Plasmodesmatal Function Is Probed Using Transgenic Tobacco Plants That Express a Virus Movement Protein

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A gene encoding a temperature-sensitive mutant (MPP154A) of the 30-kilodalton movement protein (MP) of tobacco mosaic virus (TMV) was transformed into *Nicotiana tabacum* cv Xanthi. Transgenic plants expressing the MPP154A gene complemented local and systemic movement of an MP-defective mutant of TMV (U3/12MPfs) at the permissive temperature of 24°C but not at 32°C, the nonpermissive temperature. A microinjection procedure was used to investigate the effects of the modified TMV MP on plasmodesmatal size-exclusion limits. Movement of fluorescein isothiocyanate-labeled dextran (F-dextran), with an average molecular mass of 9.4 kilodaltons, was detected between leaf mesophyll cells of the transgenic plants at 24°C; however, no movement of either 3.9-kilodalton or 9.4-kilodalton F-dextrans was detected when the transgenic plants were held for 6 hours (or longer) at 32°C. When these plants were shifted back to 24°C for 6 hours, cell-to-cell movement of the F-dextrans was again observed. Accumulation of MPP154A was not affected by the temperature regime, nor was the subcellular distribution of the MP altered. These results are consistent with a change in the protein conformation of MPP154A at the nonpermissive temperature, which gives rise to a protein that fails to modify the molecular size-exclusion limits of plasmodesmata to the same extent as wild-type MP. Surprisingly, at 32°C, movement of the F-dextrans was inhibited in transgenic plants expressing the wild-type MP gene; however, the inhibition was transient and was no longer detected after 48 hours at this elevated temperature. This transient inhibition of plasmodesmatal function was alleviated with Sirofluor, an inhibitor of callose ([1→3]-β-D-glucan) synthesis. This result provides experimental evidence that callose deposition is involved in regulating the molecular size-exclusion limit of plasmodesmata in plants. Sirofluor had no effect on the inhibition of F-dextran movement at 32°C in plants expressing the MPP154A gene, indicating that callose formation was not responsible for the failure of the temperature-sensitive mutant protein to alter the size-exclusion limit of plasmodesmata.

INTRODUCTION

The primary route for cell-to-cell spread of plant viruses is by way of plasmodesmata, plasma membrane-lined, cytoplasmic channels that extend through plant cell walls to connect adjacent cells. Because the size of virus particles and virus genomes is considerably greater than the molecular size-exclusion limit(s) of plasmodesmata (Gibbs, 1976), it is generally considered that viruses modify plasmodesmata to gain access to neighboring cells. In some virus-infected tissues, electron microscopic evidence shows viral particles in plasmodesmata (Esau et al., 1967; Allison and Shalla, 1974; Weintraub et al., 1976; Robards and Lucas, 1990). Micrographs indicating structural modifications of plasmodesmata, such as removal of the axial component along with an increase in the physical diameter of the pore, have also been reported for some virus-infected cells (Esau, 1968; Kitajima and Lauritis, 1969; Linstead et al., 1988). Although many plant viruses appear

to move from cell to cell as intact virions, others like tobacco mosaic virus (TMV) do not require the formation of intact particles for cell-to-cell spread. For example, barley stripe mosaic virus, a member of the hordeivirus group, does not require coat protein for effective cell-to-cell or long-distance movement (Petty and Jackson, 1990). Similarly, the coat protein of TMV is not required for cell-to-cell movement in an inoculated leaf (Dorokhov et al., 1983; Takamatsu et al., 1987; Dawson et al., 1988), although effective long-distance movement through the vascular system does require coat protein (Dorokhov et al., 1984). That local TMV infection does not involve viral particles is most probably the reason why this virus rarely has been detected in plasmodesmata (Robards and Lucas, 1990).

TMV is one of the most thoroughly studied plant viruses, and its single-stranded RNA genome has been shown to encode four proteins. The 126-kD and 183-kD proteins are required for virus replication (Ishikawa et al., 1986) and

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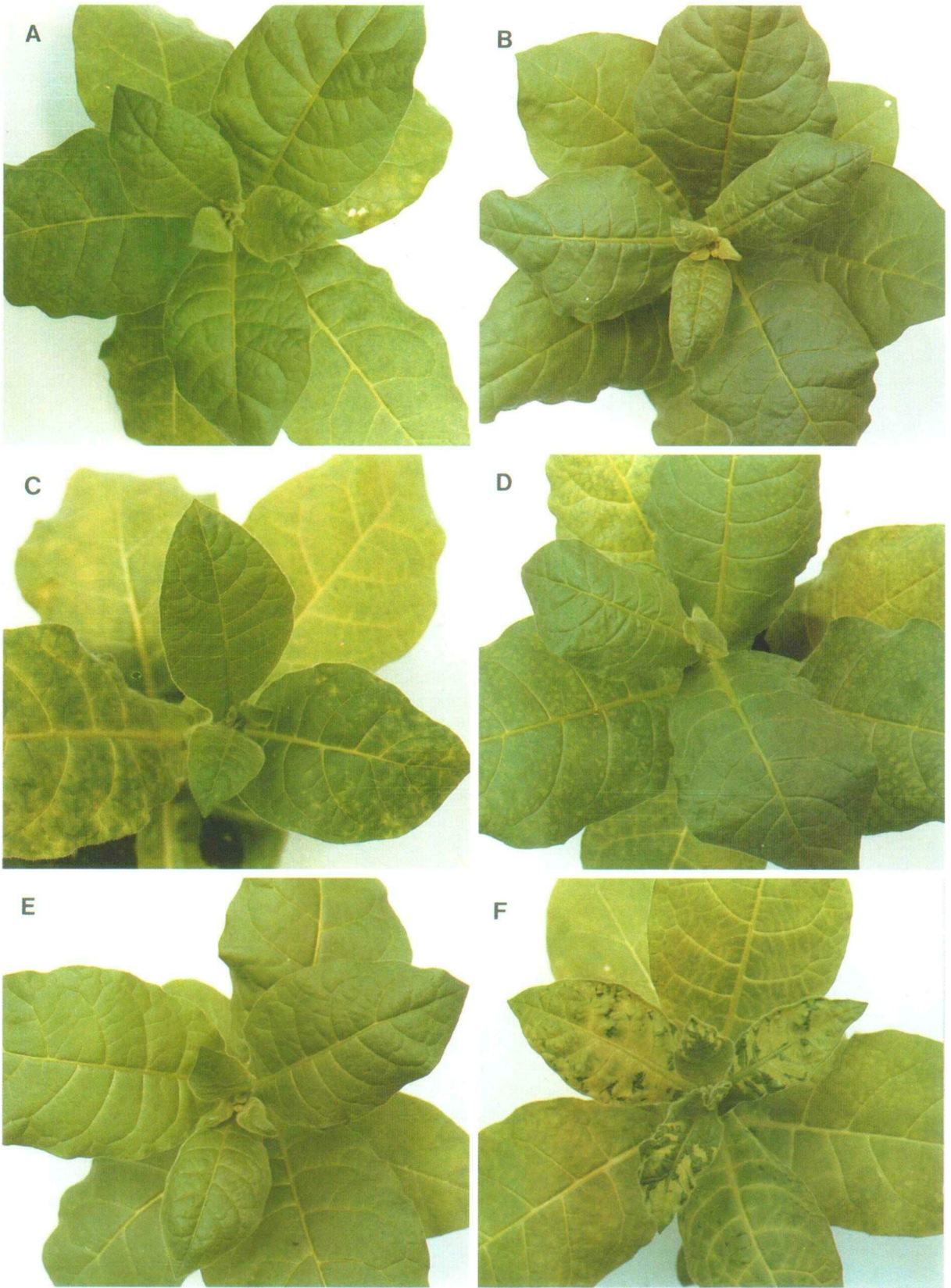[1] To whom correspondence should be addressed.

Figure 1. Effect of Temperature on the Development of Systemic Symptoms in Transgenic Tobacco Lines 2-72, 277, and 306 at 10 Days after Inoculation with either an MP-Defective Mutant of TMV (U3/12MPfs) or Wild-Type TMV.

are translated from the genomic RNA. Both proteins are postulated to be subunits of the TMV replicase (Hunter et al., 1976; Scalla et al., 1978) and have been identified in virus-infected cells (Saito et al., 1987). Translation of the 30-kD movement protein (MP) and the coat protein requires the transcription of two subgenomic RNAs (Beachy et al., 1976; Bruening et al., 1976; Hunter et al., 1976; Beachy and Zaitlin, 1977).

The TMV-encoded MP facilitates cell-to-cell spread of the virus during infection. Two different but complementary approaches were used to provide direct evidence for the function of the MP. First, transgenic Xanthi tobacco plants expressing a TMV MP gene complemented the temperature-sensitive (ts) defect in movement of the Ls1 mutant of the TMV L strain (Deom et al., 1987). At the nonpermissive temperature of 32°C, the Ls1 mutant replicates and assembles normally in inoculated leaves and leaf protoplasts but fails to move from cell to cell in inoculated leaves (Jockusch, 1968; Nishigushi et al., 1978). The ts phenotype of the Ls1 MP results from a proline-to-serine substitution at amino acid 154 (Ohno et al., 1983). At the permissive temperature of 24°C, local and systemic movement of the Ls1 virus is delayed on control tobacco plants relative to the wild-type L virus, indicating that while serine can substitute for proline, the rate of virus spread is attenuated (Nishigushi et al., 1978; Deom et al., 1987). However, in transgenic MP(+) Xanthi tobacco at 24°C, the Ls1 virus spreads as rapidly as the L virus (Deom et al., 1987). Second, when serine is substituted for proline at amino acid 154 in the MP gene of the L strain, the mutated L strain shows the same ts defect in movement as the Ls1 strain (Meshi et al., 1987). The latter study also showed that the MP is not required for replication or virus assembly.

The cellular mechanism by which the TMV MP potentiates systemic spread of the virus was studied using a novel method for delivering nonplasmalemma-permeable fluorescent probes to the cytosol of tobacco spongy mesophyll cells (Wolf et al., 1989). In transgenic plants expressing the MP gene, plasmodesmata permitted the movement of fluorescein isothiocyanate-labeled dextrans (F-dextrans) with average molecular masses of 9.4 kD. Plasmodesmata in control tobacco plants, in contrast, exhibited normal size-exclusion limits of 0.7 kD to 0.8 kD. These experiments established that the TMV MP has a direct effect on the function of plasmodesmata, and are further supported by electron microscopic immunogold studies that localize MP to plasmodesmata in infected tobacco leaves (Tomenius et al., 1987) and transgenic plants (Atkins et al., 1991; P. Moore and R. Beachy, unpublished data) as well as by the accumulation and stable association of MP with cell wall fractions obtained from either transgenic (Deom et al., 1990) or virus-infected plants (Moser et al., 1988; Deom et al., 1990).

More recently, a second mechanism has been suggested to play a role in the function of the MP. MP expressed in and purified from Escherichia coli was found to bind single-stranded nucleic acids (DNA and RNA). It was postulated that the MP might have single-stranded nucleic acid binding activity that aids, presumably by inducing an extended and unfolded RNA-protein structure, in the movement of the viral genome from cell to cell through plasmodesmata modified by the MP (Citovsky et al., 1990).

To understand better the MP and its role in virus movement, the underlying mechanism(s) responsible for the ts nature of the TMV Ls1 phenotype was further explored. A gene encoding a modified, ts MP, designated MPP154A, was used to produce transgenic tobacco plants. Dye-coupling experiments using F-dextran probes were employed to study the effect of nonpermissive temperatures on the function of plasmodesmata in these plants. These studies enabled us to establish the time course for the temperature-induced changes in molecular size-exclusion limits of the plasmodesmata. At 24°C, but not at 32°C, plants expressing the MPP154A gene modified the molecular exclusion limit of plasmodesmata similar to that detected for MP. Surprisingly, there was a transient inhibition of dye movement in plants expressing the wild-type MP at 32°C. This transient effect was alleviated with Sirofluor, an inhibitor of callose ([1→3]-β-D-glucan) synthesis (Morrow and Lucas, 1987).

RESULTS

Expression of a ts MP in Transgenic Tobacco Plants

Mutant MPP154A was generated by changing the proline residue at amino acid 154 of the TMV MP to alanine. The

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Figure 1. (continued).

(A) Transgenic tobacco plant line 2-72 held at 24°C and inoculated with U3/12MPfs.
(B) Transgenic tobacco plant line 2-72 held at 32°C and inoculated with U3/12MPfs.
(C) Transgenic tobacco plant line 277 held at 24°C and inoculated with U3/12MPfs.
(D) Transgenic tobacco plant line 277 held at 32°C and inoculated with U3/12MPfs.
(E) Transgenic tobacco plant line 306 held at 24°C and inoculated with U3/12MPfs.
(F) Transgenic tobacco plant line 2-72 held at 24°C and inoculated with wild-type TMV.

proline-to-alanine mutation was chosen because alanine is not commonly found in turn structures (Chou and Fasman, 1978) and it contains an unobtrusive, uncharged side chain. The MPP154A gene, under the control of the cauliflower mosaic virus 35S promoter, was introduced into cells of *Nicotiana tabacum* cv Xanthi. Transformed cells were selected for kanamycin resistance and regenerated into plants (Horsch et al., 1985). Four transgenic plant lines expressing the MPP154A gene were analyzed by protein gel blot analysis. Two plant lines, designated 2-72 and 2-74, expressed detectable levels of MPP154A. A quantitative analysis of the levels of MP in developmentally identical leaf tissue from lines 2-72 and 2-74 showed that the relative levels of MP in line 2-74 were approximately 30% of the levels of MP in line 2-72 (data not shown). Based on this analysis, plant line 2-72 was chosen for further study.

Figures 1A and 2 illustrate that at 24°C 90% of the seedlings from line 2-72 that expressed the mutant protein complemented the defect in movement of U3/12MPfs, a TMV mutant produced in vivo that contains a frame shift
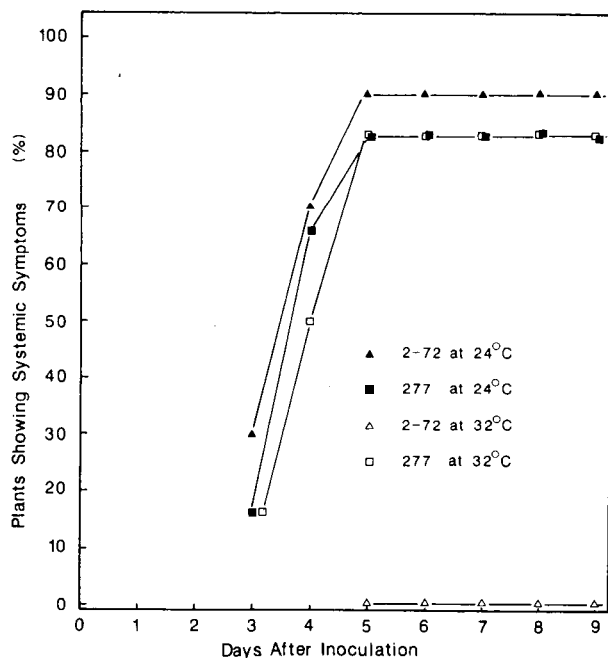
in the MP that abolishes its function (Holt and Beachy, 1991). However, as shown in Figures 1B and 2, when plant line 2-72 was inoculated with U3/12MPfs and maintained at 32°C, no disease symptoms were observed in the inoculated or systemic leaves, indicating that MP154PA is ts at 32°C. Symptoms in inoculated as well as in the upper leaves were observed in plants of transgenic line 277 (expressing the wild-type MP gene) at both temperatures, as illustrated by Figures 1C, 1D, and Figure 2 (see also Deom et al., 1987). Figure 1E demonstrates that transgenic control line 306 failed to complement U3/12MPfs at either temperature. A direct comparison between Figures 1A and 1F shows that, under identical temperature conditions (24°C), the symptoms developed in plant line 2-72 inoculated with wild-type TMV were always more severe when compared with plants inoculated with U3/12MPfs. In all cases, tissue was analyzed from inoculated and upper, noninoculated leaves for the presence of virus by immunological dot blot analysis to confirm that local and systemic disease symptoms reflected virus replication and movement. The accumulation of virus in inoculated and upper, systemic leaf tissue correlated with visual symptoms of virus movement (data not shown).

Figure 2. Time Course for the Development of Systemic Symptoms on Transgenic Tobacco Plant Lines 2-72 and 277 Maintained at either 24°C or 32°C and Inoculated with an MP-Defective Mutant of TMV (U3/12MPfs).

Seven 277 line and 10 2-72 line tobacco plants were used for each treatment. Systemic spread of U3/12MPfs virus in plants of transgenic line 2-72 at 24°C (▲) and 32°C (△) and in plants of transgenic line 277 at 24°C (■) and 32°C (□).

MPP154A Levels and Subcellular Distribution at Permissive and Nonpermissive Temperatures

To determine why MPP154A failed to provide movement function to U3/12MPfs at 32°C, the levels and subcellular distribution of MPP154A were assessed relative to wild-type MP in fully or near fully expanded leaves from transgenic plants at 24°C and 32°C. Data from these analyses are presented in Table 1. In two separate experiments, the levels of MPP154A at 32°C were 88% and 138% of the levels detected at 24°C, whereas the levels of MP were 81% and 94%. Therefore, the levels of MPP154A at 32°C do not decrease relative to MP, suggesting that the failure of MPP154A to complement U3/12MPfs is not due to lower levels of the mutant protein at 32°C. In addition, relative to MP, no correlation that could account for the ts phenotype of MPP154A was detected between the subcellular distribution of MPP154A at 24°C and 32°C. As shown in Table 1, both MPP154A and MP were distributed to a similar degree in P1, P30, and S30 subcellular fractions.

Temperature Effect on Plasmodesmatal Size-Exclusion Limits

Previous studies have established that expression of the TMV MP in transgenic tobacco plants significantly alters the molecular size-exclusion limit of plasmodesmata between leaf mesophyll cells (Wolf et al., 1989). Experiments were undertaken to determine the effect of MPP154A on

Table 1. Levels of MP in Transformed Tobacco Plants at 24 °C and 32 °C

Transgenic Plant Line	Temp (°C)	Levels of MP (cpm × 10⁻³)			
		P1	P30	S30	Total
Experiment 1					
2-72	24	429 (65)	152 (23)	81 (12)	662
	32	363 (62)	146 (25)	74 (13)	583
277	24	612 (56)	281 (26)	194 (18)	1087
	32	562 (63)	203 (23)	112 (12)	877
Experiment 2					
2-72	24	880 (57)	290 (19)	360 (24)	1531
	32	1661 (78)	266 (13)	189 (9)	2117
277	24	1956 (93)	55 (7)	87 (4)	2099
	32	1545 (78)	261 (13)	172 (9)	1978

Plants having 12 to 14 leaves were used for these experiments and the sixth leaf from the top of the plant was analyzed (the last leaf to reach a length of 5 cm was designated as leaf No. 1). Plants were maintained at 32 °C for 48 hr and leaves from 10 plants of each line were pooled for analysis. Values given are cpm × 10⁻³ of ¹²⁵I-labeled secondary antibody and represent the relative amounts of MP per gram of leaf fresh weight. Numbers in parentheses represent the percentages of the relative levels of MP in the subcellular fractions. P1, fraction enriched for cell wall components; P30, 30,000g pellet, contains membranes and other organelles; and S30, supernatant of 30,000g centrifugation cycle. Plant line 2-72 harbors a gene encoding the ts MPP154A protein; plant line 277 harbors a gene encoding the wild-type TMV MP.

the size-exclusion limit of plasmodesmata at permissive and nonpermissive temperatures. Figure 3A demonstrates that under permissive temperatures (24°C), the 9.4-kD F-dextran moved between spongy mesophyll cells of plant lines 277 and 2-72. Table 2 shows that movement of this fluorescent probe was not detected between mesophyll cells of the control tobacco line 306, which was transformed in the same way as lines 277 and 2-72 except that the MP gene was omitted. After plants were shifted to the nonpermissive temperature (32°C) for 3 hr, movement of the 3.9-kD and 9.4-kD F-dextrans was inhibited. Figure 3B indicates that, based on microinjection experiments performed on plant line 2-72 held at 32°C for 6 hr and 24 hr, the size-exclusion limits of the mesophyll plasmodesmata remained down-regulated over this period. After 48 hr at 32°C, movement of the 3.9-kD F-dextran was observed in 50% of the 2-72 plants examined, whereas the 9.4-kD F-dextran did not move from cell to cell.

To determine whether the inhibition of F-dextran movement in line 2-72 at 32°C was reversible, plants were shifted back to 24°C after 48 hr at the higher temperature. The data presented in Table 2 indicate that, within 3 hr at 24°C, both the 3.9-kD and 9.4-kD F-dextrans moved in many of the plants examined, and after 6 hr the recovery was complete, with the 9.4-kD F-dextran exhibiting cell-to-cell movement in all plants tested. Figure 3C presents

a representative dye-coupling experiment from these recovery experiments. As a control in all temperature-shift experiments, the small molecular weight fluorescent probe Lucifer Yellow CH (0.5 kD) was used to ensure that the injected cells remained viable and to determine whether normal cell-to-cell communication was affected. Table 2 demonstrates that in all such experiments, Lucifer Yellow CH moved from the injected cell into neighboring cells.

Surprisingly, a transient inhibition of probe movement was observed in 277 plants that had been held at 32°C for 6 hr and 24 hr. However, as seen from the data presented in Table 2, after 48 hr at 32°C, this inhibition was no longer detected and the 3.9-kD and 9.4-kD F-dextrans moved in all 277 plants tested.

Callose Deposition and Control of Plasmodesmatal Size-Exclusion Limits

Callose ([1→3]-β-D-glucan) synthesis has long been implicated as a mechanism for limiting the spread of virus in hypersensitive tissues (Allison and Shalla, 1974). Furthermore, temperature-associated changes in callose formation have been observed in several plant systems (Webster and Currier, 1968; Eschrich, 1975; Dinar et al., 1983), and Olesen and Robards (1990) recently suggested that callose deposition in the constricted neck region of plasmodesmata could affect the pathway available for solute movement.

To determine whether callose deposition was involved in the transient inhibition of cell-to-cell movement of the fluorescent probes in line 277 at 32°C, microinjection experiments were performed on the transgenic plants in the presence and absence of Sirofluor, an inhibitor of (1→3)-β-D-glucan synthase (Morrow and Lucas, 1987). Spongy mesophyll cells located in two separate regions on the same leaf were exposed simultaneously to either distilled water (control) or a 5 mM Sirofluor solution. Microinjection experiments were performed on these areas after a 6-hr pretreatment at 32°C. Representative dye-coupling experiments performed on control and Sirofluor-treated mesophyll cells are illustrated in Figures 3D and 3E, respectively. The combined results of these experiments are presented in Table 3. In all but one experiment, no movement of the 9.4-kD F-dextran was observed between mesophyll cells in line 277 plants that were pretreated with distilled water, whereas the 9.4-kD probe moved in five of six plants in which the mesophyll cells were pretreated with Sirofluor. Figure 3F demonstrates that in parallel control experiments performed on plant line 2-72 at 32°C, the 9.4-kD F-dextran did not move out of the injected mesophyll cell in either the distilled water or Sirofluor-pretreated tissues.
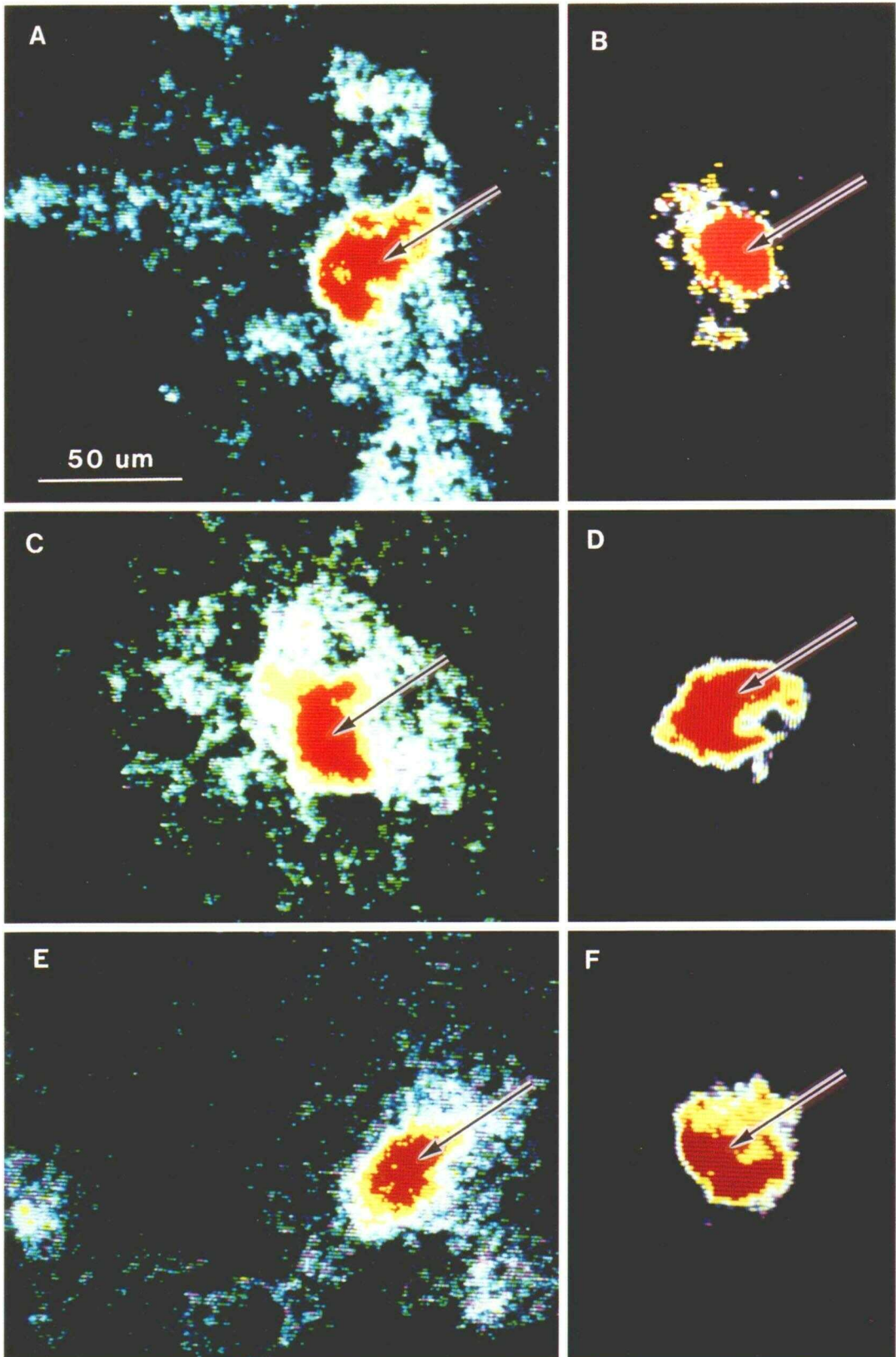
Figure 3. Movement of 9.4-kD F-Dextran in Tobacco Leaf Cells Detected after Microinjection of Liposome-Encapsulated Dye into a Spongy Mesophyll Cell, as Indicated by False-Color Imaging Obtained with a Hamamatsu Model C1966-20 Analytical System.

Table 2. Effect of Restrictive Temperature (32°C) Treatment on the Mobility of Fluorescent Probes through the Symplasmic Pathway of Mesophyll Cells of Transformed Tobacco Plants[a]

Transgenic Plant Line[b]	Probe	Molecular Mass (Daltons)	Duration of 32°C Treatment (hr)					Recovery Period (hr)	
			0	3	6	24	48	3	6
2-72	LYCH[c]	457			7 (7)	7 (7)	6 (6)		
	F-dextran	3900	10 (10)[d]	5 (8)	2 (7)	2 (7)	5 (8)	7 (8)	8 (8)
	F-dextran	9400	8 (10)	0 (8)	0 (8)	0 (8)	1 (8)	6 (8)	8 (8)
277	LYCH	457			4 (4)	5 (5)			
	F-dextran	3900	6 (6)	4 (4)	2 (4)	3 (5)	5 (5)	5 (5)	
	F-dextran	9400	6 (6)	2 (4)	2 (4)	2 (5)	5 (5)	5 (5)	
306	LYCH	457	7 (7)			7 (7)	7 (7)	5 (5)	
	F-dextran	3900	0 (7)			0 (7)	0 (7)	0 (6)	
	F-dextran	9400	0 (7)			0 (7)	0 (7)	0 (6)	

[a] Data are presented as the number of plants that showed movement of the specific probe, as determined 2 min after injection. (On average, four to five microinjections were performed, per leaf, to ascertain the extent of dye coupling.
[b] Plant lines 2-72 and 277 are as described in Table 1. Plant line 306 is transformed but does not harbor an MP gene.
[c] LYCH, Lucifer Yellow CH.
[d] Values in parentheses represent number of plants tested.

DISCUSSION

The 30-kD TMV MP modifies the size-exclusion limits of plasmodesmata in leaf tissue of transgenic tobacco plants by an unknown mechanism (Wolf et al., 1989; Deom et al., 1990, 1991). This modification corresponds with the capacity of TMV mutants that do not produce MP (MP[−] TMV) to spread from cell to cell (Holt and Beachy, 1991). In the present study, we investigated the function of a ts MP under permissive (24°C) and nonpermissive (32°C) temperatures. Transgenic tobacco plant lines that express either the wild-type MP (line 277) or the ts MP (line 2-72)

were pretreated at 32°C before injection with fluorescent high molecular weight dextrans. A 3-hr to 6-hr pretreatment significantly reduced the size-exclusion limit of plasmodesmata in line 2-72, and extended treatment at 32°C prevented the spread of MP(−) TMV. In contrast, MP(−) TMV inoculated on plant line 277 gave rise to systemic infection at both 24°C and 32°C. These results support the hypothesis that cell-to-cell spread of TMV is concomitant with modification of plasmodesmatal size-exclusion limits.

Nishiguchi et al. (1982) suggested two explanations for the inability of Ls1, which has a similar amino acid mutation

Figure 3. (continued).

Fluorescence images were captured 2 min after microinjection of the 9.4-kD F-dextran. The microinjection site is indicated with an arrow. In the situations where no dye movement occurred, the image was rechecked 10 and 20 min after the microinjection of the dye. (Fluorescence intensity scale was as follows: highest to lowest; red, yellow, white, blue, green, with black representing background.)

(A) F-dextran movement in mesophyll cells of transgenic tobacco plant line 2-72 maintained at 24°C.

(B) F-dextran molecules contained within an injected mesophyll cell of transgenic tobacco line 2-72 maintained at 32°C for 6 hr before dye injection.

(C) F-dextran movement in mesophyll cells of transgenic tobacco plant line 2-72 pretreated for 48 hr at 32°C before being returned to 24°C; 9.4-kD F-dextran microinjected 6 hr after the plant was returned to 24°C. (This is the same plant as that used for the dye-coupling experiment depicted in **[B]**.)

(D) F-dextran molecules contained within an injected mesophyll cell of transgenic tobacco plant line 277 maintained at 32°C for 6 hr before dye injection and mesophyll cells bathed in glass-distilled water.

(E) F-dextran movement in mesophyll cells of transgenic tobacco plant line 277 maintained at 32°C for 6 hr before dye injection and mesophyll cells bathed in a 5 mM Sirofluor solution.

(F) F-dextran molecules contained within an injected mesophyll cell of transgenic tobacco plant line 2-72 maintained at 32°C for 6 hr before dye injection and mesophyll cells bathed in a 5 mM Sirofluor solution.

Table 3. Effect of High Temperature Treatment on the Mobility of Fluorescent Probes between Mesophyll Cells of Transformed Tobacco Plants Treated with 5 mM Sirofluor or Distilled H_2O

Transgenic Plant Line	Distilled Water		5 mM Sirofluor	
	F-dextran	LYCH	F-dextran	LYCH
2-72	0 (6)	5 (5)	0 (5)	5 (5)
277	1 (6)	4 (4)	5 (6)	5 (5)

Dye coupling measured with 9.4-kD F-dextrans and Lucifer Yellow CH (LYCH) after a 6-hr treatment at $32 \pm 1°C$. Data are presented as the number of plants that showed movement of the specific probe, as determined 2 min after injection. (On average, four to five microinjections were performed, per leaf, to ascertain the extent of dye coupling. Values in parentheses represent number of plants tested).

in its MP, to move from cell to cell under restrictive temperatures: (1) The host plant may be induced to produce a material that blocks viral movement, or (2) the inhibition of viral movement may be caused by a functional defect in a viral-encoded protein. The decrease in movement of the 9.4-kD and 3.9-kD F-dextrans in transgenic lines 277 and 2-72 during the first 6 hr at 32°C is in agreement with the hypothesis that a blocking agent is being deposited that might modulate cell-to-cell movement of the virus. A recent model for plasmodesmatal function suggests that (1→3)-β-D-glucan synthase may be involved in the rapid deposition of callose that, by compression against the cell wall, would close an otherwise open cytoplasmic annulus (Olesen and Robards, 1990). Furthermore, temperature-associated changes in callose synthesis have been reported for several plant systems (Webster and Currier, 1968; Dinar et al., 1983). These earlier studies established that temperature-induced callose deposits in parenchyma cells were removed after a 48-hr recovery period at room temperature (Currier and Webster, 1964; Webster and Currier, 1968). In addition, these studies showed that within a population of cells not all will respond by depositing callose after a temperature perturbation. Localized domains of callose synthesis are also known to be induced in hypersensitive tissues responding to viral infection, with the result that local lesions are formed (Allison and Shalla, 1974).

The transient reduction in plasmodesmatal size-exclusion limit observed when 277 line tobacco plants were exposed to 32°C is consistent with temperature-induced synthesis of callose. As illustrated in Table 2, in the plants that exhibited this decrease in molecular size-exclusion limit, both the 3.9-kD and 9.4-kD F-dextrans failed to move from the site of injection. However, Lucifer Yellow CH was still able to pass from cell to cell, indicating that if callose deposition at the neck region of the plasmodesmata was the cause of this detected change in size-exclusion limit,

its synthesis must be well regulated, in that the cytoplasmic annulus was not completely constricted.

In comparison with plant line 277, we detected significant differences in the response of plant line 2-72 to exposure to nonpermissive temperatures. Within 3 hr at 32°C, all line 2-72 plants failed to allow the passage of the 9.4-kD F-dextran, whereas the 3.9-kD probe moved in approximately 50% of the plants examined. After 6 hr at 32°C, the number of plants in which the 3.9-kD F-dextran moved from cell to cell decreased further to about 30% and remained at this level through the 24-hr time point. Another difference between the two plant lines relates to the effect of long-term exposure to nonpermissive temperatures. After 48 hr at 32°C, the molecular size-exclusion limits of the plasmodesmata in plant line 277 had returned to control values, whereas those of plant line 2-72 showed only a partial recovery. As shown in Table 2, approximately 50% of the 2-72 tobacco plants permitted the 3.9-kD F-dextran to move from cell to cell, but the larger 9.4-kD probe was essentially retained within the injected mesophyll cells. This recovery of plasmodesmatal size-exclusion limits in line 277 (expressing the wild-type TMV MP) may have been caused by the removal of newly deposited callose by (1→3)-β-D-glucanase activity. If callose synthesis and degradation behaved similarly in line 2-72, then the inability of these plants to allow the passage of the 9.4-kD F-dextran must be associated with a change in the function of the mutant MPP154A protein.

The involvement of callose synthesis in the ts response of plasmodesmatal size-exclusion limit was examined further by using Sirofluor, an inhibitor of (1→3)-β-D-glucan synthase activity (Morrow and Lucas, 1987). As shown in Figures 3D and 3E and Table 3, plant lines 277 and 2-72 held at 32°C for 6 hr no longer allowed movement of the 9.4-kD F-dextran. However, Sirofluor maintained the higher exclusion limit in line 277 but not in line 2-72. These Sirofluor results support our hypothesis that temperature-induced callose formation in plant line 2-72 cannot form the sole basis for restricted cell-to-cell movement of the fluorescent dextrans. Thus, given the parallels between the injection experiments and viral complementation studies, these findings do not substantiate the hypothesis that the temperature sensitivity of either Ls1 or MP154PA results from callose-mediated down-regulation of plasmodesmata, i.e., increased callose synthesis or decreased callose degradation. Other cellular mechanisms of plasmodesmatal regulation involving second messengers (Erwee and Goodwin, 1983; Baron-Epel et al., 1988; Tucker, 1988) should not be discounted.

It is also important to stress that the data presented in Table 2 indicate that in plant line 277, during the first 24 hr at 32°C, approximately 50% of the injected cells did not permit the movement of the 9.4-kD F-dextran. When this experiment was repeated to investigate the effects of Sirofluor, the fraction of cells that exhibited down-regulation of plasmodesmatal size-exclusion limits increased to

approximately 80% (Table 3). This degree of variability is consistent with the all-or-none type of response that is associated with the induction of callose synthesis (Currier and Webster, 1964; Webster and Currier, 1968). Clearly the fluorescent dye-microinjection system provides an extremely sensitive method for the further study of the cellular control over callose synthesis and degradation.

The basis for the temperature sensitivity of systemic infection and modification of plasmodesmatal size-exclusion limits is not due to degradation of the ts MP. A comparison of the total amount and subcellular distribution of MP in lines 277 and 2-72 demonstrated that the temperature regimes used had no significant effect (Table 1). Hence, the time-dependent reduction in plasmodesmatal size-exclusion limit in plant line 2-72 may result from a change in the ability of the mutant TMV MP to interact with a substructural component of the plasmodesmata, whose function is to regulate the physical pore size within the cytoplasmic annulus.

This possibility would be consistent with the hypothesis proposed by Ohno et al. (1983), who suggested that the temperature sensitivity of the Ls1 mutant may result from a decrease in rigidity of the protein after the substitution of proline with serine. Secondary structural predictions of the MP indicate that proline in position 154 forms part of a β turn (Lucas et al., 1990) and its replacement, by either serine or alanine, may cause a change in tertiary structure that could alter the MP-endogenous plasmodesmatal protein interaction under nonpermissive temperatures.

Elucidating the action of the MP should aid in understanding the mechanism controlling plasmodesmatal functioning as well as developing a model to explain the mode of viral systemic infection. Although the underlying mechanism by which the MP modifies plasmodesmatal size-exclusion limits is yet to be elucidated fully, our results suggest that the TMV MP may interact with endogenous proteins within the cytoplasmic annulus of the plasmodesmata, thereby causing their physical displacement to yield a more open unoccluded structure.

METHODS

Mutagenesis and Plant Transformation with a TMV MP Gene

The cDNA encoding the MP gene was excised from pTM934SP (Oliver et al., 1986) and ligated into pBluescript KS+ (Stratagene) to produce the plasmid pTMMPKS+. This plasmid was used for oligonucleotide-directed mutagenesis (Kunkel et al., 1987). The oligonucleotide 5'GCGGGTTTCTGTGCGCTTTCTCTGG-3' was used to introduce a proline to alanine at amino acid 154 in the MP. The mutant MP gene was excised with BamHI and EcoRI, and the fragment was ligated into pMON 316 (Deom et al., 1987) at the BglII and EcoRI sites in the polylinker region between the cauliflower mosaic virus 35S promoter and the nopaline synthase

3' untranslated region. Plant transformation of the MP gene was carried out as described by Deom et al. (1987).

Plant Material and Experimental Conditions

R1 progeny of transgenic Nicotiana tabacum cv Xanthi plant lines 277 (expressing the unmodified TMV MP), 306 (transformed but without the MP gene), and 2-72 (expressing the ts MP of the U1 common strain of TMV, MPP154A) were used in the present experiments. Plant line 277 was shown previously to contain two active copies of the TMV MP (Deom et al., 1987), and segregation of nopaline synthase activity indicated that 2-72 also contained two active copies of the mutant MPP154A gene. Seeds were germinated and plants were grown in an insect-free greenhouse. Excluding the inoculation and complementation analysis, 3-week-old seedlings were transferred to a controlled-environment chamber for 1 week to 2 weeks before being employed in microinjection (dye-coupling) experiments. Temperature regime in the growth chamber was 24°C/18°C (day/night). For nonpermissive temperature treatments, plants were transferred to a second controlled-environment chamber in which the day/night temperature regime was 33°C ± 1°C; both chambers had comparable PAR flux density (400 μmol to 450 μmol m^{-2} sec^{-1}) and a 16-hr photoperiod was employed.

Virus Inoculation and Complementation Analysis

The two youngest leaves greater than 4 cm in length on tobacco plants at the five-leaf stage were inoculated (Deom et al., 1987) with a suspension (20 μg/mL) of purified TMV or U3/12MPfs. U3/12MPfs contains a defective MP gene (Deom et al. 1991) and can only spread in transgenic plants that express the MP gene (Deom et al., 1987). These inoculated plants were maintained in a controlled-environment chamber under a 14-hr photoperiod and a PAR flux density of 300 μmol to 350 μmol m^{-2} sec^{-1}. Inoculated plants were maintained either at 24°C or at 32°C and observed daily for disease symptoms. Accumulation of TMV and U3/12MPfs in inoculated and upper leaves from inoculated plants expressing the MP was determined by immunoblot analysis (Deom et al., 1987).

MP Analysis

Subcellular fractionation, extraction, and quantitation of MPP154A and MP from leaf tissue were performed as described by Deom et al. (1990).

Dye Conjugates and Liposome Preparation

Lucifer Yellow CH (Sigma) and F-dextrans (Sigma) with molecular masses of 3.9 kD and 9.4 kD were used as fluorescent probes. Probes were prepared as 1 mM solutions in 5 mM KHCO$_3$ (pH 8). Unilamellar liposomes were prepared by the freeze-thaw method described by Pick (1981) as modified by Madore et al. (1986). An

aliquot of beef brain phosphatidylserine (1.6 mg) in a chloroform:methanol (95:5) mixture was transferred, under an N_2 blanket, to a screw-cap microvolume vial that had been prechilled on ice. A stream of N_2 was then used to evaporate off the chloroform and methanol. To allow complete transferral of the resulting lipid film from the sides of the vial, the phospholipid was redissolved in 100 μL of 4°C Freon 11. A 70-μL aliquot of an aqueous solution of the fluorescently tagged molecule was then added to the dissolved lipid. The tube was flushed with N_2, capped, and thoroughly vortexed until the content reached room temperature and the Freon (boiling point 23°C) had boiled off. Small unilamellar liposomes were generated by sonicating the phospholipid suspension for 60 sec using a Branson 200 Sonifier Cell Disruptor. The sonicated mixture was again sealed under N_2 and immersed in liquid nitrogen to freeze the preparation. The frozen mixture was allowed to thaw at room temperature, briefly resonicated (30 sec), and filtered through a 0.22-μm HPLC membrane filter.

Microinjection Procedure

Liposomes were back loaded by way of capillary action into the tip of a glass micropipette that had a tip diameter of 0.5 μm to 1.0 μm. The capillary was sealed into a micropipette holder (Leitz, Rockleigh, NJ, model 520145) and injection pressures (6 psi to 15 psi) were controlled by a Pneumatic PicoPump (WPI, New Haven, CT, model PV830). Pipette movement for cell impalement was controlled by a hydraulically driven micromanipulator (Narishige, Greenvale, NY, model MO-102).

A mature tobacco leaf still attached to the plant was placed, abaxial side up, on a microscope slide and held in place with a piece of double-sided transparent tape. A small piece of modeling clay was used to form a barrier on this abaxial surface, and a portion (5 mm^2) of the lower epidermis was then removed with forceps. The exposed area was covered immediately with glass-distilled water. In some experiments, mesophyll cells were bathed with a 5 mM solution of Sirofluor prepared according to Evans and Hoyne (1982). An exposed spongy mesophyll cell was impaled with the micropipette and injected with the liposome suspension. Liposome fusion to the tonoplast and subsequent movement of dye into neighboring cells was monitored by using a Leitz Orthoplan epifluorescence microscope (equipped with a blue, BP 390-490, excitation filter) connected to a Hamamatsu (Bridgewater, NJ) image enhancement system (Model C1966-20). In the experiments calling for elevated temperatures, the room temperature was raised to 32°C ± 1°C and microinjection was carried out under these conditions.

ACKNOWLEDGMENTS

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