Biological Induction of Systemic Acquired Resistance in *Arabidopsis*

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Previously, *Arabidopsis* was shown to develop acquired resistance to a bacterial and a fungal pathogen following treatment with the activator compound 2,6-dichloroisonicotinic acid (INA). In this study, we show that a necrotizing pathogen, turnip crinkle virus (TCV), can induce systemic acquired resistance (SAR) in *Arabidopsis* against bacterial (*Pseudomonas syringae*) and viral (TCV) pathogens. The TCV-induced resistance results in both a decrease in visible disease symptoms and a reduction in the growth of the challenge pathogen. Levels of salicylic acid (SA), a likely endogenous signal for SAR, increased more than fivefold in response to TCV infection. In addition, exogenously applied SA induced resistance to the fungal pathogen *Peronospora parasitica*. Three genes induced to high levels by SA (PR-1, PR-2, and PR-5) were also induced by TCV inoculation. In addition, we show that INA treatment induces resistance to TCV, extending the previous observation of chemically induced resistance against bacterial and fungal pathogens to include protection against viral pathogens.

Many higher plants respond to necrogenic pathogen infection by becoming resistant to further infection. This plant "immunity" or acquired resistance was first reported in the early 1900s (reviewed by Chester 1933). Ross performed systematic studies demonstrating that tobacco infected with tobacco mosaic virus (TMV) becomes resistant to further TMV infection (Ross 1961a,b). Resistance that develops in the infected leaf was termed localized acquired resistance (Yarwood 1960; Ross 1961a), and resistance in distal, uninfected parts of the plant was termed systemic acquired resistance (SAR; Ross 1961b). SAR was subsequently shown to be inducible by other pathogens that cause leaf necrosis, and the resistance was shown to be effective against a broad spectrum of diseases, including those caused by viruses, bacteria, and fungi (Cruikshank and Mandryk 1960; Ross 1961b; Lovrekovich *et al.* 1968; for review, see Kuc 1982). In addition, certain chemicals that have no *in vitro* antimicrobial activity, such as 2,6-dichloroisonicotinic acid (INA), have been shown to induce the same broad-spectrum resistance (Métraux *et al.* 1991).

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In tobacco and cucumber, pathogen infection initiates a process that leads to the accumulation of high concentrations of salicylic acid (SA; Malamy *et al.* 1990; Métraux *et al.* 1990; Rasmussen *et al.* 1991). The accumulation of SA is associated with the coordinate induction of a set of at least nine SAR gene families, which include genes encoding the 10 pathogenesis-related (PR) proteins of tobacco (Van Loon and Van Kammen 1970; Ward *et al.* 1991; Yalpani *et al.* 1991). Exogenously applied SA induces the accumulation of SAR gene mRNAs in tobacco and *Arabidopsis* (White 1979; Ward *et al.* 1991b; Uknes *et al.* 1992). These results have led to the working hypothesis that one consequence of lesion formation is the accumulation of SA, which induces the expression of a set of proteins that act to limit further infection of the host (Ward *et al.* 1991). Direct support for this idea comes from the observation that transgenic tobacco plants that are unable to accumulate SA, because they express the enzyme salicylate hydroxylase, do not exhibit SAR (Gaffney *et al.* 1993). In addition, transgenic tobacco expressing high constitutive levels of tobacco PR-1a display increased disease tolerance to two oomycete fungi, *Peronospora tabacina* and *Phytophthora parasitica* (Alexander *et al.* 1993). Thus, SA is required for SAR, and at least some of the SAR genes participate directly in pathogen resistance.

The signal transduction pathways coupling the perception of pathogen infection with the acquisition of the resistant state are likely to be complex. We have chosen a molecular genetic approach to identify genes whose products function in regulating this process. Toward this end, we previously demonstrated that the synthetic compound INA can induce SAR gene expression and resistance to bacterial and fungal pathogens in *Arabidopsis thaliana* (Uknes *et al.* 1992). Here, we show that INA-induced resistance also acts on a viral pathogen. In addition, turnip crinkle virus (TCV) infection of *Arabidopsis* can induce SA, SAR gene expression, and resistance against bacterial and viral pathogens. We further demonstrate that exogenously applied SA induces resistance to the fungal pathogen *Peronospora parasitica*. Thus, all of the characteristics of acquired resistance, including biological induction, occur in *Arabidopsis*.

RESULTS

Systemic acquired resistance against *Pseudomonas syringae*.

Most ecotypes of *Arabidopsis* are susceptible to TCV, exhibiting systemic spread of the virus and a crinkled appear-

ance of the infected leaves (Simon *et al.* 1992). However, *A. thaliana* ecotype Dijon (Di-0) displays a resistance response to TCV that results in localized leaf necrosis (Simon *et al.* 1992). The viral lesions spread slowly, eventually necrotizing the entire leaf 10–20 days after infection. However, the noninoculated leaves do not show disease symptoms. To determine whether this necrosis was able to induce disease resistance, TCV was applied to two leaves of Di-0. Within 2–5 days after infection, necrotic lesions appeared on infected leaves. Seven days after TCV infection, the plants were dipped in a suspension of *Pseudomonas syringae* pv. *tomato* strain DC3000 (Whalen *et al.* 1991). Figure 1 shows *P. syringae* symptom development in TCV-infected plants compared with that in mock-inoculated controls. The controls displayed many small grayish brown lesions surrounded by spreading chlorosis, typical of bacterial speck disease, whereas the TCV-infected plants showed fewer lesions and reduced chlorosis. The bacterial titer was determined in leaves of control and TCV-infected plants in four separate experiments. Approximately 10-fold more bacteria were found in control plants than in TCV-infected plants (data not shown).

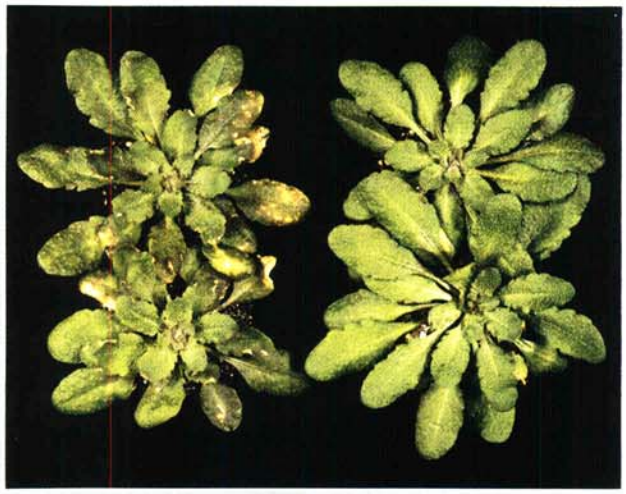

Fig. 1. Turnip crinkle virus (TCV)-induced reduction in symptoms caused by *Pseudomonas syringae*. Plants were treated with either water (left) or TCV (right) and challenge-infected 7 days later with *P. syringae*. The plants were photographed 4 days after bacterial infection.

Systemic acquired resistance against TCV.

Arabidopsis also displayed systemic resistance against TCV following a local infection with TCV. Two lower leaves were inoculated with TCV and allowed to develop lesions. Subsequently, two uninfected upper leaves of these plants were challenged with TCV, and disease symptoms were evaluated after 5 days. Figure 2 shows lesions in TCV-challenged leaves from TCV-infected plants, compared with TCV-challenged leaves from mock-inoculated controls. The controls displayed many large lesions surrounded by extensive chlorosis, whereas TCV-pretreated plants had smaller lesions with less chlorosis. To determine whether decreased symptom development corresponded to reduced virus accumulation, TCV-specific RNA was measured in the challenge leaves of control and TCV-induced plants by gel blot analysis. As shown in Figure 3A, approximately eightfold more TCV RNA was found in the challenged leaves of control plants than in TCV-induced plants.

Fig. 2. Turnip crinkle virus (TCV)-induced resistance to TCV. Leaves of Di-0 plants were treated with TCV (upper left) or bentonite buffer (upper right). Seven days later, other leaves on the same plants were challenged with TCV. Challenged leaves from TCV-treated plants (lower left) consistently showed less extensive lesion formation than challenged leaves from bentonite-treated plants (lower right). Leaves were removed from the plants for photography 5 days after the TCV challenge inoculation.

INA-induced resistance against TCV.

Since INA is able to induce resistance in *Arabidopsis* to bacterial and fungal pathogens (Uknes *et al.* 1992), we tested whether INA-induced resistance was also effective against TCV. Figure 4 shows symptom development in TCV-challenged leaves from INA-treated plants, compared with that in TCV-challenged leaves from water-treated controls. As in previous experiments, the controls displayed large, papery, grayish brown lesions, surrounded by spreading chlorosis. However, INA-treated plants had smaller lesions and less-extensive, slower-spreading chlorosis. As seen previously (Uknes *et al.* 1992), high concentrations of INA caused some phytotoxic effects in the treated plants, evident as smaller leaves with some leaf curling. To determine whether decreased symptom development was linked to a reduction in the amount of TCV present, TCV-specific RNA was measured in challenged leaves of control and INA-treated plants by RNA gel blot analysis (Fig. 3B). An average of eightfold more TCV-specific RNA was found in the challenged leaves from control plants than in INA-treated plants in three separate experiments (data not shown).

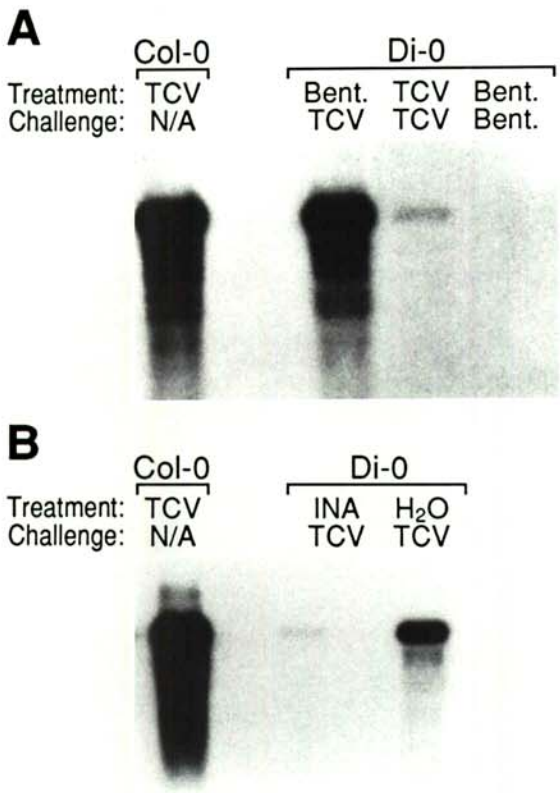

Fig. 3. Reduction in viral RNA accumulation by turnip crinkle virus (TCV)- and 2,6-dichloroisonicotinic acid (INA)-induced resistance. **A,** Di-0 plants were treated with either TCV or bentonite buffer (Bent.) and challenged with TCV or bentonite 7 days later. Challenged leaves were harvested 5 days after inoculation. **B,** Di-0 plants were sprayed with H_2O or with INA (100 μg/ml) and challenged with TCV 7 days later. Virus-treated leaves were harvested 8 days after virus inoculation for isolation of RNA. Both RNA gel blots were probed with TCV cDNA. RNA isolated from the susceptible *Arabidopsis thaliana* ecotype Columbia (Col-0) systemically infected with TCV is included as a control. N/A, not applicable.

SA-induced resistance against *Peronospora parasitica*.

Because SA is a probable endogenous signal for SAR and exogenously applied SA causes an increase in SAR gene expression in *Arabidopsis* (Uknes *et al.* 1992), we tested whether exogenously applied SA could induce resistance to *P. parasitica*. Seven-day-old plants were treated with 15 mM SA or water; 5 days later, the plants were inoculated with *P. parasitica*. Subsequent to infection, tissue samples were collected and stained to visualize fungal structures. By 6 days, dramatic differences could be observed between water-treated control plants and plants that had been treated with SA. As shown in Figure 5, only the water-treated controls showed the profuse conidiophore formation typical of downy mildew disease (Koch and Slusarenko 1990), whereas the SA-treated plants showed no evidence of infection (Fig. 5A and B). Upon microscopic examination, the control plants were found to harbor elaborate networks of fungal hyphae, with abundant haustoria, conidia, and oospores. In contrast, plants that had been treated with SA showed few hyphae and no evidence of sporulation (Fig. 5C–F). Within SA-treated plants, small regions of plant cell necrosis could be found, presumably at sites of attempted fungal penetration. Treatment of *Arabidopsis* with lower concentrations of SA gave lower levels of resistance to *P. parasitica* (data not shown).

TCV induction of SA levels.

Since endogenous SA is required for the development of SAR in tobacco (Gaffney *et al.* 1993) and SA levels increase in response to resistance-inducing pathogen infections in tobacco and cucumber (Malamy *et al.* 1990; Métraux *et al.* 1990), we measured SA levels in TCV-infected Di-0 leaves. Table 1 shows that free SA levels in Di-0 increased five- to sixfold 3 days after TCV inoculation. Free SA levels declined steadily to twofold above the level of the bentonite control 7 days after inoculation. This pattern of free SA accumulation mirrors PR-1 mRNA abundance as determined by RNA gel blot analysis of the same samples (data not shown). Levels of glucose-conjugated SA (GSA) peaked at 5 days postinfection, when the TCV-infected leaves contained 11-fold more GSA

Fig. 4. 2,6-Dichloroisonicotinic acid (INA)-induced reduction of turnip crinkle virus (TCV)-generated necrosis. Di-0 plants were treated with INA (100 μg/ml) (left), INA (50 μg/ml) (center), or water (right) and challenged with TCV 7 days later. Representative challenged leaves were removed and photographed 8 days after TCV inoculation.

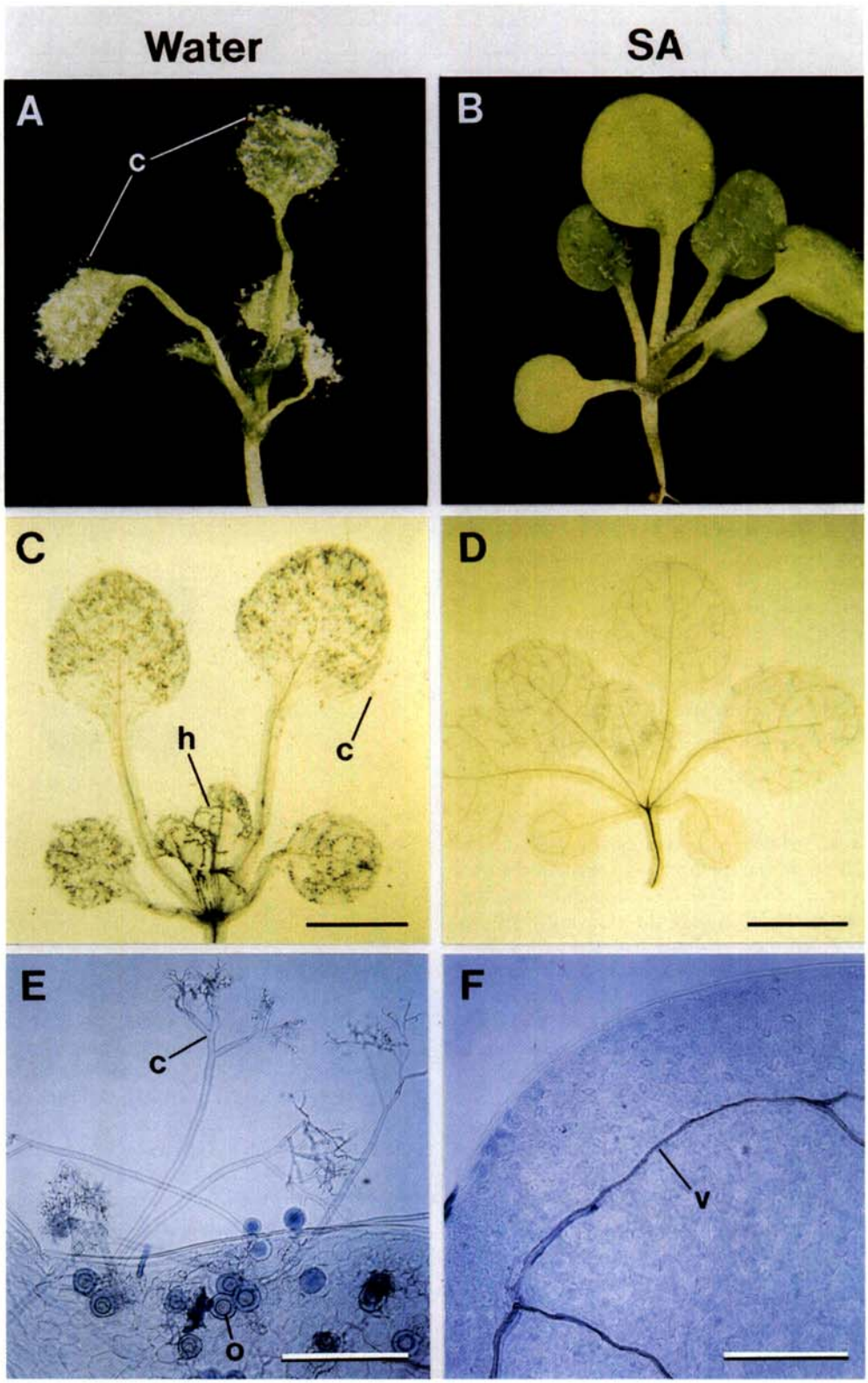

Fig. 5. Salicylic acid (SA)-induced resistance to infection by *Peronospora parasitica*. *Arabidopsis thaliana* ecotype Weiningen plants were treated with SA or water and 5 days later were inoculated with *P. parasitica*. Photographs shown are 6 days postinoculation. **A,** Disease symptom development on water-treated control; **B,** SA-treated plants. Trypan blue-stained leaves from: **C,** water-treated control; **D,** SA-treated plants (scale bar = 2.5 mm). Stained tissue at higher magnification, showing fungal development in: **E,** control and **F,** SA-treated plants (scale bar = 250 μm).c, conidiophore; h, hyphae; o, oospore; v, vascular tissue.

Table 1. Salicylic acid (SA) and glucose-conjugated SA (GSA) levels in control and turnip crinkle virus (TCV)-inoculated *Arabidopsis*

Treatment	Days postinoculation			
	0	3	5	7
SA levels				
Bentonite	35 ± 13	39 ± 6	47 ± 10	32 ± 0
TCV		218 ± 38	130 ± 51	61 ± 0
GSA levels				
Bentonite	432 ± 510	143 ± 106	184 ± 16	92 ± 45
TCV		812 ± 727	2054 ± 680	1302 ± 828

Free and glucose-conjugated SA levels in control and TCV-infected *Arabidopsis* leaves. Plants were treated with bentonite or TCV on day 0. Leaves harvested on the days indicated were extracted for SA and GSA. Day 0 samples were from noninfected plants. The averages ± standard deviation are shown (ng/g fresh weight).

than the bentonite-treated control. The level of GSA in TCV-infected tissue at day 7 declined to approximately 60% of the amount present at day 5.

Molecular responses of *Arabidopsis* to TCV infection.

In tobacco and *Arabidopsis*, INA induces the accumulation of mRNAs that encode a variety of SAR-associated proteins (Ward *et al.* 1991; Uknes *et al.* 1992). In tobacco, necrogenic pathogens cause an increase in SAR gene expression in both the infected (primary) leaves and the uninfected (secondary) leaves from infected plants (Ward *et al.* 1991). Therefore, we assayed SAR mRNA abundance from the primary and secondary leaves of Di-0 after inoculation with TCV. Figure 6 shows the result of a typical experiment. PR-1 mRNA abundance was induced more than 20-fold, and PR-2 and PR-5 mRNAs were induced approximately 10-fold in both the primary and secondary leaves 4 days after TCV infection.

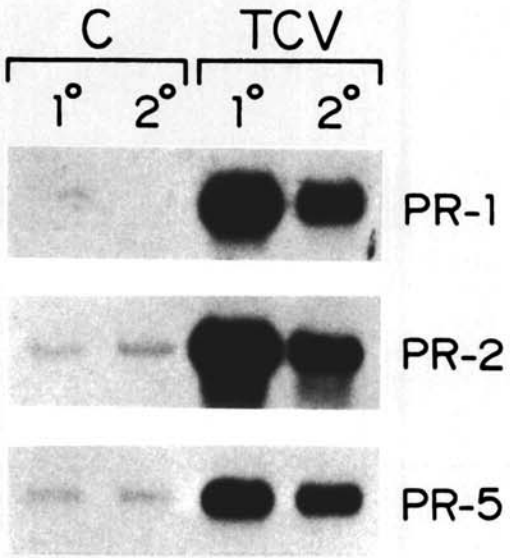

Fig. 6. Turnip crinkle virus (TCV) induction of systemic acquired resistance mRNAs in *Arabidopsis*. RNA gel blot analysis of the induction of PR-1, PR-2, and PR-5 mRNA in primary (1°) and secondary (2°) tissue. C, bentonite control; TCV, virus-inoculated tissue. Tissue was harvested for RNA extraction 4 days after treatment.

DISCUSSION

Our previous work showed that *Arabidopsis* can acquire resistance to bacterial and fungal pathogens as a result of chemical treatment (Uknes *et al.* 1992). With the aim of fully developing *Arabidopsis* as a model system for studying SAR, we have now demonstrated the remaining hallmarks of SAR in this species: 1) induction of the resistant state by pathogen infection and 2) broad-spectrum resistance. The work described here provides a basis for genetic dissection of SAR, from primary events involving the plant-pathogen interaction to expression of the disease-resistant state.

Biologically induced SAR in *Arabidopsis* was effective against both bacterial (*P. syringae*) and viral (TCV) challenge inoculation in secondary tissue. TCV- and INA–induced resistance to TCV reduced both the spread of the visible lesions and the amount of viral RNA present. Whether this reduction in pathogen accumulation was caused by inhibition of intercellular movement or by replication of the virus was not determined. Nonetheless, the TCV-infected plants seem to exert an effect on bacterial and viral pathogens analogous to the response observed after INA treatment (Uknes *et al.* 1992). Therefore, TCV on *A. thaliana* ecotype Di-0 is a suitable pathogen system for both inducing and measuring acquired resistance.

The reduced susceptibility to pathogens observed in TCV-infected plants was correlated with the accumulation of SA and mRNAs for PR-1, PR-2, and PR-5 in these plants. Following TCV infection, *Arabidopsis* showed a more than five-fold increase in SA levels. Free SA levels peaked at or before day 3 and then declined, mirroring SAR gene expression. In contrast, the glucose conjugate peaked at day 5, when PR–1 mRNA levels were already declining. This is consistent with the precursor–product relationship that has been demonstrated in tobacco (Enyedi *et al.* 1992), with free SA being the active signal molecule for SAR gene expression. In addition, exogenous SA was able to induce the expression of the SAR genes (Uknes *et al.* 1992) and resistance to *P. parasitica*. Therefore, like tobacco, *Arabidopsis* appears to use SA as an endogenous signal that mediates induction of both SAR gene expression and resistance (Gaffney *et al.* 1993; Lawton *et al.* 1993; Uknes *et al.* 1993). However, experiments in cucumber and tobacco suggest that SA, although required for resistance, is not the systemic, long-distance signal for SAR (Rasmussen *et al.* 1991; Vernooij *et al.*, unpublished).

The correlation between SAR gene expression and the development of the resistant state has led us to postulate a role for SAR gene products in resistance (Ward *et al.* 1991). Exactly how SAR gene expression causes the resistance is unknown. However, some of the SAR gene products, including β-1,3-glucanases and permatins (PR-2 and PR-5, respectively),

are known to have direct antifungal activity when assayed *in vitro* (Mauch *et al.* 1988; Roberts and Selitrennikoff 1988; Vigers *et al.* 1991; Woloshuk *et al.* 1991). A direct antifungal role for one SAR gene product has been demonstrated by using transgenic tobacco. Plants expressing high levels of tobacco PR-1a have significantly increased tolerance to the oomycete fungal pathogens *Peronospora tabacina* and *Phytophthora parasitica* (Alexander *et al.* 1993). Thus, at least part of the acquired resistance to blue mold and black shank diseases in tobacco can be attributed to the expression of PR-1a. Therefore, the high levels of PR-1 mRNA and protein found in immunized *Arabidopsis* plants may partially account for their resistance to the oomycete pathogen, *P. parasitica*.

MATERIALS AND METHODS

Cultivation of plants.

A. thaliana ecotype Di-0 was sown in 200-ml containers in an all-purpose soil mix that had been autoclaved twice for 70 min. The seeds were surface-sterilized with bleach (20% commercial bleach, 0.05% sodium dodecyl sulfate) for 5–10 min, washed several times in sterile distilled water, sprayed with 70% ethanol, and rinsed several more times before sowing. To vernalize seed, the containers were kept at 4° C in the dark for several days. Plants were grown at 20–24° C, 60% RH, 9 hr day/15 hr night. The soil surface was allowed to dry between waterings.

Infection with turnip crinkle virus.

A. thaliana ecotype Di-0 plants were treated with TCV or bentonite buffer (0.05 M glycine, 0.03 M K_2HPO_4, 0.02 g of bentonite per milliliter, pH 9.2, or aqueous 2% bentonite) on two adjacent leaves, 4 wk after planting (Simon *et al.* 1992). Bentonite buffer (5 μl) or turnip crinkle virus (0.1 μg/μl) in bentonite buffer was pipetted onto each treatment leaf. The droplet was then rubbed across the leaf surface at least five times with a gloved finger, and the treated leaves were marked with a felt-tipped pen. Only plants with distinct localized necrotic lesions were used for challenge inoculations. For the challenge application of TCV or bentonite buffer, the procedure was repeated 7 days later, using leaves opposite the primary inoculated leaves in the rosette.

Pseudomonas syringae pv. *tomato* infection conditions.

Four-week-old *A. thaliana* ecotype Di-0 plants were treated with either TCV or bentonite buffer. Seven days later, *P. syringae* infections were performed by dipping plants in a suspension of bacteria (approximately 10^8 cfu/ml) in 10 mM $MgCl_2$, 0.02% Silwet L-77 (Union Carbide, Danbury, CT), as described by Whalen *et al.* (1991). At various times after infection (ranging from 1 to 12 days), more than 20 individual leaves from more than 15 individual plants per treatment were homogenized in 10 mM $MgCl_2$ and quantified by plating on nutrient agar (Difco) containing rifampicin (50 μg/ml).

SA analysis.

Five leaves per plant were treated with aqueous 2% bentonite or TCV as described above, and duplicate or triplicate 0.5-g samples were harvested at various times (two to three leaves from each plant). The day 0 samples were collected from nontreated plants. SA was extracted from leaf tissue and analyzed essentially as described (Gaffney *et al.* 1993), with the following modifications. Methanol extracts were split into two equal parts; one part was processed for free SA analysis and one part for free SA plus GSA (Enyedi *et al.* 1992). Final samples were resuspended in 250 μl of 20% methanol, from which 50 μl was injected into the high-performance liquid chromatographic column. Step-gradient elution was performed using 20 mM sodium acetate, pH 5.0, plus 20% methanol (solvent A) and 20 mM sodium acetate, pH 5.0, plus 70% methanol (solvent B). Separation conditions were: a 15-min gradient from 5 to 30% solvent B, followed by 100% B and reequilibration at 5% B. Fluorescent detection was performed as described (Gaffney *et al.* 1993), with a 400 ± 25 nm band-pass filter. To confirm the nature of the peak that had the same retention time as pure SA, peak fractions were collected, dried down, and digested with salicylate hydroxylase (Sigma), according to the manufacturer's instructions. The salicylate hydroxylase enzyme has high specificity for SA, as it does not act on several structurally related compounds (Tom Gaffney, personal communication).

Treatment with INA.

INA (Ciba-Geigy AG, Basel, Switzerland), formulated as 25% active ingredient in a wettable powder carrier (Métraux *et al.* 1991) was suspended in distilled water at 50 (65 μM INA), 100, or 250 μg/ml. This solution, or distilled water alone, was spray-misted to the point of imminent runoff onto 4-wk-old *A. thaliana* ecotype Di-0 plants. Plants were challenged with TCV or bentonite buffer 7 days later.

Treatment with SA and inoculation with *Peronospora parasitica*.

SA (15 mM) or water alone was sprayed to imminent runoff on 1-wk-old *A. thaliana* ecotype Weiningen plants. Plants were inoculated with *P. parasitica* (WELA) 5 days later by spraying with a conidial suspension (approximately 5×10^4 spores per milliliter). Inoculated plants were incubated under humid conditions at 17° C in a growth chamber with a 14-hr day/ 10-hr night cycle. Plants were examined at 1, 6, and 10 days after inoculation for the presence of conidiophores. In addition, several plants from each treatment were randomly selected and stained with lactophenol-trypan blue (Keogh *et al.* 1980) for microscopic examination.

RNA gel blot analysis.

RNA was purified from frozen tissue samples by phenol/chloroform extraction followed by lithium chloride precipitation (Lagrimini *et al.* 1987). Samples of total RNA (5 μg) were separated by electrophoresis through formaldehyde-agarose gels and blotted to nylon membrane (GeneScreen Plus, NEN) as described by Ausubel *et al.* (1987). Ethidium bromide was included in the sample loading buffer at 40 μg/ml, which allowed photography under UV light after electrophoresis to confirm equal sample loading. *Arabidopsis* PR-1, -2, and -5 (Uknes *et al.* 1992), and TCV (a gift from A. Simon) cDNA clones were ^{32}P-labeled by random priming (Feinberg and Vogelstein 1983; International Biotechnologies, Inc., New Haven, CT). Hybridizations and washings were according to Church and Gilbert (1984). Relative amounts

of transcript were determined by detecting β-decay of ^{32}P with a Betascope 603 blot analyzer (Betagen, Waltham, MA).

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