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Salicylic Acid Is a Systemic Signal and an Inducer of Pathogenesis-Related Proteins in Virus-Infected Tobacco

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Systemic induction of pathogenesis-related (PR) proteins in tobacco, which occurs during the hypersensitive response to tobacco mosaic virus (TMV), may be caused by a minimum 10-fold systemic increase in endogenous levels of salicylic acid (SA). This rise in SA parallels PR-1 protein induction and occurs in TMV-resistant Xanthi-nc tobacco carrying the N gene, but not in TMV-susceptible (nn) tobacco. By feeding SA to excised leaves of Xanthi-nc (NN) tobacco, we have shown that the observed increase in endogenous SA levels is sufficient for the systemic induction of PR-1 proteins. TMV infection became systemic and Xanthi-nc plants failed to accumulate PR-1 proteins at 32°C. This loss of hypersensitive response at high temperature was associated with an inability to accumulate SA. However, spraying leaves with SA induced PR-1 proteins at both 24 and 32°C. SA is most likely exported from the primary site of infection to the uninfected tissues. A computer model predicts that SA should move rapidly in phloem. When leaves of Xanthi-nc tobacco were excised 24 hr after TMV inoculation and exudates from the cut petioles were collected, the increase in endogenous SA in TMV-inoculated leaves paralleled SA levels in exudates. Exudation and leaf accumulation of SA were proportional to TMV concentration and were higher in light than in darkness. Different components of TMV were compared for their ability to induce SA accumulation and exudation: three different aggregation states of coat protein failed to induce SA, but unencapsidated viral RNA elicited SA accumulation in leaves and phloem. These results further support the hypothesis that SA acts as an endogenous signal that triggers local and systemic induction of PR-1 proteins and, possibly, some components of systemic acquired resistance in NN tobacco.

INTRODUCTION

Salicylic acid (SA) was recognized as an endogenous regulator in plants after the finding that it triggers a dramatic increase in the production of metabolic heat and insect-attracting chemicals in the thermogenic inflorescences of *Arum* lilies (Raskin et al., 1987, 1989) and possibly other thermogenic plants (Raskin et al., 1990). Our subsequent research has concentrated on the role of endogenous SA in disease resistance.

The hypersensitive response (HR) is a common manifestation of plant disease resistance that is characterized by rapid cell death around the point of infection, restricting the systemic spread of pathogens. In tobacco, HR to tobacco mosaic virus (TMV) is controlled by a dominant N gene (Holmes, 1938). In contrast to nn genotype tobacco where virus spreads rapidly and systemically, N gene plants restrict TMV to a small zone of tissue where necrotic lesions are formed approximately 2 days after inoculation. The HR may lead to systemic acquired resistance (SAR), first characterized in Sweet William infected by a "carnation mosaic virus" (Gilpatrick and Weintraub, 1952). SAR is usually defined as resistance to subsequent pathogen attack, and can be detected in both infected and uninfected parts of the plant (Ross, 1961). Since its initial discovery, SAR has been demonstrated in a variety of plant-pathogen interactions. In many cases, SAR is not specific to the primary pathogen and is effective against different viruses, bacteria, or fungi (van Loon and Dijkstra, 1976; Gianinazzi, 1984). In N tobacco, smaller and fewer lesions develop in previously uninfected leaves in response to a second challenge by TMV or other necrotizing pathogens.

Commonly associated with HR and SAR is the systemic synthesis of several families of serologically distinct, low molecular weight, pathogenesis-related (PR) proteins, recently reviewed by Carr and Klessig (1989). The function of the PR-1 family of proteins, PR-1a, PR-1b, PR-1c, remains to be elucidated. In Xanthi-nc (NN) tobacco, the

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PR-2 family of proteins have β-1,3-glucanase activity (Kauffmann et al., 1987), whereas PR-3 proteins are chitinases (Legrand et al., 1987).

The phenomenon of SAR implies the existence of a signal that spreads systemically from the site of the HR to the rest of the plant. It is well established that resistance to pathogens and the production of PR proteins in tobacco and other plants can be induced by SA or acetylsalicylic acid (aspirin), even in the absence of pathogenic organisms (White, 1979; Antoniw and White, 1980; van Loon and Antoniw, 1982; Hooft van Huijsduijnen, 1986; Pennazio et al., 1987).

Suspecting that SA may be a systemic resistance signal, we have monitored endogenous levels of SA and PR-1 mRNA in TMV-inoculated resistant Xanthi-nc (NN) and susceptible Xanthi (nn) varieties of tobacco. SA levels in resistant, but not in susceptible, tobacco increased almost 50-fold in the TMV-inoculated leaves and 10-fold in the uninoculated leaves (Malamy et al., 1990). Induction of PR-1 mRNA paralleled the rise in SA levels. In contrast to TMV, SA was effective in inducing PR proteins in Xanthi (nn) tobacco (Malamy et al., 1990). Metraux et al. (1990) presented independent evidence correlating SA levels in phloem with SAR in cucumber after *Colletotrichum lagenarium* or tobacco necrosis virus infection.

Available evidence, therefore, suggests that SA may function as the natural signal for at least some components of plant disease resistance. This study further substantiates this hypothesis by providing evidence that SA moves systemically in the tobacco plant and that the levels exported from the inoculated areas are sufficient for systemic induction of PR proteins. We have also investigated the effects of various TMV components, and environmental factors such as temperature and light, on SA production in inoculated leaves of Xanthi-nc tobacco.

RESULTS

PR Protein Production and Leaf SA

To assess whether the increased levels of SA observed in TMV-inoculated Xanthi-nc tobacco leaves undergoing HR (Malamy et al., 1990) are sufficient for systemic PR-1 protein induction, excised healthy leaves were fed SA for 72 hr through the cut petiole and SA and PR-1 protein levels were analyzed in opposite half-leaves. The level of SA in a leaf was proportional to the concentration of SA in the solution in which the petiole was immersed. As shown in Figure 1, induction of PR-1 proteins was positively correlated with leaf SA. The average basal level of SA in control leaves was 34 ng of SA per gram fresh weight. A 59% increase in tissue SA levels, to 54 ng/g fresh weight, caused detectable induction of PR-1a in the extracellular fluid (Figures 1B and 1C). Because antibodies against PR-1a cross-react with PR-1b and PR-1c (Antoniw et al., 1985), we detected some induction of PR-1b at higher levels of leaf SA. TMV inoculation (1 μg of TMV per leaf) raised SA levels in the attached leaf to 333 ng/g fresh weight, resulting in a correspondingly greater induction of PR-1a and PR-1b proteins and the appearance of PR-1c. The levels of extracellular PR-1 proteins induced by SA or TMV were high enough to be visualized on silver-stained nondissociating 14% (w/v) polyacrylamide gels (Figure 1C).

SA and PR Protein Accumulation at Different Temperatures

The HR and the associated induction of PR proteins in TMV-inoculated Xanthi-nc tobacco disappear if plants are incubated at temperatures above 28°C (Kassanis, 1952; van Loon, 1975; White et al., 1983). To define further the relation between the HR to TMV, PR protein production, and SA accumulation in virus-resistant tobacco, we measured SA and PR-1 proteins in mock-inoculated or TMV-inoculated Xanthi-nc leaves at 24 and 32°C, as shown in Figure 2. Plants inoculated with TMV did not develop lesions after 3 days at 32°C, but formed numerous lesions on lower inoculated and upper uninoculated leaves 4 days after being returned to 24°C, confirming the systemic spread of TMV at 32°C. TMV-inoculated leaves of control plants incubated for 72 hr at 24°C developed 264 ± 66 (±SE) lesions, whereas SA-treated leaves developed 58 ± 19 (±SE) lesions.

SA and PR proteins were extracted from opposite half-leaves 72 hr after the leaves were sprayed with a 2 mM SA solution, inoculated with TMV, or given both treatments. For each treatment presented in Figure 2, we have averaged SA levels in four leaves, one each, from four different plants. Leaves with SA levels closest to the mean values were used for Figures 2B and 2C. As expected, at 24°C leaves inoculated with TMV contained 15 times more SA than the control leaves (Figure 2A). However, TMV-induced accumulation of SA was completely inhibited at 32°C, suggesting a correlation between SA accumulation, PR-1 induction, and HR (Figures 2B and 2C). As already observed (van Loon, 1975; White et al., 1983), PR-1 proteins did not accumulate at 32°C in the absence of SA. Treatment of plants with SA induced PR proteins at 24 and 32°C. Levels of SA were significantly lower in TMV-inoculated and/or SA-sprayed leaves at 32°C than at 24°C (Figure 2A), suggesting increased metabolic turnover or decreased biosynthesis of SA at 32°C.

SA Exudation from Inoculated Leaves

Phloem sap exuded from cut leaf petioles can be collected in solutions containing metal (Ca^{2+}) chelators (King and Zeevaart, 1974; Fellows and Zeevaart, 1983; Weibull et

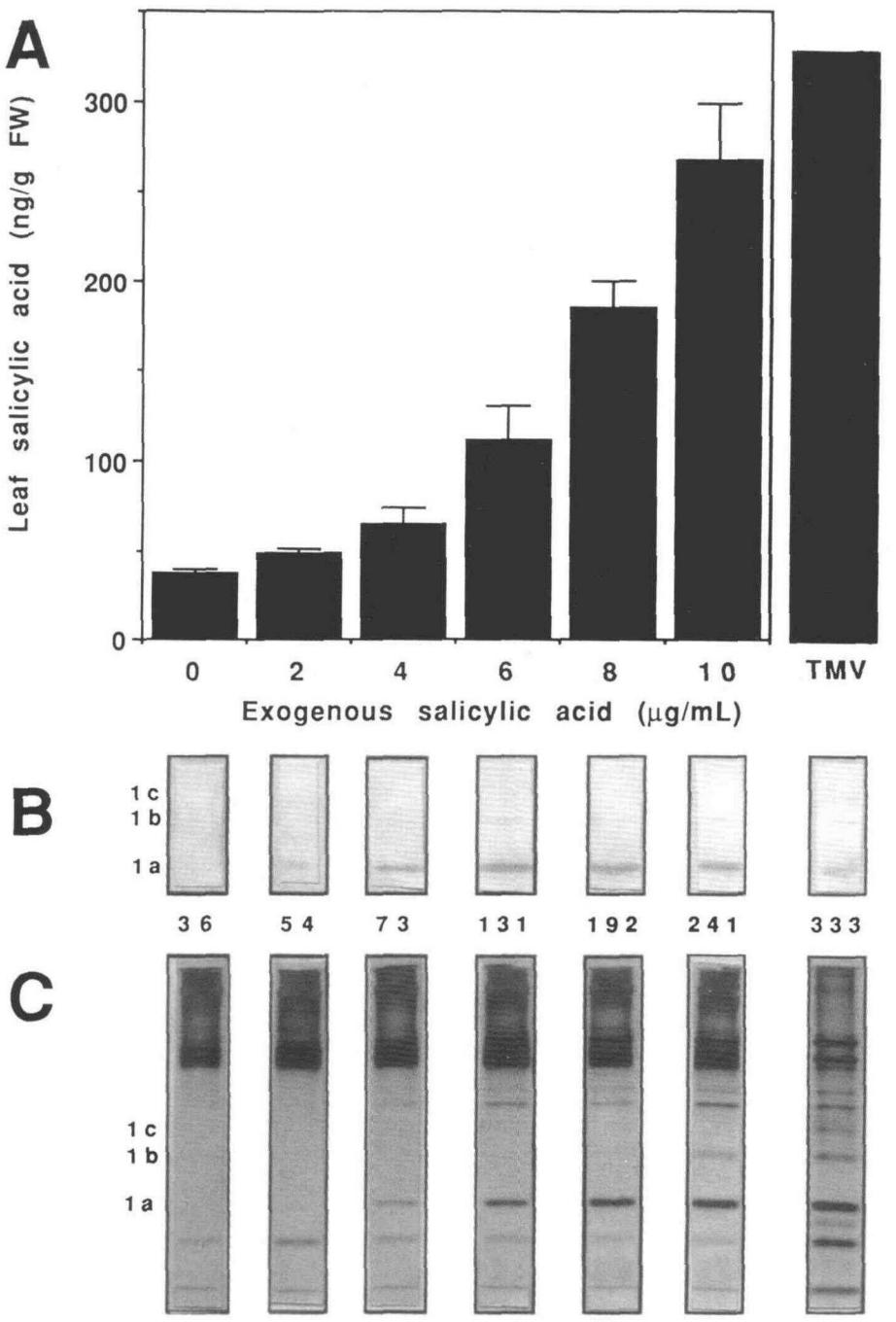

Figure 1. Induction of PR-1 Proteins as a Function of Leaf SA Levels.

(A) SA levels in excised Xanthi-nc tobacco leaves fed with different concentrations of SA for 72 hr (± SE, $n = 5$) and in one attached leaf inoculated with 1 μg of TMV. The experiment was repeated three times with similar results. FW, fresh weight.
(B) Immunoblots of PR-1 proteins extracted from the opposite halves of leaves used for **(A)**. The numbers between **(B)** and **(C)** indicate the actual SA levels (nanograms per gram fresh weight) in the individual leaves from which the proteins were extracted.
(C) Nondissociating gel electrophoresis and silver staining of extracellular proteins extracted from the opposite halves of leaves used for **(A)**.

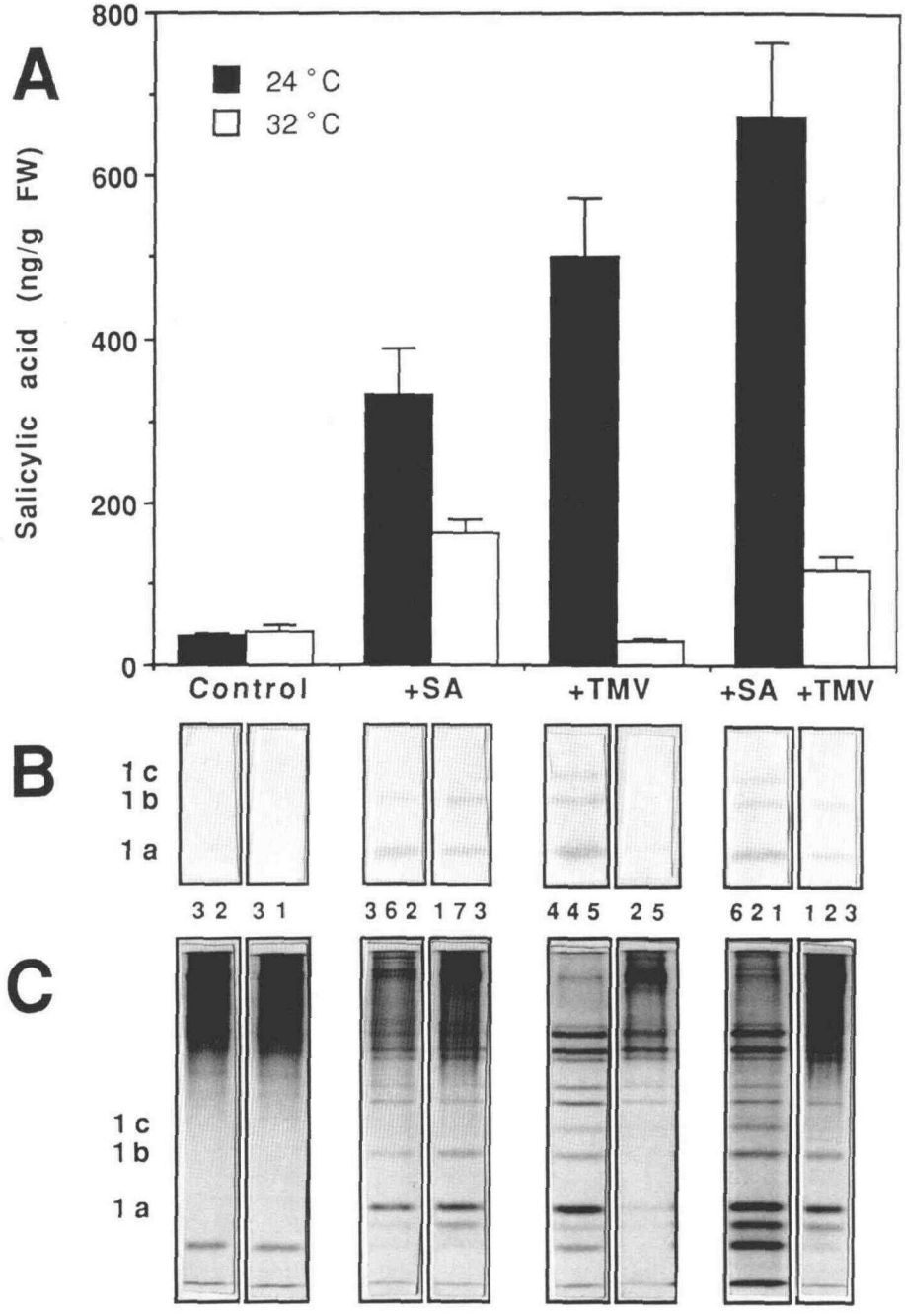

Figure 2. Effect of SA Application and TMV Infection on Leaf SA and PR-1 Protein Levels at 24 and 32°C.

(A) SA levels in the leaves of Xanthi-nc tobacco sprayed with 2 mM SA and/or inoculated with 2.3 μg TMV. Plants were incubated for 72 hr at 24 or 32°C. Each bar is a mean of four replicate treatments ± SE. The experiment was repeated twice with similar results. FW, fresh weight.

(B) Immunoblots of PR-1 proteins extracted from the opposite halves of leaves used for **(A)**. The numbers between **(B)** and **(C)** indicate the actual SA levels (nanograms per gram fresh weight) in the leaves from which the proteins were extracted.

(C) Nondissociating gel electrophoresis and silver staining of extracellular proteins extracted from the opposite halves of leaves used for **(A)**.

al., 1990). To assess the export of SA during the HR, we monitored the exudation of SA from the cut petioles of TMV-inoculated Xanthi-nc and Xanthi tobacco leaves. Figure 3 shows that very little SA, just above the minimum detection level of 10 ng/g fresh weight, was exuded over 48 hr from leaves excised immediately after TMV inoculation. However, substantial amounts of SA were detected in the EGTA solution sampled twice over a period of approximately 48 hr when leaves were excised 24 or 48 hr after inoculation. No detectable SA was exuded from the leaves of TMV-susceptible Xanthi tobacco or mock-inoculated Xanthi-nc tobacco (data not shown). Excising leaves 24 hr after inoculation followed by 48 hr of exudate collection became our standard method for measuring SA export.

Figure 4 illustrates that SA accumulation in Xanthi-nc leaves and SA exudation from the same leaves were directly proportional to the amount of TMV inoculum and the number of lesions per leaf. Tissue accumulation of SA, SA exudation, and the number of lesions produced as a result of TMV inoculation (10 μg per leaf) were approximately twofold greater in light than in darkness, as shown in Figure 5.

Elicitation of SA Accumulation by TMV Components

The following TMV (U1 strain) components were applied to the surface of Xanthi-nc tobacco leaves to determine

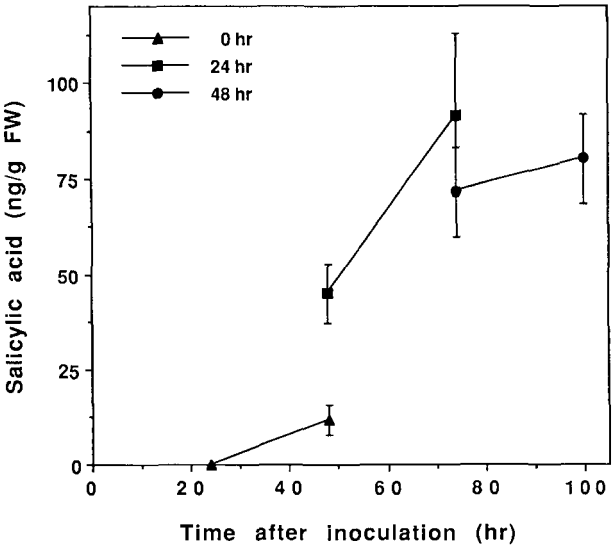

Figure 3. Exudation of SA from Xanthi-nc Tobacco Leaves Excised at 0, 24, and 48 hr after TMV Inoculation.

The time of inoculation is denoted as 0 hr on the time axis. Each point is a mean of four replicate treatments ± SE. The experiment was repeated three times with similar results. FW, fresh weight.

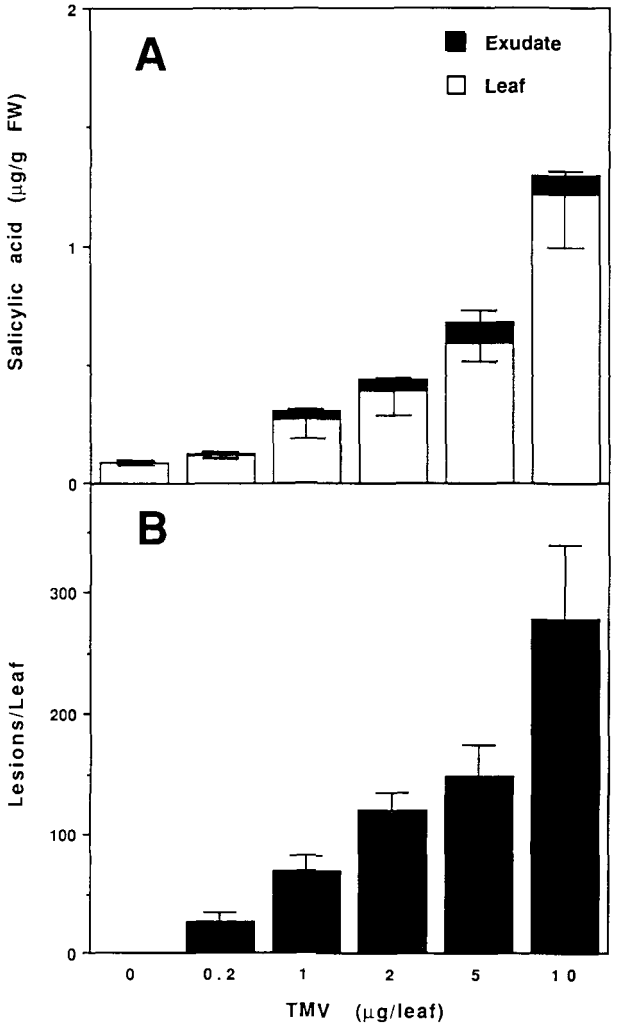

Figure 4. Effect of Different TMV Concentrations on Leaf SA Accumulation and Exudation and Lesion Number.

(A) SA accumulation and exudation. FW, fresh weight.
(B) Lesion number.
Each point is a mean of four replicate treatments ± SE. The SE bars for leaf SA and exuded SA are drawn in opposite directions. The experiment was repeated three times with similar results.

which of them elicited SA accumulation and export: (1) subunits and small (4S) aggregates of TMV coat protein; (2) 20S oligomeric aggregates of TMV coat protein (34 subunits in a 2+ layer protohelix structure); (3) long, helically aggregated subunits of coat protein alone (no RNA); and (4) unencapsidated viral RNA, which is a mild elicitor of the HR (Fraenkel-Conrat and Williams, 1955). To determine whether tissue damage stimulates SA accumulation in the absence of a pathogen, abiotic 3- to 4-mm leaf lesions were produced with dry ice chips. Also a 4-mm cork borer was used to punch 20 to 30 holes in the leaves.

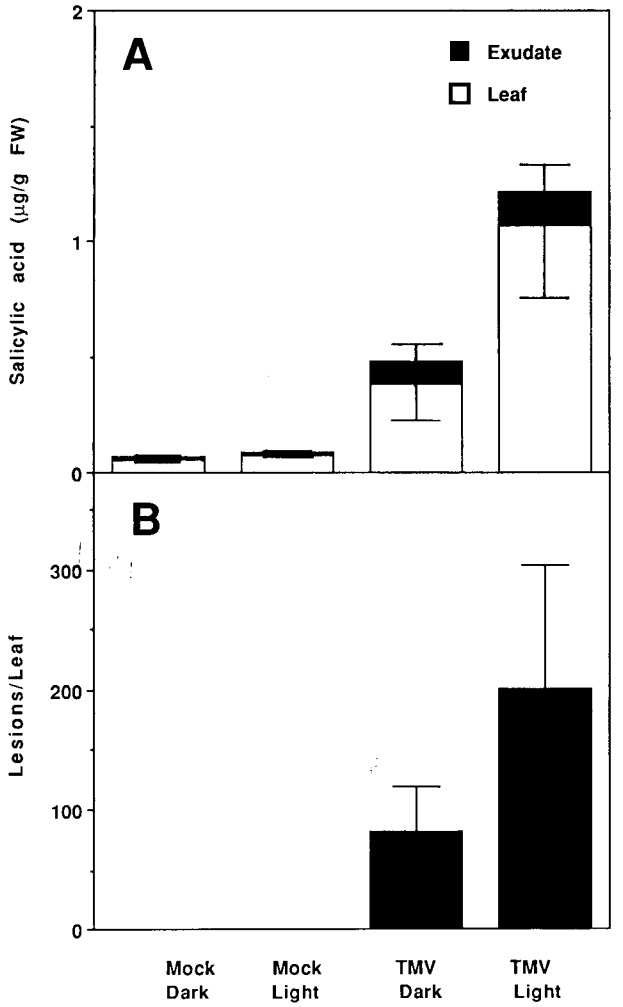

Figure 5. Effect of Illumination on Leaf SA Accumulation and Exudation and Lesion Number.

(A) SA accumulation and exudation. FW, fresh weight.
(B) Lesion number.
Each point is a mean of four replicate treatments ± SE. The SE bars for leaf SA and exuded SA are drawn in opposite directions. The experiment was repeated three times with similar results.

Figure 6 shows that in addition to TMV only the purified viral RNA induced modest and dose-dependent SA accumulation and exudation. At 10 μg per leaf, unencapsidated TMV RNA had a specific infectivity 200 times less than intact virus, inducing 10 times fewer lesions and 10 times lower levels of SA tissue accumulation and also 2.2 times lower levels of SA exudation (Figure 6). Other tested TMV components did not cause infection symptoms and did not induce SA accumulation or exudation (data not shown). Wounding was similarly ineffective in raising the basal SA levels in leaf tissue and phloem.

DISCUSSION

For SA to be a natural signal that induces PR proteins during SAR, the following conditions should be met: (1) the amount of SA present in inoculated and uninoculated tissues during SAR should be sufficient for induction of PR proteins; (2) conditions that inhibit HR, SAR, and associated PR protein induction should block SA accumulation; and (3) SA should be exported from the infected

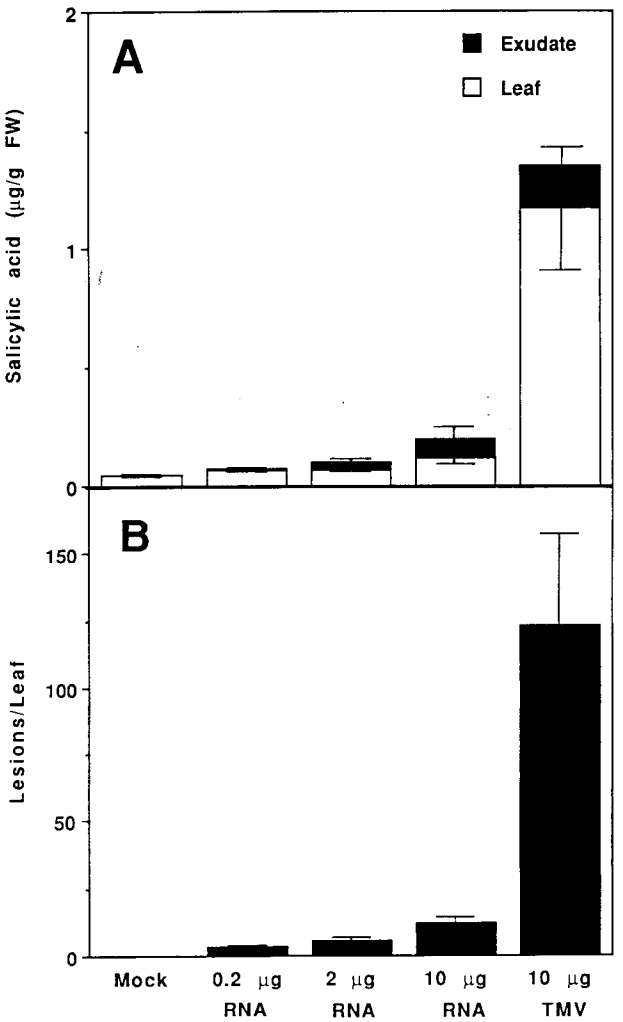

Figure 6. Effect of Unencapsidated TMV RNA on Leaf SA Accumulation and Exudation and Lesion Number.

(A) SA accumulation and exudation. FW, fresh weight.
(B) Lesion number.
Each point is a mean of four replicate treatments ± SE. The SE bars for leaf SA and exuded SA are drawn in opposite directions. The experiment was repeated three times with similar results.

leaf to the rest of the plant. The results presented in this paper show that in TMV-infected Xanthi-nc tobacco all these conditions are met.

As reported previously (Malamy et al., 1990), 72 hr after TMV inoculation the treated leaves of Xanthi-nc tobacco contain 1 μg of SA per gram fresh weight, whereas the upper, uninoculated leaves contain almost 100 ng of SA per gram fresh weight. Induction of PR-1 proteins in the inoculated leaves was much higher than in the uninoculated leaves of the same plant. Figure 1 shows that 54 ng of SA per gram fresh weight is sufficient for the induction of detectable amounts of PR-1a protein, with progressively stronger induction at higher SA levels. The general pattern of PR-1 induction in leaves treated with SA and TMV was similar. Our data suggest that SA levels in the inoculated and uninoculated leaves of NN genotype tobacco plants are sufficient for the systemic induction of PR proteins.

Our data also indicate that SA accumulation in leaf tissue and phloem is an integral part of the HR and is sufficient for the induction of PR proteins commonly observed during SAR. The fact that inhibition of SA accumulation at 32°C was accompanied by the disappearance of HR and PR proteins (Figure 2) further supports a direct connection between the HR, SA, and PR protein induction. Also, factors that increased HR in Xanthi-nc tobacco, such as high TMV inoculum levels (Figure 4) and light (Figure 5), produced a corresponding increase in SA accumulation and exudation, whereas physical injury or noninfectious components of TMV did not.

According to a recently developed mathematical model (Kleier, 1988; Hsu and Kleier, 1990), the measured physical properties of SA, $pK_a = 2.98$ (Minnick and Kilpatrick, 1939) and log K_{ow} (octanol/water partitioning coefficient) = 2.26 (Hansch and Anderson, 1967), are nearly ideal for long-distance transport in the phloem. The predictions of the theory are illustrated in Figure 7 in the form of a contour plot of the logarithm of phloem mobility as a function of acid dissociation constant (pK_a) and lipophilicity (log K_{ow}). Phloem mobility, C_f, is defined as the concentration of a compound at some remote location in a plant relative to that at the compound's source. Large values of C_f thus correspond to phloem mobile compounds and values greater than 1.0 (log $C_f > 0.0$) correspond to compounds that are predicted to concentrate in the phloem by way of acid trapping at points remote from the source. The location of SA on the log C_f contour surface suggests near ideal physical properties for phloem translocation (Figure 7).

In agreement with the model, large amounts of SA were found in the phloem of cucumber plants after *Colletotrichum* infection (Metraux et al., 1990). The movement of SA from TMV-inoculated Xanthi-nc tobacco leaves was confirmed by using the excised leaf system that we have developed (Figures 3, 4, 5, and 6). It is possible that some SA moves in plants as a sugar conjugate, because SA fed to a variety of plants forms substantial amounts of

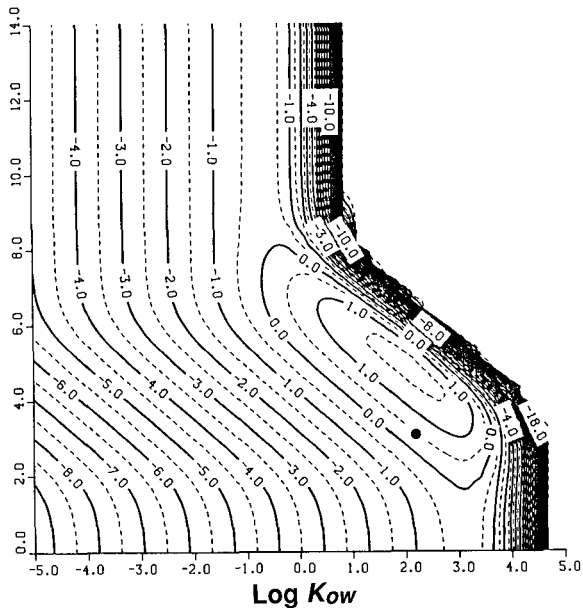

Figure 7. Contour Diagram of the Dependence of the Logarithm of Phloem Mobility (C_f) on Acidity (pK_a) and Lipophilicity (log K_{ow}) of the Undissociated Acid.

The solid square represents the position of SA on the plot.

O-glucoside (Klämbt, 1962; Cooper-Driver et al., 1972). The observed increases in SA levels in leaf and phloem may reflect an inhibition of its metabolism in addition to or instead of an increase in SA biosynthesis. More research is needed to study the changes in SA conjugation, metabolism, and biosynthesis during SAR.

Exudates collected from cut leaf petioles have been analyzed for amino acids (Weibull et al., 1990), herbicides (Groussol et al., 1986), and sugars (King and Zeevaart, 1974) in phloem sap. The use of solutions containing metal (Ca^{2+}) chelators enhances exudation, possibly due to inhibition of Ca^{2+}-dependent callose formation (Fellows and Zeevaart, 1983). Tobacco leaves exuded more SA when they remained attached to the plant for at least 24 hr after TMV inoculation (Figure 3). This is consistent with the fact that leaves excised immediately after inoculation developed fewer lesions (data not shown) and indicates that leaf attachment to the plants is required for the full manifestation of the HR. Because the amount of SA exuded from the leaf correlated with SA levels in the leaf tissue (Figures 4, 5, and 6), the excised leaf assay provides a convenient system to study the effects of plant pathogens on SA levels.

The evidence presented in this manuscript further supports the hypothesis that SA acts as an endogenous signal that activates at least some components of viral resistance

and the systemic induction of PR proteins. Unfortunately, we still do not know to what extent SA-induced resistance is based on the induction of PR proteins. It is certainly possible that SA activates additional known and unknown resistance mechanisms.

METHODS

Plant Material

Six- to eight-week-old tobacco plants, *Nicotiana tabacum* (cv Xanthi-nc NN-genotype and Xanthi nn) were grown in an environmentally controlled growth chamber at 25°C, 75% relative humidity, and 16-hr photoperiod (600 μmol m^{-2} sec^{-1}) provided by a combination of incandescent and cool-white fluorescent lights. The uppermost, nearly fully expanded leaves were abraded with wet carborundum (400 grit) and inoculated with 200 μL of a sterile aqueous suspension of TMV (U1 strain). Necrotic lesions were first apparent 40 hr after inoculation. Mock-inoculated leaves were abraded and inoculated with water alone. After inoculation, plants or excised leaves were kept at 24°C under continuous illumination provided by cool-white fluorescent bulbs (200 μmol m^{-2} sec^{-1}), unless stated otherwise.

Application of SA

The uppermost, nearly fully expanded leaf was used for all experiments. The petioles of the excised leaves were immersed in 30 mL of 10 mM K-phosphate buffer (pH 6.5) containing 1 mM EDTA and specified concentrations of SA.

For experiments on the effect of temperature, plants were preincubated at the specified temperature for 24 hr and 2.3 μg of TMV in 200 μL of water were applied to each abraded leaf. Leaves were allowed to dry, rinsed with tap water, and sprayed until runoff with 20 mM K-phosphate (pH 7.0) containing 0.025% (v/v) Triton X-100 with or without 2 mM SA. At the end of 72-hr incubations at 24°C or 32°C, leaves were sprayed again with buffer without SA and washed with copious amounts of deionized water to remove surface-bound SA. Leaves were cut longitudinally and the midrib removed. Half of each leaf was assayed for SA; the opposite half was used for extraction of PR proteins. When necessary, leaf tissue was stored at −80°C for SA analysis.

Extraction and Quantitation of SA

SA was extracted and quantified by fluorescence using HPLC as described previously (Raskin et al., 1989), except that the diol column purification step was omitted and the samples were dried by using a Model SVC 200 SpeedVac Sample Concentrator (Savant Instruments Co., Farmingdale, NY) instead of nitrogen. Fifty microliter samples were injected onto a Dynamax 60A 8-μm guard column (4.6 mm × 1.5 cm) linked to a Dynamax 60A 8-μm C-18 column (4.6 mm × 25 cm) (Rainin Instrument Co., Emeryville, CA), maintained at 40°C. SA was separated isocratically with 23% (v/v) methanol in 20 mM sodium acetate buffer (pH 5.0) at a flow rate of 1.5 mL min^{-1}. The limit of detection was 10 ng of SA per gram fresh weight. Recovery of SA, estimated by extracting tissue to which a known amount of SA had been added, averaged 45%. All results were corrected for recovery.

PR-1 Protein Analysis

Extracellular fluid was extracted from leaves as described by Parent and Asselin (1984) and stored at −80°C. One microgram of total protein was separated in nondissociating 14% (w/v) polyacrylamide electrophoresis minigels, and either silver stained (Blum et al., 1987) or electroblotted to Westran membranes (Schleicher & Schuell) using a Trans-Blot-SD (Bio-Rad) electroblotter. After transfer, membranes were blocked with 2% (w/v) Carnation nonfat dry milk in TBST buffer (20 mM Tris-HCl, 150 mM NaCl, 0.05% [v/v] Tween 20, 0.05% [w/v] sodium azide, pH 7.5) for 30 min. Polyclonal antibody against PR-1a, that also cross-reacted with PR-1b and PR-1c, was obtained from Dr. J. F. Antoniw (Rothamsted Experimental Station, Harpenden, Herts, U.K.) and used at a 500-fold dilution in TBST buffer for 30 min. Antigenic bands were visualized using the Protoblot protocol (Promega) with a 3000-fold dilution of alkaline phosphatase conjugated to anti-rabbit IgG (Sigma).

TMV Components

TMV strain U1 virions or their structural components were used in all studies. RNA was isolated by phenol/chloroform extraction, and the purity of the preparation confirmed by electrophoresis on denaturing formamide-3-(*N*-morpholino)-propanesulfonic acid agarose gels (Sambrook et al., 1989). Coat protein was isolated from TMV by the method of Durham (1972). Free subunits and small aggregates, 'A' protein (4S), were assembled to form protohelices (20S) or long helical rods (Durham, 1972). All inocula were suspended in their respective buffers and concentrations determined spectrophotometrically at 260 nm using published extinction coefficients (Zaitlin and Israel, 1975).

Collection of Leaf Exudate

The petioles of leaves inoculated with TMV were cut under 1 mM EGTA, pH 7.0, and placed in microcentrifuge tubes containing 1.5 mL of EGTA solution. Tubes with excised leaves were incubated in continuous light, 200 μmol m^{-2} sec^{-1}, 100% relative humidity, 24°C, inside 10-L plastic chambers for 48 hr, after which leaf weight and lesion numbers were determined. Leaf tissue and solutions containing phloem exudates were stored at −20°C. The solutions of phloem exudates were centrifuged at 10,000*g* for 2 min to remove debris and analyzed for SA using HPLC (see above). For the experiment presented in Figure 3, 50-μL aliquots were removed from the incubation medium at 24-hr intervals and replaced with fresh incubation solution. The data were expressed as total SA exuded per gram fresh weight after the specified incubation periods.

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