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Dissection of the Salicylic Acid Signaling Pathway in Tobacco

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Certain chemicals induce the accumulation of pathogenesis-related (PR) proteins and enhance resistance to tobacco mosaic virus (TMV) in tobacco at the application site. These compounds include polyacrylic acid (PAA), thiamine, L-α-amino butyric acid, barium chloride, 2-thiouracil, β-1,4-endoxylanase and 2,6-dichloroisonicotinic acid (INA). Because there is considerable evidence that salicylic acid (SA) is an endogenous signal leading to PR gene expression and enhanced resistance following TMV infection, we evaluated these chemicals to see if they act via SA. We report here that all of the compounds tested except INA induced stable accumulation of the glucoside of SA (SAG), probably via transient or low level production of SA. Thiamine induction of PR-1 gene expression and enhanced resistance to TMV was significantly reduced in salicylate hydroxylase-producing transgenic plants which are unable to accumulate SA. In contrast, induction of PR-1 gene expression and enhanced resistance to TMV by PAA was similar in the transgenic and wild-type plants. Furthermore, thiamine induction of SAG accumulation was blocked at elevated temperatures, whereas PAA was still effective. This suggests the presence of multiple pathways that lead to defense responses, one of which is independent of SA. As PAA, thiamine, and INA enter the pathway(s) leading to PR-1 gene expression and enhanced resistance via distinct mechanisms, they should provide chemical tools for dissection of these pathways.

Additional keywords: acquired resistance, signal transduction.

Tobacco cultivars that carry the dominant N gene (e.g., Xanthi nc) respond to a tobacco mosaic virus (TMV) infection by localizing the virus to a small region near the site of viral entry, where a necrotic lesion later appears. This hypersensitive response (HR) is a common resistance mechanism displayed by plants in response to infection. In contrast, when cultivars that lack the N gene (e.g., Xanthi) are inoculated with TMV, the virus spreads systemically, causing chlorosis and stunting. The mechanism(s) responsible for the HR and the role of the N gene are not understood (Matthews 1991).

The HR is accompanied by the systemic accumulation of pathogenesis-related (PR) proteins. The PR proteins of tobacco consist of at least five unrelated families of proteins, with most families containing both acidic and basic isoforms (for review see Cutt and Klessig 1992; Linthorst 1991). Several of the PR proteins have been demonstrated to have β-1,3 glucanase (PR-2) activity or chitinase (PR-3) activity. In combination these enzymes inhibit growth of fungal pathogens in vitro (Mauch et al. 1988). Furthermore, overexpression of chitinase and glucanase in tobacco leads to increased resistance to fungal infection (Broglie et al. 1991; Yoshikawa et al. 1993). PR-1 proteins also have antifungal activity as demonstrated in vitro (Niderman et al. 1995) and in transgenic tobacco plants constitutively expressing the PR-1a gene (Alexander et al. 1993). Antiviral activity of PR proteins has not been found (e.g., Cutt et al. 1989).

When either the inoculated or uninoculated leaves of an infected tobacco plant are exposed to a second attack by TMV, the virus is localized more effectively than during the first attack, resulting in lesions as much as 90% smaller in size. The enhanced ability to resist a second attack by TMV or even unrelated pathogens after HR is referred to as local acquired resistance (LAR) when it occurs in the previously inoculated leaf, or systemic acquired resistance (SAR) when it occurs in uninoculated tissues on the same plant (Ross 1961a,b). The mechanism behind LAR and SAR establishment is not known, although the strong correlation between the resistant state and PR gene expression and protein accumulation (Ward et al. 1991; for review see Carr and Klessig 1989) has lead to the speculation that these proteins or the pathways that induce them are involved. For this reason studies of the signal transduction pathway(s) involved in the induction of the PR genes should help to elucidate the mechanisms of LAR and SAR. Many labs, including our own, have focused on the mechanisms of PR-1 gene induction following TMV infection as a model system to study this signaling pathway (e.g., Cornelissen et al. 1986; Vernooij et al. 1994; Yalpani et al. 1991). TMV, like a large number of other pathogens, induces multiple families of PR genes in concert (Ward et al. 1991; for review see Carr and Klessig 1989). Therefore, PR-1 gene expression can serve as an indicator of the activation of this defense response.

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There is considerable evidence that salicylic acid (SA) is an endogenous signal involved in the induction of PR-1 gene expression and LAR/SAR in tobacco (for review see Malamy and Klessig 1992; Klessig and Malamy 1994). Levels of SA and its glucoside (SAG) increase after infection of resistant (Xanthi nc), but not susceptible (Xanthi) tobacco cultivars. This increase is seen in inoculated leaves and, to a lesser extent, in uninoculated leaves, consistent with the systemic appearance of PR-1 proteins and SAR (Malamy et al. 1990; Malamy et al. 1992; Yalpani et al. 1991). It has been speculated that SAG may serve as a storage form of SA for LAR, released during a challenge infection to increase the efficiency of the defense responses (Hennig et al. 1993). Elevated temperatures (above 28°C) block PR-1 protein accumulation, HR, and SA/SAG increases following TMV infection; all of these responses are restored when plants are returned to lower temperatures (Kassanis 1952; Gianinazzi 1970; Malamy et al. 1992, Enyedi et al. 1992). Finally, transgenic plants that express a bacterial salicylate hydroxylase gene (*nahG*) and hence accumulate very little SA in response to TMV infection do not display SAR (Gaffney et al. 1993). Consistent with the model that SA is an endogenous signal, the application of exogenous SA to tobacco leaves by spraying or injecting results in strong induction of the PR proteins and LAR to TMV at the site of application (White 1979; Van Loon and Antoniw 1982; Malamy and Klessig 1992 for review). SA supplied in this manner does not cause systemic effects (Gianninazzi 1984 and references therein).

Understanding the pathway(s) that leads from TMV infection to elevated levels of SA and, finally, to PR-1 gene expression, LAR and SAR is complicated by the fact that viral

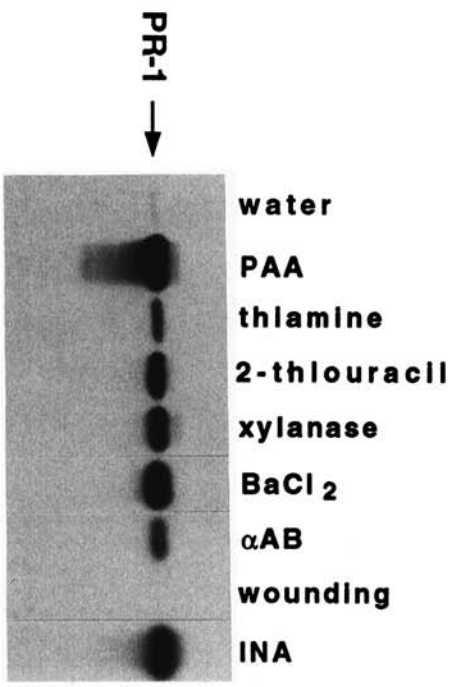

Fig. 1. Chemical induction of PR-1 protein synthesis. Shown is an immunoblot analysis of proteins extracted from tobacco leaves 6 days after their injection with inducers as labeled. Five micrograms of protein was fractionated in each lane and analyzed with anti-PR-1 mouse monoclonal antibody 33G1. The chemical concentrations used are indicated in Table 1.

Table 1. Levels of local acquired resistance induced by SA, PAA, thiamine, $BaCl_2$, αAB, and INA

Treatment	Lesion size[a]	Lesion no.[b]	Mean size (mm)	Reduction in lesion size[c](%)
Water	1.56 ± 0.44	59		
	1.50 ± 0.47	22		
	1.36 ± 0.52	94	1.47 ± 0.10	–
SA (1 mM)	0.65 ± 0.34	41		
	0.75 ± 0.26	12		
	0.66 ± 0.25	18	0.68 ± 0.06	54
PAA (0.1 mM)	0	0		
	0	0		
	0	0	0	100
Thiamine[d] (1 mM)	0	0		
	0	0		
	0	0	0	100
$BaCl_2$[d] (20 mM)	1.00 ± 0.56	6		
	0	0		
	1.00	1	0.67 ± 0.58	55 (48)
αAB (50 mM)	0.81 ± 0.37	61		
	0	0		
	0.75 ± 0.35	7	0.52 ± 0.45	65
INA (1 mM)	0.75 ± 0.35	2		
	0.69 ± 0.87	4		
	0.75	1	0.73 ± 0.03	50
Water-upper[e]	2.20 ± 0.70	17		
	2.57 ± 0.54	15		
	2.67 ± 0.58	3	2.48 ± 0.25	–
PAA-upper[e] (0.1 mM)	2.00 ± 0.82	4		
	2.10 ± 0.50	16		
	2.18 ± 0.57	11	2.09 ± 0.09	16
Thiamine-upper[e] (1 mM)	2.00 ± 0.44	15		
	2.55 ± 0.37	5		
	2.12 ± 0.59	16	2.22 ± 0.29	10

[a] Leaves of tobacco plants were injected with chemicals at the concentrations shown. For each chemical, one leaf from each of three plants were injected; thus the three sets of numbers for each treatment are from the three different plants. Six days later the injected leaves were infected with TMV. Lesion sizes were measured 7days post infection.

[b] In the experiment shown here lesions were small, even in control water-injected plants, compared to those from similar experiments (e.g. see Table 4). A result of this small lesion size is that with several treatments which enhanced resistance (PAA and thiamine), no macroscopic lesions could be detected. With other treatments ($BaCl_2$ and INA) only very few lesions were visible making it difficult to accurately assess the reduction in lesion size. In other experiments where lesions on control plants were two- to threefold larger, the percent reduction for these five chemicals ranged from 40 to 100%, with PAA and INA generally reducing lesion size the most.

[c] The percent reduction in lesion size was calculated by comparing mean lesion size on chemically-treated leaves to that of water-treated leaves.

[d] Solutions containing SA, PAA, αAB, and INA were adjusted to pH 6.5 with NaOH, which was similar to the pH of distilled water used as control. The pHs of the thiamine and $BaCl_2$ solutions were not adjusted. Therefore, to control for any effects that differences in pH might have, the pHs of these solutions (pH 4.0 and pH 5.1, respectively) were measured and a second set of control plants were injected with water adjusted to similar pHs with HCl. With water pH 4.0 or pH 5.1, the mean lesion sizes were 1.14 ± 0.35 and 1.30 ± 0.22, respectively. The percent reduction given in parentheses for $BaCl_2$ was calculated using the pH 5.1 water as control.

[e] The ability of PAA and thiamine to induce enhanced resistance to TMV in upper, untreated leaves (i.e., SAR) was monitored in leaves vertically above (2 to 3 leaves younger) the treated leaves. Similar leaves of plants with water-injected lower leaves was used as controls.

infection produces a large array of physiological and biochemical changes in the host plant. However, certain chemicals including polyacrylic acid (PAA), thiamine, barium chloride ($BaCl_2$), L-α-amino butyric acid (αAB), 2-thiouracil, β-1,4 endoxylanase (xylanase), and 2,6-dichloroisonicotinic acid (INA) have also been shown to induce local PR-1 protein accumulation and, in some cases, enhanced LAR to TMV when injected into leaves (Gianninazzi and Kassanis 1974 [PAA]; Asselin et al. 1985 [αAB, thiamine]; White et al. 1986 [$BaCl_2$, 2-thiouracil)]; Lotan and Fluhr 1990 [xylanase]; Ward et al. 1991 [INA]; this paper). The mode of action of these chemicals was unknown. We assessed whether these chemicals might act by stimulating endogenous production of SA. These experiments show that all of the above chemicals except INA induce SA production. Further characterization of three of the chemicals, PAA, thiamine, and INA, demonstrates that their induction mechanisms are clearly distinct from each other. These compounds may therefore provide chemical tools for dissection of the signal transduction pathway(s) leading to PR gene expression and resistance.

RESULTS

Abiotic elictors of defense responses induce transient SA accumulation and stable SAG accumulation.

Since the effects of the test chemicals have been assessed in different labs under different conditions and in some cases LAR was not tested, we first analyzed PR-1 protein accumulation and LAR to TMV using our conditions. Plants were injected with SA, PAA, thiamine, 2-thiouracil, $BaCl_2$, αAB, xylanase, or INA or wounded, and 6 days later the injected leaves were either harvested for PR-1 protein analysis or infected with TMV to test levels of LAR. Effects of wounding and 2-thiouracil on LAR were not assessed due to the necrosis that accompanied the treatment. At the concentrations used, all the chemicals strongly induced PR-1 protein accumulation (Fig. 1) and, where tested, LAR to TMV (Table 1). Wounding, a reported inducer of basic PR genes (Broderode et al. 1991), induced little, if any, acidic PR-1 gene expression in our hands (Fig. 1).

To assess the role of SA in chemically induced PR-1 gene expression we injected leaves of healthy Xanthi nc (genotype NN) plants with PAA, thiamine, αAB, $BaCl_2$, 2-thiouracil, xylanase, or INA and monitored levels of SA and its glucoside, SAG, 6 days postinjection (dpi) (Table 2). The effects of wounding were also tested. In general, SA levels remained at or near background levels. Only one of the 10 thiamine-injected plants and two of the 10 PAA-treated plants showed SA levels above 0.05 μg/g fresh weight. In contrast, SAG levels were elevated dramatically over background levels after

Table 2. Induction of SA and SAG accumulation 6 days after teatment with PAA, thiamine, 2-thiouracil, xylanase, $BaCl_2$, αAB, wounding, and INA[a]

Treatment	(μg/g FW) SA[b]	SAG[b]	n[c]
Water	<0.05	<0.05	5
PAA (0.1 mM)	<0.05–0.3[d]	0.8–5.5	10
Thiamine (1 mM)	<0.05–0.5[e]	1.1–4.4	10
2-Thiouracil (100 μg/ml)	<0.05	0.9-2.2	3
Xylanase (20 ng/ml)	<0.05	1.5-2.5	3
$BaCl_2$ (20 mM)	<0.05	2.4-3.2	3
αAB (50 mM)	<0.05	0.8-4.2	3
Wounding[f]	<0.05	<0.05	4
INA (1mM)	<0.05	<0.05	4
PAA (0.1 mM) 32°C[g]	<0.05	1.7-1.9	3
Thiamine (1 mM) 32°C[g]	<0.05	<0.05	3

[a] Plants were injected with chemicals at the concentrations shown. SA and SAG levels were determined 6 days after treatment.
[b] The limits of detection under our assay conditions are 0.05 μg/g fresh weight. <0.05 indicates that no peak was detected in any of the samples. Values shown represent the range of SA and SAG levels in μg/g fresh weight.
[c] n = sample size.
[d] Only two of the 10 samples had SA levels greater than 0.05.
[e] Only one of the 10 samples had SA levels greater than 0.05.
[f] Wounding was done by making parallel cuts ~5 mm apart from the midvein to near the outer edge of the leaf.
[g] Plants shifted for 2 days from 22°C to 32°C were injected with chemicals and then maintained at 32°C for 6 additional days.

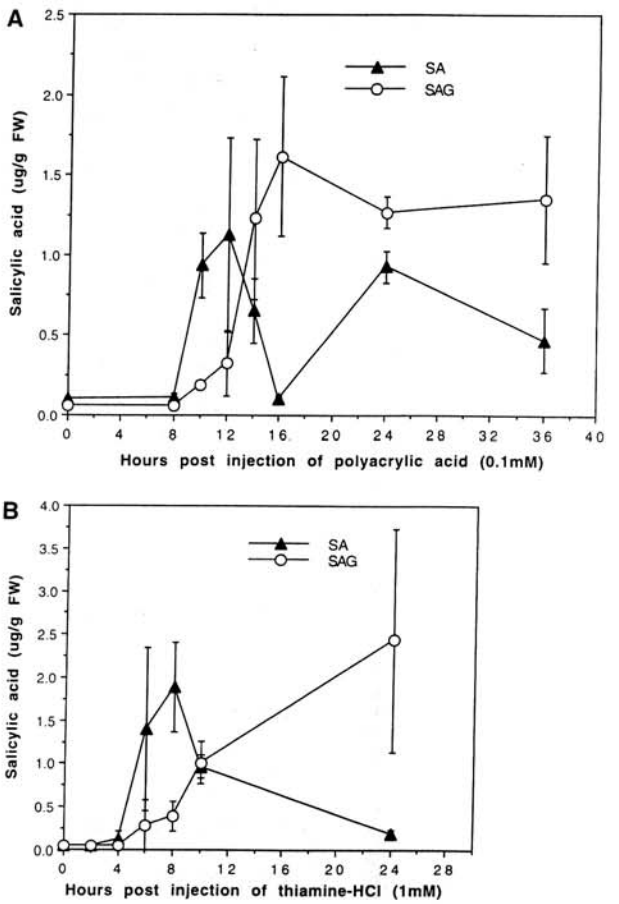

Fig. 2. Time course of SA and SAG accumulation following injection with PAA or thiamine. **A,** PAA (0.1mM) and, **B,** thiamine (1mM) were injected into healthy leaves of tobacco cv. Xanthi nc. Leaves were harvested at the indicated times after injection, and samples were taken for SA and SAG analysis. Values are represented as μg/g fresh weight. When no SA or SAG was detectable, a value of 0.05 μg/g was assigned. Each data point represents the average SA or SAG level for three leaves, one from each of three treated plants. Error bars reflect standard deviation. There is significant variation among the individual data points. It is possible that the plants were not completely synchronized in their response to the chemicals. Since each time point is taken from a different plant, this might have contributed to large differences among some data points. However, none of the samples at early time points ever showed SAG in the absence of SA. This experiment was done twice with similar results.

injection with thiamine, PAA, 2-thiouracil, xylanase, $BaCl_2$, or αAB (Table 2). There was considerable variation in the levels of SAG induced, even for a given chemical. The reason for the variation is unclear, but most likely reflects subtle variations in environmental conditions. Nevertheless, SAG levels were increased by at least 15-fold for each treatment, and occasionally over 100-fold. Only INA did not induce SAG accumulation (Table 2), confirming the recent finding of Vernooij et al. (1995). Wounding also failed to induce detectable SA or SAG accumulation (Table 2).

The presence of SAG but not SA after chemical treatment was initially surprising as TMV infection of Xanthi nc plants always results in elevated levels of *both* SA and SAG (Malamy et al. 1992). Since SAG accumulation results from the glucosylation of SA (Enyedi et al. 1992; Malamy et al. 1992), the presence of SAG suggests that SA was produced transiently during the preceding 6 days. To evaluate this hypothesis, detailed time course studies of SA and SAG levels following PAA or thiamine injection were done (Fig. 2). Indeed, a burst of SA production occurred within 10 and 6 h of injection of PAA and thiamine, respectively. This was followed by the accumulation of SAG which remained at high levels through at least 6 dpi (Table 2, Fig. 2). These data are consistent with the idea that chemically induced SAG accumulation is the result of de novo SA production following treatment. Hence, SA signaling may play a role in the induction of defense responses by all of the elicitors tested except INA.

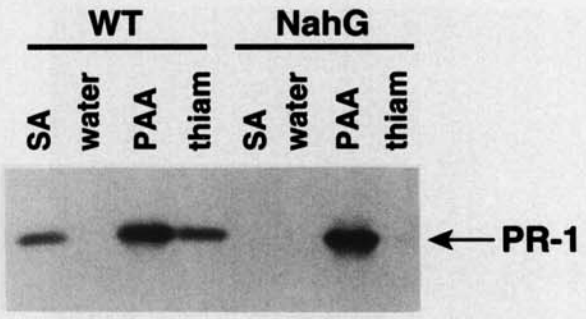

Fig. 3. Chemical induction of PR-1 genes in wild-type and NahG plants. Shown is an anti-PR-1 immunoblot analysis of proteins from leaves of wild-type (nontransgenic) or NahG (transgenic) plants 6 days after injection with various chemicals (see Fig. 1 for details). The same leaves were also used for the SA and SAG quantitation shown in Table 3. For each chemical four plants were treated. Protein from one injected leaf of each of the four plants were analyzed and gave similar results. One representative protein sample for each treatment is shown here.

Table 3. Chemical induction of SA and SAG accumulation in the treated leaves of wild-type and NahG transgenic plants

Treatment[a]	Plant	SA[b] (μg/g FW)	SAG[b] (μg/g FW)
Water	WT	<0.05	<0.05
	NahG	<0.05	<0.05
SA (1 mM)	WT	1.4–3.2[c]	9.8–14.8
	NahG	0.3–1.3[c]	0.4–0.8
PAA (0.1 mM)	WT	<0.05	2.1–5.0
	NahG	<0.05	<0.05
Thiamine (1 mM)	WT	<0.05	2.6–3.3
	NahG	<0.05	<0.05

[a] Plants were injected with chemicals at the concentrations shown. SA and SAG levels were determined 6 days after treatment.
[b] See note b to Table 2. Four plants were assayed for each trea tment.
[c] Since leaves were injected with SA, high SA levels may reflect contamination from SA on the outside of the leaf.

SA is an essential component of thiamine but not PAA induction of PR-1 gene expression.

To determine whether abiotic elicitors such as PAA and thiamine act via SA, as suggested by the above results, we utilized transgenic tobacco plants containing the bacterial *nahG* gene which encodes the degratory enzyme salicylate hydroxylase. It was previously demonstrated that these NahG plants only accumulate SA to levels two- to threefold over background in response to TMV infection, in contrast to a greater than 150-fold induction in wild-type controls (Gaffney et al. 1993). If chemical treatment of these plants leads to lower levels of PR-1 protein accumulation than in similarly treated wild-type (nontransgenic) plants, SA would be implicated as an essential component of this response. Therefore, we treated wild-type and NahG plants with PAA or thiamine and analyzed the ensuing SA/SAG levels and PR-1 protein accumulation. As controls, wild-type and transgenic plants were also injected with either water or SA.

Table 4. Chemically induced local acquired resistance in wild type and NahG transgenic plants

Treatment[a]	Plant	Lesion size (mm)	Mean size	Reduction in lesion size[b] (%)
Water	WT	4.52 ± 0.44		
		4.37 ± 0.50		
		4.50± 0.38		
		4.71 ± 0.30	4.52 ± 0.14	–
	NahG	5.84 ± 0.57		
		6.08 ± 0.45		
		6.25 ± 0.52		
		6.08 ± 0.51	6.06 ± 0.17	–
SA	WT	2.59 ± 0.38		
(1 mM)		2.97 ± 0.55		
		1.48 ± 0.66		
		1.77 ± 0.40	2.20 ± 0.69	51
	NahG	5.45 ± 0.32		
		5.60 ± 0.53		
		5.93 ± 0.54		
		5.99 ± 0.57	5.74 ± 0.26	5
PAA	WT	1.30 ± 0.28		
(0.1 mM)		1.00 ± 0.35		
		1.54 ± 0.48		
		1.32 ± 0.49	1.29 ± 0.22	71
	NahG	2.08 ± 0.75		
		3.06 ± 1.25		
		1.14 ± 0.27		
		2.34 ± 1.29	2.15 ± 0.79	65
Thiamine	WT	2.65 ± 0.57		
(1 mM)		2.78 ± 0.36		
		2.80 ± 0.41		
		2.29 ± 0.46	2.63 ± 0.24	42
	NahG	5.02 ± 0.58		
		5.41 ± 0.55		
		5.28 ± 0.53	5.24 ± 0.20	14

[a] Longitudinal halves of leaves were injected with chemicals. Six days later the entire leaf was infected with TMV. Ten lesions were measured on each treated leaf half seven days post infection.
[b] The percent reduction in lesion size was calculated by comparing mean lesion size of chemically treated leaf halves to that of water-treated leaf halves of the same genotype.

SA injection induced PR-1 protein accumulation in wild-type plants, but this induction was dramatically reduced in NahG plants (Fig. 3). This demonstrates that the degradation of SA in NahG plants is effective in blocking SA induction of the PR-1 gene. Thiamine induction of PR-1 gene expression was also substantially reduced in NahG plants compared to wild-type plants, consistent with a role for SA signaling in thiamine induction of defense responses. In contrast, the PR-1 accumulation in response to PAA was similar in wild-type and NahG plants. The results presented in Table 3 demonstrate that SA and SAG were indeed reduced to undetectable levels in NahG plants following both PAA and thiamine treatment. Therefore, the induction of the PR-1 gene by PAA appears to be independent of SA accumulation, even though PAA is a strong inducer of SA synthesis.

PAA but not thiamine induction of LAR is independent of SA accumulation.

SA is also believed to be a component of the pathway that regulates LAR. Therefore, LAR levels after PAA and thiamine treatment were also assessed in NahG plants (Fig. 4, Table 4). Leaf halves on wild-type and NahG plants were injected with thiamine, PAA, water, or SA, and 6 days later the entire leaf was inoculated with TMV. Lesions were measured 7 days after inoculation and lesion sizes on the treated half-leaf were compared to those on water-treated half-leaves. Since even untreated wild-type and NahG plants respond differently to TMV infection, comparisons were made between chemically treated and water-treated half leaves of the same genotype.

SA injection of NahG plants leads to only very low levels of LAR as compared to SA injection of wild-type plants (Table 4, Fig. 4). Again, this demonstrates the effectiveness of the NahG gene product in inhibiting SA-induced defense responses. Thiamine induction of LAR was also reduced in NahG plants as compared to wild-type (Table 4, Fig. 4). Therefore, SA appears to be a component of thiamine induction of LAR. In contrast, both NahG and wild-type plants demonstrated high levels of LAR in response to PAA (Table 4, Fig. 4). Hence, although SA signaling seems to be involved in both LAR and PR-1 gene induction in response to thiamine, the same responses to PAA are not affected by greatly reduced SA accumulation.

PAA but not thiamine is active at elevated temperatures.

In many cases the ability of a host to resist viral attack is abolished at high temperatures (Fraser 1987). When Xanthi nc

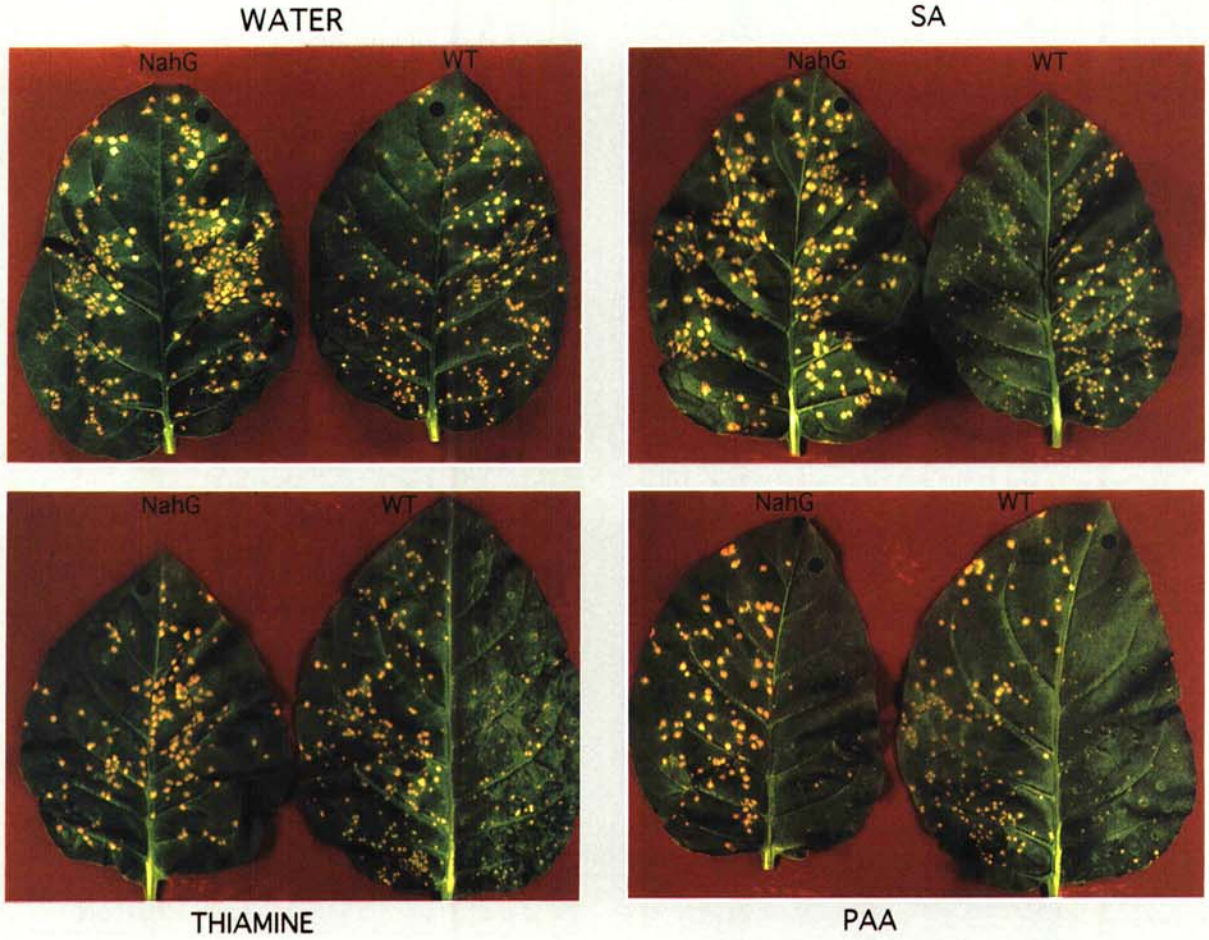

Fig. 4. Local acquired resistance induced by SA, PAA, and thiamine in wild-type and NahG plants. Half-leaves from four wild-type and four NahG plants were injected with various chemicals (see Table 4 for details). The entire treated leaves were inoculated with TMV 6 days postinjection. One representative leaf of each of the treated, TMV-infected leaves is shown here. Injected halves are identified with a black dot. Since lesions are clearly different in control, water-treated leaves of wild-type and NahG plants following TMV infection, each treated half-leaf must be compared to the water-treated half-leaf of the same genotype. Leaves were photographed 7 days postinoculation.

plants are infected with TMV and maintained at 32°C, the virus spreads sytemically and no increases in SA levels, PR gene expression or HR occur. When infected plants are transferred to lower temperatures (below 28°C), SA and PR proteins rapidly accumulate and viral spread is inhibited (Gianinazzi 1970; Kassanis et al. 1974; Malamy et al. 1992). The nature of the high temperature block that precedes SA production is not known, but can serve as a marker in the defense response pathway. Analysis of a chemical's ability to induce defense responses at 32°C may help to map its entry point in the signal transduction pathway relative to the temperature sensitive step. Therefore, PAA and thiamine were tested for their ability to induce SAG accumulation at high temperatures.

Thiamine did not induce any detectable SAG increases at 32°C by 6 days after treatment (Table 2), consistent with the demonstration by Asselin et al. (1985) that thiamine-induced PR protein accumulation is greatly reduced at elevated temperatures. Moreover, even when thiamine-injected plants were shifted to lower temperatures after 48 h (22°C), no SAG accumulation was seen (data not shown). In contrast, PAA induced SAG accumulation at 32°C, consistent with previous results showing that PAA induces PR gene expression at 32°C (Table 2; White et al. 1983). Interestingly, transferring PAA-injected plants to 22°C after 48 h resulted in further increased accumulation of SAG (data not shown). Therefore, PAA action was partially and reversibly inhibited at 32°C, while thiamine action was completely blocked.

Endogenously produced SA is not sufficient for systemic responses.

The ability of chemicals to induce SA production provides tools for the study of the metabolism and localization of endogenous SA. Such studies can also address apparently conflicting data concerning the effects of endogenous and exogenous SA. For example, endogenous SA levels increase systemically as a result of infection of tobacco with TMV and cucumber with *Pseudomonas syringae* (Malamy et al. 1990; Metraux et al. 1990), suggesting that either SA or a pathogen-triggered signal that induces SA production is mobile. Furthermore, Shulaev et al. (1995) recently demonstrated, using labeling experiments, that the majority of SA in an upper healthy leaf originates from a lower infected leaf, again suggesting SA mobility. In contrast, exogenously supplied SA exerts its effects only at the site of application (Van Loon and Antoniw 1982). We speculated that exogenously supplied SA might be modified and/or localized incorrectly, hence explaining its apparent immobility. This possibility can be tested via chemical induction of endogenous SA in the absence of other pathogen responses.

Upper leaves were monitored for SA or SAG levels after PAA or thiamine induction of endogenous SA production in lower leaves. No increases were found in the SA or SAG levels in the uninjected leaves of PAA- and thiamine-treated plants (data not shown), nor was there any accumulation of PR-1 proteins in these leaves (Fig. 5). Furthermore, only LAR was induced; little, if any, SAR was apparent, as assessed by TMV infection of upper leaves of plants whose lower leaves were injected with water, PAA, or thiamine (Table 1).

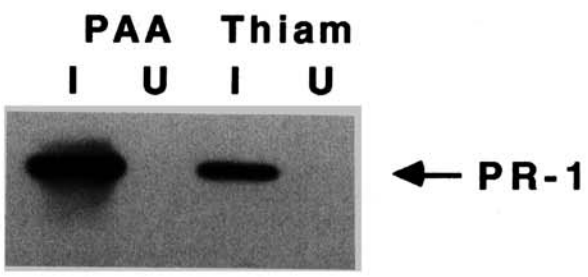

Fig. 5. Induction of PR-1 genes in injected and uninjected leaves of PAA- and thiamine-treated plants. Shown is an immunoblot analysis for PR-1 proteins of extracts from leaves 6 days after injection of PAA (0.1mM) or thiamine (1mM). See Figure 1 for details. Lanes containing protein samples from chemically treated leaves are indicated by I (Injected), while those lanes with protein samples from the combined second and third leaves above the injected leaf are designated U (Uninjected).

DISCUSSION

In this paper we report that certain chemical inducers of PR genes and LAR also induce accumulation of SAG. For PAA and thiamine (Fig. 2) it was demonstrated that the appearance of SAG was preceded by transient SA accumulation. It seems highly probable that this transient SA rise is responsible for the subsequent accumulation of SAG. SA may be transiently produced and converted in toto to SAG. This might be the result of the transient presence of the chemical inducers, which may be rapidly broken down and eliminated after injection into the plant. Alternatively, continued SA production may occur but not be detected due to rapid conversion to SAG. The latter explanation is consistent with a previous report that SA induces increased activity of a specific SA-glucosyltransferase in tobacco (Enyedi and Raskin 1993).

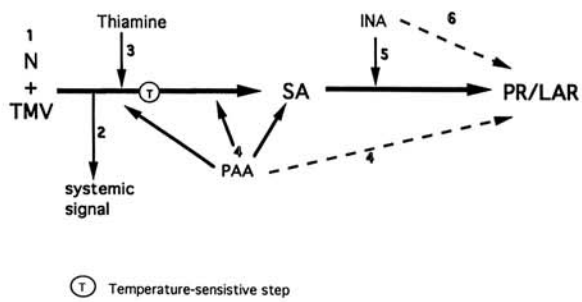

Fig. 6. Model for induction of PR genes and local acquired resistance by PAA, thiamine, and INA. This model shows the putative position of chemical inducers in the signal transduction pathway that leads to PR gene expression and LAR. Pathogen recognition (1) (i.e., N + TMV) initiates the defense response and causes a systemic signal (2) to be transferred to distal tissues. Since thiamine and PAA induce only local responses, they likely enter the pathway after the dissemination of a systemic signal. Thiamine is SA dependent and therefore must precede SA in the pathway (3). Since thiamine action is inhibited by elevated temperatures, thiamine may share a temperature-sensitive step with the TMV-induced defense pathway and therefore enter before this step as shown. Alternatively, its action may be inhibited by other unknown temperature-sensitive steps. PAA, on the other hand, is SA independent (indicated by a broken arrow) but still induces SA production. Therefore, PAA may induce two concurrent pathways for defense responses, only one of which includes SA production (4). PAA's partial sensitivity to elevated temperature suggests that PAA may enter the SA dependent pathway at a point that precedes the temperature sensitive step. INA does not induce SA increases and its effects are SA independent. Therefore, INA enters the pathway downstream of SA (5) or acts through a completely separate pathway (6).

It is intriguing that such disparate chemicals are all capable of inducing SA synthesis. It seems unlikely that the chemicals are provoking nonspecific stress responses since many compounds that are closely related to such inducers as $BaCl_2$ and αAB were inefficient or noninducers of local PR protein accumulation (Asselin et al. 1985; White et al. 1986). In addition, extensive wounding did not result in increased SA or SAG levels (Table 2), further arguing against the possibility of nonspecific stress responses.

The demonstration that several chemicals that induce PR-1 gene expression and the development of LAR also elicit SA production suggests that these inducers act through the endogenous SA signaling pathway. The chemicals can also be used as tools to dissect the signaling pathway(s) by which TMV induces defense responses. Based on the data presented, preliminary assignment of the entry points of thiamine, PAA and INA into this pathway can be made (Fig. 6). It is important to note that these assignments refer only to the effects of the inducers at the tested concentration. It is possible that at other concentrations the chemicals might cause different responses. Therefore, only well-characterized conditions should be employed when using chemical tools.

Thiamine induces PR-1 gene expression and LAR in wild-type plants, but these defense responses are greatly reduced in NahG plants. Furthermore, the induction of SA synthesis by thiamine appears to be inhibited at 32°C, as is SA synthesis, PR-1 gene expression, and the HR in response to TMV infection (Gianninazzi 1970; Malamy et al. 1992). Therefore, thiamine induction of the defense responses appears to be SA-dependent and temperature sensitive, similar to TMV induction. Thus, thiamine likely enters the signaling pathway at an early point that precedes the temperature sensitive step (3 in Fig. 6).

PAA is a strong inducer of SA synthesis, yet PR-1 and LAR induction are only slightly reduced in NahG plants. This argues that PAA induces more than one pathway leading to the defense responses, and that at least one of these pathways does not involve SA (4 in Fig. 6). This is consistent with the partial temperature sensitivity of PAA effects (the levels of SA and SAG induced are lower at 32°C than at 22°C and increase when plants are shifted to 22°C). PAA induction of SA synthesis may be partially temperature sensitive while but the SA-independent pathway is unaffected by elevated temperatures. It is also conceivable that PAA enters the pathway downstream of SA and initiates a positive feedback regulation of SA production.

The inability of thiamine and PAA to induce SA accumulation or resistance responses outside of the treated site suggests that endogenous production of SA is not sufficient for the development of these systemic effects, although the possibility that transient or very low level production of free SA somehow precludes its mobility cannot be excluded. This result suggests that despite the demonstration of SA's mobility by Shulaev and colleagues (1995), endogenously produced SA is not intrinsically mobile. If chemical induction of SA cannot cause systemic responses, some other pathogen-induced signal must spread throughout the plant triggering the mobilization or de novo production of SA in uninoculated leaves on inoculated plants. The latter model is consistent with the findings of Hammerschmidt and colleagues (Rasmussen et al. 1991; Smith et al. 1991), who showed that the removal of inoculated leaves from cucumber plants before the appearance of SA did not prevent the subsequent accumulation of SA and development of SAR in the uninoculated leaves. The existence of a systemic signal that precedes SA production is further supported by the results of Vernooij and colleagues, who demonstrated that infected NahG rootstocks, unable to accumulate SA, were still able to confer resistance to a wild-type graft (Vernooij et al. 1994). PAA and thiamine (and αAB [Malamy and Klessig, unpublished]) all exert their effects locally. Therefore, if these inducers share a common pathway with TMV, they likely enter the pathway after the dissemination of the putative systemic signal (3 and 4, Fig. 6).

In contrast to all the other tested chemicals, INA did not induce SA synthesis (Table 2; Vernooij et al. 1995). This is consistent with the fact that INA's ability to induce LAR and PR-1 expression are similar in wild type and NahG plants (Vernooij et al. 1995). Therefore, INA may act through a completely separate pathway from the other inducers (6, Fig. 6). Alternatively, INA may enter a common pathway downstream of SA synthesis (5, Fig. 6). This latter model is supported by the isolation and characterization of mutants in Arabidopsis that are insensitive to INA as well as SA (Cao et al. 1994; Delaney et al. 1995; Shah and Klessig, unpublished). It is also consistent with the observation that INA, as well as SA, inhibits catalase and ascorbate peroxidase, two key enzymes for H_2O_2 degradation (Chen et al. 1993b; Conrath et al. 1995; Durner and Klessig 1995).

Chemical inducers of SA will provide several advantages for dissection of the signal transduction pathway(s) that regulates the defense responses. First, chemical inducers stimulate in vivo SA production which may differ in processing and localization from exogenously applied SA. Secondly, when a plant host recognizes a pathogen, a large number of separate events and pathways are concurrently triggered. Chemical inducers, in contrast, may allow independent evaluation of these events. Third, chemical inducers enter the pathway at different points, providing tools for biochemical analysis of the pathway. This has been demonstrated here for thiamine, PAA, and INA based on their induction of SA, dependence on SA for elicitation of PR-1 gene expression and LAR, and response to high-temperature inhibition. Similar analyses of other chemicals will allow the construction of a more complete model. Finally, responsiveness to these chemicals will provide a biochemical approach to understanding the nature of mutants with altered responses to pathogen attack. Responsiveness to chemicals could be used as a screen to identify new mutants. The use of chemical tools complemented by genetics may be a key to understanding the complex signal transduction pathways invoked by pathogen attack.

MATERIALS AND METHODS

Plant material and growth conditions.

Tobacco plants (*Nicotiana tabacum* cv. Xanthi nc) or NahG-10 (kindly provided by John Ryals) were grown at 22°C in growth chambers programmed for a 14-h light (10,000 to 15,000 lux) cycle. For high-temperature experiments, plants were transferred to 32°C Conviron chambers 2 to 3 days before chemical treatment. After chemical treatment plants were maintained under 24-h light conditions to minimize variation due to the time of chemical treatment. TMV strain U1 was used at a concentration of 1 μg/ml in 50 mM phosphate buffer at pH 7.5 in all experiments.

Chemical treatments and wounding.

All chemicals were obtained from Sigma except for xylanase (a gift from J. Anderson, USDA, Beltsville) and INA (a gift from John Ryals, CIBA Geigy). Chemicals were dissolved in water and the pH adjusted to 6.5 with NaOH except for xylanase which was dissolved in 50 mM phosphate buffer, pH 7.0, and thiamine and $BaCl_2$, which were used without adjusting the pH. Chemical injections were performed by inserting a 18.5-gauge needle attached to a syringe below the upper leaf surface. Leaves were injected until the desired area, in most cases a longitudinal half of a leaf, appeared completely saturated. Alternatively, in some cases leaves were pricked with a 18.5-guage needle, a syringe barrel was pressed against the hole in the underside of the leaf, and chemical solutions were infiltrated into the leaf. Treatment with 2-thiouracil resulted in the appearance of small necrotic spots. $BaCl_2$ caused noticeable toughening of the leaf. For wounding, plants were sliced with a razor blade from the midvein to near the outer margin. Slices were approximately 5 mm apart.

Protein extraction and analysis.

Proteins were extracted as described (Chen et al. 1993a) and separated on 15% SDS PAG; PR-1 proteins were detected using specific anti-PR-1 mouse monoclonal antibody (33G1) (Carr et al. 1987) diluted 1:1,000 and sheep-anti-mouse antibodies conjugated to horseradish peroxidase (Amersham) diluted 1:10,000. Immunoblots were developed using the ECL detection kit from Amersham.

Quantitation of SA and SAG.

SA and SAG were extracted and analyzed as described by Malamy et al. 1992 with modifications described by Bowling et al. 1994.

Determination of local and systemic acquired resistance.

LAR was determined by injecting full or half-leaves with the test chemical and 6 days later inoculating the entire leaf with TMV. Seven days after TMV inoculation, 10 to 20 lesions were measured by hand and the average percent reduction in lesion diameter was calculated by comparison to water injected controls. To assess SAR, leaves 2 and 3 above the injected leaves were infected with TMV 6 days after treatment, 10 to 20 lesions were measured, and the average lesion diameter was compared to infected leaves above a water-treated control. Since environmental factors greatly affect both lesion size and resistance, water-injected control plants were included in each experiment.

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