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Systemic Responses in *Arabidopsis thaliana* Infected and Challenged with *Pseudomonas syringae* pv *syringae*[1]

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Attack of plants by necrotizing pathogens leads to acquired resistance to the same or other pathogens in tissues adjacent to or remotely located from the site of initial attack. We have used *Arabidopsis thaliana* inoculated with the incompatible pathogen *Pseudomonas syringae* pv *syringae* on the lower leaves to test the induction of systemic reactions. When plants were challenged with *Pseudomonas syringae* pv *syringae* in the upper leaves, bacterial titers remained stable in those preinfected on the lower leaves. However, there was a distinct decrease in symptoms that correlated with a local and systemic increase in salicylic acid (SA) and in chitinase activity. Peroxidase activity only increased at the site of infection. No changes in catalase activity were observed, either at the local or at the systemic level. No inhibition of catalase could be detected in tissue in which the endogenous levels of SA were elevated either naturally (after infection) or artificially (after feeding SA to the roots). The activity of catalase in homogenates of A. *thaliana* leaves could not be inhibited in *vitro* by SA. SA accumulation was induced by H_2O_2 in leaves, suggesting a link between H_2O_2 from the oxidative burst commonly observed during the hypersensitive reaction and the induction of a putative signaling molecule leading to system acquired resistance.

Many plants develop a lasting resistance to subsequent infections in response to pathogen attack (Ross, 1961; Madamanchi and Kuc, 1991). Resistance is expressed locally as well as distally from the attempted site of pathogen invasion and is termed SAR in the latter case. SAR is expressed against a broad range of pathogens such as fungi, bacteria, or viruses and was demonstrated to operate under field conditions (Madamanchi and Kuc, 1991). The systemic nature of SAR is postulated to be mediated by an endogenous signal released from the site of initial infection and transmitted to other parts of the plant (Ross, 1961; Guedes et al., 1980). SA was proposed to be the putative endogenous signal for SAR since pathogen infection in tobacco, cucumber, or Arabidopsis leads to the accumulation of SA (Malamy et al., 1990; Métraux et al., 1990; Rasmussen et al., 1991; Uknes et al., 1993). The increase in SA is correlated with the appearance of SAR, and application of SA induces resistance and the accumulation of defense-related genes (Ward et al., 1991; Yalpani et al., 1991; Uknes et al., 1993; Summermatter et al., 1994). Transgenic plants unable to accumulate SA and showing increased susceptibility to pathogen infection provided further support for a role of SA in SAR (Gaffney et al., 1993; Delaney et al., 1994). SA was proposed to mediate SAR by binding to and inhibiting a catalase, thus leading to an increase in H_2O_2, which may act as a second messenger to induce defense mechanisms (Chen et al., 1993; Dempsey and Klessig, 1994).

Several reports have shown that SAR can be induced in *Arabidopsis thaliana* using *Pseudomonas syringae* pv *tomato* or turnip crinkle virus to protect against the same pathogens (Uknes et al., 1993), *P. syringae* pv *tomato* carrying the avirulence gene *avrRpt2* to protect against *P. syringae* pv *tomato* and *P. syringae* pv *maculicola* (Cameron et al., 1994), or using *Fusarium oxysporum* to protect against *Pseudoperonospora parasitica* (Mauch-Mani and Slusarenko, 1994). Furthermore, treatments with SA or INA, a chemical inducer (Métraux et al., 1991), were shown to enhance resistance to *P. syringae* pv *tomato*, *P. parasitica*, turnip crinkle virus (Uknes et al., 1992, 1993), and *P. syringae* pv *syringae* (Summermatter et al., 1994).

In the present study we explored the local and systemic reactions of Arabidopsis after a primary inoculation with an incompatible pathogen. We extend our knowledge of systemic induced reactions in Arabidopsis using *P. syringae* pv *syringae* to protect against the same organism. We show that SAR is expressed as a suppression of symptoms and is accompanied by local or systemic increase in endogenous SA, as well as in peroxidase and chitinase activities. Unlike peroxidase and chitinase, catalase was not affected by pathogen attack. Furthermore, catalase activity did not change in plants treated with SA or INA. We also report a direct effect of H_2O_2 on the accumulation of SA.

MATERIALS AND METHODS

Plant Material, Bacterial Strain, and Growth Conditions

Arabidopsis thaliana cv RLD was sown in commercial potting soil and grown in trays in a growth chamber under the following conditions: 12-h photoperiod, 22/18°C (day/night) temperatures, and 55/90% RH. Twenty to 25 d after sowing, individual plants were transferred to plastic pots (three plants/pot; 10 cm diameter).

Pseudomonas syringae pv *syringae* strain D20 (kindly provided by Ray Hammerschmidt, Michigan State University, East Lansing, MI) was cultured either on solid LB medium or in liquid medium as described by Maniatis et al. (1982).

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Abbreviations: ABTS, 2,2′-azino-di-(3-ethylbenzthiazoline)-6-sulfonate; HR, hypersensitive response; INA, 2,6-dichloroisonicotinic acid; LB, Luria Bertani; SA, salicylic acid; SAR, systemic acquired resistance.

Plant Inoculations

Plants were used 14 to 18 d after transplanting. Six fully expanded healthy leaves (lower position of the rosette) were marked on one side with a nontoxic pen and infiltrated with about 50 μL of a water suspension of *P. syringae* (4×10^8 cells/mL) as previously described (Whalen et al., 1991). Control plants were infiltrated with deionized water. At regular intervals, leaves were collected and analyzed for SA content and for enzyme activities. SAR was determined after challenge inoculation of five upper leaves in the rosette using *P. syringae* (10^7 cells/mL). Leaves were analyzed 1 week after the challenge inoculation and the extent of symptoms was determined by weighing the necrotized surface cut out from a copy of the leaf on transparent paper.

Quantification of Bacterial Growth in Planta

Control and infected leaves were washed gently with a soap-water solution, rinsed in sterile water, and washed once in 100% ethanol, followed by sterile water rinses. After the leaves were homogenized in 4 volumes of sterile, distilled water, appropriate dilutions were made and the suspensions were plated on solid LB medium. After 48 h, bacterial titers were determined by counting the colonies.

SA Analysis

Free and bound SA in leaves were quantified by HPLC using *o*-anisic acid as the internal standard as previously described (Meuwly and Métraux, 1993). The contribution of bacteria to the SA content measured in inoculated leaves was estimated as follows. Bacteria were grown in LB medium with or without leaf homogenates of Arabidopsis. The leaves were homogenized in liquid N_2, and the powdered material was added to the LB medium before autoclaving. The total amount of SA produced in a leaf by the infiltrated bacteria was estimated by multiplying the SA production per bacterium with the number of colony-forming units per leaf 24 h after infection.

Enzyme Assays

Leaf tissue was collected, frozen in liquid N_2, and stored at −70°C. Leaves were extracted by grinding in 2 volumes of ice-cold sodium phosphate buffer (50 mM, pH 6.5). Homogenates were centrifuged in microfuge tubes at 4°C for 10 min at 14,000*g*, and the supernatant was used to measure enzyme activities. Protein concentration was determined as described by Bradford (1976) with BSA as the standard.

Peroxidase Activity Assay

Peroxidase activity was estimated using guaiacol as the substrate. The oxidation of the substrate was measured spectrophotometrically at 450 nm as previously described (Hammerschmidt et al., 1982). The assay solution contained sodium phosphate (10 mM, pH 6), 0.25% (v/v) guaiacol, 100 mM H_2O_2, and leaf extract.

Chitinase Activity Assay

[^{3}H]Chitin (kindly provided by T. Boller, University of Basel, Switzerland) was used as the substrate to measure chitinase activity in homogenates as previously described (Métraux and Boller, 1986).

Catalase Activity

Catalase activity was measured as previously described (Fracheboud, 1986): 50 μL of homogenate diluted 5 times in 50 mM sodium phosphate buffer, pH 6.5, were incubated for either 1 or 5 min with 50 μL of 100 mM H_2O_2. The reaction was stopped by adding 12% (w/v) TCA to give a final concentration of 5% (w/v). Twenty microliters of the reaction mixture were added to 1 mL of 1 mg/mL ABTS (Boehringer Mannheim) containing 0.8 unit/mL of peroxidase (POD, Boehringer Mannheim). The following reaction takes place:

$$\text{ABTS}_{\text{red.}} + H_2O_2 \xrightarrow{\text{POD}} \text{ABTS}_{\text{ox.}} + H_2O$$

Oxidation of ABTS is measured by the increase in A_{610}, which is proportional to the amount of H_2O_2 and inversely proportional to catalase activity. The amount of H_2O_2 was determined using standard curves obtained with known concentrations of H_2O_2. SA was tested as a putative inhibitor of catalase as follows. SA was dissolved either in a sodium phosphate buffer (50 mM, pH 8.0) or in citrate buffer (20 mM, pH 6.5). Leaf homogenates prepared as described above were incubated with different concentrations of SA during 5 min at room temperature and catalase activity was then determined as described above. Peroxidase present naturally in leaf homogenates might decrease H_2O_2 and affect the catalase determination. However, at the dilutions used for catalase activity measurements, no peroxidase activity was detected in the homogenates.

Treatment with INA and SA

INA (kindly provided by H. Kessmann, CIBA AG, Basel, Switzerland) or SA was applied as a soil drench at 1 mM. Leaves were collected 48 h after the beginning of the treatment and assayed for enzyme activities as described above.

SA Induction by H_2O_2

Plants were infiltrated with different concentrations of H_2O_2 at appropriate dilutions from a 30% (w/v) stock solution. After 48 h the treated leaves were extracted and their SA content was determined.

RESULTS

Induction of SAR by *P. syringae*

Figure 1A shows the appearance of necrotic lesions with associated chlorosis caused by *P. syringae* pv *syringae*, a wheat pathogen, in Arabidopsis 4 d after inoculation. The interaction of *A. thaliana* ecotype RLD with *P. syringae* pv *syringae* is incompatible as indicated by the limited growth in vivo associated with a strong HR (Figs. 1 and 3A). This

Figure 1. *P. syringae*-induced resistance to *P. syringae*. The photograph shows upper leaves of plants inoculated either with water (A) or with *P. syringae* (4×10^8 cells/mL) (B) on the lower leaves of the rosette. Two days after infection of the lower leaves, upper leaves were challenged with the same pathogen (10^7 cells/mL). Leaves were photographed 4 d after challenge.

is unlike other reported incompatible interactions such as *A. thaliana* ecotype Pi-O and the *P. syringae* pv *maculicola* isolate m1 (Debener et al., 1991), in which no symptoms were visible. The hypersensitive symptoms formed rapidly and were clearly visible 2 d after infection. When upper leaves of plants pretreated with the pathogen on the lower leaves were infected with the same pathogen, there was little damage (Fig. 1B). Lesion size decreased by 90% in these systemically protected leaves 2 d after challenge and SAR was observed up to 8 d after the first infection (Fig. 2). The decrease in necrosis taking place in upper leaves after preinoculation of lower leaves did not correspond to a decrease in bacterial titer; in fact, initial bacterial titers remained stable in upper leaves from both control and preinfected plants (Fig. 3B).

Local and Systemic Induction of SA by *P. syringae*

Free and bound SA increased locally and systemically after infection of the lower leaves of the rosette compared to controls (Fig. 4). The highest concentrations of free and bound SA were present in the infected lower leaves, reaching up to 1 μg/g fresh weight for free SA and 20 μg/g fresh weight for bound SA. In the upper leaves, the highest concentrations were 0.6 μg/g fresh weight for free SA and 8 μg/g fresh weight for bound SA. We also determined SA concentrations within infected leaves and compared the necrotized and the noninfected parts. No significant difference in free SA was measured between infected and noninfected areas 8 d after infection, but the bound form increased up to 25 μg/g fresh weight in necrotized tissue compared to 4 μg/g fresh weight in the noninfected parts.

To determine the relative contribution of SA produced by *P. syringae*, we measured SA levels in the culture medium of *P. syringae* with or without addition of *A. thaliana* leaf homogenates. The estimated contribution of SA produced by *P. syringae* to the leaf tissue levels is presented in Table I. As can be seen by comparing Figure 4 and Table I, this contribution is negligible whether or not *P. syringae* was grown in the presence of *A. thaliana* leaf homogenate or not.

Induction of Enzyme Activities after Infection with *P. syringae*

Infected and upper noninfected leaves of plants infected on the lower leaves with *P. syringae* were analyzed for changes in peroxidase, chitinase, and catalase activities. Peroxidase activity in infected leaves increased up to 6-fold 8 d after infection, whereas no significant changes were found in upper, noninfected leaves (Fig. 5, A and B). Chitinase activity was 2-fold higher in infected leaves from 2 to 8 d postinoculation (Fig. 5C). In upper leaves of induced plants the activity of chitinase increased slightly compared to controls (Fig. 5D).

Since catalase was postulated to be inhibited by endogenous SA and shown to be a binding site for SA (Chen et al., 1993), we determined catalase activity locally and systemically after infection. Whereas catalase activity was higher in the upper than in the lower leaves, no differences were found between control and preinfected plants (Fig. 5, E and F).

To test whether catalase can be inhibited by higher concentrations of SA than those present in infected leaves, we applied SA by soil drench. No inhibition of catalase was found despite free SA levels reaching up to 60 ± 6 μg/g

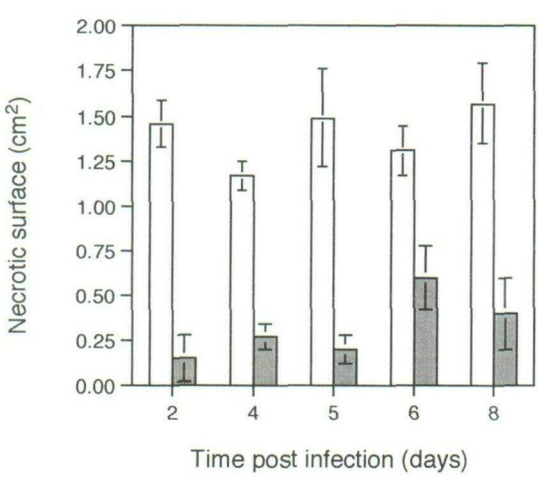

Figure 2. SAR in upper leaves of *A. thaliana* challenged at different times with *P. syringae*. Bacterial inoculations were the same as in Figure 1. The necrotic surface was measured in the upper leaves 1 week after the challenge. Each bar represents the mean from 30 leaves from 6 individual plants (±SE). Filled bars, Preinfected plants; open bars, control plants.

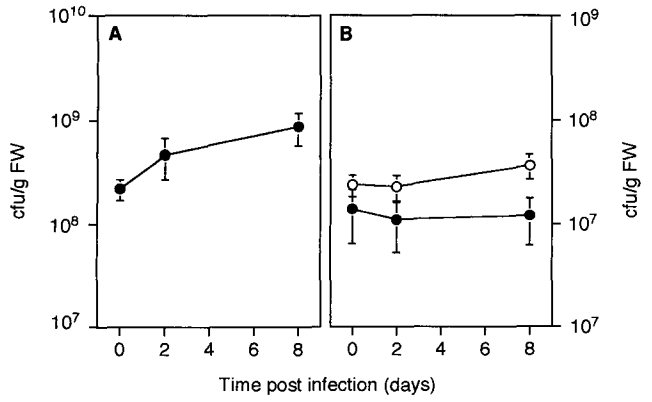

Figure 3. Determination of bacterial titer in *A. thaliana* leaves after infection with *P. syringae*. A, Bacterial growth in the infected lower leaves; B, multiplication of bacteria in the upper leaves of either control plants (○) or plants preinfected on the lower leaves (●). Bacterial inoculations were the same as for Figure 1. Results are means ±SE of five replicates; cfu, colony-forming units; FW, fresh weight.

fresh weight (n = 3) (Fig. 6). Soil drench of plants with INA induced a small increase in free SA accumulation (1 ± 0.1 μg/g fresh weight; n = 3) and left the levels of catalase activity unchanged (Fig. 6). Since the inhibition of catalase by SA might be decreased during tissue homogenization, for example by dilution of SA or loss of compartmentation, we tested the effect of SA in tissue homogenates in vitro. SA had no direct effect on catalase activity even at the highest SA concentration tested (Table II). Furthermore, the inhibitor 3-amino-1,2,4-triazole (used at 5 mM; preincubation time 5 or 30 min with the plant extract; substrate concentration 10 mM H_2O_2) decreased the activity of catalase by 81% (±4.6%; n = 3). Under the same conditions SA (used at 5 mM) had no effect.

SA Induction by H_2O_2

Since active oxygen species accumulate locally during HR (see reviews by Sutherland, 1991; Mehdy, 1994) and can lead to an increase in accumulation of pathogenesis-related protein (Chen et al., 1993), we tested the effect of H_2O_2 on the accumulation of SA in Arabidopsis leaves. Injection of H_2O_2 into the leaves did not lead to changes in the levels of free SA after 48 h. However, changes in bound SA were observed at 50 mM H_2O_2 and at higher concentrations (Fig. 7). Time-course experiments showed an increase in free SA during the first 24 h. SA concentrations then returned to basal levels, suggesting a conversion to the bound form (data not shown). Phytotoxic side effects became visible at concentrations equal to or higher than 0.5 M H_2O_2.

DISCUSSION

The use of an incompatible pathogen as challenge organism led to the discovery of an interesting facet of SAR, a process commonly assumed to operate by limiting the spread of a pathogen in the tissue. We show here that *A. thaliana* preinfected with the incompatible *P. syringae* pv *syringae* can be protected against the effect of subsequent inoculations with the same bacteria (Figs. 1 and 2). SAR is expressed by a reduction in symptoms caused by the bacteria in the leaf tissue (Fig. 2) rather than by a reduction in bacterial titer that remained constant in both controls and induced plants (Fig. 3). It appears that the necrosis itself is not a prerequisite for blocking the bacterium in the tissue,

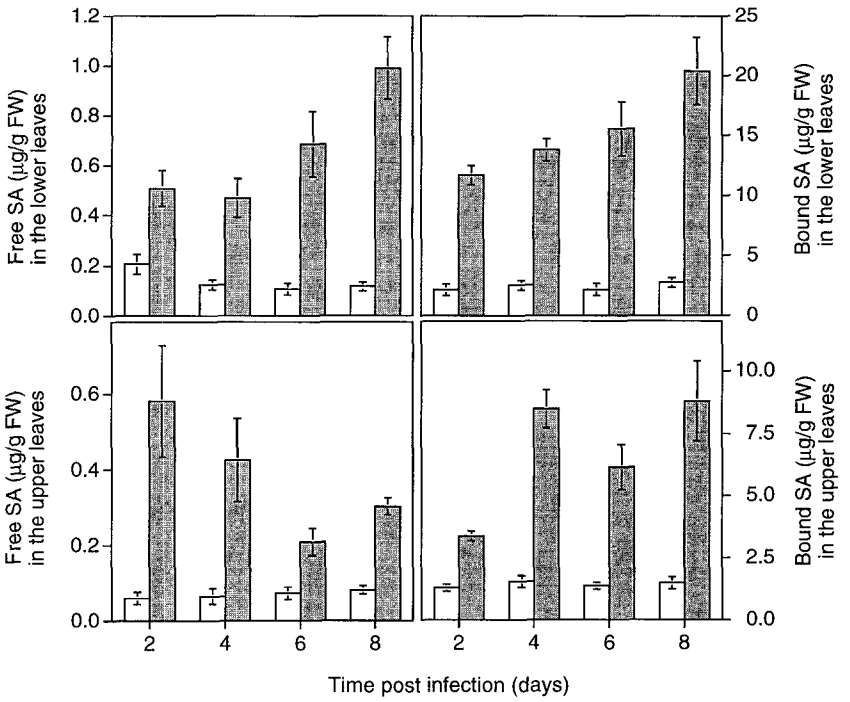

Figure 4. Time course of local and systemic accumulation of free and bound SA in *A. thaliana* after infection. Plants were infected on the lower leaves with *P. syringae* (4 × 10^8 cells/mL) and SA was analyzed in inoculated leaves and in upper, untreated leaves. Control plants were mock inoculated with water. The experiment was repeated twice. Results are means ± SE of three replicates. Filled bars, Infected plants; open bars, control plants. FW, Fresh weight.

Table I. *Contribution of SA produced by* P. syringae *to the SA level in infected* A. thaliana *tissue*.

Values represent the product of the production in vitro (in ng/bacteria) and of the number of bacteria in the tissue 24 h after infection. Results are means ± SE of three determinations.

Medium	Free SA	Bound SA
	ng/g fresh wt leaf	
LB	0.24 ± 0.059	0.11 ± 0.008
LB + 1% Arabidopsis leaf homogenate	0.19 ± 0.07	0.12 ± 0.016

since in upper leaves of plants preinfected on the lower leaves little or no bacterial multiplication takes place in vivo, despite the absence of HR symptoms. Similar observations were made earlier in tobacco infected with incompatible strains of *P. syringae* or *Pseudomonas pisi*, in which HR but not bacterial multiplication could be suppressed by albumin injection (reviewed by Kiraly et al., 1991). It has been proposed that H_2O_2 produced during the oxidative burst could trigger hypersensitive cell death, whereas lower doses of H_2O_2 might serve to activate cellular protectant genes such as GSH peroxidase or GSH S-transferase in adjacent cells (Levine et al., 1994). The suppression of lesions observed here in the upper leaves (Figs. 1 and 2) could suggest the participation of antioxidative mechanisms, as in a superoxide-resistant tobacco strain exhibiting enhanced resistance to symptoms caused by bacterial, viral, and fungal pathogens and which contains high levels of nonenzymatic antioxidants such as GSH or ascorbic acid (Gullner et al., 1991; Kiraly et al., 1991). The activation of protective mechanisms against oxidative cellular damage might thus be another manifestation of the SAR syndrome that requires further investigation.

SAR in Arabidopsis has essentially the same features as those described in other plants, such as cucumber or tobacco. The decrease in disease symptoms is associated with a local and systemic increase in the accumulation of SA (Fig. 4) as well as increases in chitinase (Fig. 5) or pathogenesis-related proteins (Métraux and Boller, 1986; Ye et al., 1990; Ward et al., 1991; Yalpani et al., 1991). Levels of

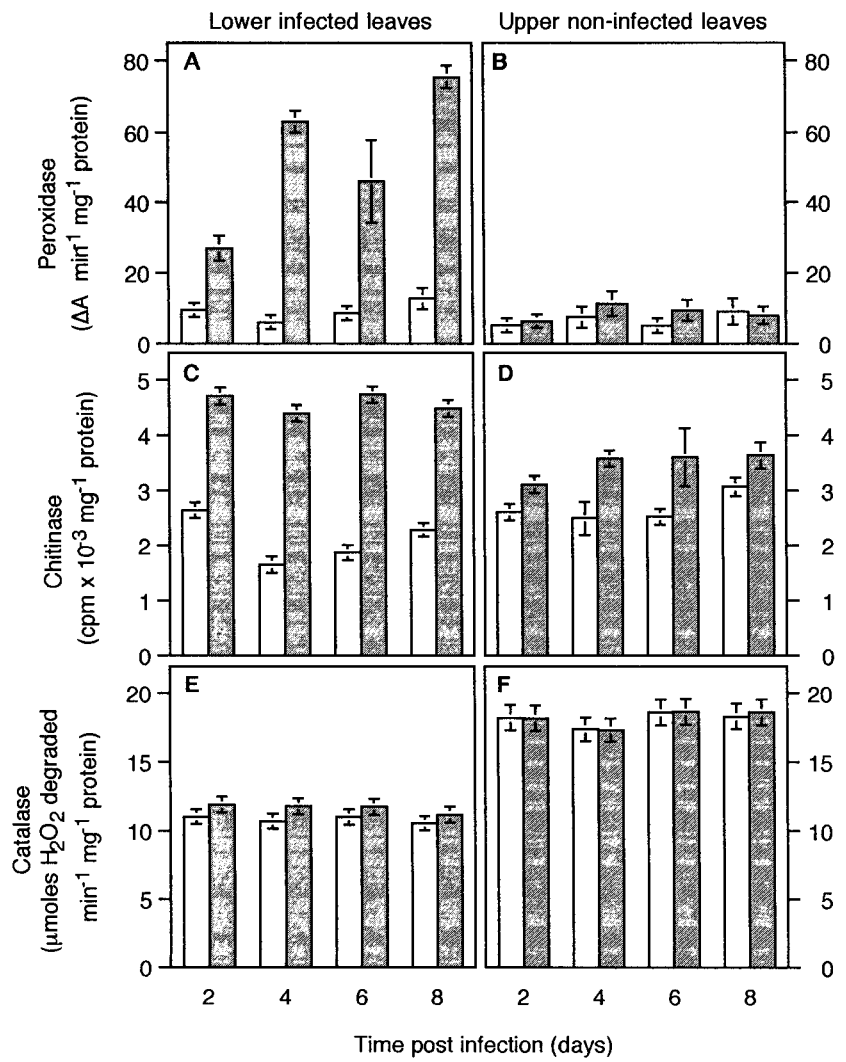

Figure 5. Changes in enzyme activities during SAR. Peroxidase (A and B), chitinase (C and D), and catalase (E and F) activities were measured in homogenates from infected or control plants. Experimental details are the same as described in Figure 4. Results are means ± SE of three replicates. Filled bars, Lower or upper leaves from infected plants; open bars, lower or upper leaves from water-injected plants.

Table II. *Effect of SA on catalase activity*

Values are means ± SE of three determinations.

Buffer	Concentration of SA	Catalase Activity
	mM	μmol H_2O_2 degraded mg^{-1} protein min^{-1}
Citrate, pH 6.5	0	65.3 ± 6.9
	0.05	64.8 ± 5.8
	0.5	65.4 ± 6.4
	2.5	64.4 ± 6.3
Phosphate, pH 8	0	64.9 ± 6.1
	0.5	65.1 ± 6.2
	5	63.7 ± 5.4
	12.5	65.5 ± 6.3

free SA in upper leaves from preinfected plants were in the same order of magnitude as in the infected parts (Fig. 4), in contrast with tobacco or cucumber, in which SA levels in the upper induced leaves were lower by one order of magnitude (Malamy et al., 1990; Meuwly and Métraux, 1993). As in tobacco or in cucumber, levels of free SA in Arabidopsis are maintained within a certain concentration range and free excessive SA is removed by conjugation (Figs. 4 and 7) (Enyedi et al., 1992; Meuwly and Métraux, 1993). Bound SA is also found to accumulate in upper leaves of infected plants (Fig. 4) in contrast with Xanthi-nc tobacco (Enyedi et al., 1992).

Whereas peroxidase activity strongly increased in infected leaves, it remained unchanged in the upper leaves (Fig. 5), unlike the situation in tobacco (Ye et al., 1990). Preinfection had no detectable effect on catalase, which showed a higher activity in the younger than in older leaves (Fig. 5). SA has been proposed to inhibit catalase activity, leading to the generation of H_2O_2, which acts as a second messenger and activates plant defense during SAR (Chen et al., 1993). Our data show that catalase activity was not inhibited by the high endogenous SA levels reached after infection or after SA drench applications (Figs. 5 and 6). Interestingly, high concentrations of SA (12.5 mM) had no effect on total catalase activity in vitro (Table II). Although it is difficult to determine how much free SA catalase is confronted with in a cell, the endogenous levels after infection or after SA drench can be estimated to reach 1 and 310 μM, respectively, if we assume an even distribution of SA. This is below the concentration (1 mM) required to inhibit the SA-binding catalase (Chen et al., 1993). However, since catalases occur in different isoforms (Willekens et al., 1994), the SA-binding catalase might represent only a minor fraction of total catalase activity, thus escaping our analysis. Its relevance in the oxidative burst during HR and in the systemic induction of resistance remains to be elucidated.

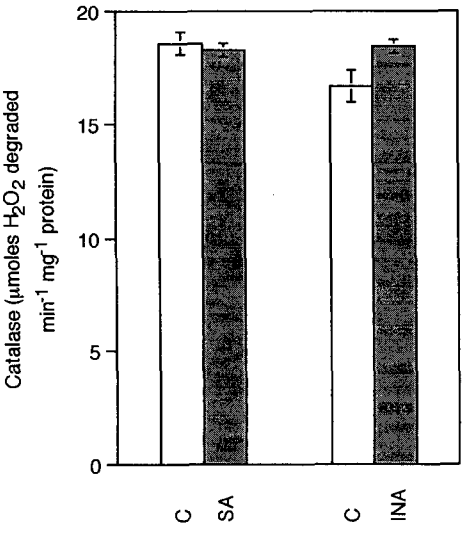

Figure 6. Catalase activity in homogenates 2 d after treatments with SA or INA. SA or INA were applied by soil drench at a concentration of 1 mM. Results are means ± SE of three replicates. C, Control plants.

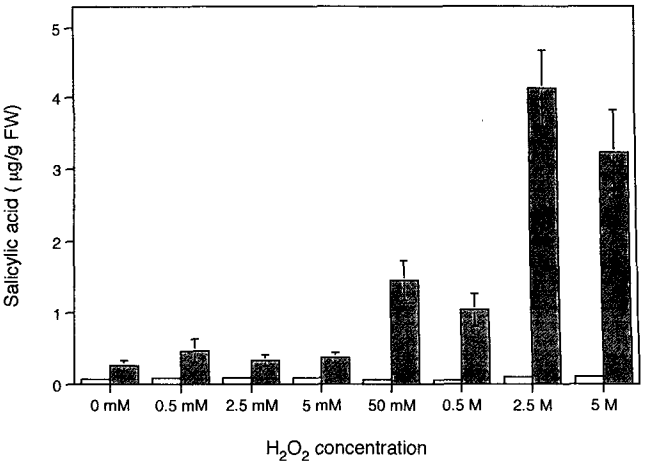

Figure 7. Levels of free and bound SA after treatment with different H_2O_2 concentrations. Plants were injected with H_2O_2 and analyzed 48 h later. Results are means ± SE of three replicates. Open bars, Free SA; filled bars, bound SA. FW, Fresh weight.

Finally we showed that H_2O_2 treatment can also induce the accumulation of SA in *A. thaliana* leaves (Fig. 7). Since H_2O_2 as well as SA stimulate the expression of PR-1 (Chen et al., 1993; Uknes et al., 1993), our results raise the possibility that resistance and concomitant defense-related gene expression arise from pathogen-induced H_2O_2, which activates SA production during HR. In such a model, SA would operate downstream of H_2O_2 in the signal transduction pathway.

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