Salicylic Acid Has Cell-Specific Effects on *Tobacco mosaic virus* Replication and Cell-to-Cell Movement[1]

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Tobacco mosaic virus (TMV) and *Cucumber mosaic virus* expressing green fluorescent protein (GFP) were used to probe the effects of salicylic acid (SA) on the cell biology of viral infection. Treatment of tobacco with SA restricted TMV.GFP to single-epidermal cell infection sites for at least 6 d post inoculation but did not affect infection sites of *Cucumber mosaic virus* expressing GFP. Microinjection experiments, using size-specific dextrans, showed that SA cannot inhibit TMV movement by decreasing the plasmodesmatal size exclusion limit. In SA-treated transgenic plants expressing TMV movement protein, TMV.GFP infection sites were larger, but they still consisted overwhelmingly of epidermal cells. TMV replication was strongly inhibited in mesophyll protoplasts isolated from SA-treated nontransgenic tobacco plants. Therefore, it appears that SA has distinct cell type-specific effects on virus replication and movement in the mesophyll and epidermal cell layers, respectively. Thus, SA can have fundamentally different effects on the same pathogen in different cell types.

Salicylic acid (SA) is a component of the signal transduction pathway needed for induction of systemic acquired resistance (SAR), a plant-wide enhancement of resistance against a broad spectrum of pathogens (Dempsey et al., 1999; Murphy et al., 1999). The trigger for SA synthesis and induction of SAR is the recognition of an invading microorganism by the product of a resistance gene (Baker et al., 1997). Often, this recognition is accompanied by the hypersensitive response (HR), a form of rapid programmed host cell death in a region around the point of pathogen entry (Hammond-Kosack and Jones, 1996).

Tobacco (*Nicotiana tabacum*) plants that possess the *N* gene (Whitham et al., 1994) are resistant to *Tobacco mosaic virus* (TMV) and exhibit the HR after inoculation with that virus. The HR is followed by an increase in SA (Malamy et al., 1990) and induction of SAR throughout the plant (Ross, 1961a, 1961b). In these plants, the TMV is localized to the vicinity of the necrotic lesions. However, the tissue necrosis that occurs is not the sole cause of virus localization. For example, studies with green fluorescent protein (GFP)-tagged TMV (TMV.GFP) have shown that live cells around the HR contain TMV for significant periods of time after lesion formation (Wright et al., 2000; Murphy et al., 2001). This indicates that processes other than cell death are limiting virus spread. In addition, *NN*-genotype transgenic tobacco plants, which have been transformed with a bacterial salicylate hydroxylase gene and, therefore, cannot accumulate SA, do not limit virus spread. Although the cells of these plants can still undergo HR-type cell death, the plants exhibit a spreading necrosis after TMV inoculation (Mur et al., 1997; Darby et al., 2000), showing that SA accumulation is required to localize TMV. Additionally, treatment of susceptible tobacco with aspirin (acetyl-SA) or SA caused a profound reduction in accumulation of TMV in the absence of any macroscopic cell death at all (White et al., 1983; Chivasa et al., 1997).

Successful development of local infection by plant viruses requires the replication and subsequent cell-to-cell movement of the virus from the initially inoculated cell to adjacent healthy cells via plasmodesmata (PD). TMV is a plus-sense, single-stranded RNA virus that encodes at least five polypeptides (Palukaitis and Zaitlin, 1986). One of these, the 30-kD movement protein (MP), is sufficient and essential for TMV cell-to-cell spread (Deom et al., 1987; Meshi et al., 1987). In contrast, *Cucumber mosaic virus* (CMV) requires two viral proteins for cell-to-cell movement, the 3a MP and the coat protein (CP; Suzuki et al., 1991; Canto et al., 1997).

The TMV MP possesses at least two activities that allow it to mediate cell-to-cell movement of the virus. First, TMV MP opens or "gates" PD linking mesophyll and epidermal cells from a basal size exclusion limit (SEL) that is below 1 kD to an SEL of 10 kD or greater (Wolf et al., 1989; Waigmann et al., 1994; Oparka et al., 1997). Second, TMV MP cooperatively binds to and unfolds single-stranded nucleic acid to form long and very thin nucleoprotein complexes in vitro (Citovsky et al., 1990, 1992). Thus, it has been proposed that in vivo the TMV MP chaperones the TMV RNA as a viral ribonucleoprotein complex through gated PD (Citovsky et al., 1992). Similarly,

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the 3a MP of CMV can modify the SEL of PD in both mesophyll and epidermal cells in tobacco (Vaquero et al., 1994; Ding et al., 1995; Canto et al., 1997). Also, the 3a MP has nucleic acid and NTP-binding properties indicating that the 3a MP can also chaperone CMV RNA through PD (Li and Palukaitis, 1996). However, it differs from the TMV MP in that it can form tubules, a phenomenon that may be important for cell-to-cell movement through PD interconnecting tobacco epidermal cells (Canto and Palukaitis, 1999).

In SA-treated tobacco, the accumulation of at least two viruses, TMV and *Potato virus X* (PVX), is inhibited at the site of inoculation (Chivasa et al., 1997; Naylor et al., 1998). But at least one virus, CMV, is able to evade this effect (Naylor et al., 1998; Ji and Ding, 2001). However, it was found that SA is able to inhibit CMV movement out of the inoculated leaf to the rest of the plant (Naylor et al., 1998; Ji and Ding, 2001). Thus, in tobacco, SA can induce resistance to viruses by triggering at least two different defensive mechanisms.

In our previous studies on the effect of SA on TMV infection of tobacco, it was concluded that SA induced resistance to TMV in large part by inhibiting virus replication (Chivasa et al., 1997; Chivasa and Carr, 1998). However, since that time, viruses that express free GFP have been used successfully to investigate both natural (Wright et al., 2000) and genetically engineered (Goregaoker et al., 2000) forms of virus resistance. In the present study, we used TMV.GFP (Lacomme and Santa Cruz, 1999) and CMV.GFP (Canto et al., 1997) to observe and re-assess the effects of SA on virus accumulation and local cell-to-cell virus movement in tobacco.

RESULTS

SA Interferes with the Distribution of TMV.GFP, But Not CMV.GFP, in SA-Treated Tobacco

The use of GFP-tagged viruses allowed the effects of SA treatment on inoculation efficiency to be monitored. CMV.GFP or TMV.GFP was inoculated onto SA-treated or control tobacco leaf tissue. The number of CMV.GFP infection sites was counted on inoculated leaves at 22 h post inoculation (hpi) and TMV.GFP infection sites were counted on inoculated leaf discs at 22 hpi (experiment 1) or 2 d post inoculation (dpi; experiment 2; Table I). SA treatment did not cause a statistically significant decrease in the number of infection sites established by either of the viruses (Table I). This result shows that SA-induced resistance to viruses is unlikely to be due to a reduction in the number of infection sites in the inoculated tissue.

The development of TMV.GFP infection sites on untreated and SA-treated tobacco plants was examined by epifluorescent microscopy at various time points between 2 and 6 dpi. Although by 2 dpi, TMV.GFP infection sites were visible in SA-treated plants, all of these consisted of single epidermal cells (Fig. 1, A–C). In contrast, infection sites in control plants ranged from single epidermal cells (Fig. 1E) to multiple cell sites that frequently showed TMV.GFP movement into the palisade mesophyll cells (Fig. 1, F and G). By 3 dpi, infection sites in control plants expanded rapidly through epidermal and palisade mesophyll cells and when viewed using epifluorescent optics appeared as solid, intensely fluorescent discs (Fig. 1H). Yet, even by 6 dpi in SA-treated plants, TMV infection was predominantly limited to single cells (Fig. 1D) and very rarely to groups of two or three epidermal cells (data not shown).

Table I. *The effect of SA treatment on the mean number of infection sites in tobacco tissue directly inoculated with either TMV or CMV engineered to express free GFP*

Discs and leaves were floated on or sprayed with 1 mM SA for 5 d prior to inoculation.

	Control	SA
	Mean no. of infection sites/ leaf disc ± SD	
TMV.GFP		
Experiment 1 (n = 5 leaf discs per treatment)	12 ± 5.3	8.4 ± 4
Experiment 2 (n = 10 leaf discs per treatment)	8.8 ± 4.9	7.5 ± 4.9
CMV.GFP (n = 3 leaves per treatment)	31 ± 4.6	21 ± 14

Previous studies demonstrated that SA interferes with TMV RNA accumulation in directly inoculated tobacco tissue (Chivasa et al., 1997). In the present study, using the observed intensity of GFP fluorescence as an approximate measure of virus replication, it appeared that the levels of virus replication in single-cell inoculation sites in SA-treated and control plant tissue were similar (compare Fig. 1, A and E). This result indicated that although SA was having relatively little effect on TMV replication in epidermal cells, it might be inhibiting TMV cell-to-cell movement.

In parallel experiments, the development of CMV infection sites was observed in control and SA-treated tobacco using a CMV.GFP construct (Canto et al., 1997). We observed no difference in the development of CMV.GFP infection sites between SA-treated and untreated tobacco plants (Fig. 2). This result is consistent with previous work showing that SA does not affect CMV accumulation in directly inoculated tissues but inhibits long-distance movement (Naylor et al., 1998). Taken together with the results presented in Figure 1, this data serves to emphasize that SA can specifically interfere with the accumulation and cell-specific distribution of TMV, while having no such effects on CMV in the inoculated tissue.

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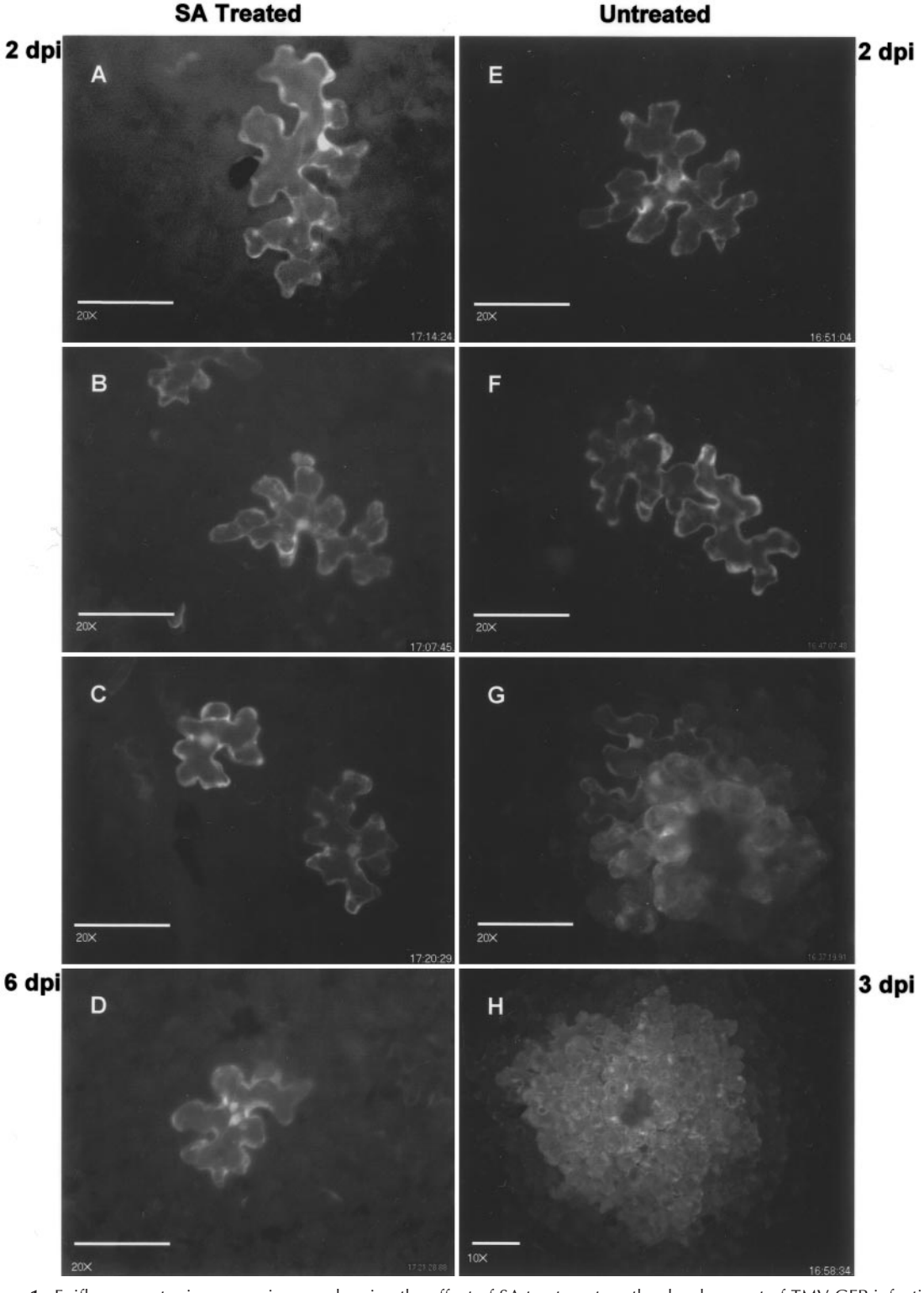

Figure 1. Epifluorescent microscope images showing the effect of SA treatment on the development of TMV.GFP infection sites in tobacco. A through D, Infection sites in leaves from SA-treated tobacco consisted predominantly of single epidermal cells at 2 dpi (A–C) and 6 dpi (D). E through H, Infection sites in leaves from untreated tobacco at 2 dpi (E–G) and 3 dpi (H). Within 2 dpi, single (E) and multiple (F) epidermal cell infection sites were observed in untreated tobacco and some infection sites consisted of epidermal and palisade mesophyll cells (G). By 3 dpi, most infection sites had expanded dramatically and were composed of hundreds of epidermal and mesophyll cells. All scale bars = 100 μm.

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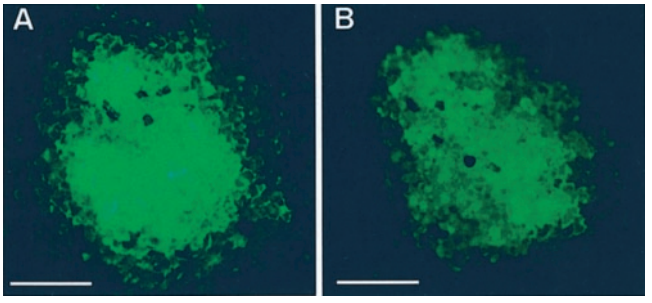

Figure 2. Epifluorescent microscope images of CMV.GFP infection sites on leaves of untreated and SA-treated tobacco. A, CMV.GFP infection site in untreated tobacco 4 dpi. B, CMV.GFP infection site in SA-treated tobacco at 4 dpi. There is no significant difference in the size or appearance of infection sites developing in leaves of untreated or SA-treated tobacco plants. Scale bar = 500 μm.

SA Does Not Inhibit PD Gating

Microinjection of fluorescently tagged dextrans of defined M_r into tobacco epidermal cells of control and SA-treated tobacco was carried out to determine whether or not SA has any direct effects on PD function. Mature leaves were used for all microinjection experiments as developing leaves show a shift in PD permeability after the carbon sink-source transition (Oparka et al., 1999).

Surprisingly, we found that SA treatment alone could, to a limited extent, actually increase the SEL of PD linking tobacco epidermal cells (Table II). The basal SEL of tobacco epidermal cell PD has been reported to be 1 kD or less (Derrick et al., 1990), but we found, in line with the data of Poirson et al. (1993), that PD of tobacco epidermal cells frequently permitted the passage of a 3-kD dextran (Table II; movement occurred in 52% of impalements). Furthermore, SA treatment caused the cell-to-cell movement of 3-kD dextran in almost all injections (Table II; movement occurred in 93% of impalements). In contrast, movement of microinjected 10-kD dextran between tobacco epidermal cells never occurred, even in SA-treated plants (Table II). Thus, SA can increase the proportion of epidermal cells possessing PD with an SEL allowing movement of a 3-kD dextran, but it cannot increase the SEL of PD to the extent of allowing movement of a 10-kD dextran (Table II). That is, to an extent correlated with the requirement for virus movement (Wolf et al., 1989).

Table II. *Movement of fluorescent dye or fluorescently tagged dextrans in epidermal cells of tobacco leaves from plants that were either untreated or pretreated with SA*

Data are expressed as free dye (fluorescein data is shown) or fluorescein (F)- or Texas Red (R)-tagged dextran movement/total no. of injections.

Fluorescent Probe	Control Treatment	SA Treatment
Fluorescein (>1 kD)	10/10 (100%)	10/10 (100%)
3-kD R-dextran	11/21 (52%)	13/14 (93%)
10-kD F-dextran	0/10 (0%)	0/20 (0%)

Therefore, we conclude that SA does not have any direct effect on PD function that is likely to either help or hinder viral cell-to-cell movement.

SA Does Not Inhibit TMV MP-Mediated Gating between Epidermal Cells

TMV.GFP appeared to be severely restricted in its ability to move out from the initially inoculated epidermal cell in SA-treated tobacco (Fig. 1). This suggested that SA may inhibit the activity of TMV MP. Because the TMV MP is essential and sufficient to potentiate TMV cell-to-cell movement (Deom et al., 1987; Wolf et al., 1989), we investigated whether or not SA affects the gating function of the MP. Therefore, we tested this possibility using transgenic tobacco plants that constitutively express the TMV MP under the control of the *Cauliflower mosaic virus* 35S promoter (transgenic tobacco line 277; Deom et al., 1987). The transgenic MP tobacco can complement TMV mutants lacking a functional MP (Deom et al., 1987), and the mesophyll cell PD in these plants have an increased SEL allowing the cell-to-cell movement of microinjected 10-kD dextrans (Wolf et al., 1989). Thus, we treated the transgenic tobacco with SA to determine whether the chemical had any effect on the gating activity of the TMV MP expressed in trans.

First, northern-blot analysis was carried out to confirm that the steady-state level of the transcript of the *MP* transgene was not affected by SA treatment (data not shown). The activity of the MP was assessed by microinjection of a 10-kD, fluorescently labeled dextran into epidermal cells of transgenic MP tobacco that had been either SA treated or left untreated. In agreement with previous work on these transgenic plants (Wolf et al., 1989), constitutive expression of the TMV MP potentiated the cell-to-cell movement of 10-kD dextran (in 72.5% of injections; Table III). Pretreatment of MP transgenic tobacco plants with SA before microinjection did not appear to significantly affect the ability of the MP to potentiate movement of a 10-kD dextran because movement occurred in 66% of injections (Table III). Thus, SA does not appear to inhibit the gating function of TMV MP in tobacco epidermal cells, and this, therefore, cannot account for the apparent inhibition of cell-to-cell movement of TMV.GFP in SA-treated tobacco.

SA Disrupts the Normal Tissue Distribution Pattern of TMV.GFP in MP-Expressing Transgenic Tobacco

Because SA had no apparent effect on the gating function of the TMV MP, we anticipated that supplying TMV MP in trans would have little effect on SA-induced resistance to TMV. However, we found that although the MP transgenic plants were still able to exhibit SA-induced resistance to TMV, the accumulation and distribution of TMV.GFP in these

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Table III. *Movement of 10-kD dextran in epidermal cells of transgenic tobacco expressing the TMV movement protein (MP)*

Data are expressed as dextran movement/total number of injections. Between one and three injections were made on each of 15 separate SA-treated or 16 untreated plants.

Fluorescent Probe	Control Treatment	SA Treatment
10-kD R-dextran	23/34 (68%)	29/44 (66%)

plants differed significantly from that in wild-type tobacco.

Leaf discs from MP transgenic tobacco were floated on 1 mM SA or water for 4 d before inoculation with wild-type TMV U1. Accumulation of virus-specific RNA was examined at 2 dpi by northern-blot analysis. Pre-incubation with SA caused a significant reduction in the accumulation of TMV RNA (Fig. 3) in inoculated MP transgenic tobacco and in nontransgenic tobacco. This result demonstrated that supplying MP in trans does not abolish SA-induced resistance to TMV.

However, microscopic examination using epifluorescent optics showed that supplying MP in trans did increase the spread of virus. At 3 dpi this was true both in control and SA-treated MP transgenic tobacco. In untreated plants (Fig. 4A), TMV.GFP infection sites composed of both epidermal and mesophyll cells developed. These were larger than the TMV.GFP infection sites that develop in nontransgenic tobacco at this time point and this difference in the rate of movement could easily be seen by eye at 7 dpi (Fig. 5). In SA-treated MP transgenic tobacco at 3 dpi (Fig. 4B) TMV.GFP moved further through the epidermal cell layer than in SA-treated nontransgenic tobacco (Fig. 1) but it still did not enter the palisade mesophyll cells.

Confocal laser scanning microscopy was used to better resolve the TMV.GFP infection sites in control and SA-treated MP transgenic tobacco and serial optical sections were taken through infection sites 3 and 4 dpi. In untreated MP transgenic tobacco, TMV.GFP had already spread extensively into the mesophyll cells by 3 dpi (Fig. 4, C–F). In contrast, in SA-treated MP transgenic tobacco TMV.GFP was typically confined to the epidermal cell layer and could not be detected in the underlying palisade mesophyll layer at 3 and 4 dpi (Fig. 4, G–J and K–N). However, by 4 dpi in a small number of infection sites, TMV.GFP had spread to a few palisade mesophyll cells under the infection center. An example of this is shown in Figure 4, O through R.

Thus, the constitutive expression of the TMV MP in the transgenic plants could partly relieve the inhibition of TMV movement seen in SA-treated nontransgenic tobacco (Fig. 4), even though it did not prevent the overall decrease in TMV RNA accumulation caused by SA in the inoculated tissue (Fig. 3). Taken together, these results show that although movement of TMV out of the primary inoculated cell is slowed in SA-treated tobacco, this effect is not sufficient by itself to mediate SA-induced resistance to the virus.

SA Inhibits TMV Replication in Tobacco Mesophyll Protoplasts

The imaging studies using TMV.GFP showed that in both untransformed and MP transgenic tobacco TMV.GFP was, for at least 4 d, limited to the epidermal cells in SA-treated plants. We wanted to investigate the possibility that SA was inhibiting replication in the mesophyll cells. To do this, protoplasts were generated from tobacco plants that had been either treated with 1 mM SA or watered normally for the previous 4 d. Microscopic examination confirmed that the vast majority (at least 98%) of the protoplasts in the preparations were derived from mesophyll cells (data not shown). Aliquots of protoplasts made from control and SA-treated tobacco were placed in an oxygen electrode to confirm that both sets of protoplasts had similar rates of respiration and were, therefore, equally viable (data not shown).

Protoplasts from control and SA-treated leaves were electroporated with either TMV RNA or CMV RNA. Total RNA from the protoplasts was extracted 15 h after electroporation. Accumulation of TMV-specific RNA was decreased in the protoplasts from SA-treated tobacco (Fig. 6A). Because the vast majority of protoplasts generated from intact tobacco leaves are mesophyll cells, this result shows that SA strongly interferes with TMV replication in these

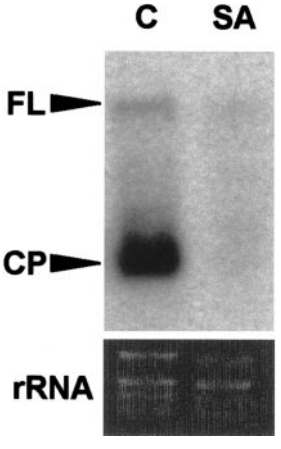

Figure 3. RNA gel-blot analysis of the effect of SA on the accumulation of TMV-specific RNAs in TMV-inoculated leaf tissue of transgenic tobacco expressing the TMV MP. Equal amounts of total RNA extracted from 10 pooled leaf discs (10 mm diameter, pretreatment) were loaded in each gel lane and, after electrophoresis, were transferred to nitrocellulose for hybridization with a ^{32}P-labeled plus-sense, strand-specific riboprobe. Leaf discs had been floated on water (lane C) or 1 mM SA (lane SA) for 4 d before inoculation with wild-type TMV (strain U1). RNA was extracted from leaf discs 48 h later. In this autoradiograph, the bands corresponding to TMV full-length RNA and CP mRNA are indicated by FL and CP, respectively. The ethidium bromide-stained ribosomal bands photographed before blotting are shown below.

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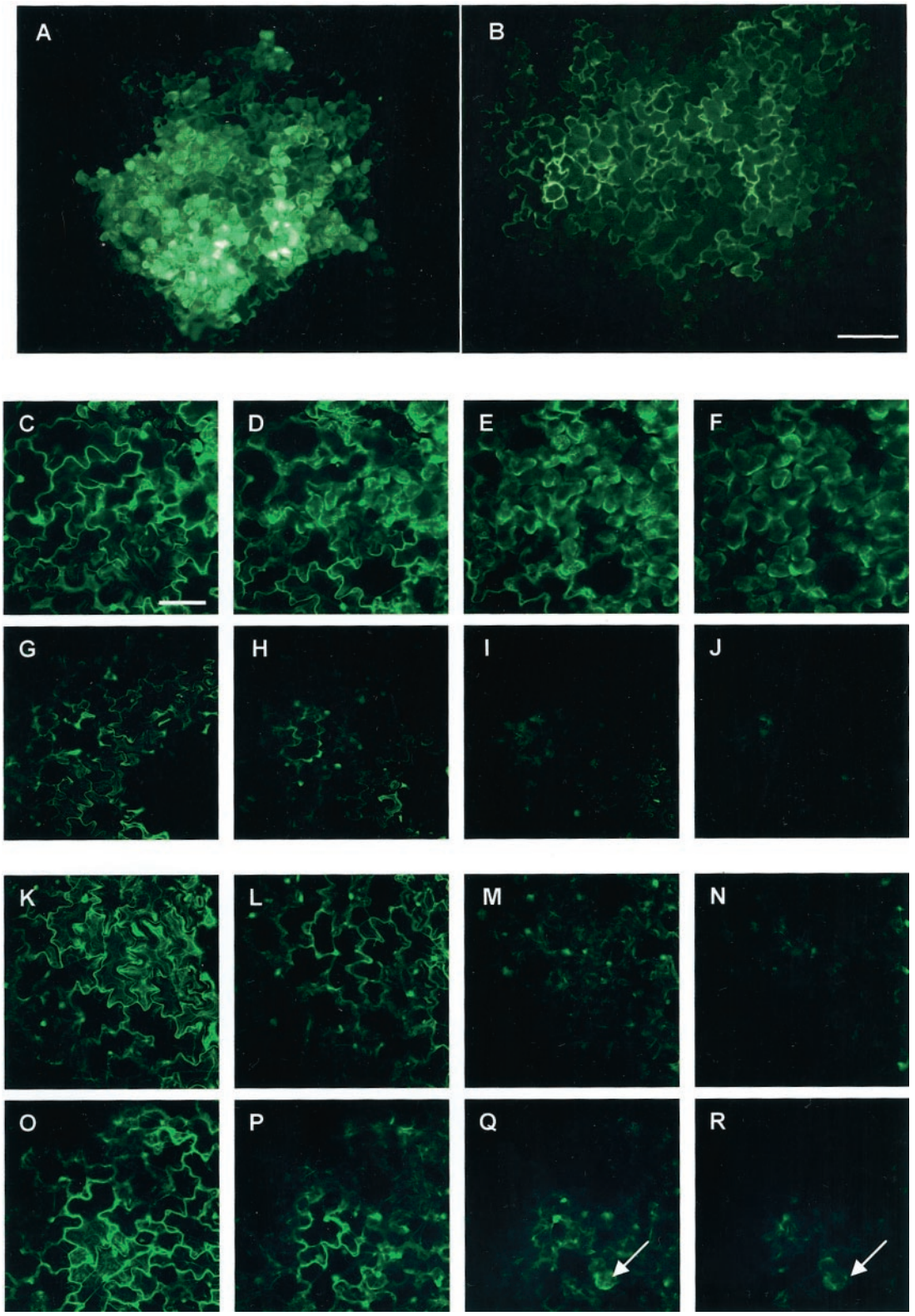

Figure 4. Effect of SA treatment on the development of TMV.GFP infection sites in transgenic tobacco expressing the TMV MP. A and B, Epifluorescent microscope images of TMV.GFP infection sites 3 dpi on tobacco leaves from untreated (A) and SA-treated (B) MP transgenic tobacco (scale bar = 200 μm). In the untreated plants, TMV.GFP infection sites were visualized as intensely fluorescent discs due to the GFP signal from the palisade cells (A). In contrast, GFP fluorescence was only seen in the epidermal cell layer in infection sites in SA-treated plants (B). C through R, Confocal laser scanning microscopic

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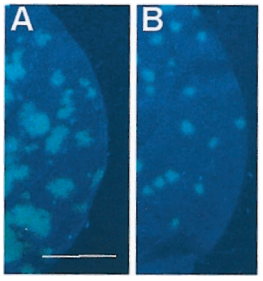

Figure 5. The spread of TMV.GFP in transgenic tobacco expressing the TMV MP and in nontransformed tobacco. TMV.GFP was inoculated onto transgenic tobacco expressing the TMV MP (A) and nontransformed tobacco (B). After 7 d, infection sites were visualized with a hand-held UV lamp. TMV.GFP infection sites had spread further in the transgenic tobacco expressing the TMV MP (A) compared with the nontransformed control (B). Scale bar = 1 cm.

cells. Inhibition of TMV replication was also seen when protoplasts from control-treated leaves were incubated in vitro with 50 μM SA for 40 min before electroporation both in the present study (Fig. 6) and in preliminary experiments (Naylor, 1999).

Accumulation of CMV-specific RNA was not affected by SA in protoplasts prepared at the same time (Fig. 6B). This result was expected, because it was previously shown that SA did not affect CMV replication in directly inoculated tobacco tissue (Naylor et al., 1998). This result also demonstrated that the protoplasts from SA-treated tobacco used in this experiment were capable of supporting virus replication. Thus, the interference with TMV replication in protoplasts was a specific effect of SA treatment and was not because of loss of protoplast viability.

DISCUSSION

The Inhibitory Effect of SA on TMV Replication Occurs in the Mesophyll Cells

Chivasa et al. (1997) observed that SA treatment reduced TMV RNA accumulation in directly inoculated tobacco and concluded that SA can induce resistance to TMV replication. Our protoplast study (Fig. 6) shows that SA-induced resistance to TMV can operate at the single-cell level. This demonstrates that SA can induce interference with TMV replication, as concluded by Chivasa et al. (1997). However, when GFP fluorescence in TMV.GFP infection sites in control and SA-treated tobacco leaves are compared (Fig. 1), it is clear that SA is having relatively little if any effect on TMV replication in the epidermal cells. Taken together, the protoplast data and the microscopic appearance of TMV.GFP infection sites indicate that the inhibitory effect of SA on TMV replication occurs almost entirely in the mesophyll cells.

Although replication of TMV.GFP does not appear to be significantly inhibited in the initially inoculated epidermal cells of SA-treated tobacco, no movement of TMV.GFP to neighboring epidermal cells was observed. This apparent constraint to movement could be partially relieved in TMV MP transgenic tobacco where TMV.GFP infection sites could expand radially through the epidermal cell layer but did not penetrate into the underlying mesophyll cells. This suggests indirectly that in the epidermal cells of SA-treated tobacco the quantity of MP synthesized in the infected cells is insufficient to facilitate virus movement.

However, this superficially compelling idea does not fit in well with what is known about the properties of TMV MP. For example, Szécsi et al. (1999) demonstrated that even a 10- to 12-fold reduction in MP synthesis during TMV replication has no effect on the rate of cell-to-cell movement. In addition, transcription of TMV MP mRNA from the 35S promoter in transgenic tobacco is easily sufficient to complement a strain of TMV that lacks a functional *MP* gene (Arce-Johnson et al., 1997). This is despite the fact that the transcription of MP mRNA driven by the *Cauliflower mosaic virus* 35S promoter is only a fraction (as little as 2%) of that produced in a wild-type TMV infection (Arce-Johnson et al., 1995). Altogether, these data (Arce-Johnson et al., 1995, 1997) show that relatively little TMV MP is required to facilitate a normal rate of TMV cell-to-cell movement. Thus, although we have seen in our experiments that SA treatment inhibits TMV.GFP epidermal cell-to-cell movement in nontransgenic tobacco, this may not be due solely to a decrease in the amount of MP synthesized in the infected cell.

Figure 4. (*Legend continued from preceding page.*)
images of TMV.GFP infection sites (scale bar = 100 μm). C through F, Serial transverse optical sections descending through a TMV.GFP infection site in untreated plants 3 dpi from the surface of the epidermal cell layer (C) to the interface between the epidermal and palisade mesophyll cell layers (F). TMV.GFP is already present in all of the palisade mesophyll cells beneath fluorescing epidermal cells. G through J, Serial transverse optical sections descending through a TMV.GFP infection site in SA-treated plants 3 dpi from the surface of the epidermal cell layer (G) to the interface between the epidermal and palisade mesophyll cell layers (J). No GFP fluorescence was apparent in the palisade cells. K through N, Serial transverse optical sections going down through a TMV.GFP infection site in SA-treated plants 4 dpi from the surface of the epidermal cell layer (K) to the interface between the epidermal and palisade mesophyll cell layers (R). In this example, no GFP fluorescence was apparent in the palisade cells. O through R, Serial transverse optical sections going down through a TMV.GFP infection site in SA-treated plants 4 dpi from the surface of the epidermal cell layer (O) to the interface between the epidermal and palisade mesophyll cell layers (N). In this example GFP fluorescence was apparent in a few palisade cells at the center of the infection site, which are indicated by white arrows.

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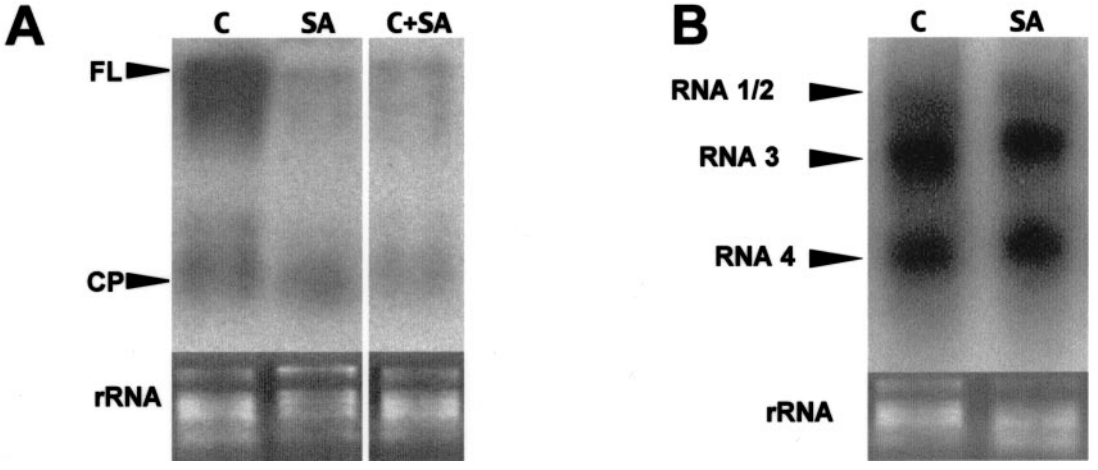

Figure 6. The effect of SA on the accumulation of virus-specific RNA in tobacco leaf protoplasts. A, Protoplasts were generated from whole leaves of tobacco plants that had either been untreated (lane C and lane C+SA) or pretreated with SA (lane SA) for 4 d. Protoplasts were inoculated with RNA of wild-type TMV (strain U1) using electroporation. One aliquot of control protoplasts was incubated in 50 μM SA for 40 min before inoculation with TMV (lane C+SA). At 15 hpi, total RNA was extracted from the protoplasts and subjected to RNA gel-blot analysis and hybridization with a ^{32}P-labeled TMV plus-sense, strand-specific riboprobe. In this autoradiograph, the bands corresponding to TMV full-length RNA and CP mRNA are indicated by FL and CP, respectively. These RNA bands do not occur in mock-inoculated protoplasts (data not shown). B, Protoplasts prepared in the above experiment were also inoculated with RNA from CMV (strain Fny). At 15 hpi, total RNA was extracted from the protoplasts and subjected to RNA gel-blot analysis and hybridization with a ^{32}P-labeled riboprobe complementary to the 3′-terminal sequences common to the genomic CMV RNAs 1, 2, and 3 and the subgenomic RNA 4. The ethidium bromide-stained ribosomal bands photographed before blotting are shown beneath each autoradiograph.

SA Does Not Block PD or Inhibit the Gating Properties of TMV MP

We investigated whether SA-treatment affected the properties of PD in tobacco leaves. Decreasing the basal SEL of tobacco PD has been shown to impede virus movement (Beffa et al., 1996; Iglesias and Meins, 2000). This was shown with transgenic tobacco expressing an antisense β-1,3-glucanase gene sequence. In these plants, callose accumulated around PD, which decreased the SEL of the PD (Iglesias and Meins, 2000), and reduced the rate of TMV and PVX movement (Beffa et al., 1996; Iglesias and Meins, 2000). In our investigation, we found that SA treatment did not decrease the SEL of PD in tobacco leaves. In fact, SA appeared to enhance the SEL of PD. This is consistent with the data of Beffa et al. (1996), because SA induces the expression of several extracellular pathogenesis-related proteins including some with β-1,3-glucanase activity (for review, see Van Loon and Van Strien, 1999). It is conceivable that these enzymes may increase the SEL of PD by decreasing the amount of callose that normally surrounds and constricts them. It is difficult to imagine how the relatively modest increase in the SEL of PD could in any way directly affect virus cell-to-cell movement. However, one might speculate that the increase in SEL may contribute to defense by enhancing the trafficking of host intercellular signaling molecules.

We also demonstrated that SA did not affect the ability of transgenically expressed TMV MP to gate PD between tobacco epidermal cells (Table III). Thus, the apparent inhibition of TMV.GFP cell-to-cell movement in SA-treated tobacco (Fig. 1) is unlikely to be due to impairment of MP gating function. However, TMV MP has other functions in addition to its ability to potentiate movement of TMV RNA through PD. For instance, the TMV MP was recently shown to be an integral membrane protein of the endoplasmic reticulum (Reichel and Beachy, 1998) that may anchor the TMV replication complex to this organelle (Mas and Beachy, 1999; Reichel et al., 1999). Furthermore, the MP appears to be capable of redirecting endoplasmic reticulum organization (Reichel and Beachy, 1998). If SA treatment interferes with subcellular localization of the TMV MP, then this may account for the apparent inhibition of TMV.GFP movement in SA-treated tobacco without necessarily affecting the gating properties of the MP. To determine whether this is true, a further series of experiments using TMV expressing a GFP:MP fusion will be required.

Could RNA Silencing Play a Role?

More speculatively, it is conceivable that the restriction of TMV.GFP seen in SA-treated plants may be due in some part to RNA silencing (also known as post-transcriptional gene silencing; for review, see Li and Ding, 2001). Two recent papers indicate that SA-induced resistance to viruses and RNA silencing may be connected either in the way that the two

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processes are induced, or in the way they function. Ji and Ding (2001) showed that the CMV suppressor of RNA silencing, the 2b protein, is needed to allow that virus to overcome SA-induced resistance to replication and/or cell-to-cell movement in the directly inoculated tissue. When introduced into genetically modified TMV, the 2b protein enhanced the accumulation of the virus in the inoculated tissue of SA-treated plants. In addition, expression of the 2b protein in transgenic *Nicotiana* spp. also inhibited SA-induced expression of the alternative oxidase gene (Ji and Ding, 2001), a marker for SA-induced resistance to viruses (Murphy et al., 1999, 2001). These findings suggest a linkage between the signal transduction pathways responsible for induction of SA-induced resistance to viruses and RNA silencing.

The possibility that a functional connection exists between SA-induced resistance to viruses and RNA silencing arose from the discovery that a tobacco gene encoding an RNA-dependent RNA polymerase (RdRp) was induced by SA (Xie et al., 2001). The significance of this is that host RdRp enzymes are key components of the RNA silencing mechanism (Morel and Vaucheret, 2000). Although it was found, using antisense transgenic plants, that the SA-inducible RdRp was not needed for SA-induced resistance to TMV (Xie et al., 2001), it is possible that it may be required for SA-induced resistance to other viruses. If RNA silencing does play a role in the restriction of TMV local movement in SA-treated plants, our experiment with the MP transgenic tobacco could imply that the MP can inhibit the silencing, at least to some extent. However, this is speculative because, although it is known that a PVX MP (p25; Voinnet et al., 2000) can suppress the propagation of RNA silencing, there is currently no evidence that the TMV MP can counter the induction or maintenance of RNA silencing.

Relevance of SA-Induced Resistance to Plants Expressing SAR

In resistant, *N*-gene containing tobacco plants, SAR against TMV is manifested by production of fewer and smaller HR lesions in plants that have previously encountered the virus (Ross, 1961a, 1961b). Treatment of *N*-gene tobacco plants with SA or aspirin also results in fewer, smaller HR lesions compared with untreated plants (White, 1979). In our experiments, we investigated the effects of SA-treatment on resistance to TMV in tobacco plants that do not contain the *N*-gene and that, therefore, do not display the HR in response to TMV. However, we believe that our results do throw some light on why fewer and/or smaller visible necrotic lesions appear on SA-treated, or SAR-expressing, *NN* genotype tobacco plants when they are challenged with TMV.

Specifically, we found that TMV.GFP was limited almost exclusively to single-cell infection sites in SA-treated tobacco for up to 6 dpi. This is significant because it has been shown that the HR mediated by the *N*-gene, unlike many other pathogen-induced cell death phenomena (for review, see Heath, 2000) cannot occur at the single-cell level. For example, TMV does not cause necrosis of TMV-infected protoplasts from *N*-gene tobacco (Otsuki et al., 1972). More recently, it was shown that a movement deficient TMV.GFP construct that could only infect single epidermal cells did not elicit cell death in an *N*-gene containing host (Wright et al., 2000). These studies indicate that *N*-gene-mediated death can occur only when the virus has infected a group of cells although, so far, the minimum number of cells that constitutes a "doomed quorum" is still not known. Thus, in SA-treated, or SAR-expressing, *NN*-genotype tobacco leaves the reduction in the number of HR lesions produced after challenge with TMV may be due, at least in part, to limitation of the virus to single cells, or to groups of cells that are too small in number to trigger the HR.

CONCLUSION

In summary, in the leaf mesophyll cells of SA-treated plants the replication of TMV is greatly decreased. In contrast, SA does not appear to significantly decrease TMV replication in initially inoculated epidermal cells. Instead, it induces resistance to movement between the epidermal cells. However, inhibition of movement between epidermal cells was not because of a SA-induced reduction in the SEL of epidermal cell PD, nor was it because of inhibition of the TMV MP plasmodesmal gating function by SA. The wider significance of these results is that they show that SA, rather than having a uniform effect on pathogen resistance throughout all the cells and tissues of a plant, can have profoundly different effects on the same pathogen in different cell types. We conclude from this that cell and tissue development exerts a powerful influence over the "design" of the defensive signaling pathways and the resistance mechanisms that they trigger.

MATERIALS AND METHODS

Plant Growth Conditions

Tobacco (*Nicotiana tabacum*) cvs Xanthi (*nn* genotype) and Xanthi-nc (*NN* genotype), *Nicotiana benthamiana*, and cv Xanthi tobacco transformed with the TMV 30-kD MP gene (line 277; Deom et al., 1987) were maintained under greenhouse conditions with supplementary lighting in winter. Virus-inoculated plants were also maintained under greenhouse conditions. SA treatment was carried out by watering plants with 1 mM SA for 5 d.

Plant Inoculation

For infection of plants with TMV.GFP, infectious RNA transcripts were synthesized from pTMV.GFP linearized

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with *Kpn*I (Lacomme and Santa Cruz, 1999) using a T7 transcription kit (Ambion, Austin, TX) and inoculated directly onto leaves of 8-week-old *N. benthamiana* with carborundum as an abrasive. During replication, TMV.GFP generates GFP mRNA from an introduced subgenomic CP promoter. The virus still directs production of CP from its own CP promoter and can assemble into infectious virions making it possible to use infected leaves as an inoculum. After 7 d, inoculated leaves were ground up in 100 mM potassium phosphate buffer (pH 7) and stored in aliquots at −80°C. TMV.GFP sap was defrosted immediately before inoculation with carborundum onto tobacco plants. For CMV.GFP, RNA transcripts were synthesized from *Spe*I-linearized pFny109, pFny209, pF:GFP/CP, and pF:3a/GFP representing Fny-CMV RNAs 1 and 2 and a combination of RNA 3-derived transcripts with the GFP gene replacing either the MP gene or the CP gene (Canto et al., 1997). The transcripts were pooled and inoculated directly onto tobacco cv Xanthi-nc. Inoculations for both TMV.GFP and CMV.GFP were always onto the first and second fully expanded leaves above the cotyledons.

Microinjection

Small volumes (50–100 μL) of 5 mM 3- and 10-kD fluorescein isothiocyanate-labeled dextrans (F-dextran, Molecular Probes Inc., Eugene, OR) were mixed with a small amount of Sephadex G10 (Pharmacia, Piscataway, NJ) to remove any free fluorescein isothiocyanate. Removal of free dye was checked by thin layer chromatography (Oparka et al., 1997). Ten-kilodalton F-dextran and 10-kD Texas red dextran (R-Dextran, Molecular Probes) were spun through ultrafiltration membranes (Whatman, Clifton, NJ) with a M_r cut-off (MWCO) of 100 kD (to remove any particles), followed by repeated concentration through filters with a 10-kD MWCO (to remove contaminating low-M_r dextrans). Similarly, the 3-kD R-dextran was repeatedly concentrated through an ultrafiltration membrane with a MWCO of 3 kD (Pall Filtron, Northborough, MA).

Leaves were detached from untreated and SA-treated plants and the petiole was immersed in 100 mM Suc in a 0.5-mL microfuge tube (after Oparka et al., 1997). Leaves were fixed to microscope slides using double-sided adhesive tape.

Microinjection was performed using a hydraulic micromanipulator (Narashege Co., Tokyo) attached to a coarse positioner (Narashege) that was mounted onto an Optiphot-2 epifluorescence microscope (Nikon, Tokyo). Micropipettes (Eppendorf, Hamburg, Germany) with a tip size of approximately 1 μm were attached to a pressure injection device connected to a pressure probe (constructed by Denton Prior, SCRI, Invergowrie, Dundee, Scotland).

Microinjection was into epidermal cells on the upper side of the leaf. Cell-to-cell movement of dextrans was monitored by epifluorescence. F-dextrans and CMV.GFP infection sites were visualized using a filter block (Nikon) containing a 450- to 490-nm excitation filter, a 510-nm dichroic mirror, and a 520-nm barrier filter. R-dextrans were visualized using a filter block (Nikon) containing a 510- to 560-nm excitation filter, a 580-nm dichroic mirror, and a 590-nm barrier filter. TMV.GFP infection sites were visualized using a 405-nm excitation filter, a 450-nm dichroic mirror, and a 520-nm barrier filter (Omega Optical, Glen Spectra Ltd, Stanmore, UK).

Fluorescent images of TMV.GFP infection sites were captured using Metamorph software (Universal Imaging Corp., West Chester, NY) and a cooled, CCD camera (model RTE/CCD 1317-K, Princeton Instruments, Marlow, Buckinghamshire, UK). Where similar time points are compared, exposure times for the CCD camera were the same. Images of CMV.GFP infection sites were photographed with a Nikon UFX-DX camera system using Fujichrome ISO 400 color print film.

Protoplast Preparation and Infection

Protoplasts were prepared from leaves of control and SA-pretreated tobacco cv Xanthi-nn plants as previously described (Hills et al., 1987; Carr and Zaitlin, 1991). The protoplasts (10^6 cells mL^{-1}) were inoculated by electroporation with viral RNA prepared from TMV U1 or CMV Fny. Electroporation was performed in a final volume of 0.5 mL of sterile 0.7 M mannitol, using a ring electrode (2.5 mm high, 1-cm gap) connected to a ProGenitor 1 electroporation apparatus (Hoefer Scientific Instruments, San Francisco), by applying two 5-ms pulses of 300 V. Protoplasts were electroporated with 5 μg of viral RNA. All experiments included a set of mock-inoculated protoplasts. After electroporation, protoplasts were incubated in low light at 25°C in wells of multiwell sterilin plates (Bibby Sterilin Ltd., Stone, UK) coated with 1% (w/v) noble agar in incubation medium described in Carr et al. (1994).

Analysis of RNA

Fifteen hours after electroporation, protoplasts were harvested by centrifugation at 1,000*g*. The cells were resuspended in RNA extraction buffer (UltraSpec, Biotecx Inc., Houston), and RNA was extracted after the manufacturer's instructions. RNA was separated by agarose gel electrophoresis and blotted to nitrocellulose as previously described (Chivasa et al., 1997). TMV-specific RNA was detected using ^{32}P-labeled riboprobes as described by Carr and Zaitlin (1991) and CMV-specific RNA was detected using ^{32}P-labeled riboprobes as described by Gal-On et al. (1994).

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