Endogenous Salicylic Acid Levels Correlate with Accumulation of Pathogenesis-Related Proteins and Virus Resistance in Tobacco

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ABSTRACT

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Salicylic acid (SA) is hypothesized to be an endogenous regulator of local and systemic disease resistance and an inducer of pathogenesis-related (PR) proteins among plants. High levels of PR proteins have been observed in an uninoculated amphidiploid hybrid of *Nicotiana glutinosa* × *N. debneyi*, which is highly resistant to tobacco mosaic virus (TMV). Fluorescence, UV, and mass spectral analysis established that the levels of SA in healthy *N. glutinosa* × *N. debneyi* leaves were 30 times greater than in *N. tabacum* 'Xanthi-nc' tobacco, which does not constitutively express PR proteins and is less resistant to TMV. Upon TMV-inoculation, SA levels increased at least 70-fold in leaves of Xanthi-nc but rose only slightly in the hybrid. Phloem exudates of *N. glutinosa* × *N. debneyi* contained at least 500 times more SA than those of Xanthi-nc. SA treatment caused the appearance of PR-1 protein in Xanthi-nc but did not affect constitutively high levels of PR-1 protein in *N. glutinosa* × *N. debneyi*. In contrast to Xanthi-nc tobacco, TMV-inoculated *N. glutinosa* × *N. debneyi* kept at 32 C accumulated more than 0.5 μg SA/g fresh weight, maintained high levels of PR proteins, and developed a hypersensitive response to TMV. PR proteins have previously been shown to accumulate in the lower leaves of healthy, flowering Xanthi-nc tobacco, which exhibited increased resistance to TMV. These developmentally induced increases in resistance and PR-1 proteins positively correlated with tissue levels of SA. These results affirm the regulatory role of SA in disease resistance and PR protein production.

The hypersensitive response (HR), characterized by necrosis of host cells in the immediate vicinity of the site of pathogen penetration, is a frequently observed form of resistance of plants to pathogens. HR is accompanied by complex biochemical changes that strengthen the physical and chemical barriers to pathogen spread (26). Often, some of these resistance responses are also activated in uninoculated parts of the plant. As a result, the whole plant becomes more resistant to a subsequent challenge. This form of induced resistance, called systemic acquired resistance (SAR; 23), is associated with HR or certain compatible interactions and may be effective against further infections by viruses, bacteria, and fungi (7).

Recent evidence suggests that salicylic acid (SA), a phenolic with ubiquitous distribution among plants (18), is a systemic signal responsible for the induction of SAR. The application of SA increases the resistance of a number of plants to viral, bacterial, and fungal pathogens (14,28,31). During SAR, SA activates the expression of genes coding for pathogenesis-related (PR) proteins (27). While the function of the PR-1, 4, and 5 families of PR proteins is unknown, members of the PR-2 and 3 families have β-1,3-glucanase and chitinase activities, respectively (reviewed by 9), suggesting their involvement in plant defense. The extent to which PR proteins play a role in SA-mediated disease resistance is not fully determined.

Endogenous levels of SA increase systemically in tobacco mosaic virus (TMV)-inoculated Xanthi-nc tobacco that carries the N resistance gene to TMV, but not in a susceptible nn genotype cultivar (4,11). The presence of microgram levels of SA in TMV-inoculated Xanthi-nc leaves is accompanied by the accumulation of a SA-conjugate, presumably β-*O*-D-glucosylsalicylic acid (4,12). This compound does not appear to be a signal in SAR because it cannot be detected in phloem exudates of TMV-inoculated Xanthi-nc leaves (4). SA produced during SAR is phloem mobile (13,20,30) and is produced in quantities sufficient to stimulate accumulation of PR proteins and greater resistance to subsequent TMV-infection (4,30). Incubation of TMV-inoculated Xanthi-nc tobacco at 32 C blocks HR, SAR, and the associated PR protein accumulation and also prevents the accumulation of SA (12,30). However, not all SA detected throughout the plant during SAR may be produced in the inoculated leaf, opening the possibility for the existence of additional systemic signals (20).

In previous studies (4,11,30), SA in tobacco leaves was increased by feeding SA or inoculating with TMV, and the effect of SA on PR protein accumulation and resistance to TMV was assessed. In the present investigation, we measured the levels of SA in healthy tobacco tissues previously reported to have high levels of TMV-resistance and to accumulate PR proteins. *Nicotiana glutinosa* L. × *N. debneyi* Domin, an amphidiploid hybrid of *N. glutinosa* (N genotype) and the susceptible *N. debneyi* (nn genotype), exhibits a higher degree of resistance (measured as smaller lesions) to TMV infection than either of its parental species or Xanthi-nc tobacco (2). The high level of resistance of *N. glutinosa* × *N. debneyi* to TMV extends also to tobacco necrosis virus and to HR-inducing strains of *Pseudomonas syringae, Cercospora nicotianae, Chalara elegans*, and *Phytophthora parasitica* (3). Associated with high disease resistance are constitutively high levels of PR protein b1″, PR-5, chitinase, β-1,3-glucanase, and peroxidase (2,3,16). These proteins are usually absent in healthy, unstressed *N. glutinosa* and *N. debneyi* but accumulate during development of induced disease resistance (3,16). As is the case for the serologically related PR-1 proteins of Xanthi-nc, the role of b1″ protein in SAR is not known (1).

PR protein accumulation is also developmentally regulated in healthy tobacco. Most notably, PR-1, 2, and 3 proteins are expressed in tobacco flowers (10) and in lower leaves of flowering tobacco (6,15). As these proteins accumulate in the foliage of mature plants, there is also an increase in resistance to infection by viral (5,24) and fungal (21,29) pathogens.

Here we report a correlation between high levels of endogenous SA and TMV-resistance in *N. glutinosa* × *N. debneyi* hybrid

and lower leaves of mature Xanthi-nc. These results further support the regulatory role of SA in disease resistance and PR protein accumulation.

MATERIALS AND METHODS

Plant material. Seeds of *N. tabacum* L. 'Xanthi-nc' (gift from D. F. Klessig, Rutgers University) and of the amphidiploid *N. glutinosa* × *N. debneyi* hybrid (gift from P. Ahl-Goy, Agricultural Division, Ciba-Geigy Ltd., Basel, Switzerland) were germinated and grown as previously described (30). In experiments comparing *N. glutinosa* × *N. debneyi* to Xanthi-nc tobacco, the uppermost nearly fully expanded leaves of 8-wk-old plants were used. For studies on the levels of SA as a function of leaf position and plant age, greenhouse-grown Xanthi-nc plants at each of the following stages were used: stage 1, plants were 6 wk after seeding and had 13 leaves (>3 cm); stage 2, plants were 10 wk after seeding and had 29 leaves and one or two open flowers; stage 3, plants were 12 wk after seeding, had 29 leaves, and were at seed filling stage. None of the leaves selected for the experiments showed signs of senescence or chlorosis. Inoculations with the U1 strain of TMV (2.5 μg/leaf) were as described (30). The diameter of necrotic lesions, a measure of resistance (22), was determined by using a stereoscopic microscope. Results for each treatment are presented as the mean diameter of at least 50 randomly selected lesions from four different plants.

Extraction and quantitation of SA. SA was extracted from leaf samples (0.5 g) and quantified by HPLC using spectrofluorescence (4). Hydrolysis of leaf extracts with almond β-glucosidase (Sigma Chemical Co., St. Louis, MO) was performed as previously described (4). All data were corrected for SA-recovery, which ranged from 46 to 70% for *N. glutinosa* × *N. debneyi* and from 34 to 62% for *N. tabacum*.

Identification of SA in extracts from *N. glutinosa* × *N. debneyi*. β-Glucosidase-treated extracts from leaves of uninoculated *N. glutinosa* × *N. debneyi*, prepared for HPLC as previously described (4), 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 Instr. Co., Emeryville, CA), maintained at 40 C. The sample was fractionated isocratically with 23% (v/v) methanol in 20 mM sodium acetate buffer (pH 5.0) at a flow rate of 1.5 mL/min. Detection by fluorescence was as described previously (19). A fluorescing compound that coeluted with authentic SA was collected from multiple injections, pooled, and dried under vacuum. The residue was resuspended in ethylacetate, and insoluble buffer salts were removed by centrifugation at 5,000 *g* for 15 min. The supernatant was dried under vacuum and used for further analysis. It is termed HPLC-purified leaf extract.

The HPLC-purified leaf extract was resuspended in 55% (v/v) methanol in water, and an emission spectrum was obtained with a fluorescence spectrophotometer (Model FL 750 HPLC Plus, McPherson Instr. Co., Acton, MA) by using an excitation wavelength of 315 nm and scanning at 50 nm/min from 350–475 nm.

A portion of the dried residue of the HPLC-purified leaf extract was dissolved in a small volume of 100% methanol, and an excess of diazomethane in ether was added. After 30 min at room temperature, samples were dried under N_2. The residue was dissolved in dichloromethane, and the sample was analyzed by gas chromatography/mass spectrometry using a Varian 3400 gas chromatograph (Varian Walnut Creek Instrument Div., Walnut Creek, CA) interfaced to a Finnigan MAT 8230 high-resolution magnetic sector double-focusing mass spectrophotometer (Finnigan Corp., San Jose, CA). A DB-5 capillary column (30 m × 0.32 mm, 0.25 μm film thickness; J. & W. Instruments, Inc., New Brighton, MN) was used with a column temperature program (50 C for 3 min, 50–300 C at 10 C/min). The mass spectrum of the fraction coeluting with SA-methyl ester (retention time: 9.5 min) was determined in the electron impact mode by scanning the mass spectrum from 35 to 350 atomic mass units at 1 sec/decade and using a Finnigan MAT SS 300 data system for acquisition and data processing.

An aliquot of the HPLC-purified leaf extract was resuspended in 55% (v/v) methanol in water and injected onto a Beckman Ultrasphere ODS 5 μm C-18 guard column (4.6 mm × 4.5 cm; Beckman Instruments, Inc., Fullerton, CA) linked to a Beckman Ultrasphere ODS 5 μm C-18 column (4.6 mm × 25 cm) and a Beckman System Gold HPLC. Sample constituents were separated using a linear 40-min gradient of 0–100% (v/v) acetonitrile against 1% (v/v) acetic acid in water at a flow rate of 1 ml/min. An absorption spectrum of a fraction coeluting with authentic SA was obtained with a Beckman Model 168 diode array detector module.

PR protein analysis. Leaf tissue (1 g) was pulverized in liquid N_2 and suspended in 60 mM sodium phopshate-citrate buffer, pH 2.8 (1 ml/g tissue), containing 6 mM L-ascorbic acid and 14 mM 2-mercaptoethanol. After thawing, the homogenate was centrifuged at 10,000 *g* for 30 min. The soluble proteins in the supernatant were analyzed by nondenaturing polyacrylamide gel electrophoresis (PAGE) as previously described (30). The identity of b1″ protein was confirmed by probing Western blots with antiserum raised against PR-1a from Xanthi-nc. This immuneserum is known to cross-react with b1″ protein (1). Protein content was determined using the Bio-Rad protein assay dye reagent with bovine serum albumin as a standard (Bio-Rad Laboratories, Inc., Richmond, CA).

RESULTS

Resistance and accumulation of SA and PR proteins in Xanthi-nc and *N. glutinosa* × *N. debneyi*. Extracts from uninoculated leaves of healthy 8-wk-old *N. glutinosa* × *N. debneyi* plants contained a fluorescent metabolite that cochromatographed with authentic SA (Fig. 1A and B). This substance was identical to SA as confirmed by its fluorescence emission spectrum (Fig. 1C and D), its coelution with authentic SA under different chromatographic conditions (data not shown), its UV-absorption spectrum (Fig. 1E and F), and by gas chromatography-mass spectral analysis (Fig. 1G and H). Furthermore, analysis of identical leaf extracts by fluorescence emission and UV-absorption yielded similar estimates of leaf levels of SA: 2.31 μg/g fresh weight. SA levels were about 30-fold higher in uninoculated leaves of *N. glutinosa* × *N. debneyi* than in the leaves of *N. tabacum* 'Xanthi-nc' (Fig. 2A, day 0, Table 1).

Previously it was shown that a SA-conjugate, presumably *O*-β-D-glucosylSA, accumulates in TMV-inoculated or SA-treated tissues of Xanthi-nc tobacco (4). Levels of conjugated SA can be estimated as the difference between SA contents of β-glucosidase-hydrolyzed and unhydrolyzed leaf extracts. β-Glucosidase hydrolysis of extracts from healthy leaves of *N. glutinosa* × *N. debneyi* liberated an additional 6.85 μg SA/g fresh weight (Fig. 2, day 0). No *O*-β-D-glucosylSA was detected in uninoculated leaves of Xanthi-nc.

Exudation of SA with the phloem sap of TMV-inoculated Xanthi-nc leaves was previously shown to accompany the appearance of HR, PR proteins, and SAR (30). SA was below the level of detection (0.005 μg/g fresh weight) in exudates of healthy Xanthi-nc leaves. However, *N. glutinosa* × *N. debneyi* leaves exuded 0.52 μg SA/g fresh weight/48 h.

The effect of TMV-inoculation on lesion diameter and levels of SA and PR proteins in *N. glutinosa* × *N. debneyi* and Xanthi-nc was examined. Total numbers of lesions per unit leaf area were similar in the hybrid and in Xanthi-nc. The levels of free SA and its conjugate increased dramatically in leaves of Xanthi-nc tobacco 3 days after TMV inoculation (Fig. 2A and B). In *N. glutinosa* × *N. debneyi*, however, free and conjugated SA levels remained relatively constant. TMV-inoculation strongly induced accumulation of PR-1 proteins in Xanthi-nc but did not significantly affect the amount of b1″ protein in the hybrid (Fig. 3). Five days after inoculation, the mean diameter of individual TMV-induced lesions on *N. glutinosa* × *N. debneyi* was 0.2 ± 0.1 (±SE) mm compared to 1.8 ± 0.1 mm on Xanthi-nc.

Spraying healthy Xanthi-nc with SA (2.5 mM) resulted in induction of PR-1 proteins, but in *N. glutinosa* × *N. debneyi*

no major changes in the levels of b1″ protein were observed (Fig. 4). This suggests that the response to SA in the hybrid is not sensitive to increasing tissue SA beyond the basal levels.

Effect of temperature on resistance and accumulation of SA and PR proteins in Xanthi-nc and *N. glutinosa* × *N. debneyi*. In TMV-inoculated Xanthi-nc tobacco, the HR and associated accumulation of SA and PR proteins is blocked if plants are kept above 28 C (12,30). However, *N. glutinosa* × *N. debneyi* develops HR lesions even if incubated at 31 C (2). We observed that by 3 days after inoculation, TMV-inoculated *N. glutinosa* × *N. debneyi* kept at either 24 or 32 C developed lesions with an individual diameter of 0.2 (±0.1) mm. In contrast, Xanthi-nc showed no HR at 32 C but developed lesions with a diameter of 1.6 (±0.1) mm at 24 C.

The levels of SA and its conjugate in mock-inoculated *N. glutinosa* × *N. debneyi* and Xanthi-nc were not significantly affected by incubation temperature (Table 1). However, the content of SA and its conjugate was lower in TMV-inoculated plants

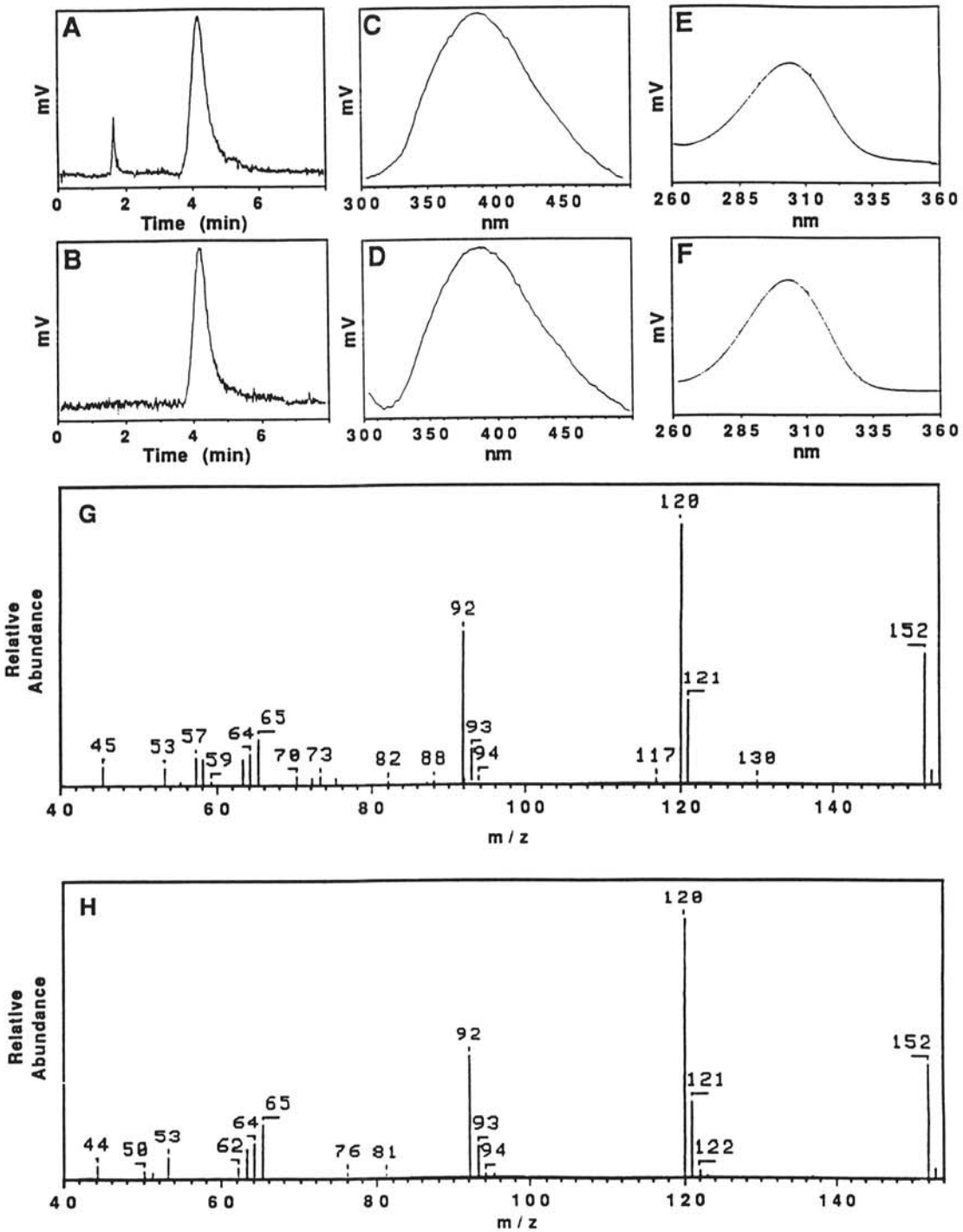

Fig. 1. Identification of salicylic acid in leaf extracts of uninoculated *Nicotiana glutinosa* × *N. debneyi* hybrid. **A,** HPLC-elution profile of *N. glutinosa* × *N. debneyi* leaf extract (fluorescence at 405 nm; excitation at 315 nm). The material eluting between 4 and 5 min was collected and termed HPLC-purified leaf extract. **B,** Same as in **A** but for authentic SA. **C,** Fluorescence emission spectrum from 350–475 nm of the HPLC-purified leaf extract upon excitation at 315 nm. **D,** Same as **C** but for authentic SA. **E,** UV-absorption spectrum of a fraction that coeluted with authentic SA after a second chromatographic fractionation of the HPLC-purified leaf extract. **F,** Same as in **E** but for authentic SA. **G,** Electron impact mass spectral analysis of HPLC-purified leaf extract. Diazomethane-treated HPLC-purified leaf extract used for **C** and **E** was injected onto a GLC interfaced with a mass spectrometer. The mass spectrum of the fraction coeluting with authentic 2-hydroxybenzoic acid methyl ester (SA-methyl ester) is shown. **H,** Same as in **G**, but for authentic SA-methyl ester.

of both genotypes kept at 32 C compared to those kept at 24 C. The levels of free SA in TMV-inoculated *N. glutinosa* × *N. debneyi* kept at 32 C were 88% lower than in TMV-inoculated *N. glutinosa* × *N. debneyi* at 24 C and 73% lower than levels found in mock-inoculated leaves exposed to 32 C. However, the amount of glucosylSA present in the TMV-inoculated leaves of *N. glutinosa* × *N. debneyi* kept at 32 C was only 36% lower than that of mock-inoculated leaves exposed to the same temperature. Consistent with previous reports, PR protein accumulation in TMV-inoculated Xanthi-nc was inhibited by incubation at 32 C but was not affected in *N. glutinosa* × *N. debneyi* (Fig. 5).

Effect of tobacco age and leaf position on constitutive levels of SA, PR-1 proteins, and resistance to TMV. We investigated whether the previously reported presence of PR proteins and elevated TMV-resistance in the lower leaves of flowering tobacco correlates with increased SA levels. Three developmental stages of Xanthi-nc tobacco were chosen: plants at stages 1, 2, and 3 were 6, 10, and 13 wk old, respectively. At stage 2, the first blossoms were opening. At stage 3, the inflorescence was beginning to form seeds. Upper, middle, and lower nonsenescing leaves from healthy plants at these three developmental stages were analyzed for SA, PR-1 proteins, and resistance to TMV.

At stage 1, levels of SA in the foliage of Xanthi-nc were about 0.07 μg/g fresh weight and were independent of leaf position (Fig. 6). However, a basipetal gradient in SA levels was detected in plants at stages 2 and 3. Levels of SA in the lower leaves were about two to three times higher than in apical or middle positioned leaves. The increased levels of SA in the lower leaf correlated with the accumulation of PR-1 proteins (Fig. 7). Leaves that showed elevated levels of SA and PR proteins also exhibited increased resistance to TMV, measured as a reduction in lesion diameter (Fig. 8). When comparing upper leaves of stage 1 plants with the same leaves of older plants, we observed some decrease in lesion diameter with plant age that was not accompanied by an increase in SA or PR-1 proteins (Figs. 6 and 7). Not only size but morphology of TMV-induced lesions was affected by

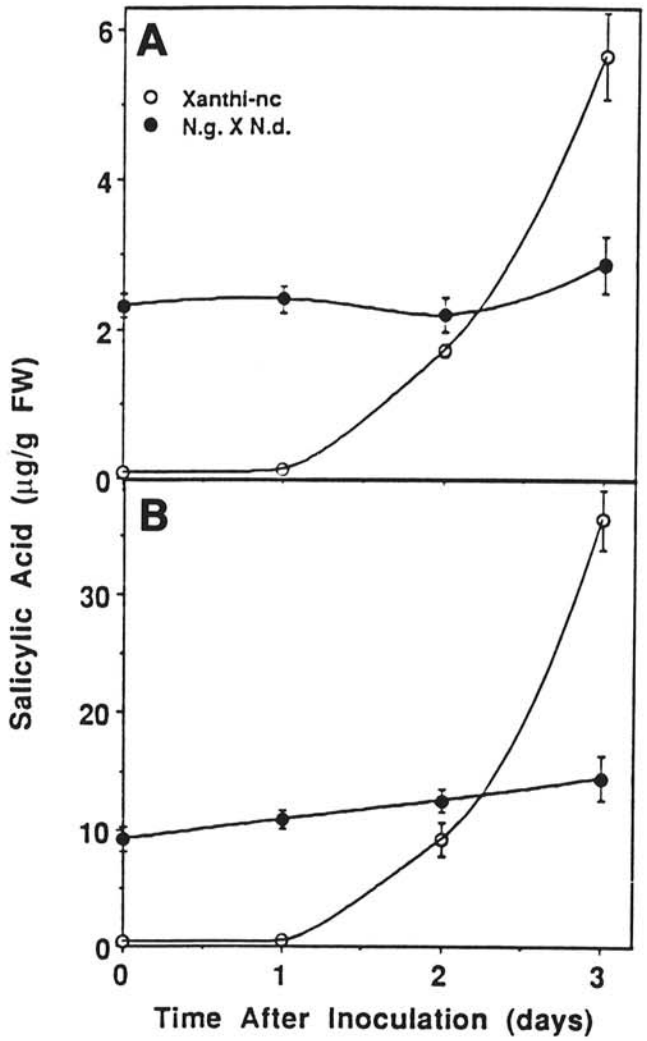

Fig. 2. Effect of TMV inoculation on levels of free and total salicylic acid in Xanthi-nc and *Nicotiana glutinosa* × *N. debneyi* tobacco. Plants were inoculated with TMV on day 0. Each bar is the mean of four replicate leaf extracts (+SE). The experiment was repeated once with similar results. FW, fresh weight. **A,** Free SA. **B,** Total SA.

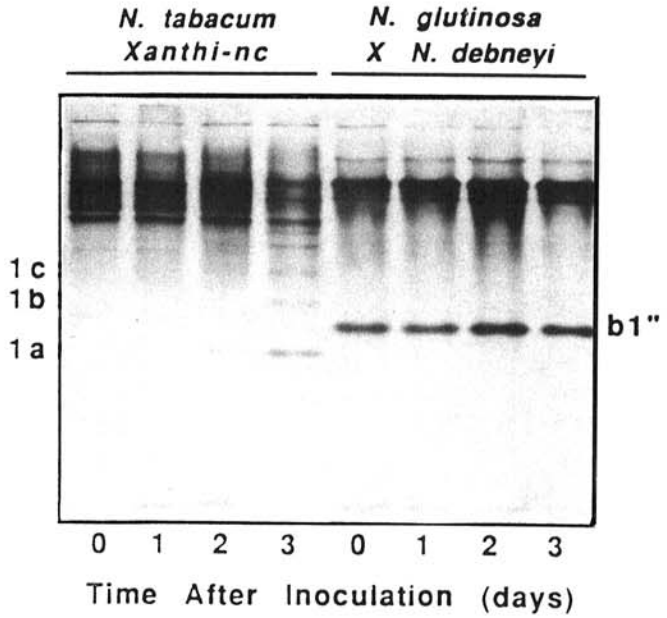

Fig. 3. Effect of TMV inoculation on pathogenesis-related protein accumulation in Xanthi-nc and *Nicotiana glutinosa* × *N. debneyi* tobacco. Nondenaturing gel electrophoresis and silver-staining of acidic proteins from leaves that were used for Figure 2A and B.

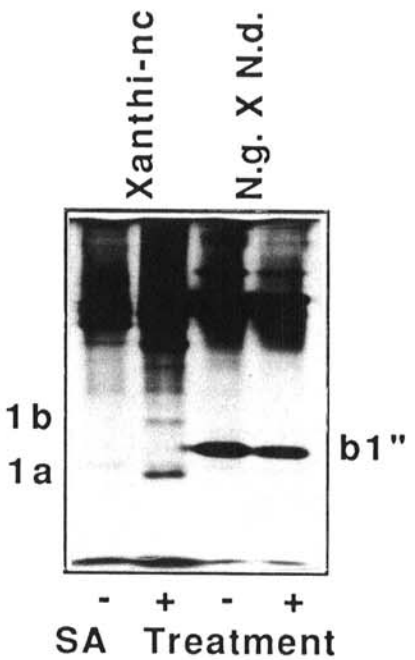

Fig. 4. Nondenaturing gel electrophoresis and silver-staining of acidic proteins from Xanthi-nc and *Nicotiana glutinosa* × *N. debneyi* tobacco treated with salicylic acid. Leaves of Xanthi-nc and *N. glutinosa* × *N. debneyi* tobacco seedlings were lightly rubbed with Carborundum and then sprayed with 10 mM potassium phosphate buffer, pH 6.5 with (+) or without (−) 2.5 mM SA and sampled 5 days later.

leaf position and developmental stage. Lesions on upper leaves of stage 1 plants showed a broad dark-brown zone at the periphery, whereas in older leaves or flowering plants no such zone was apparent (Fig. 9).

DISCUSSION

Systemic increases in SA, which follow local inoculation with necrotizing pathogens, are believed to trigger the induction of PR proteins and systemic resistance throughout the plant (4,11, 13,30). We showed earlier that SA produced naturally during TMV-inoculation is sufficient for the induction of TMV resistance and PR protein accumulation observed during SAR (4,30).

SA levels in healthy leaves of the highly TMV-resistant *N. glutinosa* × *N. debneyi* hybrid, which constitutively express PR proteins, were 30 times higher than the SA levels in the young, unchallenged leaves of Xanthi-nc tobacco, which show lower resistance to TMV and no detectable PR proteins (Figs. 2 and 3). The amount of SA present in the leaves of a healthy hybrid is much greater than needed for the induction of PR proteins and TMV-resistance in Xanthi-nc tobacco (4). No qualitative or quantitative changes in the acidic proteins were induced by treating *N. glutinosa* × *N. debneyi* with TMV (Fig. 3) or SA (Fig. 4). This suggests that the induction of PR proteins by SA is close to saturation in this genotype.

In Xanthi-nc tobacco, SA levels increased 70-fold 3 days after TMV inoculation. This increase in endogenous SA was accompanied by a corresponding increase in glycosylSA (Fig. 2A and B). However, the levels of free and conjugated SA in the *N. glutinosa* × *N. debneyi* plants were not greatly affected by TMV (Fig. 2 and Table 1). This may indicate that the plant's ability to produce SA is almost independent of the HR. Alternatively, the lack of large SA increases in the TMV-inoculated hybrid may be explained by the fact that the HR lesions in *N. glutinosa* × *N. debneyi* were 80 times smaller than in Xanthi-nc tobacco. Earlier we showed that SA is produced in the vicinity of the HR lesions (4).

At this time we do not know the reasons for the high basal levels of SA in the hybrid. It is unlikely that the metabolism of SA is inhibited in the *N. glutinosa* × *N. debneyi* plants, since the leaves of the hybrid have a ratio of free/conjugated SA that is similar to that observed in the TMV-inoculated Xanthi-nc

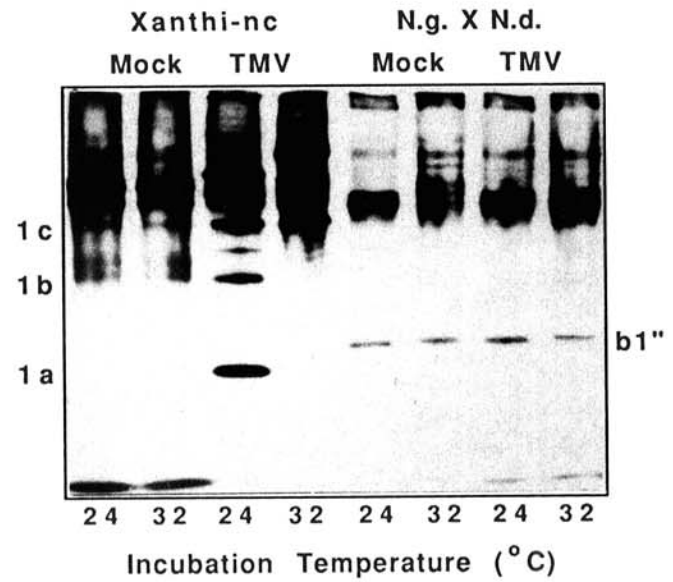

Fig. 5. Effect of incubation temperature and TMV inoculation on accumulation of pathogenesis-related proteins in Xanthi-nc and *Nicotiana glutinosa* × *N. debneyi* tobacco. Nondenaturing gel electrophoresis and silver-staining of acidic proteins from leaves that were also used for Table 1.

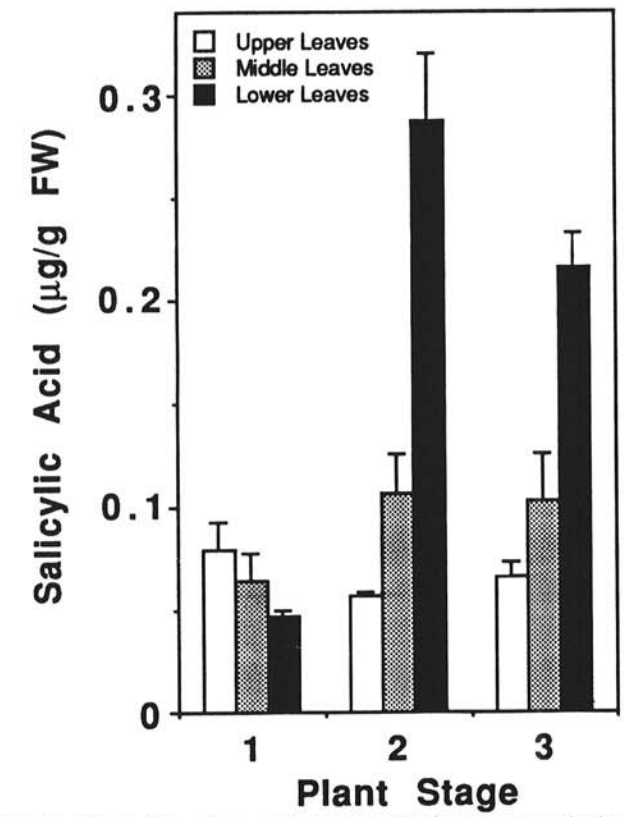

Fig. 6. Effect of developmental stage and leaf position on the levels of salicylic acid in Xanthi-nc tobacco. Upper, middle, and lower leaves from plants at the developmental stages described in Results were analyzed for levels of free SA. The experiment was repeated four times with similar results. Each bar is the mean of extracts from four replicate leaves (+SE).

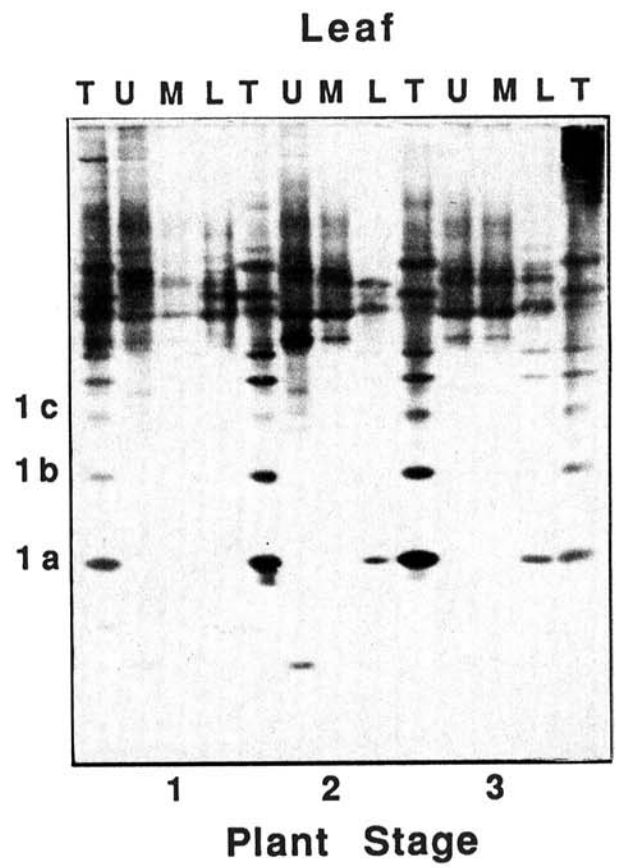

Fig. 7. Effect of developmental stage and leaf position on accumulation of pathogenesis-related proteins. Nondenaturing gel electrophoresis and silver-staining of acidic proteins extracted from uninoculated plants that were also used for Figure 6. U, upper, M, middle, L, lower, T, TMV-inoculated leaves.

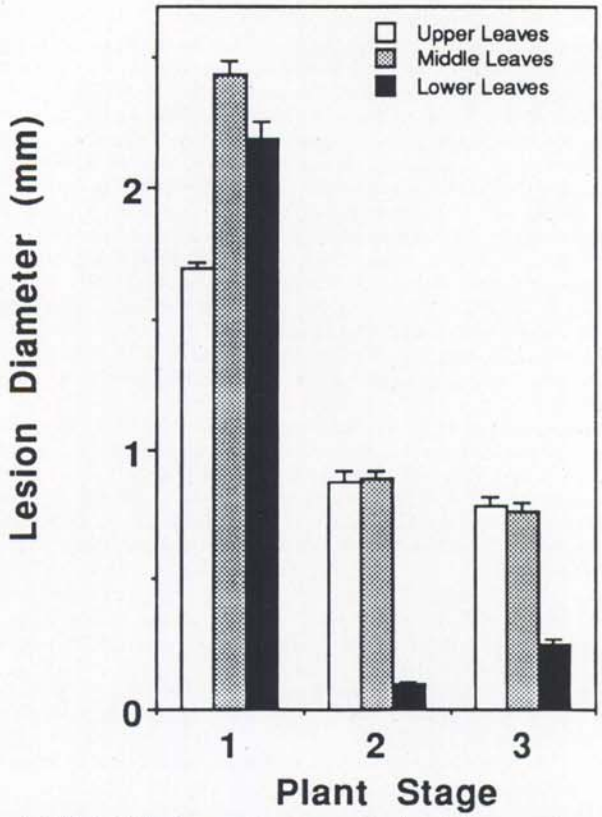

Fig. 8. Effect of developmental stage and leaf position on resistance of Xanthi-nc tobacco to TMV. Upper, middle, and lower leaves from plants at the developmental stages described in Results. Diameter of TMV-induced HR lesions 5 days after inoculation with 2.5 μg TMV/leaf. Each bar is the mean (+SE) of at least 50 lesion measurements on leaves from four different plants.

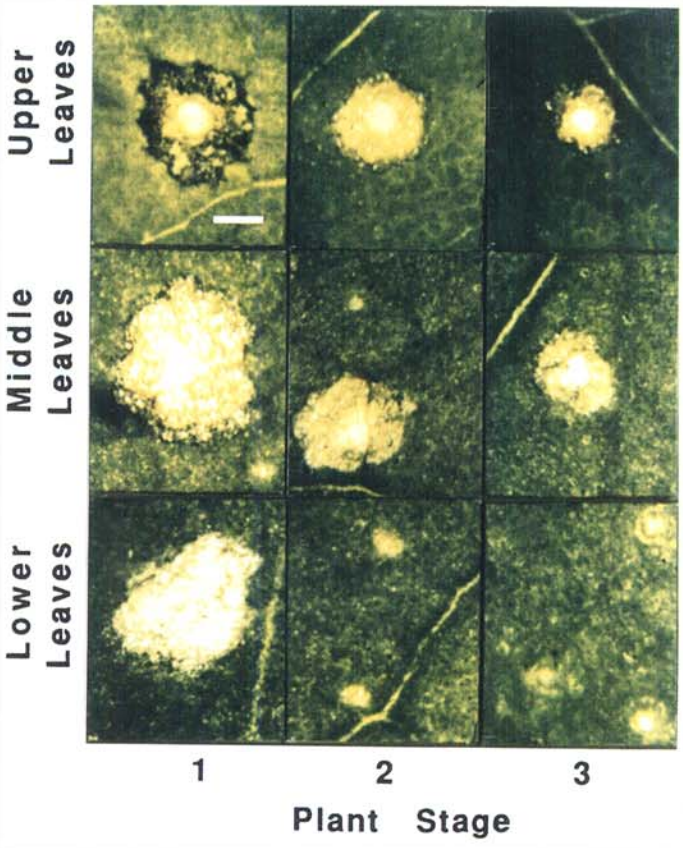

Fig. 9. Developmental and leaf-position variations in TMV-induced lesions. Micrographs of representative lesions analyzed in Figure 8 were taken 5 days after treatment with TMV. Bar = 0.5 mm.

tobacco during the HR. It is possible, however, that high levels of endogenous SA in *N. glutinosa* × *N. debneyi* result from the constitutively higher activity of the rate-limiting enzymes which convert *trans*-cinnamic acid to SA (17). This was shown to be true for TMV-inoculated Xanthi-nc tobacco (Leon, Yalpani, Raskin, Lawton, *unpublished data*).

SAR, HR, and the accumulation of PR proteins is inhibited if TMV-inoculated Xanthi-nc tobacco is kept at 32 C (8,25). Unlike plants kept at 24 C, inoculated Xanthi-nc tobacco plants kept at 32 C fail to accumulate SA (12,30). While the content of SA in TMV-inoculated *N. glutinosa* × *N. debneyi* kept at 32 C was lower than at 24 C (Table 1), TMV resistance and PR protein accumulation in this plant were not affected by higher temperature (Figs. 2 and 5). This may be because the lower levels of SA present at 32 C (0.57 μg/g fresh weight) are still sufficient to induce PR proteins (30) and resistance (4) in *N. glutinosa* × *N. debneyi* plants. Alternatively, the PR proteins and other factor(s) which maintain high TMV resistance in the hybrid kept at 24 C may have persisted after transfer to 32 C. We do not have an explanation for the reduction in the SA levels produced by the TMV-inoculation of *N. glutinosa* × *N. debneyi* leaves at 32 C. Mock-inoculated leaves of *N. glutinosa* × *N. debneyi* contained the same amount of SA at 24 and 32 C. It is possible that SA conjugation and further metabolism of the conjugate is stimulated by the HR to TMV. This stimulation may explain our observation that in infected leaves of *N. glutinosa* × *N. debneyi* kept at 32 C, the ratio of free SA to total SA was 2.4 times lower than in mock-inoculated plants kept at the same temperature.

Coincident with the increase in resistance and accumulation of PR proteins in the lower leaves of flowering Xanthi-nc tobacco, there was up to a fivefold increase in the levels of SA (Figs. 6–9). Compared to the levels observed upon TMV infection, the increase in SA associated with aging was relatively small. However, we found that even a 50% increase in SA above levels found in healthy leaves of 6 to 8 wk-old Xanthi-nc plants can induce significant PR protein accumulation (30). This, together with the observation that tissue accumulation of SA increases resistance (4), suggests that the developmental changes in resistance to TMV (manifested as the severity of the HR) are mediated by SA. It is possible that sensitivity of tobacco to SA changes under the influence of environmental factors or during development. This may explain the occasional lack of perfect correlation between the SA levels, PR proteins, and lesion size. A developmental change of tissue sensitivity to SA has already been observed in the thermogenic inflorescence of voodoo lilies where SA induces heat production through the activation of the cyanide-insensitive respiration (19). In addition to SA-mediated resistance, a number of other factors could affect the ability of

TABLE 1. Effect of incubation temperature and tobacco mosaic virus inoculation on free and total salicylic acid levels in *Nicotiana tabacum* 'Xanthi-nc' and *N. glutinosa* × *N. debneyi* tobacco

	Leaf SA[b] (μg/g fresh weight)			
	Xanthi-nc		N.g. × N.d.[c]	
SA level[a]	24 C	32 C	24 C	32 C
Free SA				
Mock	0.07 ± 0.01	0.08 ± 0.03	2.20 ± 0.21	2.12 ± 0.23
TMV[d]	2.28 ± 0.79	0.12 ± 0.03	4.67 ± 0.71	0.57 ± 0.03
Total SA				
Mock	0.11 ± 0.01	0.09 ± 0.01	8.91 ± 0.85	11.03 ± 0.76
TMV	17.05 ± 1.26	0.65 ± 0.04	15.56 ± 0.71	7.18 ± 0.43

[a] SA = salicylic acid.
[b] Plants were preincubated for two days at 24 or 32 C. Upon TMV inoculation they were further incubated at the same temperature and sampled three days later. Each value is the mean of four replicate leaf extracts (± SE). Similar results were obtained in three sets of replicated experiments. The results of experiment 1 are shown.
[c] *N. glutinosa* × *N. debneyi*.
[d] Tobacco mosaic virus.

the virus to infect and propagate in leaf tissue. For example, older leaves are more heavily lignified, providing additional barriers for pathogen multiplication and spread.

Our results further support the hypothesis that SA plays a regulatory role in the induction of PR proteins and disease resistance. *N. glutinosa* × *N. debneyi* hybrid and lower leaves of flowering Xanthi-nc tobacco may serve as examples of plants or tissues which use SA to activate resistance mechanisms. Therefore, it is tempting to speculate that crops with elevated levels of endogenous SA should have greater pathogen resistance and increased yield. The elucidation of the pathway of SA biosynthesis in plants will lead to the isolation of genes responsible for the rate-limiting steps in SA production. Eventually, transgenic plants with higher levels of SA will be created, allowing a direct testing of the role of SA in disease resistance.

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