Salicylic Acid, Ethylene, and Pathogen Resistance in Tobacco

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Received 9 April 1993. Accepted 1 July 1993.

Salicylic acid (SA) plays an important regulatory role in the resistance response of N-gene tobacco to tobacco mosaic virus (TMV). To determine whether SA accumulation following inoculation with a necrotizing pathogen is a generalized phenomenon, endogenous SA levels were quantified following inoculation of two species of *Nicotiana* with viral, bacterial, and fungal pathogens. In Xanthi-nc (NN) and Xanthi (nn) cultivars of *Nicotiana tabacum*, tobacco necrosis virus produced necrotic lesions and a more than 28-fold increase in total SA (the sum of free and β-*O*-D-glucosyl SA) within 96 hr. Significant increases in SA were also observed in *Nicotiana sylvestris* inoculated with a mutant TMV strain capable of producing necrotic lesions in this tobacco species. Infiltration of Xanthi-nc and Xanthi tobacco leaves with *Pseudomonas syringae* pv. *tomato* also produced a 100-fold increase in total SA within 72 hr. Stem injection with blue mold (*Peronospora tabacina*) sporangia produced 3.6- and 18.8-fold increases of free and total SA, respectively, in previously uninfected leaves, which coincided with an increase in resistance. Exposure of TMV-inoculated tobacco leaf disks to ethylene (10 μl/L) resulted in a reduction in SA accumulation. However, an inhibitor of ethylene action, 2,5-norbornadiene, did not produce a significant change in SA accumulation in TMV-inoculated leaf tissues. The relatively minor negative effect of ethylene on SA production suggests that ethylene is not directly involved in the signal transduction pathway that leads to SA accumulation and export from the tissues infected with necrotizing pathogens.

Additional keywords: pathogenesis-related proteins, systemic acquired resistance.

During an incompatible plant-pathogen interaction, a localized necrosis, often called the hypersensitive response (HR), may develop (Klement 1982). Following the HR, a state of heightened resistance to subsequent pathogen attack appears in pathogen-free parts of the plant. This inducible resistance, termed systemic acquired resistance (SAR) by Ross (1961), is effective against viruses, fungi, and bacteria. SAR implies the existence of a signal molecule produced in the hypersensitively responding tissues that moves throughout the plant activating resistance (Ross 1966). Recent evidence suggests that salicylic acid (SA) may serve as one of the signals in SAR (for review, see Raskin 1992).

It is well established that resistance to pathogens and the production of pathogenesis-related proteins (PR proteins) in plants can be induced by SA or acetylsalicylic acid, even in the absence of pathogenic organisms. The discovery of the protective function of salicylates was made in tobacco (*Nicotiana tabacum* L. 'Xanthi-nc') by White (1979). The involvement of endogenous SA in tobacco SAR was suspected after the discovery of local and systemic increases in SA in tobacco mosaic virus (TMV)-resistant N-gene tobacco leaves (cv. Xanthi-nc) following inoculation with TMV (Malamy *et al.* 1990; Enyedi *et al.* 1992a). A 20-fold increase in free SA in TMV-inoculated tobacco leaves is accompanied by the appearance of an SA-glucose conjugate, β-*O*-D-glucosyl SA (Enyedi *et al.* 1992a; Malamy *et al.* 1992; Enyedi and Raskin 1993). This conjugate, adjacent to the HR lesion, is present at much higher levels than free SA. In spite of the fact that a substantial amount of SA is sequestered in a conjugated form, the levels of free SA remain sufficient for the systemic induction of PR-proteins and resistance to a subsequent challenge by TMV (Yalpani *et al.* 1991; Enyedi *et al.* 1992a).

A separate set of experiments implicated SA as a phloem-transported signal for SAR in cucumbers. Following inoculation of cucumber with necrotizing bacteria, fungus, or virus, SA levels in the phloem increased dramatically (Metraux *et al.* 1990; Rasmussen *et al.* 1991). Increases in phloem SA were observed as early as 8 hr after inoculation with *Pseudomonas syringae* pv. *syringae*, and increased resistance was observed within 24 hr (Rasmussen *et al.* 1991; Smith *et al.* 1991). The increases of SA levels in the phloem are correlated with SAR (Metraux *et al.* 1990), which may last for 3–6 wk in cucumber (Kuc and Richmond 1977). However, the role of SA as the primary signal in cucumber SAR was recently questioned (Rasmussen *et al.* 1991).

Ethylene, a gaseous plant hormone, is also produced in tissues undergoing the HR (De Laat *et al.* 1981). Ethylene was suspected as a possible SAR signal because its application induces lignification (Rhodes and Wooltorton 1973), and treatment with ethephon, an ethylene-releasing compound, induces some PR proteins (Brederode *et al.* 1991). Although treatment of tobacco with ethylene can reduce disease severity (Van Loon 1977), ethylene synthesis does not increase systemically during SAR. It is also unclear whether ethylene is produced in levels sufficient for the induction of even a localized resistance response (for review, see Enyedi *et al.* 1992b). Nothing is known about the interactions between ethylene and SA during pathogenesis. However, in pear cell

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MPMI Vol 6, No.6, 1993, pp. 775-781

suspension culture, high levels of exogenously applied SA inhibited the conversion of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid to ethylene (Leslie and Romani 1986).

SA is emerging as an important signal molecule in SAR in spite of the few systems in which it has been investigated. So far, increases in SA during pathogenesis have been measured only in the Xanthi-nc tobacco-TMV system (both tissue and phloem) and in cucumber (phloem only) (for review, see Yalpani and Raskin 1993). The present investigation was undertaken in part to determine whether changes in SA levels always accompany the establishment of SAR in different tobacco cultivars and *Nicotiana* species inoculated with necrotizing viral, bacterial, and fungal pathogens. Three cultivars of *N. tabacum*, with or without the N-gene, and *N. sylvestris* Speg. & Comes, carrying the N′ resistance gene to TMV, were used in this study. Since ethylene may serve as another endogenously produced inducer of plant defense systems, we investigated the correlations between SA accumulation/conjugation and ethylene levels in tobacco. Our goal was to establish whether ethylene precedes SA in the signal transduction chain leading to SAR.

RESULTS

Induction of SA by viral pathogens of tobacco.

The various pathogens used in this study are summarized in Table 1. TMV does not induce the HR, SAR, PR proteins, or SA production in nn-genotype tobacco, *N. tabacum* (reviewed by Yalpani and Raskin 1993). However, tobacco necrosis virus (TNV) induces an HR in both nn (cv. Xanthi) and NN (cv. Xanthi-nc) genotypes. To determine the connection between the N-gene and the ability of plant viruses to induce SA production, both Xanthi-nc and Xanthi tobacco cultivars were inoculated with TNV.

Figure 1 illustrates the TNV-induced SA accumulation observed in the inoculated leaves of both tobacco cultivars. Forty-eight hours postinoculation (PI), the levels of free and total SA had increased fourfold and 12.9-fold, respectively, in Xanthi-nc tobacco and 3.3- and 19.7-fold, respectively, in Xanthi tobacco. Furthermore, accumulation of free and total SA in nn and NN genotype tobaccos followed similar kinetics, with the level of total SA increasing over 28-fold in both cultivars by 96 hr PI. Both TNV-inoculated cultivars developed morphologically similar HR lesions about 48 hr PI. The TNV-induced lesions continued to expand throughout the experiment (to 96 hr).

Table 1. List of pathogens used in this study

Pathogen	Source	Reference
TMV Strain U1	T. M. A. Wilson, Scottish Crop Research Institute	Yalpani *et al.* (1991)
TMV Ni 118 (U1)	T. M. A. Wilson	Wittmann *et al.* (1965)
Tobacco necrosis virus strain A	P. Romaine, Penn State Univ.	Babos and Kassanis (1963)
Pseudomonas syringae pv. *tomato* (PT-23)	D. Kobayashi, Rutgers Univ.	Kobayashi *et al.* (1989)
Peronospora tabacina	J. Kuć, Univ. of Kentucky	Tuzun *et al.* (1989)

N. sylvestris containing the N′ gene does not respond hypersensitively to the U1 (*vulgare*) strain of TMV. However certain nitrous acid-induced mutants of U1 TMV induce the HR in *N. sylvestris* (Wittmann *et al.* 1965). To see whether pathogen-induced SA increases occur in different *Nicotiana* species, we inoculated *N. sylvestris* leaves with 25 µg per leaf of the TMV muant Ni 118. Within 48 hr of inoculation and concurrent with the appearance of HR lesions, free and total SA levels each increased about 4.5-fold above the levels in mock-inoculated leaves (Fig. 2). By 96 hr PI, free and total SA levels in inoculated leaves increased sixfold above the levels present in mock-inoculated tissues sampled at the same time (Fig. 2B). No significant changes in SA levels were observed in the mock-inoculated leaves throughout the experiment.

Induction of SA by bacterial pathogens on tobacco.

The levels of free and total SA increased in Xanthi and Xanthi-nc tobacco leaves following inoculation with a bacte-

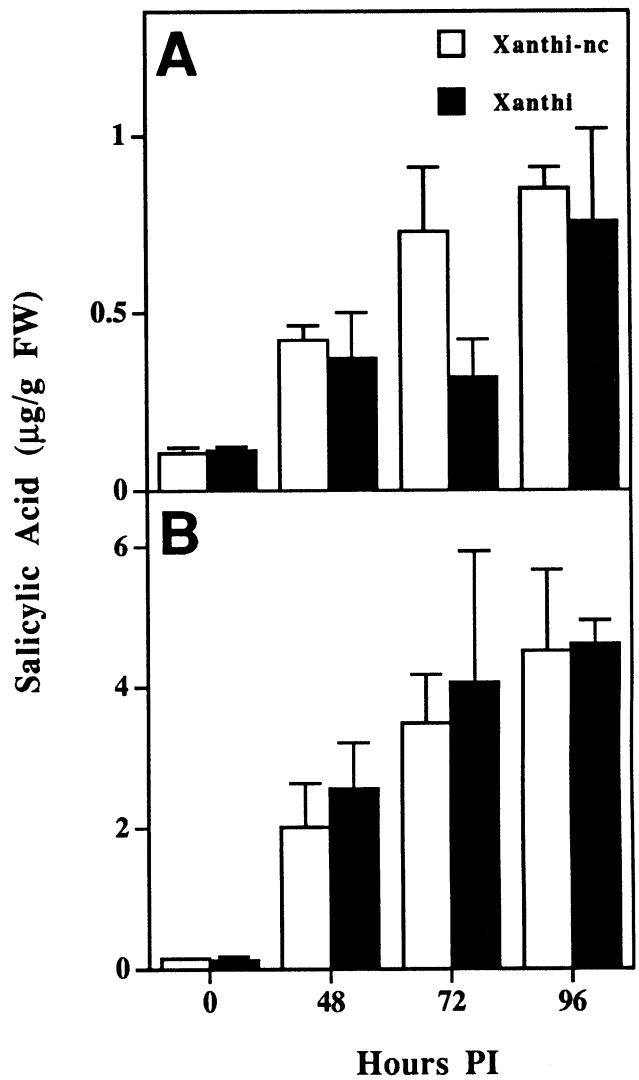

Fig. 1. Effect of inoculation with tobacco necrotic virus (TNV) (33 µg of TNV per leaf) on the levels of **A,** free and, **B,** total salicylic acid in Xanthi and Xanthi-nc tobacco. Each bar is the mean of four replicate leaf extracts (± SE). The experiment was repeated twice with similar results. FW, fresh weight; PI, postinoculation.

rial pathogen of tomato, *P. s.* pv. *tomato* (Fig. 3). Following bacterial inoculation, both cultivars developed similar HR lesions at the site of pathogen inoculation; these were first apparent at 18 hr PI and continued to expand to 15–20 mm in diameter. Twenty-four hours PI, the levels of free SA in the vicinity of the HR lesions had increased 38-fold in Xanthi tobacco and 13-fold in Xanthi-nc (compared to levels in mock-inoculated tissues) (Fig. 3A). This difference in free SA levels between the two cultivars may be explained by the smaller lesion size produced in Xanthi-nc tobacco in response to *P. s.* pv. *tomato*. Seventy-two hours PI, levels of total SA had increased more than 100-fold in both tobacco cultivars (Fig. 3B), with 92% of SA in Xanthi-nc tobacco and 73% in Xanthi tobacco present as a conjugate. The differences in the levels of total and free SA in both cultivars were not significant within $P = 0.05$ using a Student's *t*-test (Steel and Torrie 1980). Total SA accumulation in the vicinity of the *P. s.* pv. *tomato*-induced lesions by 72 hr PI produced a concentration of about 1 mM, using a leaf water content value of 82%, as previously measured for tobacco leaves.

Induction of SA by fungal pathogens of tobacco.

Stem injection of tobacco with a sporangial suspension of *Peronospora tabacina*, the causal agent of blue mold, gives rise to the SAR response by 21 days PI (Madamanchi and Kuc 1991). We observed that stem injection of the tobacco cultivar KY-14 with *P. tabacina* sporangia elevates SA levels in the third and fourth systemically protected leaves (Fig. 4). By 13 days PI, the level of free SA had nearly doubled, while the level of total SA increased 8.7-fold compared to that in leaves of mock-inoculated plants (Fig. 4B). Twenty-one days after stem injection, resistance to challenge with blue mold was 75% greater in *P. tabacina* stem-injected plants than in water-injected controls. At the same time, the levels of SA in the leaves of *P. tabacina*-protected plants increased 3.6- and 18.8-fold for free and total SA, respectively, above the levels

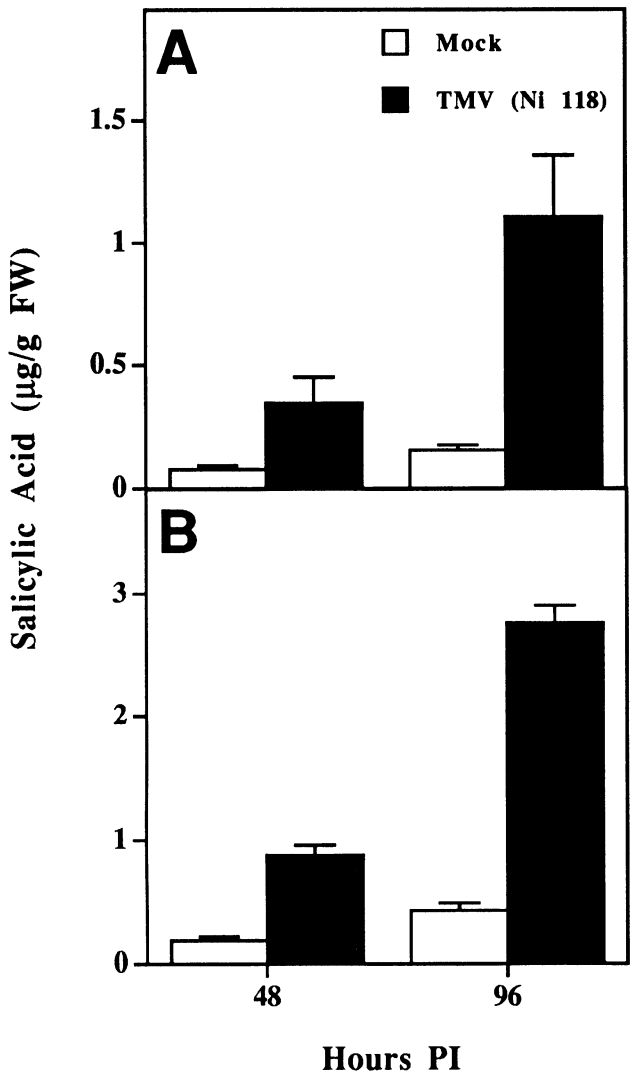

Fig. 2. Effect of inoculation with a tobacco necrosis virus (TMV) coat protein mutant (Ni 118) (25 µg per leaf) on levels of **A,** free and, **B,** total salicylic acid in *Nicotiana sylvestris*. Each bar is the mean of four replicate leaf extracts (± SE). The experiment was repeated twice with similar results. FW, fresh weight; PI, postinoculation.

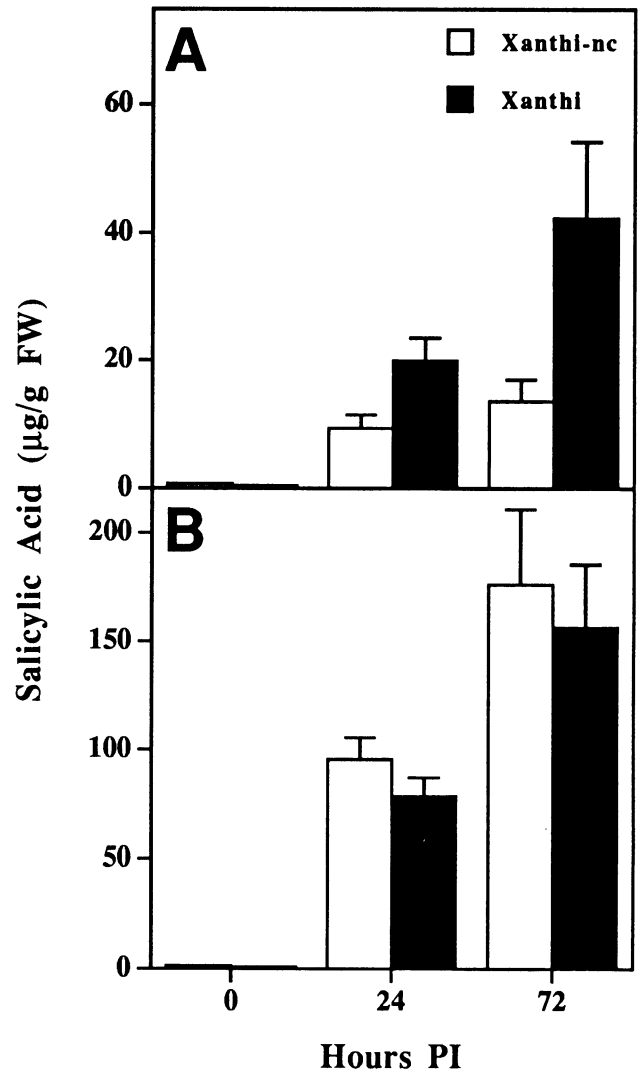

Fig. 3. Effect of infiltration with *P. syringae* pv. *tomato* on levels of **A,** free and **B,** total salicylic acid in the vicinity of hypersensitive response lesions in Xanthi and Xanthi-nc tobacco. The area around the infiltration site was harvested with a 21-mm cork borer at the times indicated. Each bar is the mean of four replicate leaf extracts (± SE). The experiment was repeated twice with similar results.

in water-injected controls (Fig. 4). Thus, resistance to *P. tabacina* induced by stem injection of *P. tabacina* sporangia is correlated with increased endogenous SA levels.

Ethylene and SA.

Ethylene may play a role in the induction of pathogen defense in plants. To investigate a possible interaction between ethylene and SA in and around HR lesions, TMV-inoculated Xanthi-nc tobacco leaf disks were incubated in the presence or absence of ethylene (10 μl/L). Ethylene treatment resulted in a 2.8-fold reduction in free SA levels 72 hr PI and a 3.7-fold reduction by 120 hr PI (Table 2). By 120 hr PI, ethylene had reduced the total SA accumulation in TMV-inoculated leaf disks by 2.2-fold. Treatment of entire Xanthi-nc tobacco plants (TMV- or mock-inoculated on one leaf) with 10 μl/L of ethylene supplied in the stream of air produced results similar to these reported here for the leaf disks (data not shown).

Leaf disks incubated in the presence of 10 μl/L of ethylene appeared thicker than controls and slightly chlorotic by 120 hr PI. TMV-induced lesions on leaf disks were larger and more diffuse than lesions formed on attached leaves. The ethylene treatment did not visibly change the appearance of the TMV-induced lesions when compared to those of the air control.

Incubation of tobacco leaves with an inhibitor of ethylene action, 2,5-norbornadiene, did not significantly affect the levels of free and total SA in both mock- and TMV-inoculated tobacco leaves (Fig. 5). However, mock-inoculated tissues treated with 2,5-norbornadiene showed consistently higher variability in the levels of total SA than tissues incubated in air (Fig. 5A). None of the 2,5-norbornadiene-induced changes in SA levels (free and total) were significant at $P = 0.05$ using a Student's *t*-test. Treatment with 2,5-norbornadiene did not visibly affect tobacco leaves or interfere with the development of the HR. In addition, 2,5-norbornadiene did not significantly affect the accumulation of PR-1 proteins in TMV- and mock-inoculated Xanthi-nc tobacco leaves (Fig. 6).

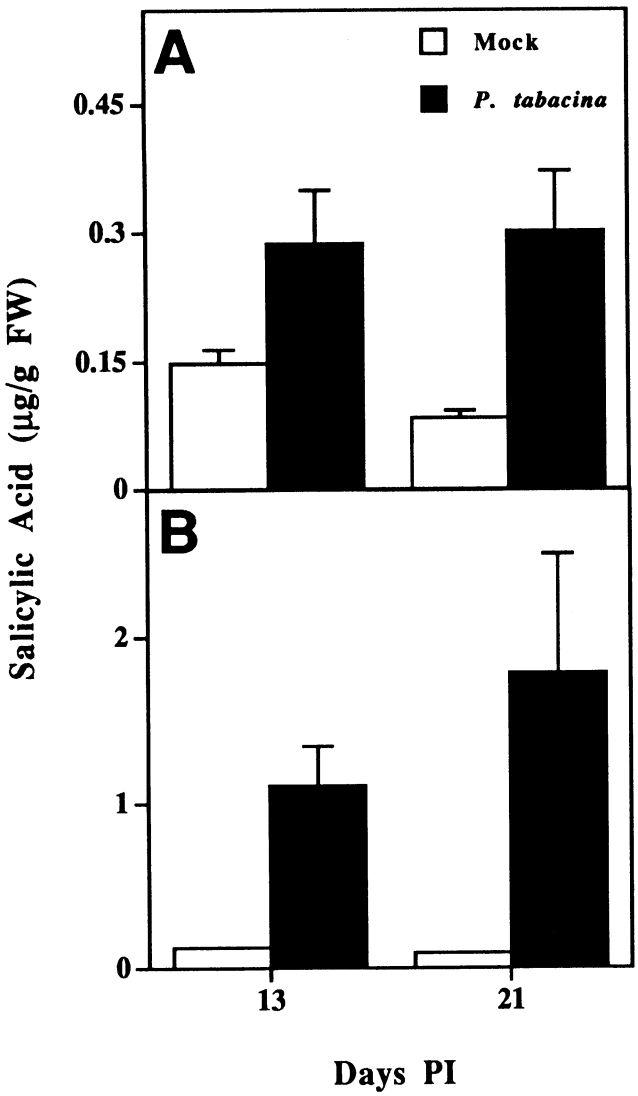

Fig. 4. Levels of **A,** free and, **B,** total salicylic acid in KY-14 tobacco following stem injection with *Peronospora tabacina*. Plants were stem-injected with either sterile water or 1×10^6 sporangia of *P. tabacina*. The third and fourth leaves were harvested 13 and 21 days after stem injection. Each bar is the mean of eight replicate leaf extracts (± SE). The experiment was repeated twice with similar results.

DISCUSSION

Our results indicate that increases in the level of SA in two *Nicotiana* species and different genotypes of *N. tabacum* can be induced by a range of viral, bacteria, and fungal pathogens capable of eliciting a necrotizing response and SAR. We have previously demonstrated that N-gene-carrying Xanthi-nc tobacco leaves or plants accumulate SA recognition in response to localized TMV inoculation (Malamy *et al.* 1990). Here we report that, regardless of the presence or absence of the N-gene, induction of the HR by either TNV (Fig. 1) or *P. s.* pv. *tomato* (Fig. 3) resulted in large increases in endogenous SA. The SA increases observed in Xanthi (nn) tobacco during the TNV- or *P. s.* pv. *tomato*-induced HR contrast with the lack of SA accumulation observed during the compatible interaction of Xanthi (nn) tobacco with TMV (Malamy *et al.* 1990). However, the magnitude and timing of free and total SA increases elicited in Xanthi-nc tobacco by TMV and TNV were similar (compare Malamy *et al.* 1990; Enyedi *et al.* 1992a, and Fig. 1). These results indicate that, in tobacco, the

Table 2. Effect of ethylene on tobacco mosaic virus (TMV)-induced salicylic acid (SA) accumulation

	Free SA[a] (μg/g FW)		Total SA[a] (μg/g FW)	
Treatment	**72 hr**	**120 hr**	**72 hr**	**120 hr**
Control-air	0.10 ± 0.02	0.11 ± 0.04	0.13 ± 0.04	0.45 ± 0.10
TMV-air	5.67 ± 0.38	4.68 ± 0.45	12.03 ± 3.19	13.50 ± 2.99
Air + 10 ppm C_2H_4	0.06 ± 0.01	0.03 ± 0.01	0.12 ± 0.03	0.15 ± 0.02
TMV + 10 ppm C_2H_4	1.99 ± 0.65	1.25 ± 0.05	10.81 ± 0.54	6.19 ± 0.05

[a] Each number is the mean of four replicate leaf extracts (± SE). This experiment was repeated twice with similar results. FW = fresh weight.

N-gene is not primarily involved in SA accumulation but rather functions in a TMV recognition pathway, which triggers the HR and the accumulation of SA and PR proteins. These results also indicate that SA production in tobacco is not species- or pathogen-specific. However, it is tightly linked to the development of the HR and is triggered by yet unknown events occurring during the development of a necrotic lesion. This conclusion is further supported by the fact that inhibition of TMV-induced HR in Xanthi-nc tobacco by high temperature (32° C) blocks SA accumulation but not virus proliferation (Yalpani *et al.* 1991). It is important to note that wounding by itself does not induce SA accumulation in NN or nn tobacco (Yalpani *et al* 1991).

The presence of the N′ gene confers hypersensitivity to TMV strain U1 mutants with an altered coat protein gene (Wittmann *et al.* 1965; Knorr and Dawson 1988). We observed that inoculation of *N. sylvestris* (N′) with the HR-inducing TMV mutant Ni 118 caused an increase in the level of endogenous SA, which coincided with the development of a HR (Fig. 2). This observation, taken together with the induction of SA by TNV and *P. s.* pv. *tomato* in *N. tabacum*, indicates that the ability of different *Nicotiana* species to accumulate SA is primarily determined by their capacity to respond in a hypersensitive manner to a pathogen. We hypothesize that SA production in and around the HR lesions leads to systemic increases of SA, which, in turn, trigger systemic PR-protein accumulation and SAR. The appearance of SAR in tobacco plants inoculated on one leaf with TMV (Ross 1961) or *P. s.* pv. *syringae* (Ahl *et al.* 1980) has been demonstrated previously.

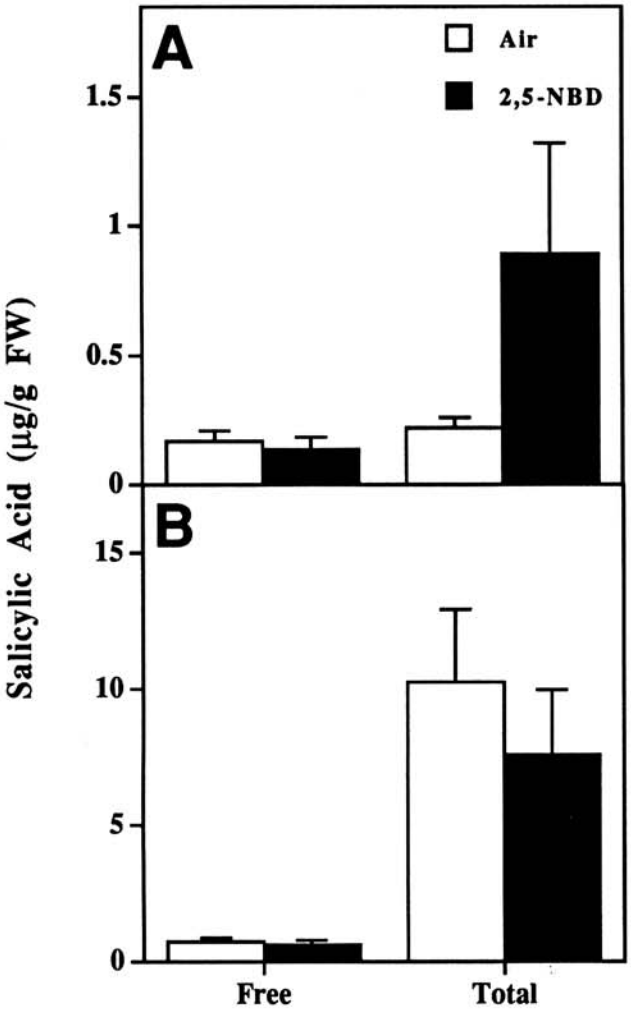

Fig. 5. Effect of 2,5-norbornadiene (2,5-NBD) on accumulation of free and total salicylic acid in **A,** mock-inoculated and, **B,** tobacco mosaic virus-inoculated Xanthi-nc tobacco leaves. The excised leaves were incubated at 100% RH for 48 hr with or without 800 µl/L of 2,5-NBD. Each bar is the mean of four replicate leaf extracts (± SE). This experiment was repeated twice with similar results.

Stem inoculation of tobacco with a *P. tabacina* (blue mold) sporangial suspension induces SAR, chitinases, β-1,3-glucanases, and other SA-inducible PR proteins in the leaves (Tuzun *et al.* 1989; Ye *et al.* 1989; Madamanchi and Kuc 1991). Although aspirin (acetylSA) treatment has been shown to induce resistance to *P. tabacina* and TMV in KY-14 tobacco (Ye *et al.* 1989), the endogenous levels of leaf SA in tobacco stem injected with *P. tabacina* sporangia has never been determined. We observed that the levels of SA increased in tobacco leaves 13 and 21 days after stem injection with *P. tabacina* (Fig. 4). These increases coincided with the appearance of a greater resistance to *P. tabacina* infection. Taken together, these observations suggest that SA may not only act as a leaf-to-leaf resistance signal but may also be involved in stem-to-leaf communications during the establishment of SAR.

Ethylene produced during HR may be involved in the activation of some local resistance mechanisms (reviewed in Enyedi *et al.* 1992b). We found that ethylene was mildly antagonistic to SA accumulation in TMV-inoculated tobacco leaf disks (Table 2). Treatment of excised leaves with 2,5-

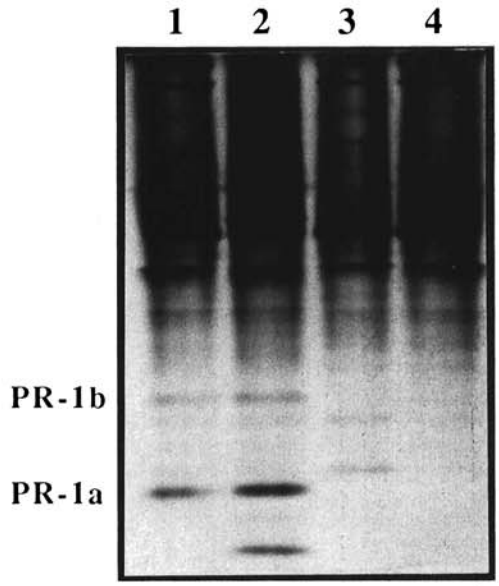

Fig. 6. Effect of 2,5-norbornadiene on accumulation of pathogenesis-related (PR) proteins in tobacco mosaic virus (TMV)-inoculated and mock-inoculated Xanthi-nc tobacco leaves. The excised leaves were incubated at 100% RH for 48 hr with or without 800 ml/L of 2,5-norbornadiene. Acidic proteins were extracted from leaves, separated by nondenaturing gel electrophoresis, and silver stained. Lane 1, TMV-inoculated tobacco leaf incubated in air; lane 2, TMV-inoculated tobacco leaf incubated in the presence of 2,5-norbornadiene; lane 3, mock-inoculated tobacco leaf incubated in air; lane 4, mock-inoculated tobacco leaf incubated in the presence of 2,5-norbornadiene.

norbornadiene, an ethylene action inhibitor, did not result in significant changes in SA levels (Fig. 5) and PR-1 protein accumulation (Fig. 6). Our experiments indicate that ethylene is not directly involved in a signal transduction pathway that leads to SA accumulation and export from the tissues surrounding HR lesions. The relatively weak inhibitory action of ethylene on SA accumulation may be explained by the indirect effects of this hormone on leaf physiology and metabolism. Our results also indicate that ethylene is not involved in the signal transduction pathway that leads to the accumulation of PR-1 proteins during the HR, since 2,5-norbornadiene did not affect PR-1 protein accumulation in TMV-inoculated leaves. Earlier experiments with inhibitors of ethylene biosynthesis indicated that ethylene serves more as a symptom than a signal in the induction of chitinase, another PR protein (Mauch *et al.* 1984). This conclusion was questioned by Raz and Fluhr (1992), who suggested that ethylene may act as an intermediary in the SA induction of chitinase in Samsun NN tobacco. It is currently believed that ethylene does not play an important role in SAR (Enyedi *et al.* 1992b).

Our results further support the hypothesis that, at least in tobacco, SA may be involved in the establishment of SAR to a broad range of pathogens. We have also determined that SA accumulation is not mediated by increased ethylene production in the infected tissues.

MATERIALS AND METHODS

Plant culture.

Tobaccos (*N. tabacum* 'Xanthi' and 'Xanthi-nc') were grown as previously described (Yalpani *et al.* 1991). Seeds of *N. sylvestris* were kindly provided by T. M. A. Wilson, Scottish Crop Research Institute, Dundee, Scotland, U.K., and the plants were grown and maintained as for *N. tabacum*. Fully expanded nonsenescing leaves of 8- to 10-wk-old tobacco plants were used in all studies, except where noted. Tobacco cv. KY-14, used in *P. tabacina* experiments, was grown as described previously (Tuzun *et al.* 1989).

Pathogens and plant inoculations.

The pathogens used in this study are described in Table 1. In all studies involving plant viruses, purified virus preparations were used, and plants were inoculated as previously described (Yalpani *et al.* 1991). TMV strain U1 was applied at a concentration of 5 μg per leaf, TMV Ni 118 at 25 μg per leaf, and TNV at 33 μg per leaf. Overnight cultures of P. s. pv. *tomato* were pelleted at 9,820 *g*, washed in sterile water, repelleted, and resuspended in sterile water to a final concentration of OD_{600} = 1.0. Dilution plating was used to determine the final inoculum concentration, which averaged 1×10^9 cfu/ml. Approximately 50 μl of bacterial suspension was infiltrated per inoculation into the abaxial surface of tobacco leaves using a sterile plastic syringe. Whole inoculated leaves or leaf disks were harvested for determinations of tissue SA levels except in experiments using *P. s.* pv. *tomato*. Following bacterial inoculation of tobacco leaves, 21-mm leaf disks immediately surrounding the infiltrated areas were sampled. Mock-inoculations for viral inoculation used 100 mM phosphate buffer, pH 6.8, and controls for *P. s.* pv. *tomato* were infiltrated with sterile water.

All experiments using *P. tabacina* were conducted at the University of Kentucky, Lexington, as previously described (Ye *et al.* 1989). Briefly, tobacco plants (cv. KY-14) were stem injected with 1 ml (1×10^6 sporangia) of the blue mold pathogen, *P. tabacina,* isolate 82, which resulted in a stem lesion. The third and fourth leaves from the apex were analyzed for SA content at either 13 or 21 days after stem injections. The plants were then challenged by spray inoculation with a sporangial suspension of *P. tabacina*, isolate 79, and the symptoms of disease of the top five fully expanded leaves were rated 7 days PI as previously described (Tuzun *et al.* 1989).

Ethylene.

Studies involving ethylene or the ethylene action inhibitor 2,5-norbornadiene (Sisler and Yang 1984) were conducted in sealed glass dessicators (8-L volume). Ethylene gas was applied at a concentration of 10 μl/L. This concentration was verified by gas chromatography of the gas samples removed from the headspace. The ethylene adsorbent Purafil (Purafil Co., Atlanta, GA) was used in the leaf disk assay to absorb endogenously produced ethylene (in control treatments only). The leaf disks, 21 mm in diameter, used in ethylene studies were excised with a cork borer from surface-sterilized leaves of Xanthi-nc tobacco at 24 hr PI. Leaves were surface-sterilized by immersing them for 15 min in a 5% bleach solution and then for 15 sec in 70% ethanol. The excised leaf assay was adaped from Yalpani *et al.* (1991). The concentration of 2,5-norbornadiene used in these studies was 800 μl/L.

Extraction and quantitation of SA.

SA was extracted from the leaf tissues as previously described (Raskin *et al.* 1989; Yalpani *et al.* 1991). Total SA (the sum of free and glucose-conjugated SA) was determined following enzymatic hydrolysis with β-glucosidase (EC 3.2.1.21; almond, Sigma) as previously described (Enyedi *et al.* 1992a; Enyedi and Raskin 1993).

PR protein analysis.

Leaf tissue (1 g) was pulverized in liquid N_2 and suspended in 60 mM sodium phosphate-citrate buffer, pH 2.8 (0.5 ml/g of 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 (2 μg per lane) were analyzed by nondenaturing polyacrylamide gel electrophoresis as previously described (Yalpani *et al.* 1991). Protein content was determined using the Bio-Rad protein assay dye reagent with bovine serum albumin as a standard.

ACKNOWLEDGMENTS

This research was supported in part by the U.S. Department of Agriculture/Competitive Research Grants Office Grant 90-37261-5659, Division of Energy Biosciences of U.S. Department of Energy Grant DE-FG05-91ER20049, New Jersey Commission for Science and Technology Grant 93-240380-1, and the New Jersey Agricultural Experiment Station.

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