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2,6-Dichloroisonicotinic Acid-Induced Resistance to Pathogens Without the Accumulation of Salicylic Acid

Bernard Vernooij, Leslie Friedrich, Patricia Ahl Goy,[1] Theodor Staub,[1] Helmut Kessmann,[1] and John Ryals

Agricultural Biotechnology, Ciba-Geigy Corporation, P.O. Box 12257, Research Triangle Park, NC 27709-2257. [1]Crop Protection Division, Ciba-Geigy Limited, CH-4002 Basel, Switzerland

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Systemic acquired resistance (SAR) is a pathogen-inducible defense mechanism effective against a wide range of pathogens. Salicylic acid (SA) is an essential component of this pathway, as transgenic plants unable to accumulate salicylic acid are incapable of developing SAR. Here, we show that the synthetic chemical 2,6-dichloroisonicotinic acid (INA) acts via the SAR signal transduction pathway. However, it does not induce SA accumulation during the time required for the induction of SAR gene expression or resistance to TMV. Furthermore, INA can induce both resistance and SAR gene expression in transgenic tobacco and Arabidopsis plants that cannot accumulate SA. Thus, INA apparently activates a component of the SAR signaling pathway downstream of SA accumulation.

Additional keywords: activator, chemical inducer, nahG, pathogen resistance, salicylic acid deficient.

Plants have several inducible defense mechanisms that act to limit pathogen infection, including increased lignification and cell wall cross-linking (Bowles 1990), production of small antibiotic molecules (i.e., phytoalexins) (Dixon 1986), host cell death at the site of infection (i.e., the hypersensitive response) (Slusarenko *et al.* 1991), and the production of reactive oxygen species (Mehdy 1994). Many plants also develop an increased resistance against subsequent pathogen infection in uninfected tissues. This systemic acquired resistance (SAR) can be effective against viruses, bacteria, and fungi and is accompanied by the systemic expression of a group of genes termed SAR genes (Kuc 1982; Ward *et al.* 1991). Evidence is emerging that these SAR genes play an important role in affecting the resistant state. For instance, tobacco plants constitutively overexpressing PR-1 have increased resistance against oomycete fungi (Alexander *et al.* 1993).

Several chemicals have been reported to induce resistance to pathogens when applied to plants (Kessmann *et al.* 1994). For example, when exogenously applied to tobacco or Arabidopsis, salicylic acid (SA) induced resistance to the same spectrum of pathogens as biological inducers of SAR, as well as expression of the SAR genes (White 1979; Ward *et al.* 1991; Uknes *et al.* 1992, 1993). Subsequently, SA was shown to be an endogenously synthesized compound critical for the induction of the SAR signaling pathway (Malamy *et al.* 1990; Métraux *et al.* 1990; Gaffney *et al.* 1993). 2,6-Dichloroisonicotinic acid (INA) also appears to be an activator of the SAR pathway. Like SA, INA induces broad-spectrum disease resistance (Métraux *et al.* 1991; Staub *et al.* 1992). This resistance is apparently due to an indirect mechanism, since neither INA nor its metabolites have in vitro antibiotic activity (Métraux *et al.* 1991). The idea that INA activates the SAR pathway is supported by the observation that INA induces expression of the same set of SAR genes that are induced by either SA treatment or various infectious agents (Ward *et al.* 1991; Uknes *et al.* 1992).

The accumulation of SA is the only known biochemical step in the pathway leading to the onset of SAR (Malamy *et al.* 1990; Métraux *et al.* 1990; Gaffney *et al.* 1993). In this study, we describe experiments designed to determine if INA acts through the accumulation of SA or downstream of it. The results demonstrate: 1) that INA does not induce SA accumulation; and 2) that INA is effective in transgenic plants that are unable to accumulate SA. Taken together, the results suggest that INA induces the SAR signal transduction pathway by acting either at the same site or downstream of SA accumulation.

RESULTS

INA induction of SAR in tobacco.

INA was first reported as a synthetic chemical that was capable of inducing a broad-spectrum disease resistance in several plant species (Métraux *et al.* 1991). It has been suggested that INA activates some step leading to the establishment of SAR and, if this is correct, the compound could be useful in the dissection of the signal transduction pathway. A well-characterized example of SAR is the induction of resistance in tobacco by tobacco mosaic virus (TMV) infection (e.g., Ross 1961; Ward *et al.* 1991). Broad-spectrum disease resistance is induced, and this is accompanied by the accumulation of high levels of mRNA from nine gene families, termed SAR genes (Ward *et al.* 1991). If INA actually induces SAR, then it should protect against the same spectrum of pathogens as a TMV infection, and it should induce the same type of gene expression.

We compared the spectrum of pathogen resistance induced by INA to that induced by viral treatment. Figure 1 and Table 1 show that INA treatment of tobacco leaves induces resis-

Corresponding author: John Ryals.

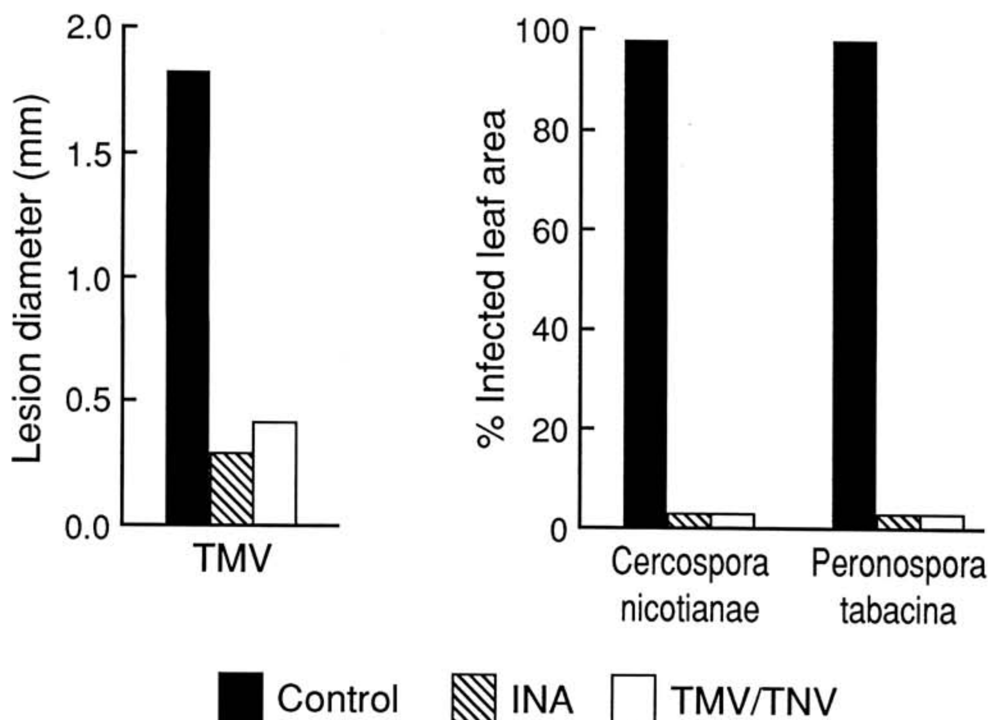

Fig. 1. Protection of tobacco against various pathogens as a result of either 2,6-dichloroisonicotinic acid (INA) or tobacco mosaic virus (TMV) pretreatment (tobacco necroșis virus [TNV] for *Peronospora tabacina*).

Table 1. Disease symptoms in control and induced tobacco leaves[a]

	Disease severity		
Pathogen	**Water**	**INA**	**TMV**
Pseudomonas syringae	4.3	0.1	1.3
Phytophthora parasitica	5.0	0.3	2.0

[a] Plants were treated with water, INA, or TMV. Seven days later plants were challenged with pathogen and subsequently rated for disease severity on a 5-point scale (see Materials and Methods).

tance to the same pathogens as does treatment with TMV or tobacco necrosis virus (TNV). This includes significant protection against TMV, *Cercospora nicotianae* Ellis & Everh., *Peronospora tabacina* D.B. Adam, *Phytophthora parasitica* Dastur. var. *nicotianae* (Breda de Haan) Tucker, and *Pseudomonas syringae* pv. *tabaci*. We previously demonstrated that INA treatment induced expression of the same nine SAR genes in tobacco that are induced by TMV treatment; but genes that are not routinely induced in systemic tissues by TMV treatment, including the class I glucanase and class I chitinase, are also not induced by INA (Ward *et al.* 1991).

We published similar results for Arabidopsis (Uknes *et al.* 1992, 1993). For example, INA treatment leads to protection against the same spectrum of pathogens and induction of the same SAR genes as inoculation of plants with turnip crinkle virus (TCV). Thus, based on the criteria that define SAR, namely the spectrum of both pathogen resistance and gene induction, INA treatment induces an equivalent response, presumably by activating the SAR pathway.

INA does not induce SA accumulation.

Because SA accumulation has been confirmed as an intermediate step in the signal transduction pathway leading to

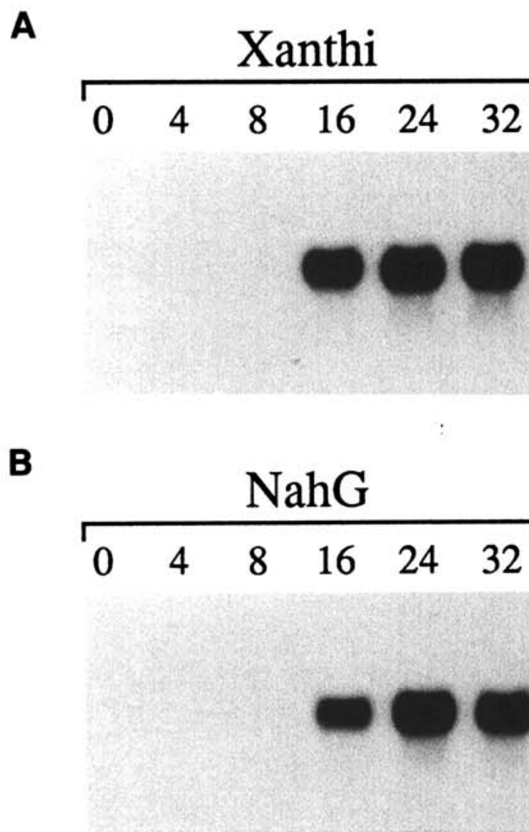

Fig. 2. Time course of PR-1 gene expression in 2,6-dichloroisonicotinic acid treated plants. **(A)** Xanthi.nc plants. **(B)** NahG plants. Time is indicated in hours.

SAR, we sought to place the action of INA relative to the accumulation of SA. First, we tested whether INA induced SA accumulation during the time required for SAR gene expression. Figure 2A shows that in INA-treated tissues, PR-1, one of the SAR genes, was induced between 8 and 16 h after application. Accumulation of PR-1 mRNA reached a plateau within 24 h and remained constant for another 8 h. In these same tissues, SA levels did not increase relative to either uninjected tissues (t = 0 h), or water-injected controls (Fig. 3A). Free SA is conjugated, mostly into a stable SA-glucoside (SAG) form (Malamy *et al.* 1992; Enyedi *et al.* 1992); therefore, increased SAG levels serve as a signature for transient increases in free SA. As with free SA, INA did not increase the level of total SA (free SA + SAG) in the time required for maximal induction of PR-1 gene expression (Fig. 3B). Next, we tested whether SA levels increased at extended times after INA application. To this end, two leaves were injected per plant (with INA or water), and one leaf was harvested for SA analysis at indicated times. The remaining leaf was challenged with TMV 7 days after chemical application. Figure 3C shows that SA levels do not rise in INA-injected leaves between 1 and 7 days after treatment. Likewise, total SA levels (free SA + SAG) did not increase in this time (Fig. 3D). We tested whether the INA application induced both PR-1 gene expression and resistance in these plants and found that INA, but not the water control, induced both PR-1 gene expression (data not shown) and resistance (lesion sizes were reduced 81% relative to the water controls). Taken together, these data suggest that INA does not induce SAR via SA accumulation.

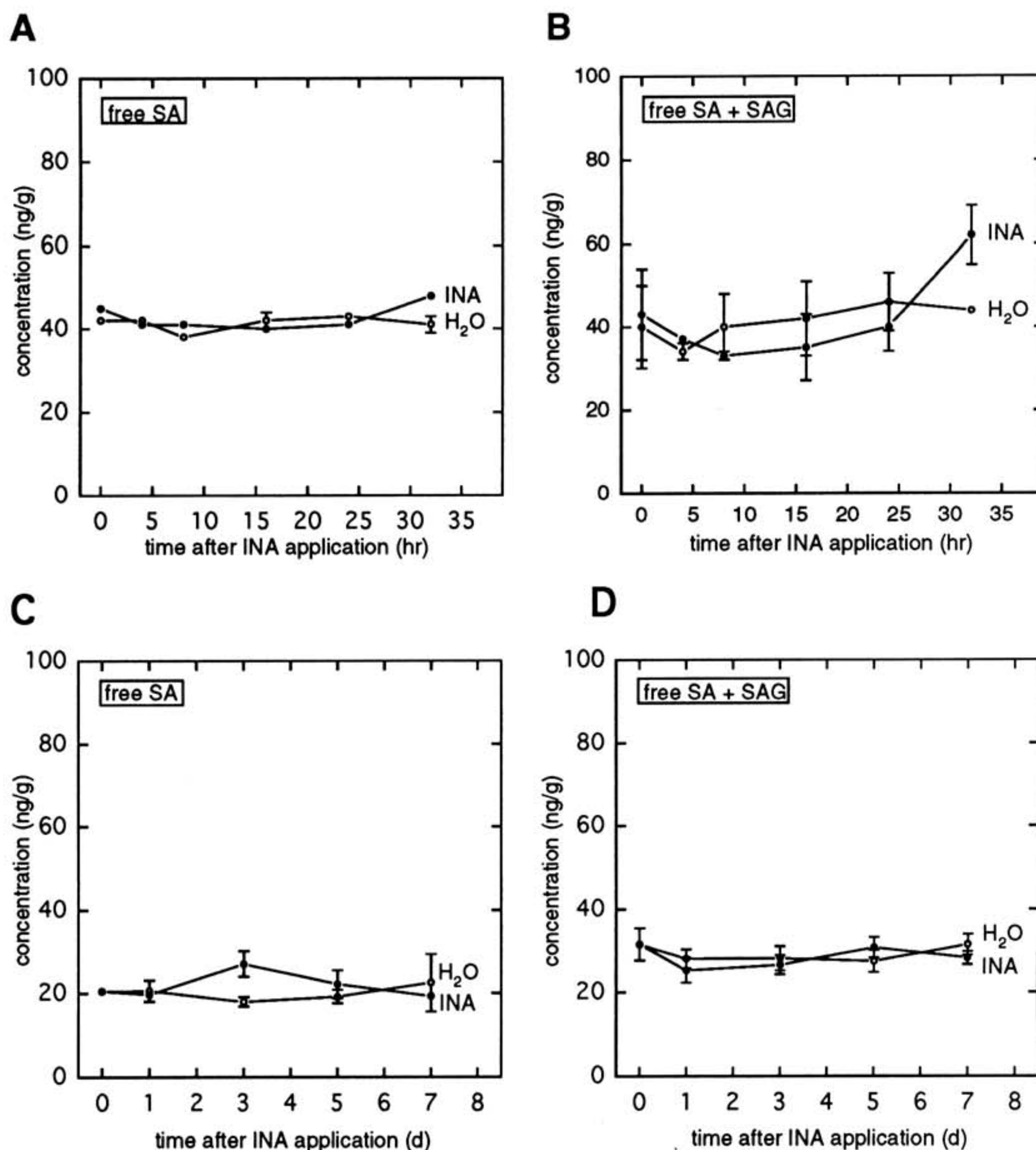

Fig. 3. Salicylic acid (SA) levels in Xanthi.nc plants treated with either water or 2,6-dichloroisonicotinic acid (INA). **(A)** and **(B)** 32-h time course. **(C)** and **(D)** 7-day time course. SAG, SA-glucoside.

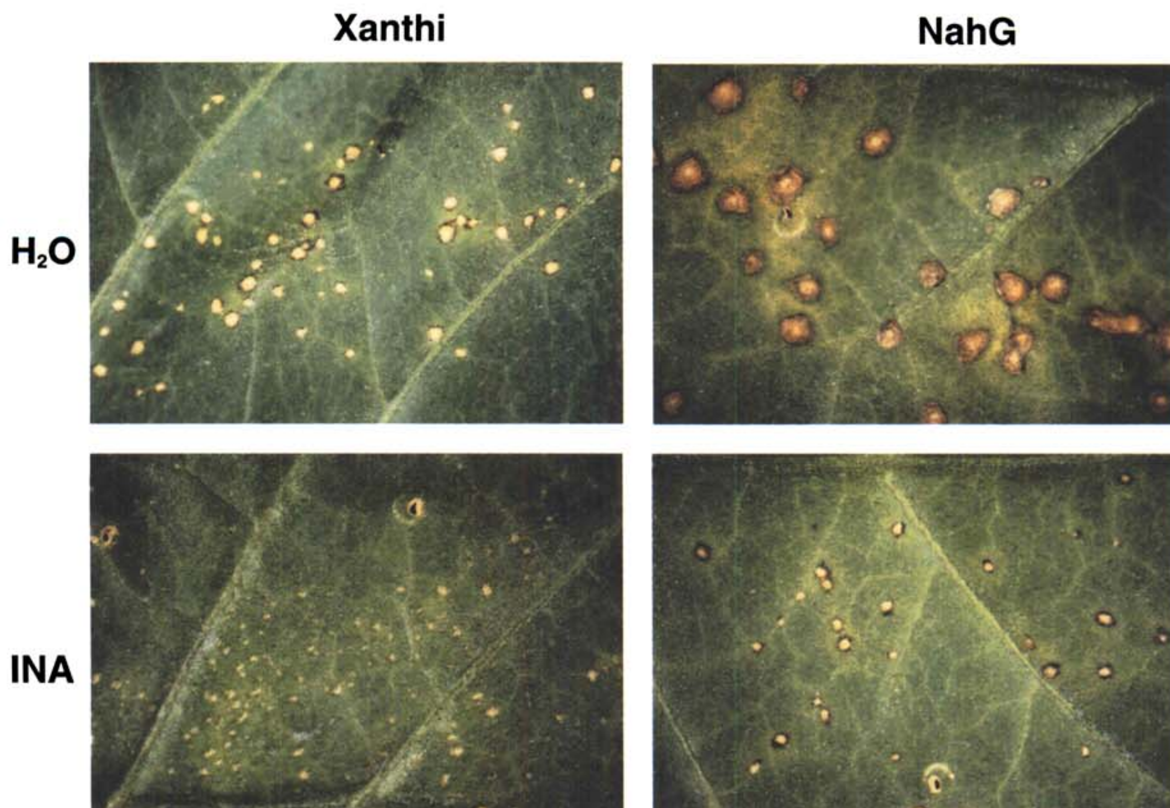

Fig. 4. 2,6-Dichloroisonicotinic acid (INA) induced resistance in Xanthi.nc and NahG tobacco. Photos were taken 7 days after INA- and water-pretreated plants were inoculated with tobacco mosaic virus (TMV). The magnification of the leaves is equal in all photos.

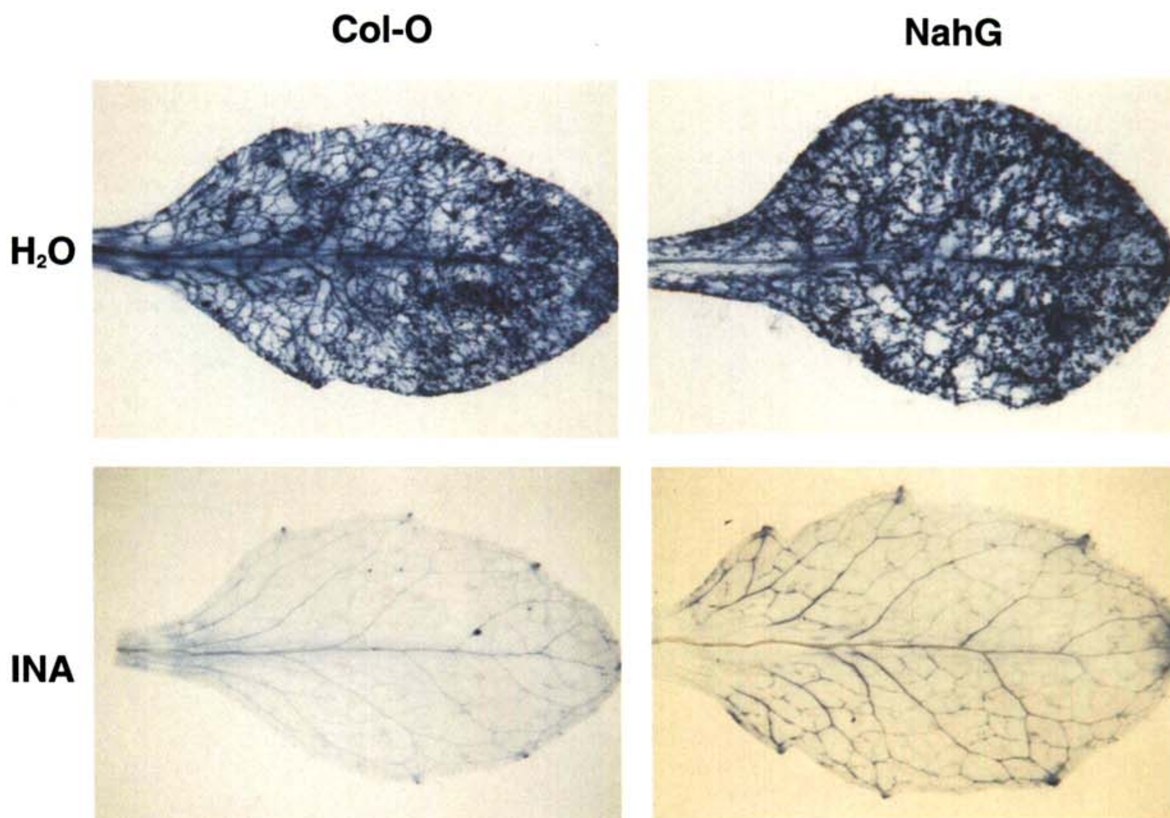

Fig. 5. 2,6-Dichloroisonicotinic acid (INA) induced resistance in Arabidopsis. Plants were sprayed with water or INA and challenged with spores of *Peronospora parasitica* isolate Noco 4 days later. After 10 days, individual leaves were stained with trypan blue, which stains fungal structures and the plant vascular system.

INA induction of resistance in NahG tobacco plants.

We have shown previously that transgenic tobacco plants expressing the bacterial salicylate hydroxylase (*nahG*) gene are unable to accumulate SA following pathogen infection and that they are incapable of inducing SAR (Gaffney *et al.* 1993). We also found that the exogenous application of SA will not induce resistance in the NahG tobacco plants, presumably due to the action of salicylate hydroxylase on SA (L. Friedrich, *unpublished*). If INA works either at the same step as SA (i.e., an analogue or mimic) or downstream of SA action, then it should be an effective inducer of acquired resistance even in NahG plants. To determine if INA could induce resistance in NahG tobacco plants, we injected leaves with INA or water and then inoculated these leaves with TMV 6–7 days later. Lesion sizes were measured 5–7 days after the challenge inoculation. In both NahG and Xanthi.nc (untransformed control) plants, INA pretreatment reduced lesion sizes by approximately 50%, as shown in Figure 4 and Table 2. Consistent with our previous results, TMV lesion sizes in control NahG plants are larger than those in control Xanthi.nc plants (Gaffney *et al.* 1993; Delaney *et al.* 1994). Interestingly, the lesions in the leaves of the INA-treated NahG plants are also larger than those in the INA-treated Xanthi plants. In the nontreated leaves of INA-injected Xanthi.nc and NahG plants, resistance is also induced, to a similar extent as in the injected leaves (Table 2). This could be due to the systemic movement of the injected INA (Métraux *et al.* 1991). Thus, INA induces resistance in wild type as well as in non-SA-accumulating plants, indicating that the ability to accumulate SA is not required for INA-induced pathogen resistance.

INA induction of PR-1 gene expression in NahG tobacco.

Induction of SAR is accompanied by the coordinate expression of the SAR genes (Ward *et al.* 1991). To test whether INA also induces SAR gene expression in NahG tobacco, plants were injected with INA and the leaves were harvested at various times after INA application. These tissues were analyzed for PR-1 gene expression by Northern blot analysis (Fig. 2B). The accumulation of PR-1 mRNA after INA was similar in both wild type (Fig. 2A) and NahG plants. In tissues of both genotypes, PR-1 expression was induced between 8 and 16 h post-INA application and remained constant up to 32 h.

INA induction of resistance in NahG Arabidopsis plants.

To determine if the action of INA was similar in species other than tobacco, Arabidopsis plants expressing salicylate hydroxylase were also evaluated following chemical treatment. *Arabidopsis thaliana* (L.) Heynh. ecotype Colombia (Col-O) and Col-O plants harboring a *nahG* transgene (K. Lawton, *unpublished*), were sprayed with INA or water and challenged with spores of the fungus *Peronospora parasitica* (Pers.:Fr.) Fr. isolate Noco, which causes downy mildew disease. Ten days after the challenge inoculation, plants were scored for the presence and absence of conidiophores on the leaf surfaces (Table 3). Leaves pretreated with water showed an extensive distribution of downy mildew in both Col-O and NahG plants. Trypan blue staining and microscopic evaluation of the inoculated tissues revealed extensive growth of fungal hyphae and the production of oospores in these leaves (Fig. 5). On leaves pretreated with INA, however, fungal growth and disease development were inhibited in both wild type and NahG plants (Fig. 5).

INA induction of PR-1 mRNA accumulation in NahG Arabidopsis.

NahG and wild type Arabidopsis plants were also tested for the induction of PR-1 mRNA in water- and INA-treated plants at various times following application. Figure 6 shows a Northern blot probed with labeled Arabidopsis PR-1 cDNA. PR-1 mRNA levels begin to increase within 4 h in both NahG and Col-O tissues, and maximal expression is obtained by 72 h after application.

DISCUSSION

INA has been described as a resistance-inducing chemical effective in many plant species (Kessmann *et al.* 1994). Available data suggested that INA induced systemic acquired resistance, a well-characterized response to pathogen infection in both tobacco and Arabidopsis. In Arabidopsis, infection of leaves with TCV was shown to induce resistance against *Pseudomonas syringae* DC3000 and TCV. At the same time that resistance is established, mRNA from three SAR genes, PR-1, PR-2, and PR-5, coordinately accumulates to high levels in uninfected tissues (Uknes *et al.* 1993). Exogenous treatment of Arabidopsis with INA also induced

Table 2. TMV lesion measurements in control and induced tobacco leaves

	TMV lesion sizes (mm)			
	Xanthi		NahG	
	Water	INA	Water	INA
Injected leaves				
Exp. 1	1.07 ± 0.30	0.58 ± 0.10 (45%)	2.29 ± 0.36	0.99 ± 0.25 (57%)
Exp. 2	3.83 ± 0.43	2.76 ± 0.34 (28%)	5.41 ± 0.52	3.74 ± 0.36 (31%)
Exp. 3	2.71 ± 0.39	0.76 ± 0.24 (72%)	3.77 ± 0.57	0.86 ± 0.37 (77%)
		48%		55% mean
Systemic leaves				
Exp. 1	1.40 ± 0.43	0.58 ± 0.18 (26%)	2.64 ± 0.41	1.24 ± 0.69 (32%)
Exp. 2	3.92 ± 0.32	2.90 ± 0.33 (58%)	5.07 ± 0.32	3.46 ± 0.38 (53%)
Exp. 3	2.90 ± 0.32	0.64 ± 0.11 (78%)	3.76 ± 0.31	1.62 ± 0.87 (57%)
		54%		47% mean

[a] Seven days after water or INA injections, treated and untreated (systemic) leaves were challenged with TMV. Lesions were measured (mm) 7 days later. A minimum of 10 lesions per leaf on nine leaves were measured for each treatment. The relative reduction in lesion sizes induced by INA pretreatment is indicated in parentheses and averaged over three independent experiments. These data are from different experiments than the one shown in Figure 1.

resistance against *Pseudomonas syringae* DC3000, TCV, and *Peronospora parasitica,* and concomitantly induces mRNA accumulation of the SAR genes (Uknes *et al.* 1992). In tobacco, we show here, both viral and INA treatment induce resistance to the same five pathogens. In previous studies, we showed that either TMV, INA, or SA treatment of plants induces accumulation of the same nine SAR genes. Furthermore, the treatments do not induce expression of other defense genes such as the class I chitinase or class I glucanase (Ward *et al.* 1991). Thus, based on the criteria that define SAR in either Arabidopsis or tobacco (i.e., spectrum of resistance and gene expression), INA induces the SAR pathway.

The only confirmed step in the signal transduction pathway leading to SAR is the accumulation of SA (Malamy *et al.* 1990; Métraux *et al.* 1990; Gaffney *et al.* 1993). Here, we show that INA treatment does not result in SA accumulation in tobacco. Furthermore, INA is capable of inducing pathogen resistance and gene induction in transgenic plants expressing the bacterial salicylate hydroxylase gene. Together, these results strongly argue that INA acts either at the same step or downstream of SA.

It is of particular interest that even though INA can reverse the effect of *nahG* on inhibiting SAR, it does not totally compensate. For example, while the percent reduction of lesion size is similar in Xanthi and NahG tobacco, the size of the lesions in both control and induced plants is larger in NahG plants. There are several plausible explanations for this result. One possibility is that SA may have two roles in disease resistance; one involved in the hypersensitive response and the second in the signal transduction pathway leading to SAR. If this is the case, then INA may only compensate in SAR signal transduction. In any event, the roles of SA and INA in disease resistance remain an interesting area for future study.

MATERIAL AND METHODS

Plant material and treatments.

Nicotiana tabacum L. 'Xanthi.nc' and 'NahG-10' tobacco plants (Gaffney *et al.* 1993) were grown as described (Payne *et al.* 1990) and treated at 6–8 wk. For the RNA and SA analysis and the TMV assays in Table 2 and Figure 4, three leaves per plant from three plants were completely injected with water or 0.7 mM INA (formulated as 25% active ingredient in a wettable powder). Tissues collected for biochemical analysis were harvested, pooled, frozen in liquid nitrogen, and stored at –80° C.

Arabidopsis plants were grown as previously described (Uknes *et al.* 1992) and treated at 3–4 wk. Water and INA (325 μM, as a wettable powder) were sprayed to runoff. Tissues for PR-1 Northern analysis were harvested at the indicated times and frozen prior to RNA extraction (Uknes *et al.* 1992).

Pathogen assays.

For each of the pathogen assays shown in Figure 1 and Table 1, three Xanthi.nc plants were injected with INA (the methylester derivative of INA was injected for the *Phytophthora parasitica* assay) or with water on two half-leaves per plant. Seven days later, plants were challenged with the pathogen. TMV and *C. nicotianae* assays were performed as described (Vernooij *et al.* 1994). Other assays were performed as follows: *Pseudomonas syringae* pv. *tabaci* was injected at 10^6 cfu/ml, and plants were kept in a humid chamber for 2 days. After 4–6 days, plants were rated for disease severity on a 5-point scale (0 = no symptoms; 5 = total necrosis) (Delaney *et al.* 1994). *Phytophthora parasitica* var. *nicotianae* zoospores (8×10^4 per milliliter, 2 ml per plant) were pipetted around the stem base and washed into the soil with 50 ml of water. Wilting was rated 21 days later (Alexander *et al.* 1993). A spore suspension of *Peronospora tabacina* (10^3 per milliliter) was sprayed on plants that were subsequently kept in the dark at 25° C at 100% humidity for 24 h. After another 11–13 days, the plants were rated for severity of disease.

For Arabidopsis disease resistance assays, spores of *Peronospora parasitica* isolate Noco were harvested from leaves of Col-O plants that were infected 1 wk earlier and resuspended in water at $5–10 \times 10^4$ spores per milliliter. This suspension was sprayed onto plants 4 days after the chemical pretreatment. Seven to 10 days later, leaf surfaces were examined for the presence of conidiophores. Representative leaves were stained with trypan blue (Keogh *et al.* 1980), examined under a microscope, and photographed.

Biochemical analysis.

RNA extractions, Northern blot analysis, and SA extractions were performed as previously described (Gaffney *et al.* 1993; Uknes *et al.* 1993). SA levels were not corrected for recovery.

Table 3. Disease severity in control and induced Arabidopsis plants[a]

	Infected plants[b] (%)			
	Col-O		NahG	
	Water	INA	Water	INA
Exp. 1	100% (27)	0% (24)	100% (42)	0% (44)
Exp. 2	93% (46)	0% (40)	100% (36)	0% (34)
Exp. 3	100% (22)	0% (32)	97% (31)	7% (28)

[a] Ten days after infection with fungal spores, the percentage of infected plants (plants with conidiophores) was determined in water and INA pretreated plants.

[b] The number of plants in each group is indicated in parentheses.

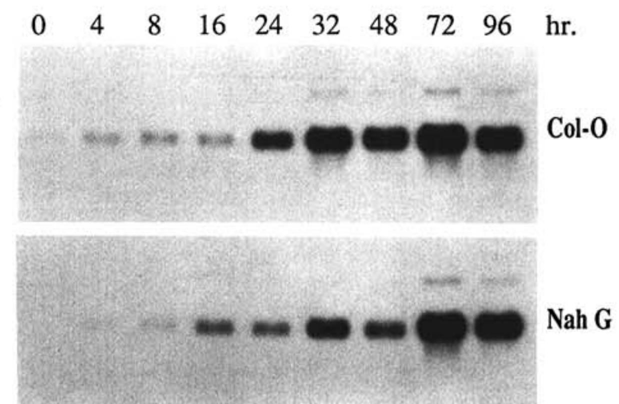

Fig. 6. Time course of 2,6-dichloroisonicotinic acid (INA) induced PR-1 mRNA accumulation in Col-O and NahG plants following INA injection (time in hours).

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