The Plant Cell, Vol. 9, 547–557, April 1997 © 1997 American Society of Plant Physiologists

Salicylic Acid Interferes with Tobacco Mosaic Virus Replication via a Novel Salicylhydroxamic Acid–Sensitive Mechanism

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Salicylic acid (SA) induces resistance to all plant pathogens, including bacteria, fungi, and viruses, but the mechanism by which SA engenders resistance to viruses is not known. Pretreatment of tobacco mosaic virus (TMV)–susceptible (nn genotype) tobacco tissue with SA reduced the levels of viral RNAs and viral coat protein accumulating after inoculation with TMV. Viral RNAs were not affected equally, suggesting that SA treatment interferes with TMV replication. Salicylhydroxamic acid (SHAM), an inhibitor of the mitochondrial alternative oxidase, antagonized both SA-induced resistance to TMV in nn genotype plants and SA-induced acquired resistance in resistant (NN genotype) tobacco. SHAM did not inhibit induction of the PR-1 pathogenesis-related protein or induction of resistance to *Erwinia carotovora* or *Botrytis cinerea* by SA. This indicates that SA induces resistance to TMV via a novel SHAM-sensitive signal transduction pathway (potentially involving alternative oxidase), which is distinct from that leading to resistance to bacteria and fungi.

INTRODUCTION

Salicylic acid (SA; 2-hydroxybenzoic acid) can modulate changes in gene expression in plants resisting pathogen infection (Raskin, 1992; Klessig and Malamy, 1994). When a plant possessing a resistance gene for a specific pathogen is inoculated with that pathogen, the resistance gene product recognizes the presence of the invading microorganism and activates a signal transduction pathway (or pathways) that mobilizes the plant to protect itself against disease (Staskawicz et al., 1995). In many plants, the first defensive measure that occurs is rapid programmed host cell death at the inoculation site (the hypersensitive response [HR]). This is followed by the induction of systemic acquired resistance (SAR; reviewed in Ryals et al., 1996) throughout the plant, as manifested by production of fewer and smaller necrotic lesions in response to a second challenge with the pathogen (Ross, 1961a, 1961b).

SA functions in plants as a key component of the signal transduction pathway leading to the induction of SAR and plays a role in resistance to all microbial pathogens, including fungi, bacteria, and viruses (Delaney et al., 1994). Catalase and ascorbate peroxidase have been identified as receptors for SA, which implies that H_2O_2 follows SA in signal transduction (Chen and Klessig, 1991; Durner and Klessig, 1995). However, results of studies with transgenic plants expressing the SA-degrading enzyme salicylate hydroxylase (Bi et al., 1995; Neuenschwander et al., 1995) and experiments on defense gene expression in untransformed plants exposed to UV-B radiation (Green and Fluhr, 1995) indicate that SA-induced gene activation may occur via at least two distinct pathways, of which only one is H_2O_2 dependent. Although it appears that the host-encoded antifungal and antibacterial pathogenesis-related (PR) proteins induced by SA may contribute to resistance against bacterial and fungal pathogens (Carr and Klessig, 1989; Bowles, 1990), the mechanisms by which SA mediates resistance to viruses remain a mystery.

Tobacco plants possessing the *N* gene (Whitham et al., 1994) for resistance to tobacco mosaic virus (TMV) respond to inoculation with that virus by exhibiting hypersensitivity, accompanied by an increase in SA levels in the tissue (Malamy et al., 1990) and induction of SAR (Ross, 1961a, 1961b). However, the macroscopic tissue necrosis that occurs during the HR may not be the sole cause of virus localization. For example, treatment with an SA derivative, aspirin (acetylsalicylic acid), caused a profound reduction in accumulation of TMV, even in tobacco plants that do not possess the *N* gene (nn genotype) and therefore that do not respond hypersensitively to the virus (Antoniw and White, 1980; White et al., 1983). Also, results from a study with alfalfa mosaic virus (AlMV) indicate that SA treatment can entirely suppress replication of that virus in cowpea protoplasts, even though they do not undergo an HR in response to AlMV (Hooft Van Huijsduijnen et al., 1986). More recently, Freidrich et al. (1996) showed that the SAR-inducing chemical

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benzothiadiazole can reduce levels of TMV RNAs in nn genotype tobacco. Taken together, these studies with nonhypersensitive plant material indicate that host cell death cannot be the only factor involved in limitation of virus spread.

When the early studies of White et al. (1983) and Hooft Van Huijsduijnen et al. (1986) were conducted, it was thought that the reduction in virus replication seen in these nonhypersensitive hosts was associated with the induction of PR protein gene expression. However, subsequent work using transgenic tobacco constitutively expressing PR proteins showed that PR proteins play no role in combating viral infection in either hypersensitively (Cutt et al., 1989; Linthorst et al., 1989) or nonhypersensitively (Cutt et al., 1989) responding plants. However, certain PR proteins can function in resistance against cellular pathogens. For example, constitutive expression of the gene for the antifungal pathogenesis-related protein PR-1 (Niderman et al., 1995) in transgenic tobacco plants can protect against fungal infection (Alexander et al., 1993). Despite this, PR-1 protein gene expression is often used as a molecular marker for induction of resistance to all pathogens, including viruses (Ryals et al., 1996).

We have followed up on the early study of White et al. (1983) by attempting to determine the mechanism(s) underlying the effect of SA on TMV. TMV is a typical plant virus with a plus-sense single-stranded RNA genome of 6395 nucleotides that encodes five polypeptides (Palukaitis and Zaitlin, 1986). The genomic RNA acts as the mRNA for synthesis of the 126- and 183-kD proteins that are components of the viral RNA–directed RNA polymerase (RdRp) or replicase complex. The initial product of TMV replicase activity is a minus-sense RNA complementary to the full-length TMV genomic RNA (Palukaitis and Zaitlin, 1986). This RNA serves as the template for synthesis of new copies of the genomic RNA and for the transcription of three subgenomic mRNAs (Palukaitis and Zaitlin, 1986). The subgenomic mRNAs are as follows, in order of increasing size: the viral coat protein (CP) mRNA; the I_2 RNA, which directs synthesis of the viral movement protein; and the I_1 RNA, which may function in synthesis of a putative third virus-coded RdRp protein of 54 kD (Sulzinski et al., 1985; Palukaitis and Zaitlin, 1986; Golemboski et al., 1990). Of these subgenomic RNAs, the CP mRNA is produced in the greatest quantity. In addition, the RdRp complex generates double-stranded forms of the viral RNAs, which are by-products of replicase activity that are not thought to play any direct role in replication (Palukaitis and Zaitlin, 1986).

In addition to engendering resistance to pathogens, SA induces a wide range of other biochemical changes in plant tissues (Raskin, 1992). One of the most notable changes in plant metabolism caused by SA is the induction of the so-called alternative respiratory pathway (Laties, 1982; Raskin, 1992; McIntosh, 1994; Day et al., 1995). In the alternative respiratory pathway, the potential energy of the mitochondrial proton gradient is coupled to ATP synthesis at only one site, in contrast to the conventional cytochrome pathway in which ATP synthesis can occur at three sites (Laties, 1982; McIntosh, 1994; Day et al., 1995). The unused potential energy is lost as heat—a phenomenon that is most obvious in the case of thermogenic plants such as Arum lilies (Raskin et al., 1987); however, it can also be detected to a lesser degree in plants that are not obviously thermogenic (Van Der Straeten et al., 1995).

Synthesis of the terminal oxidase of the alternative respiratory pathway (the alternative oxidase [AOX]) is induced by stress, developmental cues, or SA (Kapulnik et al., 1992; Rhoads and McIntosh, 1993), but the specific metabolic importance of AOX and the alternative pathway in plants that are not overtly thermogenic remain unclear (Laties, 1982; McIntosh, 1994; Day et al., 1995). Noting that SA is important both for induction of resistance to viruses and for AOX gene induction, we wondered whether SA-induced resistance to TMV and the activity of AOX might be connected. Therefore, we initiated studies on the accumulation of viral RNA and CP in TMV-inoculated tobacco treated with SA in the presence or absence of salicylhydroxamic acid (SHAM), a well-characterized inhibitor of AOX activity (Laties, 1982; McIntosh, 1994; Day et al., 1995).

RESULTS

SA Interferes with the Normal Pattern of TMV RNA Accumulation in Inoculated nn Genotype Tobacco Leaf Tissue

To compare events in TMV-inoculated tissue with or without SA treatment, tobacco leaf discs were floated on 1 mM SA, 3-hydroxybenzoic acid (HBA), an isomer of SA that is unable to bind the SA receptor catalase or induce SAR (Chen and Klessig, 1991), or water for various periods before inoculation with TMV. Accumulation of the full-length plus- and minus-sense TMV RNAs and the CP mRNA was examined 2 days postinoculation by RNA gel blot analysis, using ^{32}P-labeled RNA probes specific for plus- or minus-sense TMV RNAs (Carr and Zaitlin, 1991).

A 3-day preincubation with SA caused a significant reduction in the accumulation of all TMV RNAs (compared with preincubation on water or HBA) over the subsequent 2-day postinoculation incubation (Figure 1A). With preincubation periods on SA of 5 or 7 days, an increased degree of suppression of accumulation of plus-sense viral RNAs was observed (Figure 1A). Even though no appreciable reduction in accumulation of any TMV RNA species was seen in tobacco leaf discs pretreated with HBA, a slight increase in TMV RNA accumulation was noted in HBA-preincubated tissue compared with the levels in tissues preincubated on water (Figure 1A). Although this observation is puzzling, our results with HBA indicate that the suppression of TMV RNA accumulation seen in SA-treated tissue was induced specifically by SA.

Examination of longer autoradiographic exposures of the RNA gel blots showed that SA appeared to reduce

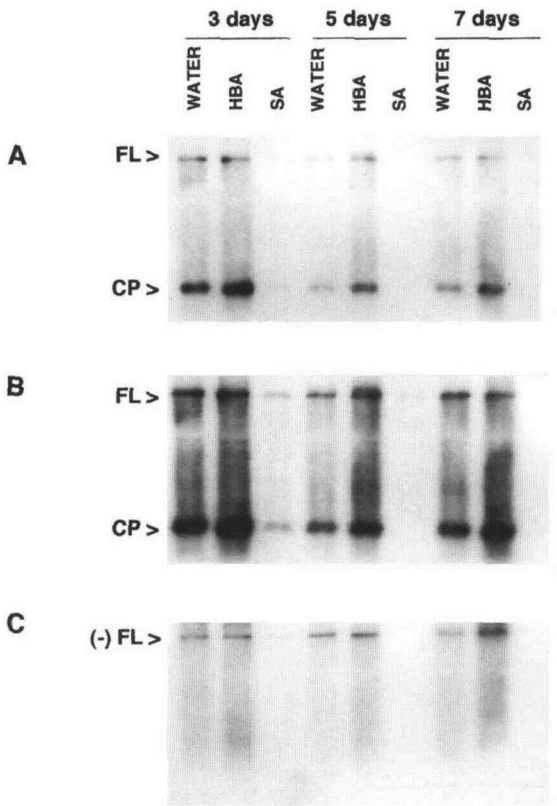

Figure 1. Effect of SA on Accumulation of TMV-Specific RNAs in TMV-Inoculated Leaf Tissue of Xanthi-nn Tobacco.

(A) RNA gel blot analysis of plus-sense single-stranded TMV RNAs in TMV-inoculated leaf discs. Equal amounts of total single-stranded RNA extracted from pooled leaf discs (10 discs [10-mm in diameter] per solution per time point) were loaded on each gel lane and, after electrophoresis, were transferred to nitrocellulose for hybridization with a ^{32}P-labeled plus-sense strand-specific riboprobe. Leaf discs were floated for 3, 5, or 7 days on water, 1 mM HBA, or 1 mM SA. Incubation was performed in a growth chamber at 25°C with a 16-hr photoperiod. After this pretreatment period, discs were briefly removed from the solutions and inoculated with TMV (strain U1). Discs were replaced in their original solutions and incubated for an additional 2 days before RNA extraction. The bands in this autoradiograph (16-hr exposure), corresponding to TMV full-length RNA and CP mRNA, are indicated by FL and CP, respectively.

(B) A longer autoradiographic exposure (48 hr) of the RNA gel blot blot shown in **(A)** to illustrate the presence of low but detectable amounts of TMV-specific plus-sense RNAs in SA-treated leaf disc tissue.

(C) RNA gel blot detection of TMV full-length, single-stranded, minus-sense RNA (indicated by (−)FL) in the leaf disc total RNA by using a minus-sense RNA-specific ^{32}P-labeled riboprobe.

accumulation of the CP mRNA to a greater extent than the reduction of full-length plus-sense RNA (Figure 1B). Quantitation by liquid scintillation spectrometry of bound, radiolabeled probe from excised RNA bands (Carr and Zaitlin, 1991) supported this observation. For example, 5 days of preincubation with SA resulted in an approximately sevenfold suppression of genomic RNA accumulation, whereas the suppression of accumulation of CP mRNA was ~14-fold (data not shown). No attempt was made to quantitate signals for the ss forms of the I_1 and I_2 subgenomic mRNAs, because these RNA species are much less abundant than is the CP mRNA and are not easily imaged by autoradiography.

The level of full-length minus-sense TMV RNA was also reduced in tissues preincubated on SA solutions (Figure 1C). Quantitation by liquid scintillation spectrometry of bound radiolabeled probe in excised RNA bands (Carr and Zaitlin, 1991) indicated that full-length minus-sense TMV RNA was reduced ~80-fold in tissue preincubated on SA for 5 days as compared with the level in TMV-inoculated tissue preincubated on water (data not shown). RNA gel blot analysis of denatured double-stranded forms of the viral RNAs indicated that the level of double-stranded RNA corresponding to the subgenomic CP mRNA was suppressed to a greater extent than was the full-length double-stranded genomic RNA when inoculated tissue had been preincubated on SA (Figure 2).

SHAM Antagonizes the Effect of SA on TMV in Inoculated nn Genotype Tobacco Leaf Tissue

To address the possibility that AOX may play a role in SA-induced resistance to TMV, we compared the accumulation

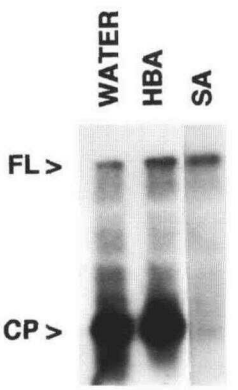

Figure 2. RNA Gel Blot Analysis of the Effect of SA on Accumulation of TMV Double-Stranded RNAs in Xanthi-nn Tobacco Leaf Discs.

Leaf discs (10 discs per treatment) were pretreated by flotation on water, 1 mM HBA, or 1 mM SA for 5 days before inoculation with TMV U1, followed by 2 additional days of incubation before extraction of RNA. Equal amounts of double-stranded enriched RNA extracted from tissues of pooled leaf discs were denatured and loaded onto adjacent lanes of the same gel before electrophoresis and RNA gel blotting. Hybridization was with a ^{32}P-labeled, plus-sense strand-specific riboprobe. The positions of the double-stranded forms of full-length and the CP subgenomic RNA are indicated by FL and CP, respectively. The water and HBA lanes were taken from an autoradiographic exposure of 24 hr, and the SA lane is from an autoradiographic exposure of 3 weeks.

of AOX transcripts in leaf discs floated on water or 1 mM SA by RNA gel blot analysis (Figure 3). Under our experimental conditions, the level of AOX transcripts was increased by incubation on SA (Figure 3), in line with earlier work (Kapulnik et al., 1992; Rhoads and McIntosh, 1993). This suggested that AOX gene induction and resistance may be correlated. To examine this possibility further, we performed experiments with the AOX inhibitor SHAM.

Consistent with the results of our RNA studies (Figures 1 and 2), TMV CP accumulation (monitored by protein gel blotting) was suppressed in inoculated leaf discs pretreated with SA but not in those floated on water (Figure 4A). However, when leaf discs were pretreated with solutions containing the AOX inhibitor SHAM as well as SA, no reduction in CP accumulation was observed (Figure 4A). The results are consistent with a role for AOX activity in SA-mediated induction of resistance to TMV. In contrast, SA-induced accumulation of the antifungal protein PR-1 is not inhibited by SHAM (Figure 4B). In fact, SHAM itself appears to be able to induce PR-1 accumulation (Figure 4B).

SHAM Antagonizes SA-Induced Resistance to TMV in nn Genotype Tobacco Plants

We wanted to determine whether SHAM, in addition to preventing SA-induced suppression of TMV replication in directly inoculated tissue, can interfere with SA-induced resistance to TMV in the whole plant. Xanthi-nn tobacco plants, which are susceptible to systemic infection with TMV, were watered for 3 consecutive days with 1 mM SA or a mixture of 1 mM SA plus 1 mM SHAM. These plants, together with untreated control plants, were each inoculated on one lower leaf with TMV (5 μg mL^{-1}), placed in a growth chamber with continuous illumination to enhance development of systemic TMV disease (Matthews, 1991), and examined daily for disease symptoms. SA-treated plants remained symptom free for up to 14 days postinoculation with TMV (data not shown). However, by 5 to 6 days postinoculation, untreated control plants and plants treated with the SHAM plus SA mixture displayed the classic TMV disease symptoms (vein clearing and stunting of upper, uninoculated leaves; Figures 5A to 5C).

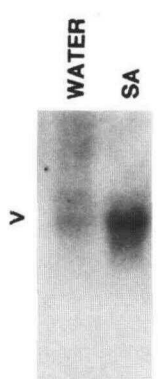

Figure 3. Increase in Accumulation of AOX Transcripts in SA-Treated Tobacco Tissue.

RNA gel blot analysis of equal amounts of RNA extracted from Xanthi-nn tobacco leaf tissue floated for 5 days on water or 1 mM SA. The blot was hybridized with a ^{32}P-labeled AOX-specific DNA probe. The position of the AOX transcript is indicated by the arrowhead.

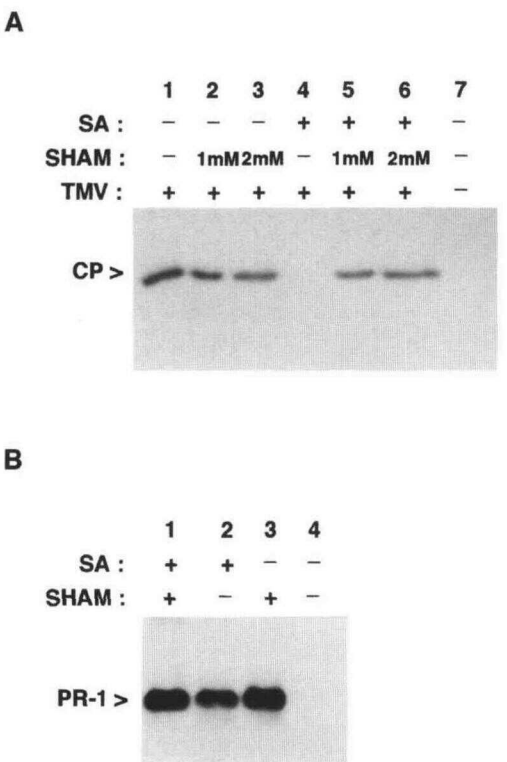

Figure 4. Effect of SHAM on SA-Induced Changes in TMV CP and PR-1 Protein Accumulation in Xanthi-nn Tobacco Leaf Tissue.

(A) Leaf discs were floated for 5 days on water (lanes 1 and 7), solutions of 1 mM SA (lane 4), SHAM at 1 or 2 mM (lanes 2 and 3, respectively), or 1 mM SA and 1 or 2 mM SHAM combined (lanes 5 and 6, respectively). After inoculation with TMV (lanes 1 to 6) or mock inoculation (lane 7), the discs were incubated for an additional 2 days on the same solutions before extraction of soluble proteins. Proteins were analyzed by SDS-PAGE and protein gel blotting, using a polyclonal rabbit TMV CP antiserum. CP indicates the position of the TMV CP band.

(B) PR-1 protein accumulation in extracts from chemically treated leaf disc extracts used in **(A)** were examined. Protein gel blot analysis was performed using a polyclonal rabbit PR-1 antiserum. Lane 1 contains 1 mM SHAM plus 1 mM SA; lane 2, 1 mM SA; lane 3, 1 mM SHAM; and lane 4, water. PR-1 indicates the position of the PR-1 protein band.

(+) indicates presence and (–) indicates absence of a chemical in the incubation solution.

Protein gel blot analysis of proteins extracted from upper leaves of plants at 5 days postinoculation showed that compared with accumulation in untreated control plants, accumulation of TMV CP in the upper uninoculated leaves of SA-treated plants was greatly reduced (Figure 6). However, the TMV CP accumulated to similar levels in the upper uninoculated leaves of plants treated with SA plus SHAM and in control plants (Figure 6). Thus, SHAM inhibited the delay in development of systemic TMV disease symptoms and the reduction in virus accumulation induced by SA in TMV-susceptible tobacco.

SHAM Abolishes SA-Induced Acquired Resistance to TMV in Leaves of Resistant (NN Genotype) Tobacco Plants

Experiments were performed to determine whether the SHAM-sensitive, SA-induced suppression of TMV replication that we had observed in susceptible (nn genotype) tobacco tissue plays a role in virus localization or induction of acquired resistance in genetically resistant (NN genotype) tobacco. Detached leaves from Xanthi-NN tobacco plants were fed via the petioles with water, 1 mM SA, or 1 mM SA plus 1 mM SHAM for 3 days before inoculation with TMV U1. After 2 days, the leaves were examined for the presence of visible necrotic lesions. As expected due to results of earlier studies (White, 1979; Antoniw and White, 1980), SA-treated leaves had fewer lesions compared with water-fed control leaves (Table 1 and Figure 7A). In contrast, the leaves treated with the mixture of SA plus SHAM developed local necrotic lesions in similar numbers to the water-fed leaves (Figures 7B and 7C). There were no obvious differences in morphology between the lesions on the SA plus SHAM-treated leaves and those on the water-treated leaves (data not shown). Thus, it appears that although SHAM treatment cannot prevent the virus-induced HR in resistant (NN) tobacco plant tissue, it can interfere with its enhancement, that is, the induction of acquired resistance by SA.

SHAM Does Not Abolish SA-Induced Resistance to *Erwinia carotovora* and *Botrytis cinerea* in Tobacco Plants

The finding that SHAM antagonizes SA-induced resistance to TMV but does not block SA-induced PR-1 accumulation suggested that there may be a branch in the defensive signal transduction pathway downstream of SA, with one branch leading to resistance to the virus and the other leading to the activation of PR protein genes. If this were indeed the case, we might expect that SHAM would not block SA-induced resistance to bacterial or fungal pathogens because the PR proteins have been strongly implicated in resistance to these types of pathogen. To test this hypothesis, we examined the effects of SA and SHAM on the resistance

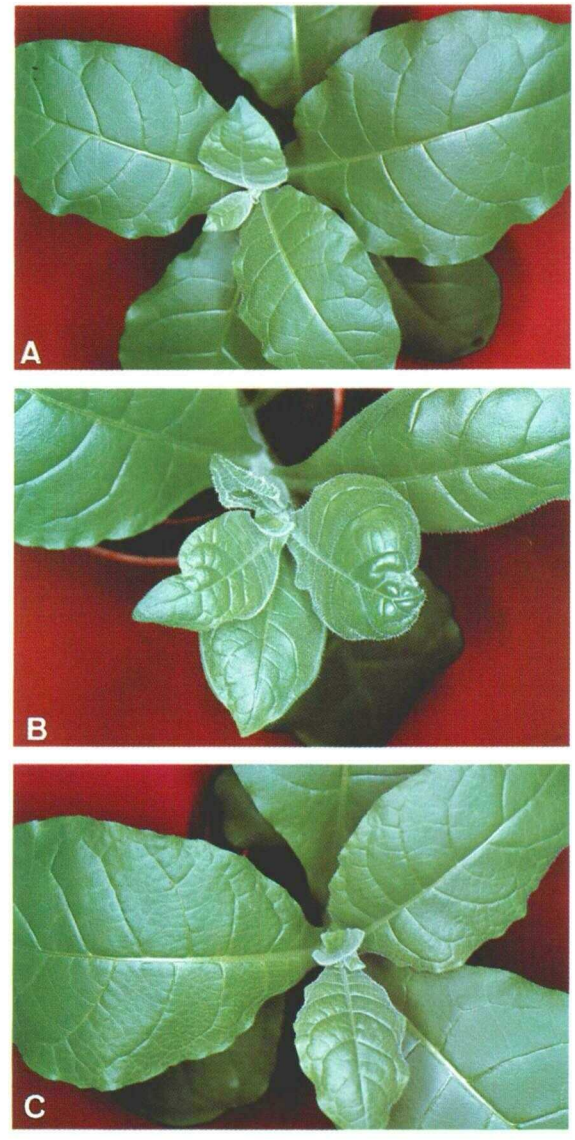

Figure 5. Effect of SHAM on the SA-Induced Delay of Systemic TMV Disease in nn Genotype Tobacco Plants.

(A) Appearance of the upper uninoculated leaves of a Xanthi-nn tobacco plant 1 week postinoculation with TMV U1 on a lower leaf after pretreatment with SA. At this time point, the plant was asymptomatic.

(B) Disease symptoms (curling, distortion, and vein clearing) on the upper uninoculated leaves of a Xanthi-nn tobacco plant 1 week postinoculation with TMV U1 on a lower leaf after pretreatment with SA plus SHAM.

(C) Disease symptoms on the upper uninoculated leaves of a Xanthi-nn tobacco plant 1 week postinoculation with TMV U1 on a lower leaf with no pretreatment (water-only control).

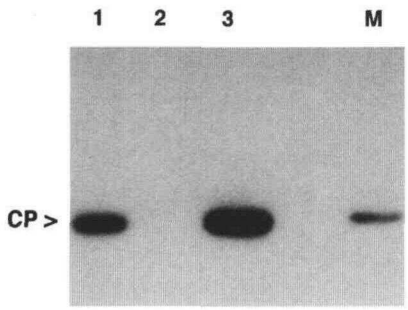

Figure 6. Effect of SHAM on the SA-Induced Reduction of Accumulation of the TMV CP in nn Genotype Tobacco Plants.

Gel blot analysis of proteins extracted from upper uninoculated leaves of Xanthi-nn tobacco 1 week postinoculation on a lower leaf with TMV U1, after no pretreatment (lane 1), pretreatment with SA (lane 2), or pretreatment with SA plus SHAM (lane 3). The protein gel blot was probed with a polyclonal rabbit anti-TMV CP antiserum. Each of lanes 1 to 3 was loaded with a combined extract of total soluble proteins from three plants (3 μg total). Lane M was loaded with 0.1 μg of authentic TMV CP as a marker.

of tobacco plants to two nonviral pathogens: the bacterial pathogen *E. c.* subsp *carotovora*, which was already known to be inhibited on SA-treated plants (Palva et al., 1994), and *B. cinerea*, a necrotrophic fungal pathogen with a wide host range (Jarvis, 1977).

When tobacco plants previously watered with HBA, SA, SHAM, or SA plus SHAM were inoculated with *E. c. carotovora*, it was found that symptom development on the plants treated with SA and SHAM differed markedly from that observed on the control (HBA-treated) plants (Figure 8). When plants were examined at 24 hr postinoculation, the majority of HBA-treated plants (eight of 11) displayed visible evidence of tissue maceration (watery, collapsing lesions) on inoculated leaves (Figure 8, leaf B, for example), whereas none of the SA plus SHAM–treated plants showed any visible evidence of disease (Figure 8, leaf E). Most of the SA- and SHAM-treated plants showed no tissue damage greater than that caused by the inoculation procedure. However, a minority of plants pretreated with SA (two of 13) and SHAM (three of 13) showed evidence of tissue maceration on inoculated leaves at 24 hr postinoculation (examples of which are shown in Figure 8), but the degree of damage appeared to be much less than that observed in the inoculated leaves of HBA-treated plants. By 48 hr postinoculation, leaves on HBA-treated plants that had displayed tissue maceration by 24 hr had completely collapsed (data not shown). In contrast, the smaller lesions seen on some of the SA- and SHAM-treated plants did not expand further and dried up by 72 hr postinoculation (data not shown), at which point the experiment was concluded.

Tobacco plants were watered for 5 days with SA, SHAM, or SA plus SHAM or left untreated before spraying with spores of *B. cinerea*. At 48 hr postinoculation, all of the untreated plants had collapsed, whereas all of the treated plants had survived (Figure 9). However, by 5 days postinoculation, some individual plants in the chemically treated groups were begining to wilt, and by 10 days postinoculation (at which point the experiment was terminated), the majority of plants in the chemically treated groups had died (data not shown).

DISCUSSION

The results of our studies of TMV RNA and CP accumulation in inoculated leaf tissue from nn genotype tobacco are consistent with those of White et al. (1983), who observed large reductions in the accumulation of TMV virions in acetylsalicylic acid–treated nn genotype tobacco leaves. In inoculated tissue, SA caused not only an overall reduction in the levels of TMV RNA and CP but also changed the balance of plus- to minus-sense and full-length to subgenomic TMV RNAs being produced. This suggests that SA can induce interference with the normal functioning of the viral RdRp complex. We conclude that the reduction in viral RNA and protein seen in SA-treated nn genotype tobacco tissue and the delay in the onset of disease symptoms seen in SA-treated nn genotype plants are due in large part to interference with viral replication and transcription, although secondary effects, for example, on the movement of TMV through plant tissue, cannot be ruled out at this time. Recently, Osman and Buck (1996) described the purification of a functional in vitro RdRp preparation from TMV-infected plant tissue. This may make it possible to perform a more detailed analysis of the mechanism(s) underlying SA-mediated interference with TMV RdRp activity.

Curiously, we consistently saw a slight enhancement in accumulation of TMV-specific RNA accumulation in in-

Table 1. Effect of SA and SHAM on Lesion Numbers[a] on TMV-Inoculated Xanthi-NN Tobacco Leaves

Treatment	Experiment 1	Experiment 2
Water	86.3	44.7
1 mM SA	34.0[b]	8.7[b]
1 mM SA + 1 mM SHAM	75.3	38.7

[a] The petioles of detached leaves (three per treatment) of 5-week-old (experiment 1) and 7-week-old (experiment 2) Xanthi-NN plants were submerged in SA, SA plus SHAM, or water for 3 days before TMV inoculation. Two days after inoculating with 1 μg/mL TMV U1, the necrotic lesions on all leaves were counted. Each value is a mean of the lesion numbers on three leaves.

[b] The mean lesion numbers for the SA-treated leaves in each experiment differ significantly from the mean lesion numbers for leaves treated with water or SA plus SHAM ($P < 0.05$).

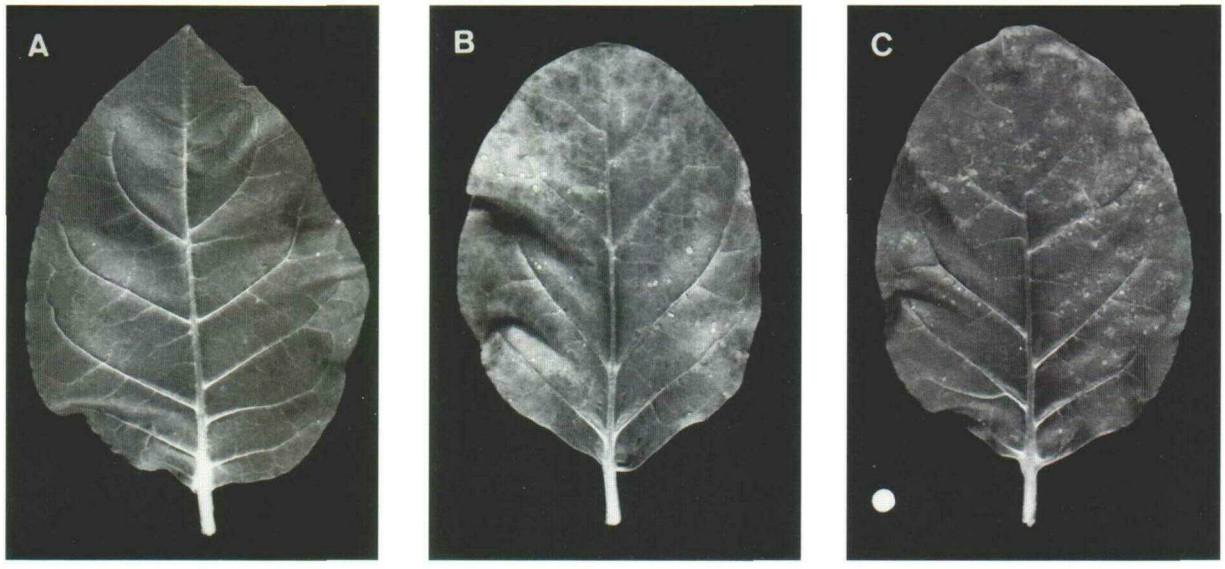

Figure 7. SHAM Inhibits SA-Induced Acquired Resistance to TMV in NN Genotype Tobacco.

(A) A leaf detached from a Xanthi-NN tobacco plant fed via the petiole for 3 days with 1 mM SA before inoculation with TMV U1 (1 μg mL^{-1}). The leaf (photographed 2 days postinoculation) had very few visible necrotic lesions.
(B) A leaf detached from a Xanthi-NN tobacco plant fed via the petiole for 3 days with water alone before inoculation with TMV U1 (1 μg mL^{-1}) and photographed 2 days postinoculation. A large number of necrotic lesions are visible on this control leaf.
(C) A leaf detached from a Xanthi-NN tobacco plant fed via the petiole for 3 days with SA plus SHAM (at 1 mM each) before inoculation with TMV U1 (1 μg mL^{-1}) and photographed 2 days postinoculation. In contrast to the SA-treated leaf in **(A)**, this leaf has a similar degree of visible necrosis to the water control **(B)**. The white dot in the lower left of **(C)** was an orientation mark for photography.

oculated nn genotype tobacco tissue that previously had been treated with HBA (Figure 1). Although we can offer no explanation for this effect at present, the observation suggests that although HBA, unlike its isomer SA, cannot induce resistance to TMV, it may not be entirely without biological activity, as was thought previously.

The results of our study of TMV RNA accumulation in SA-treated leaf discs differ in certain respects from the results

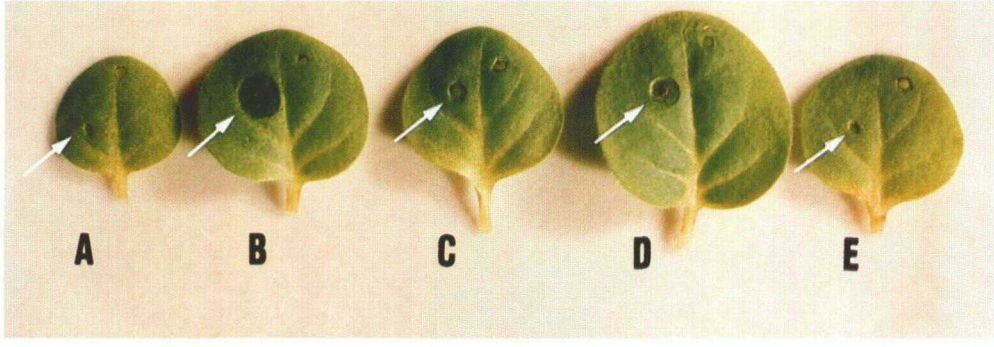

Figure 8. Effect of SA and SHAM on the Response of Tobacco Plants to the Bacterial Pathogen *E. c. carotovora.*

Six-week-old Xanthi-NN tobacco seedlings were pretreated with 1 mM solutions of HBA (leaves A and B), SA (leaf C), SHAM (leaf D), or SA plus SHAM (leaf E) for 5 days before inoculation with *E. c. carotovora* (leaves B to E), or mock inoculation (leaf A, uninfected control). Plants were incubated for an additional 24 hr to allow development of soft rot symptoms before inoculated leaves were removed from the plants for photography. The points of inoculation or mock inoculation are indicated by an arrow. The small holes close to the tip of each leaf were made to identify inoculated leaves while still attached to the plants.

Figure 9. Effect of SA and SHAM on the Response of Tobacco Plants to the Fungal Pathogen *B. cinerea.*

Six-week-old Xanthi-NN tobacco seedlings were photographed 48 hr postinoculation.
(A) Untreated control plants.
(B) Plants pretreated with 1 mM SA.
(C) Plants pretreated with 1 mM SHAM.
(D) Plants pretreated with 1 mM SA plus SHAM.
Plants were pretreated for 5 days before being sprayed with a suspension of *B. cinerea* spores.

of Hooft Van Huijsduijnen et al. (1986) in their study of AlMV RNA accumulation in cowpea protoplasts. For example, even though TMV RNA accumulation was significantly reduced by SA treatment, it was not completely abolished even after several days of incubation with SA, whereas AlMV replication in cowpea protoplasts was completely suppressed after only 24 hr of exposure to SA (Hooft Van Huijsduijnen et al., 1986). The contrasts between our results and those of Hooft Van Huijsduijnen et al. (1986) suggest that SA does not inhibit replication of all viruses in all plant systems to exactly the same degree. However, the inhibition by SHAM of the induction of acquired resistance (fewer visible necrotic lesions in response to TMV) by SA in NN genotype tobacco plants showed that SHAM-sensitive interference with TMV replication is a requirement for the manifestation of SAR against TMV.

Although we cannot conclude that induction of any of the known PR proteins plays any role in the SA-mediated resistance to TMV, the requirement of a preincubation period of several days on SA before resistance can be observed indicates that the resistance mechanism may well require changes in host gene expression, either positive or negative, to occur. The results obtained in our experiments with SHAM strongly suggest that one such change in host gene expression is the activation of the nuclear gene for the plant mitochondrial AOX. We found that SHAM, an inhibitor of AOX (Laties, 1982; McIntosh, 1994; Day et al., 1995), antagonized the SA-mediated induction of resistance to TMV in inoculated nn genotype tobacco tissue, in whole nn genotype tobacco plants, and in NN genotype tobacco tissue. Although consistent with a role for AOX in resistance to TMV, these results must be interpreted with caution. SHAM is a specific inhibitor of AOX in intact tissues (Laties, 1982; McIntosh, 1994; Day et al., 1995), but it can also inhibit other enzymes under certain circumstances. For example, studies with SHAM have implicated lipoxygenase activity in elicitation of phytoalexins in potato tuber slices (Preisig and Kuc, 1987). Our observation that SHAM alone can induce PR-1 protein accumulation does not argue against a role for AOX in resistance, but it does suggest that a proportion of the applied SHAM may be degraded to SA in planta, and it further reinforces the need to move beyond the present "pharmacological" approach to examining the potential role of AOX in resistance. Therefore, further experiments, specifically using transgenic plants with modified levels of AOX activity, are needed to test definitively the link between AOX activity and resistance to TMV when these plants can be made available (Vanlerberghe et al., 1994) .

Whether or not AOX is SHAM's authentic target, this chemical can inhibit selectively the induction of SA-mediated resistance to TMV in tobacco, while having no apparent effect on the SA-mediated induction of the antifungal PR-1 protein. This difference in SHAM sensitivity between the two processes indicates that SA is either a component of two separate defensive signal transduction pathways, one of which is SHAM sensitive and leads to resistance to viruses whereas the other is SHAM insensitive and leads to PR protein gene induction, or a component of a single defensive signal transduction pathway that diverges downstream of SA into SHAM-sensitive and SHAM-insensitive branches.

We predicted from this model that SA-induced resistance to fungi and bacteria, which is mediated at least in part by PR proteins, would not prove to be SHAM sensitive. Our results obtained using a bacterial pathogen, *E. c. carotovora*, and a fungal pathogen, *B. cinerea*, appear to support our contention. In the case of *E. c. carotovora*, SA prevented the spread of soft rot symptoms throughout the inoculated leaf, as previously described by Palva et al. (1994). SA was also able to delay markedly the onset of disease in tobacco plants inoculated with *B. cinerea.* Consistent with both our PR-1 protein accumulation data (Figure 4B) and our model was the observation that SA-induced resistance to these pathogens was not inhibited by SHAM and that, in fact, SHAM alone was able to promote resistance to them.

In conclusion, it appears that SA functions in a novel defensive signal transduction pathway that leads specifically to a complex inhibition of TMV replication. The pathway is distinct from that leading to PR protein gene activation in that it requires the activity of AOX (or some other SHAM-sensitive activity). Furthermore, this pathway is required for the expression of acquired resistance to TMV.

METHODS

Plant Growth Conditions

Tobacco (*Nicotiana tabacum*) cultivars Xanthi-nn and Xanthi-NN were maintained under greenhouse conditions with supplementary lighting in winter. Tobacco mosaic virus (TMV)–inoculated Xanthi-nn plants (previously watered with solutions of salicylic acid [SA] or SA plus salicylhydroxamic acid [SHAM]) were incubated in a Fitotron cabinet (Sanyo Ltd., Loughborough, UK) at 25°C under continuous illumination. Leaf discs excised from Xanthi-nn leaves (using a 1-cm-diameter cork borer) and detached leaves of Xanthi-NN tobacco were incubated under a 16-hr-light and 8-hr-dark regime.

Inoculations with TMV

Leaf discs (Xanthi-nn tobacco) were floated for 3, 5, or 7 days on water, 1 mM 3-hydroxybenzoic acid (HBA), 1 mM SA, or a solution of SA plus SHAM (both at 1 mM). After this pretreatment period, discs were briefly removed from the solutions and inoculated with TMV (strain U1) at 10 μg mL^{-1} in 5 mM potassium phosphate buffer, pH 7.2, with carborundum as an abrasive, applying the inoculum over the entirety of each disc's adaxial surface with a cotton swab. Discs were replaced in their original solutions and incubated for an additional 2 days before RNA or protein extraction. Inoculation of Xanthi-nn and Xanthi-NN leaves was performed using 5 and 1 μg mL^{-1} of TMV U1, respectively.

Inoculation of Tobacco Plants with *Erwinia carotovora* subsp *carotovora*

E. c. carotovora SCRI 193 was maintained on Luria-Bertani agar at 30°C. Groups of Xanthi-NN tobacco plants (11 to 13 per group) were watered with either HBA, SA, SHAM, or SA plus SHAM (all at a concentration of 1 mM) for 5 days. Each plant was spot inoculated with 1 μL of bacterial suspension (10^9 colony-forming units per mL) on the upper surface of one leaf per plant by using a micropipettor (Palva et al., 1994). Some treated plants were also mock inoculated to control for tissue damage caused by the inoculation procedure. Inoculated plants were incubated at 30°C. Soft rot symptoms were scored at 12 hr postinoculation.

Inoculation of Tobacco Plants with *Botrytis cinerea*

B. cinerea cultures were maintained on half-strength potato dextrose agar at 20°C. Groups of Xanthi-NN tobacco plants (minimum of 15 per group) were watered with SA, SHAM, or SA plus SHAM (all at a concentration of 1 mM) for 5 days or left untreated. All 60 plants were inoculated simultaneously with *B. cinerea* by spraying with a spore suspension at a density of 10^5 spores per mL. After inoculation, plants were incubated at 20°C under natural illumination.

Protein and RNA Extractions

Soluble proteins were extracted, separated by SDS-PAGE, electroblotted to nitrocellulose, and subjected to protein gel blot analysis by using polyclonal rabbit antisera raised against the TMV coat protein (CP) and tobacco PR-1, using previously described methods (Carr et al., 1987; Carr and Zaitlin, 1991). Extraction of total leaf tissue RNA and lithium chloride fractionation of total RNA into single-stranded and double-stranded enriched RNA fractions was performed using the methods of Berry et al. (1985) and Carr and Zaitlin (1991). RNA gel blot analysis of TMV-specific RNAs by using strand-specific ^{32}P-labeled riboprobes and liquid scintillation counting of bound phosphorus-32 in RNA bands were performed using previously published methods (Carr and Zaitlin, 1991).

Alternative oxidase (AOX) transcript was detected on RNA gel blots by hybridization with a ^{32}P-labeled AOX DNA fragment. The DNA was synthesized by polymerase chain reaction (Sambrook et al., 1989) using the primers GTGATACCCAATTGGTGC and GAAACAGTGGCTGCAGTGCC, designed to amplify the conserved regions of the two published tobacco AOX sequences (Vanlerberghe and McIntosh, 1994; Whelan et al., 1995), and checked for authenticity by automated sequencing.

ACKNOWLEDGMENTS

We thank Milton Zaitlin and Michael Wilson for antisera raised against the TMV CP; George Salmond for the *E. carotovora* culture; Adrienne Pate for the culture of *B. cinerea*; and Jim Berry, Peter Palukaitis, and David Pate for advice and suggestions. This work was supported by grants from the Biotechnology and Biological Sciences Research Council (Grant No. P03659) and the Leverhulme Trust. S.C. was supported by a studentship from the Cambridge Commonwealth Trust, and M.N. was supported by a studentship from the Biotechnology and Biological Sciences Research Council. We dedicate this paper to the memory of the late Tom ap Rees.

Received September 26, 1996; accepted February 10, 1997.

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