette with a large tip diameter (about 130 μm) against the side of the cell opposite to that selected for injection. Before impalement, pressure within the injector was increased to counteract cell turgor (Oparka et al., 1991).

Incubation of Inoculated Leaves

After inoculation, leaves were placed in an incubator with the cut petiole submerged in a vial containing one-tenth strength Murashige and Skoog salts (Flow Laboratories, McLean, VA) and maintained at 24°C under high humidity and continuous illumination at 3100 lux.

Estimation of the Time Scale of TRV Cell-to-Cell Movement

Virus inoculum (containing fluorescein) was microinjected into the cytoplasm of either cell 1, 2, or 3 of one trichome per leaf. Leaves were then incubated for periods up to 15 hr after which the inoculated trichome was removed by cutting through the base of cell 1 using a scalpel blade (Figure 1A). Leaves were returned to the incubator for about 5 days to assess whether or not virus had spread from the inoculated trichome into the leaf lamina by observing symptoms of virus infection.

Examination of Plasmodesmatal Size Exclusion Limit TRV-Infected Trichomes

Virus particles were injected into the cytoplasm of cell 2 (Figure 1A) of one trichome per leaf. After 5 hr, the inoculated cell was reinjected with a 3 mM solution of fluorescent-labeled macromolecule and observed under blue illumination for brief periods at intervals up to 20 min after injection. For this experiment, sulforhodamine B replaced fluorescein in the inoculum. Sulforhodamine B aided in the observation of the progress of microinjection but is not visible under blue illumination and so did not interfere with observation of the subsequent microinjection of fluorescent macromolecules. Photographs were taken using Kodak T-MAX film rated at 400 ASA.

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