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Systemic Acquired Resistance in *Arabidopsis* Requires Salicylic Acid but Not Ethylene

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Systemic acquired resistance (SAR) is an inducible plant response to infection by a necrotizing pathogen. In the induced plant, SAR provides broad-spectrum protection against not only the inducing pathogen, but also against other, unrelated pathogens. Both salicylic acid (SA) and SAR-gene expression have been implicated as playing important roles in the initiation and maintenance of SAR. Here, we describe the characterization of transgenic *Arabidopsis* plants that express the bacterial *nahG* gene encoding salicylate hydroxylase, an enzyme that can metabolize SA. Strong, constitutive expression of this gene prevents pathogen-induced accumulation of SA and the activation of SAR by exogenous SA. We show that SAR in *Arabidopsis* can be induced by inoculation with *Pseudomonas syringe* pv. *tomato* against infection by a challenge inoculation with *Peronospora parasitica*. This response is abolished in transgenic, *nahG*-expressing *Arabidopsis*, but not in ethylene-insensitive mutants. These experiments support the critical role of SA in SAR and show that ethylene sensitivity is not required for SAR induction. The NahG *Arabidopsis* plants will be important for future studies aimed at understanding the role of SA in plant disease resistance mechanisms.

Additional keyword: pathogen resistance.

Systemic acquired resistance (SAR) is an inducible, broad-spectrum, systemic resistance response that is triggered in many plants following infection by certain necrotizing pathogens. While the response has been known for almost 100 years (reviewed by Chester 1933) and many descriptive and physiological studies have been reported, relatively little is known about the biochemical basis of SAR (Ryals et al. 1994). Recently, however, there has been increased interest in understanding the biochemical and molecular genetic basis for SAR since this could lead to the development of novel mode-of-action fungicides as well as genetically engineered crops with enhanced disease resistance.

SAR can be divided conceptually into two phases: initiation and maintenance (Ryals et al. 1994). The initiation phase begins immediately following pathogen infection and includes the transient changes that lead to the establishment of a quasi-steady-state in which resistance is maintained. During the maintenance phase of SAR in cucumber, tobacco, and *Arabidopsis* a number of genes, called SAR genes, are highly expressed (Métraux 1989; Ward et al. 1991; Uknes et al. 1992). In tobacco, the products of these genes accumulate to levels of about 3 to 5% of the total mRNA and protein in the pathogen-infected leaves and about 0.3 to 1% of the total mRNA and protein in uninfected leaves of pathogen-infected plants (J. Ryals, unpublished results). These genes encode proteins that constitute some of the pathogenesis-related (PR) proteins. A number of these proteins have in vitro antifungal activity (Mauch et al. 1988; Roberts and Selitrennikoff 1988; Roberts and Selitrennikoff 1990), which has led to the suggestion that they may play an active role in maintaining the resistant state (Ryals et al. 1992). In support of this idea, it has been shown that transgenic plants expressing SAR genes or related genes have enhanced tolerance to fungal and bacterial pathogens (Alexander et al. 1993; Broglie et al. 1991; Lawton et al. 1993; Liu et al. 1995).

Currently, only two steps of the signaling pathway during SAR initiation have been clearly defined. First, as stated earlier, the SAR genes are expressed at a time that correlates with the onset of resistance (Ward et al. 1991). Second, salicylic acid (SA) has been shown to accumulate to high levels in pathogen-infected leaves of tobacco, cucumber, and *Arabidopsis*, and to much lower but still significant levels in the uninfected leaves of pathogen-infected plants (Malamy et al. 1990; Métraux et al. 1990; Uknes et al. 1992; Vernooij et al. 1994). In addition, the concentration of SA is correlated with both the accumulation of SAR marker proteins such as the tobacco PR-1 protein and enhanced resistance against pathogens (Yalpani et al. 1991). Because exogenous SA application to either cucumber, tobacco, or *Arabidopsis* leaves causes the accumulation of the same set of proteins and mRNAs as in SAR, SA has been implicated in the SAR signal transduction pathway (Lawton et al. 1994a; Métraux et al. 1989; Uknes et al. 1992; Ward et al. 1991). In support of this idea, transgenic tobacco plants that express salicylate hydroxylase (SAH), an enzyme produced by *Pseudomonas putida* that metabolizes SA to catechol (You et al. 1991), do not exhibit SAR in response to pathogen infection (Gaffney et al. 1993).

Along with its involvement in SAR signal transduction, SA has been shown to have a central role both in disease susceptibility and genetically determined disease resistance (Delaney et al. 1994). NahG tobacco plants do not accumulate significant levels of SA and have enhanced susceptibility to pathogens. Transgenic *Arabidopsis* plants that express *nahG* also have enhanced pathogen susceptibility and become sus-

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ceptible to avirulent bacterial and fungal pathogens. Thus, it appears that SA mediates both SAR and genetically determined disease resistance.

While SA is required for SAR, several lines of evidence indicate that it is not the systemically transmitted signal molecule. In cucumber, SAR could be established in uninfected leaves even though the pathogen-inoculated leaves were removed from the plant prior to detectable SA accumulation (Rasmussen et al. 1991). Likewise, in tobacco, reciprocal grafts between Xanthi-nc and transgenic *nahG*-expressing tobacco plants demonstrated that SAR could be established in Xanthi-nc scions grafted to NahG rootstocks (Vernooij et al. 1994). Thus, it appeared that while SA was required for signal transduction in the target (i.e., uninfected, systemic) tissues, it was not the systemic signal molecule. Furthermore, these results implicated the involvement of other signal molecules in SAR.

The phytohormone ethylene, it has also been suggested, plays a role in disease resistance (Ecker and Davis 1987; Raz and Fluhr 1993; Van Loon 1977). Ethylene has been shown to be produced during pathogen infection (Mauch et al. 1984; Ross and Williamson 1951), exogenous ethylene application induces enzymes such as glucanase and chitinase, which have antifungal properties (Mauch et al. 1992), and treatment of cell cultures with fungal elicitors induces ethylene biosynthesis (Spanu and Boller 1989). However, when plants are exposed to gaseous ethylene they do not show heightened disease resistance but instead sometimes show increased susceptibility (Hoffman and Heale 1987; Hughes and Dickerson 1989). Recently, ethylene has been implicated as a plant-produced signal that is utilized by the post-harvest fungal pathogens *Colletotrichum gloeosporioides* and *C. musae* to stimulate spore germination in ripening fruit (Flaishman and Kolattukudy 1994). Furthermore, certain ethylene-insensitive *Arabidopsis* plants show reduced disease symptoms following inoculation with an avirulent bacterial pathogen (Bent et al. 1992). While ethylene sensitivity is not required for chemically induced acquired resistance in *Arabidopsis*, it may serve as a modulator of tissue responsiveness to low levels of salicylate (Lawton et al. 1994b).

In the work presented here we show that SAR in *Arabidopsis* can be induced by primary inoculation with a bacterial pathogen to confer systemic resistance against challenge inoculation with a fungal pathogen. We also further characterize transgenic *Arabidopsis* plants that constitutively express high levels of *nahG* mRNA and protein. These plants do not accumulate significant levels of SA following pathogen inoculation and do not exhibit SAR or accumulate PR-1 mRNA in systemic tissue. Thus, as in tobacco, SA is crucial for establishment of SAR in *Arabidopsis*. Finally, using the ethylene-insensitive mutant *etr1*, we show that in addition to chemically induced acquired resistance, ethylene is not required for biologically induced SAR.

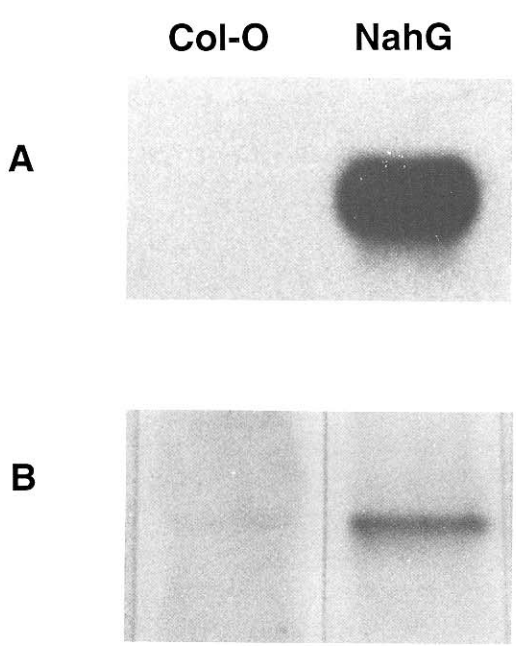

Fig. 1. Expression of *nahG* in transgenic *Arabidopsis*. The levels of *nahG* mRNA (**A**) and protein (**B**) in homozygous, transgenic *Arabidopsis* plants were determined by RNA gel blot analysis (**A**) and Western blotting (**B**).

RESULTS

Expression of the *nahG* gene in transgenic *Arabidopsis*.

The bacterial *nahG* gene encodes salicylate hydroxylase (SAH), an SA-metabolizing enzyme that catalyzes the decarboxylation of SA (You et al. 1991). *Arabidopsis* ecotype Columbia-O (Col-O) was transformed by *Agrobacterium*-mediated root transformation with the SAH coding region under the control of the enhanced 35S promoter as previously described (Delaney et al. 1994). Four independent transformants were obtained and homozygous lines were identified by testing T3 progeny for kanamycin resistance and analyzing expression of *nahG* mRNA and protein. All 10 homozygous transformed lines showed high levels of both *nahG* mRNA and protein. One line, B15, was chosen for further experiments. Shown in Figure 1 is the analysis of the *nahG* mRNA (Fig. 1A) and protein (Fig. 1B) levels in Columbia wild-type and transgenic NahG *Arabidopsis*. Interestingly, the expression of both the mRNA and protein was considerably higher than was observed in transgenic NahG tobacco and, unlike tobacco, pathogen inoculation did not affect the level of either *nahG* message or protein (data not shown).

Pathogen induction of salicylic acid accumulation is suppressed in *nahG*-expressing plants.

To determine the effect of SAH expression on salicylic acid accumulation, leaves of wild-type and transgenic NahG plants were inoculated with either buffer or *Pseudomonas syringe* pv. *tomato* strain DC3000 *avr*Rpt2*(Pst)*. Two days following inoculation both free SA and total SA (free SA plus glucosylated salicylic acid) levels were measured in the inoculated and uninoculated leaves of mock- or *Pst*-inoculated plants. These results are shown in Figure 2. In wild-type plants both free SA and total SA increased dramatically in the inoculated leaves. In the untreated leaves from pathogen-inoculated plants a slight but significant increase in SA was also observed. In contrast, the levels of free SA and total SA were substantially reduced in the inoculated leaves of the transgenic NahG plants relative to inoculated leaves of wild-type plants. Moreover, neither free SA nor total SA increased in the untreated leaves of pathogen-inoculated NahG plants.

Thus, salicylate hydroxylase was very effective in metabolizing endogenous SA, thereby preventing SA accumulation in NahG plants.

Chemical induction of acquired resistance in NahG *Arabidopsis*.

We have previously shown that NahG *Arabidopsis* can respond to 2,6-dichloroisonicotinic acid (INA), a synthetic activator of acquired resistance (Delaney et al. 1994; Vernooij et al. 1995). To determine if transgenic NahG plants can also respond to exogenous SA treatment, wild-type and NahG plants were treated with either SA or INA, and both PR-1 mRNA accumulation and resistance to *Peronospora parasitica* were determined. Accumulation of PR-1 mRNA is tightly correlated with disease resistance in tobacco and *Arabidopsis* (Uknes et al. 1992; Ward et al. 1991). As shown in Figure 3, PR-1 mRNA accumulated in wild-type plants following both SA and INA treatment. In contrast, transgenic NahG plants did not accumulate PR-1 message following exogenous SA application while INA induced the accumulation of PR-1 mRNA to essentially the same levels observed in wild-type plants. Thus, the SAH enzyme was capable of inactivating exogenously applied SA.

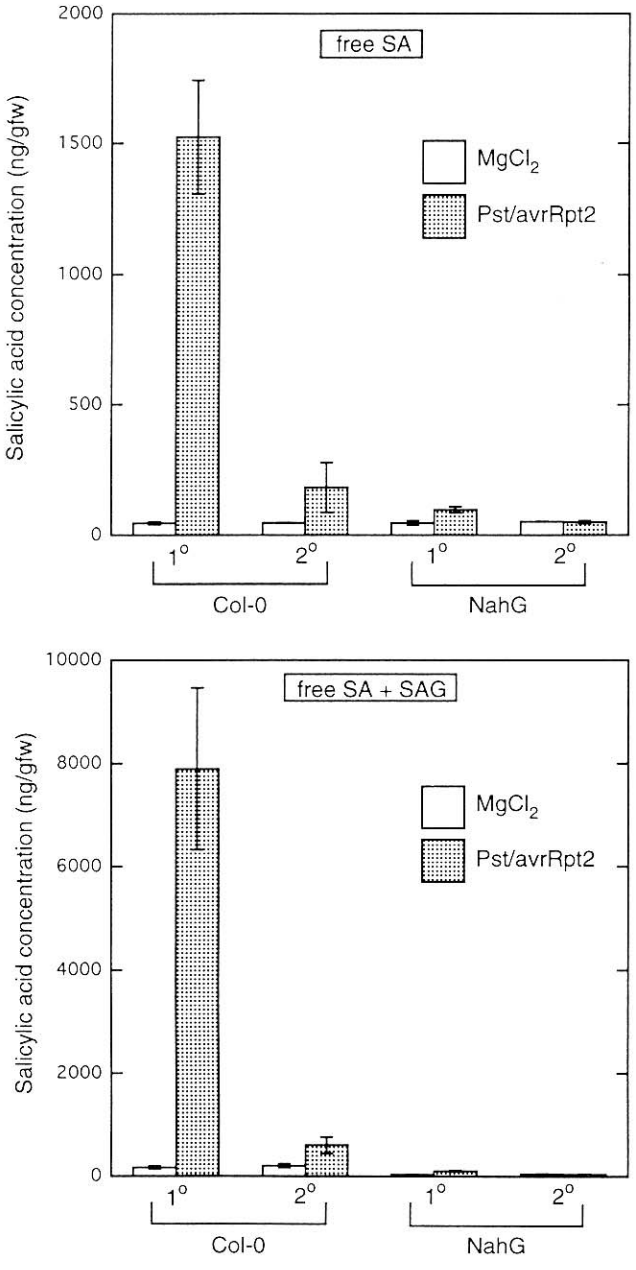

Fig. 2. Salicylic acid (SA) levels in inoculated and uninoculated leaves of Columbia (Col-O) and NahG *Arabidopsis* plants. Two days postinoculation with buffer or *Pst* DC3000 *avr*Rpt2 inoculated (1°) leaves or uninoculated (2°) leaves were harvested for SA extraction and analysis.

To determine the effects of chemical applications on disease resistance in NahG plants, both wild-type and NahG plants were inoculated with the fungal pathogen *P. parasitica* following treatment with SA or INA. As shown in Figure 4, exogenous application of SA resulted in disease resistance in wild-type plants but did not induce resistance in NahG transgenic plants. However, INA treatment induced resistance to *P. parasitica* in both wild-type and NahG plants. Taken together, these results show again a strong correlation between expression of SAR genes and resistance. These results are also consistent with the idea that INA acts at the same step as or downstream of SA in the SAR signal transduction pathway (Vernooij et al. 1995).

Pathogen-induced SAR in SA- and ethylene-compromised plants.

SAR in *Arabidopsis* has been previously demonstrated (Cameron et al. 1994; Mauch-Mani and Slusarenko 1994; Uknes et al. 1993). To further develop the SAR model in *Arabidopsis*, we investigated the possibility of using *Pst* to induce resistance against *Peronospora parasitica*. Leaves were inoculated with either the *Pst* strain containing the plasmidborne *avr*Rpt2 gene or mock-inoculated with 10 mM $MgCl_2$. At 2 days postinoculation the inoculated leaves as well as uninoculated leaves on the same plant were harvested for RNA extraction and analysis. At the same time, a group of

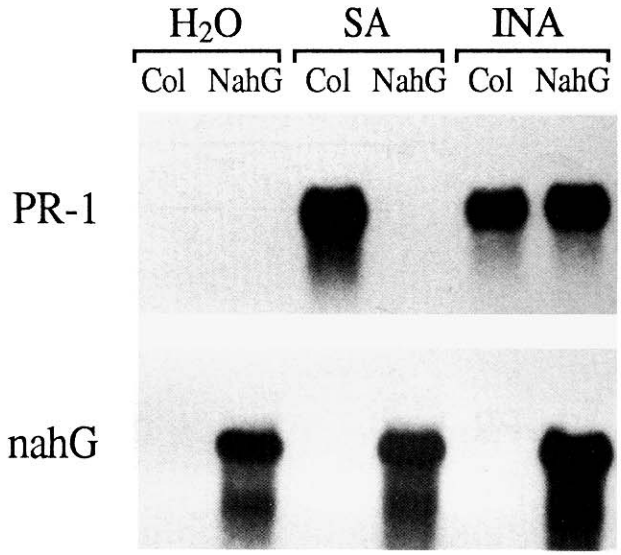

Fig. 3. PR-1 mRNA accumulation in wild-type (Col) and NahG plants following treatment with salycylic acid or 2,6-dichloroisonicotinic acid (INA). At 24 h post-treatment, total RNA was isolated and analyzed by RNA gel blot hybridization using cDNA to PR-1 and *nahG* as probes.

these plants was challenge-inoculated with the fungal pathogen *P. parasitica* pv. *Noco*.

Shown in Figure 5 is an RNA blot hybridized with a cDNA to PR-1. *Pst* inoculation of Columbia wild-type plants resulted in a dramatic accumulation of PR-1 mRNA in both the inoculated and uninoculated leaves. Relative to the buffer-treated control, the level of PR-1 increased 280-fold in *Pst* inoculated leaves and 85-fold in uninfected leaves of the pathogen-inoculated plants. Similar results were observed when the plants were inoculated with the virulent *Pst* strain without the Rpt2 gene (K. Lawton and K. Weymann, unpublished data). Thus, as observed in cucumber and tobacco, a pathogen that results in a necrotic response in the host induced SAR in *Arabidopsis*.

The effect of *Pst* inoculation on resistance against subsequent infection with a fungal pathogen is shown in Figure 6. The plants were analyzed for *P. parasitica* infection 7 days after the challenge inoculation with the fungus. Untreated leaves of plants that had been mock-inoculated with buffer supported extensive hyphal growth and development of conidiophores (Fig. 6A). In dramatic contrast, plants that were pre-inoculated with the bacterial pathogen did not support fungal growth (Fig. 6D). Fungal mycelia and conidiophores were not observed on either the *Pst*-inoculated (data not shown) or uninoculated leaves of these plants. Thus, a bacterial pathogen can induce resistance against a fungal pathogen in *Arabidopsis*.

To more clearly define the roles of salicylic acid and ethylene in biologically induced SAR, these experiments were carried out in the ethylene-insensitive *etr1* mutant and the SA-deficient transgenic NahG plants. As shown in Figure 5, *Pst*-inoculation of leaves of the *etr1* mutant resulted in PR-1

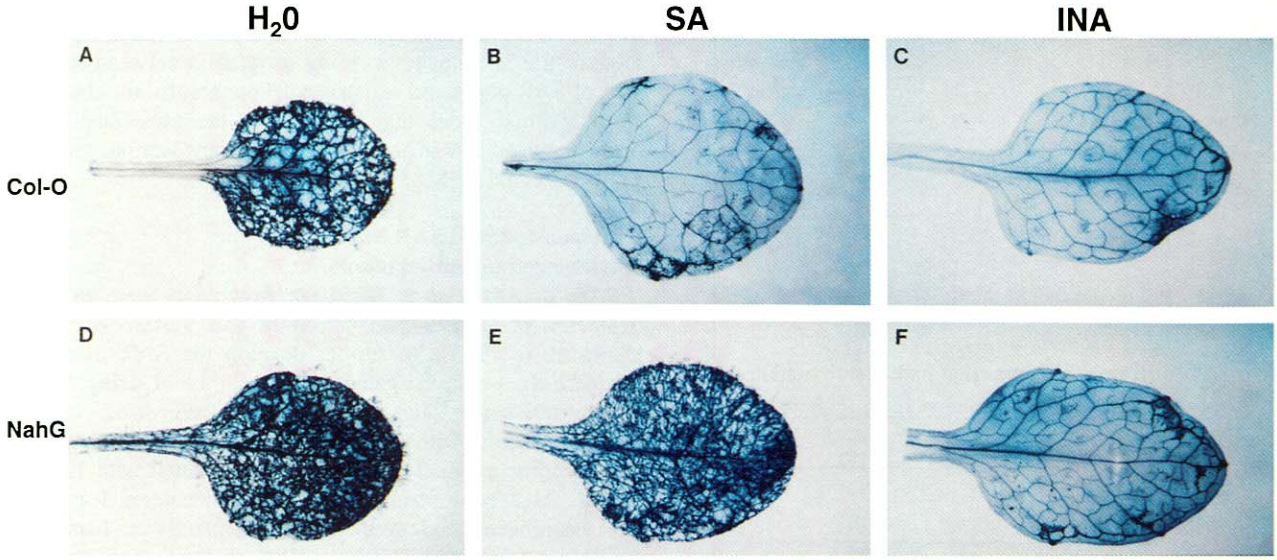

Fig. 4. Chemically induced resistance in wild-type (Col-O) and NahG plants. Wild-type **(A, B, C)** or NahG **(D, E, F)** plants were treated with water **(A, D)**, 5mM salycylic acid **(B, E)** or 0.25 mg 2,6-dichloroisonicotinic acid per ml **(C, F)** 2 days prior to challenge-inoculation with *Peronospora parasitica*. Plants were examined for symptoms and leaves were harvested for analysis of fungal growth by trypan blue staining 7 days after fungal inoculation.

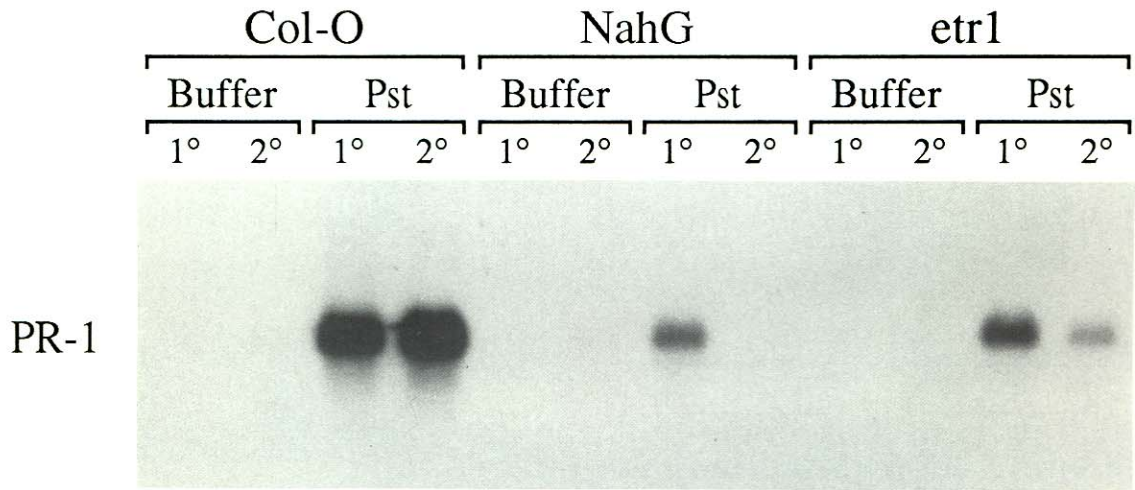

Fig. 5. PR-1 mRNA accumulation in response to pathogen inoculation. Total RNA was extracted from inoculated (1°) and uninoculated (2°) leaves of Columbia, NahG, and *etr1* plants at the time of challenge inoculation with *Peronospora parasitica*. Primary leaves were inoculated with *Pst* DC3000 *avr*Rpt2 (*Pst*) or 10mM $MgCl_2$ (Buffer) 2 days prior to tissue harvest.

mRNA accumulation in both the inoculated and uninoculated leaves. PR-1 message abundance in *Pst*-inoculated leaves was induced 172-fold over buffer-treated control leaves, while uninoculated leaves of *Pst*-treated plants accumulated 31-fold more PR-1 mRNA than did the leaves of the buffer control plants. In NahG plants the increase in PR-1 mRNA abundance in *Pst*-inoculated leaves was an order of magnitude less (24-fold) than observed in wild-type plants (280-fold) and no PR-1 message was detected in the uninoculated leaves of these plants. This low-level induction of PR-1 mRNA in pathogen-inoculated leaves is consistent with the level of SA observed in these tissues (Fig. 2).

To determine the effect of either SA or ethylene on pathogen-induced resistance, *etr1* and NahG plants were challenge-inoculated with the fungal pathogen *P. parasitica* 2 days after receiving an initial treatment with buffer or the bacterial pathogen *Pst* DC3000/*avr*Rpt2. Disease symptoms on leaves that had not received an initial buffer or bacterial treatment were analyzed 7 days post-challenge-inoculation. These results are shown in Figure 6. Leaves from mock-inoculated control plants (Fig. 6B, C) show extensive growth of fungal mycelia that ramified the entire leaf. However, leaves of *etr1* plants that had been pre-inoculated with *Pst* do not show fungal hyphae (Fig. 6F), similar to wild-type plants (Fig. 6D). Thus, SAR was operable in both wild-type and ethylene-insensitive mutants. Moreover, the transgenic NahG plants did not show resistance; the leaves from both the mock- and *Pst*-inoculated plants were heavily colonized by the fungus (Fig. 6E). Taken together these data indicate that SA is critical for biological induction of SAR in *Arabidopsis*, while ethylene sensitivity is not. Further, *etr1* leaves that had been inoculated with *Pst* prior to fungal challenge were resistant to infection, as observed with wild-type plants (data not shown). Fungal resistance was not observed in NahG leaves that had been treated with bacteria; however, these leaves were necrotic by the time of fungal inoculation.

DISCUSSION

Previously, we have shown that SA is required for SAR in tobacco (Gaffney et al. 1993; Vernooij et al. 1995). SA has also been shown to play a central role in disease susceptibility and resistance in both tobacco and *Arabidopsis* (Delaney et al. 1994). We show here that *Arabidopsis* plants transformed with the bacterial gene *nahG*, which encodes the SA-metabolizing enzyme, salicylate hydroxylase, constitutively express high levels of *nahG* mRNA and protein. These plants do not accumulate high levels of SA systemically following pathogen inoculation. Furthermore, PR-1 mRNA does not accumulate in uninfected, systemic tissues following pathogen inoculation and these tissues are not protected against subsequent pathogen infection. These observations extend the correlation between the accumulation of SA, PR-1 mRNA accumulation, and resistance, and strongly suggest SA is required for SAR in *Arabidopsis*. Thus, in two divergent species, SA is required for SAR and general disease resistance.

In contrast to this finding, ethylene does not appear to be required for SAR in *Arabidopsis*. Previously, we have shown that chemical activation of SAR is not compromised in ethylene-insensitive *Arabidopsis* mutants (Lawton et al. 1994b). The data presented here extend those findings to biologically induced SAR. In *etr1 Arabidopsis*, pathogen inoculation of primary leaves results in accumulation of PR-1 mRNA in uninoculated leaves. Furthermore, these plants are resistant to subsequent pathogen infection. Following inoculation with a bacterial pathogen, the *etr1* plants did not support fungal growth in either the bacterially inoculated leaf (data not shown) or in uninoculated leaves of inoculated plants, as ob-

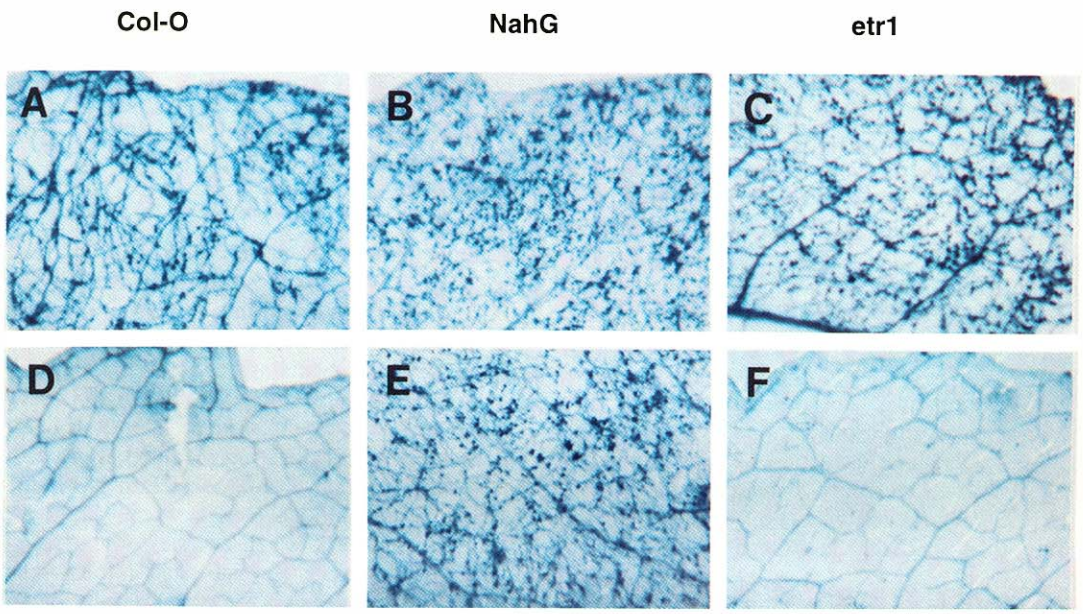

Fig. 6. Bacterially induced systemic acquired resistance against the fungal pathogen *Peronospora parasitica* in *Arabidopsis*. Photomicrographs of trypan blue stained leaves harvested from Columbia wild-type **(A, D),** NahG **(B, E)** and *etr1* **(C, F)** plants 7 days after challenge inoculation with *Peronospora parasitica*. Shown are the uninoculated challenge leaves from plants that were pre-inoculated with buffer (**A, B, C**) or *Pst* DC3000 *avr*Rpt2 (**D, E, F**) 2 days prior to challenge inoculation with *P. parasitica*.

served with the wild-type *Arabidopsis*. Thus, SAR signal transduction does not require ethylene sensitivity. However, because the level of PR-1 mRNA was reduced in *etr1* plants, ethylene may function to enhance the SA-dependent signal transduction pathway, as previously suggested (Lawton et al. 1994b).

We have recently reported that the resistance-activating compound INA does not induce the accumulation of SA in tobacco (Vernooij et al. 1995). Further, we have shown that INA induces both PR-1 accumulation and disease resistance in both tobacco and *Arabidopsis* (Vernooij et al. 1995). Here we extend those observations and show that exogenous application of SA does not induce PR-1 or *P. parasitica* resistance in NahG *Arabidopsis*. This is most likely due to the inefficient uptake of exogenously applied SA combined with the efficiency of the SAH enzyme in degrading any SA that enters the cell. Thus, in NahG plants exogenous SA is unable to activate SAR.

Biological induction of SAR in *Arabidopsis* has been demonstrated by a number of groups. Turnip crinkle virus (TCV) inoculation of Dijon, an ecotype that responds hypersensitively to TCV, induced resistance to both viral (TCV) and bacterial (*Pst*) pathogens as well as PR gene expression (Uknes et al. 1993). Similarly, inoculation with the fungal pathogen *Fusarium oxysporum* induced resistance against subsequent infection by another fungal pathogen, *P. parasitica* , and was associated with the accumulation of PR-1 and PR-2 mRNA (Mauch-Mani and Slusarenko 1994). In other experiments, an avirulent strain of bacteria (*Pst* carrying *avr*Rpt2) induced SAR against the virulent bacteria (*Pst*) (Cameron et al. 1994). Here we have extended those observations to include bacterial-induced SAR against subsequent infection with a fungal pathogen. Inoculation of *Arabidopsis* with *Pst* (*avr*Rpt2), resulted in both the systemic accumulation of PR-1 mRNA and resistance against infection by *P. parasitica*. Injection of bacterial pathogens to induce SAR, followed by *P. parasitica* challenge, provides an effective and technically facile means of evaluating pathogen resistance. A major advantage of this system is the ability to assay SAR in a range of *Arabidopsis* accessions. *Pst*DC3000 widely infects *Arabidopsis* and a number of *P. parasitica* pathovars of *Arabidopsis* are available. Furthermore, as in the classically defined system of cucumber/*Colletotrichum lagenarium*, the presence of an R-gene/avr-gene interaction is not required for inducing SAR. However, while the virulent *Pst*DC3000 induces necrosis and SAR, the presence of the *avr*Rpt2 gene enhances the reproducibility of *Pst* DC3000 as an inducer since the kinetics of the necrotic response are more consistent between experiments, facilitating the timing of the challenge inoculation.

Salicylic acid is important in plant disease resistance and susceptibility. However, our understanding of the role of SA in disease and other physiological responses is just beginning. The availability of well characterized *Arabidopsis* plants that are blocked in their ability to accumulate SA will be valuable for future studies of the SAR signal transduction pathway.

MATERIALS AND METHODS

Transgenic Arabidopsis.

Arabidopsis plants were transformed with the *nahG* coding sequence under the control of an enhanced 35S promoter (Gaffney et al. 1993) by the *Agrobacterium*-mediated root transformation procedure as previously described (Delaney et al. 1994). Four individual transformation events resulted in 10 independent lines that were homozygous for *nahG*, based on resistance of the T3 progeny to kanamycin. These homozygous lines were analyzed for *nahG* protein by Western blot analysis. All lines expressed a high level of salicylate hydroxylase protein constitutively and did not accumulate PR-1 mRNA in response to exogenous SA application. The progeny from one event (B3, B4, B5, and B15) accumulated slightly higher levels of *nahG* mRNA and protein. One of these lines (B15) was selected for further experimentation and analysis.

Protein analysis.

Total protein was extracted from leaf tissue of wild-type and transgenic NahG *Arabidopsis* in sample buffer containing 125 mM tris, 10% β-mercaptoethanol, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 0.002% bromphenolblue. Protein extracts were separated by SDS–polyacrylamide gel electrophoresis on a 10 to 20% tris-tricine gel (Novex). The polypeptides were then electroblotted and immunostained as previously described (Gaffney et al. 1993).

RNA analysis.

RNA was isolated from frozen tissue samples by phenol-chloroform extraction followed by lithium chloride precipitation (Lagrimini et al. 1987). Total RNA samples (10 μg) were electrophoretically separated through formaldehyde-agarose gels and blotted to hybridization membrane (GeneScreen Plus; DuPont-New England Nuclear Research Products, Boston, MA) as described previously (Ausubel et al. 1987). Equal loading of samples was determined by including 40 μg ethidium bromide per ml in the sample loading buffer allowing visualization of RNA by photography under UV light. ^{32}P-labeled cDNA probes were synthesized by random priming of isolated insert DNA using the random primers DNA labeling system (Gibco BRL, Gaithersburg, MD). The PR-1 cDNA was described by Uknes et al. (1992). Hybridization and washing conditions were as described previously (Church and Gilbert, 1984). Relative amounts of transcript were determined by detecting β-decay of ^{32}P with a Betascope analyzer (Betagen, Waltham, MA).

Chemical application and *P. parasitica* inoculation.

Columbia wild-type, the ethylene-insensitive mutant, *etr1*, in the Columbia background, and *nahG*-expressing transgenic Columbia seeds were surface sterilized and sown in autoclaved growing media as described previously (Uknes et al. 1992). For analysis of chemical induction of PR-1 gene expression and acquired resistance, 2- to 3-day-old wild-type or transgenic *nahG*-expressing seedlings were sprayed to imminent run-off with 5 mM SA or 0.25 mg INA (25% active ingredient, wettable powder) per ml. Control plants were sprayed with sterile water. Two days after SA or INA application, plants were either harvested for RNA extraction and analysis or sprayed with a conidial suspension of *P. parasitica* containing 4 to 5 × 10^4 spores/ml. Following inoculation, plants were placed in a covered flat to maintain high humidity and grown at 17°C with a 14 h light/10 h dark cycle in a moist growth chamber to encourage fungal sporulation. Fun-

gal growth on plants was analyzed 7 days after inoculation by viewing with a dissecting microscope. In addition, several plants were randomly chosen from each treatment and stained with lacto-phenol trypan blue (Keogh et al. 1980) for microscopic examination. Inoculations were repeated at least three times with essentially the same results each time.

Bacterial inoculation and analysis of systemic acquired resistance.

Three- to four-week-plants were inoculated on four to five leaves per plant using a syringe containing 10 mM $MgCl_2$ or *Pseudomonas syringe* pv. *tomato* DC3000 (~10^7 CFU/ml) harboring the plasmidborne *avr*Rpt2 resistance gene or *Pst* DC3000 alone. Approximately 50 µl was injected into each leaf. Two days postinoculation, the inoculated and uninoculated leaves were harvested from some of the plants for SA and RNA extraction and analysis. To assay SAR, the plants were challenge inoculated with the fungal pathogen *Peronospora parasitica* pv. *Noco* as described above. Experiments were repeated several times with similar results and representative experiments are presented.

Salicylic acid extraction and analysis.

Free and total SA analysis was conducted on mock- and *Pst*-inoculated leaves as well as uninoculated leaves as previously described (Yalpani et al. 1991) with modifications described (Gaffney et al. 1993).

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LITERATURE CITED

Alexander, D., Goodman, R. M., Gut-Rella, M., Glascock, C., Weymann, K., Friedrich, L., Maddox, D., Ahl Goy, P., Luntz, T., Ward, E., and Ryals, J. 1993. Increased tolerance to two oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. Proc. Natl. Acad. Sci. USA 90:7327-7331.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. 1987. Current Protocols in Molecular Biology. John Wiley & Sons, New York.

Bent, A. F., Innes, R. W., Ecker, J. R., and Staskawicz, B. J. 1992. Disease development in ethylene-insensitive *Arabidopsis thaliana* infected with virulent and avirulent *Pseudomonas* and *Xanthomonas* pathogens. Mol. Plant-Microbe Interact. 5:372-378.

Broglie, K., Chet, I., Holliday, M., Cressman, R., Biddle, P., Knowlton, C., Mauvais, C. J., and Broglie, R. 1991. Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science 254:1194-1197.

Cameron, R. K., Dixon, R. A., and Lamb, C. J. 1994. Biologically induced systemic acquired resistance in *Arabidopsis thaliana*. Plant J. 5:715-725.

Chester, K. S. 1933. The problem of acquired physiological immunity in plants. Q. Rev. Biol. 8:275-324.

Church, G. M., and Gilbert, W. 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA 81:1991-1995.

Delaney, T. P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut-Rella, M., Kessmann, H., Ward, E., and Ryals, J. 1994. A central role of salicylic acid in plant disease resistance. Science 266:1247-1250.

Ecker, J. R., and Davis, R. W. 1987. Plant defense genes are regulated by ethylene. Proc. Natl. Acad. Sci. USA 84:5202-5206.

Flaishman, M. A., and Kolattukudy, P. E. 1994. Timing of fungal invasion using host's ripening hormone as a signal. Proc. Natl. Acad. Sci. USA 91:6579-6583.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H., and Ryals, J. 1993. Requirement of salicylic acid for the induction of systemic acquired resistance. Science 261:754-756.

Hoffman, R., and Heale, J. B. 1987. Cell death, 6-methoxymellein accumulation, and induced resistance to *Botrytis cinerea* in carrot root slices. Physiol. Mol. Plant Pathol. 30:67-75.

Hughes, R. K., and Dickerson, A. G. 1989. The effect of ethylene on phenylalanine ammonia lyase (PAL) induction by a fungal elicitor in *Phaseolus vulgaris*. Physiol. Mol. Plant Pathol. 34:361-378.

Keogh, R. C., Deverall, B. J., and McLeod, S. 1980. Comparison of histological and physiological responses to *Phakopsora pachyrhizi* in resistant and susceptible soybean. Trans. Br. Mycol. Soc. 74:329-333.

Lagrimini, L. M., Burkhart, W., Moyer, M., and Rothstein, S. 1987. Molecular cloning of complementary DNA encoding the lignin-forming peroxidase from tobacco: Molecular analysis and tissue-specific expression. Proc. Natl. Acad. Sci. USA 84:7542-7546.

Lawton, K., Uknes, S., Friedrich, L., Gaffney, T., Alexander, D., Goodman, R., Métraux, J.-P., Kessmann, H., Ahl Goy, P., Gut-Rella, M., Ward, E., and Ryals, J. 1993. The molecular biology of systemic acquired resistance. Pages 422-432 in Mechanisms of Defense Responses in Plants. B. Fritig and M. Legrand, eds. Kluwer Academic Publishers, Dordrecht.

Lawton, K. A., Beck, J., Potter, S., Ward, E., and Ryals, J. 1994a. Regulation of cucumber class III chitinase gene expression. Mol. Plant-Microbe Interact. 7:48-57.

Lawton, K. A., Potter, S. L., Uknes, S., and Ryals, J. 1994b. Acquired resistance signal transduction in *Arabidopsis* is ethylene independent. Plant Cell 6:581-588.

Liu, D., Raghothama, K. G., Hasegawa, P. M., and Bressan, R. A. 1995. Osmotin overexpression in potato delays development of disease symptoms. Proc. Natl. Acad. Sci. USA 91:1888-1892.

Malamy, J., Carr, J. P., Klessig, D. F., and Raskin, I. 1990. Salicylic acid: A likely endogenous signal in the resistance response of tobacco to viral infection. Science 250:1002-1004.

Mauch, F., Hadwiger, L. A., and Boller, T. 1984. Ethylene: Symptom, not signal for the induction of chitinase and β-1,3-glucanase in pea pods by pathogens and elicitor. Plant Physiol. 76:607-611.

Mauch, F., Mauch-Mani, B., and Boller, T. 1988. Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. Plant Physiol. 88:936-942.

Mauch, F., Meehl, J. B., and Staehelin, L. A. 1992. Ethylene-induced chitinase and β-1,3-glucanase accumulate specifically in the lower epidermis and along vascular strands of bean leaves. Planta 186:367-375.

Mauch-Mani, B., and Slusarenko, A. J. 1994. Systemic acquired resistance in *Arabidopsis thaliana* by a predisposing infection with a pathogenic isolate of *Fusarium oxysporum*. Mol. Plant-Microbe Interact. 7:378-383.

Métraux, J.-P., Burkhart, W., Moyer, M., Dincher, S., Middlesteadt, W., Williams, S., Payne, G., Carnes, M., and Ryals, J. 1989. Isolation of a complementary DNA encoding a chitinase with structural homology to a bifunctional lysozyme/chitinase. Proc. Natl. Acad. Sci. USA 86: 896-900.

Métraux, J.-P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., Raschdorf, K., Schmid, E., Blum, W., and Inverardi, B. 1990. Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. Science 250:1004-1006.

Rasmussen, J. B., Hammerschmidt, R., and Zook, M. N. 1991. Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae* pv. *syringae*. Plant Physiol. 97:1342-1347.

Raz, V., and Fluhr, R. 1993. Ethylene signal is transduced via protein phosphorylation events in plants. Plant Cell 5:523-530.

Roberts, W. K., and Selitrennikoff, C. P. 1988. Plant and bacterial chitinases differ in antifungal activity. J. Gen. Microbiol. 134:169-176.

Roberts, W. K., and Selitrennikoff, C. P. 1990. Zeamatin, an antifungal protein from maize with membrane-permeabilizing activity. J. Gen. Microbiol. 136:1771-1778.

Ross, A. F., and Williamson, C. E. 1951. Physiologically active emanations from virus-infected plants. Phytopathology 41:431-438.

Ryals, J., Uknes, S., and Ward, E. 1994. Systemic acquired resistance.

Plant Physiol. 104:1109-1112.
Ryals, J., Ward, E., and Métraux, J.-P. 1992. Systemic acquired resistance: an inducible defense mechanism in plants. Pages 205-229 in: Inducible Plant Proteins: Their Biochemistry and Molecular Biology. J. L. Wray, ed. Cambridge University Press, Cambridge.
Spanu, P., and Boller, T. 1989. Ethylene biosynthesis in tomato infected by *Phytophthora infestans*. Pages 255-260 in: Biochemical and Physiological Aspects of Ethylene Production in Lower and Higher Plants. H. Clijsters, ed. Kluwer Academic Publishers, Dordrecht.
Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E., and Ryals, J. 1992. Acquired resistance in *Arabidopsis*. Plant Cell 4:645-656.
Uknes, S., Winter, A. M., Delaney, T., Vernooij, B., Morse, A., Friedrich, L., Nye, G., Potter, S., Ward, E., and Ryals, J. 1993. Biological induction of systemic acquired resistance in *Arabidopsis*. Mol. Plant-Microbe Interact. 6:692-698.
Van Loon, L. C. 1977. Induction by 2-chloroethylphosphonic acid of viral-like lesions, associated proteins, and systemic resistance in tobacco. Virology 80:417-420.
Vernooij, B., Friedrich, L., Ahl Goy, P., Staub, T., Kessmann, H., and Ryals, J. 1995. 2,6-dichloroisonicotinic acid-induced resistance to pathogens without the accumulation of salicylic acid. Mol. Plant-Microbe Interact. 8:228-234.
Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditz-Jawhar, R., Ward, E., Uknes, S., Kessmann, H., and Ryals, J. 1994. Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. Plant Cell 6: 959-965.
Ward, E. R., Uknes, S. J., Williams, S. C., Dincher, S. S., Wiederhold, D. L., Alexander, D. C., Ahl Goy, P., Métraux, J.-P., and Ryals, J. A. 1991. Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell 3:1085-1094.
Yalpani, N., Silverman, P., Wilson, T. M. A., Kleier, D. A., and Raskin, I. 1991. Salicylic acid is a systemic signal and an inducer of pathogenesis-related proteins in virus-infected tobacco. Plant Cell 3:809-818.
You, I.-S., Ghosal, D., and Gunsalus, I. C. 1991. Nucleotide sequence analysis of the Pseudomonas *putida* PpG7 salicylate hydroxylase gene (*nahG*) and its 3′-flanking region. Biochemistry 30:1635-1641.