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Inhibition of ascorbate peroxidase by salicylic acid and 2,6-dichloroisonicotinic acid, two inducers of plant defense responses

(reactive oxygen species/enhanced disease resistance/pathogenesis-related proteins/plant signal transduction/horseradish peroxidase)

JÖRG DURNER AND DANIEL F. KLESSIG*

Waksman Institute and Department of Molecular Biology and Biochemistry, Rutgers, The State University of New Jersey, P.O. Box 759, Piscataway, NJ 08855

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ABSTRACT **In recent years, it has become apparent that salicylic acid (SA) plays an important role in plant defense responses to pathogen attack. Previous studies have suggested that one of SA's mechanisms of action is the inhibition of catalase, resulting in elevated levels of H_2O_2, which activate defense-related genes. Here we demonstrate that SA also inhibits ascorbate peroxidase (APX), the other key enzyme for scavenging H_2O_2. The synthetic inducer of defense responses, 2,6-dichloroisonicotinic acid (INA), was also found to be an effective inhibitor of APX. In the presence of 750 μM ascorbic acid (AsA), substrate-dependent IC_{50} values of 78 μM and 95 μM were obtained for SA and INA, respectively. Furthermore, the ability of SA analogues to block APX activity correlated with their ability to induce defense-related genes in tobacco and enhance resistance to tobacco mosaic virus. Inhibition of APX by SA appears to be reversible, thus differing from the time-dependent, irreversible inactivation by suicide substrates such as *p*-aminophenol. In contrast to APX, the guaiacol-utilizing peroxidases, which participate in the synthesis and crosslinking of cell wall components as part of the defense response, are not inhibited by SA or INA. The inhibition of both catalase and APX, but not guaiacol peroxidases, supports the hypothesis that SA-induced defense responses are mediated, in part, through elevated H_2O_2 levels or coupled perturbations of the cellular redox state.**

Plants utilize both passive (e.g., cuticle and cell wall) and active defenses to protect themselves against microbial pathogens. Defenses induced after infection (active) include synthesis of phytoalexins, antiviral factors, proteinase inhibitors, peroxidases, hydrolytic enzymes, and pathogenesis-related (PR) proteins (1). During the past several years, a large body of evidence has been obtained that indicates that salicylic acid (SA) is an important component in one or more signal transduction pathway(s) leading to activation of some of these defenses (for reviews, see refs. 1–3). The identification and characterization of a SA-binding protein, whose binding affinity and specificity indicated that it may be the receptor for SA, has provided considerable insight into the SA-mediated signaling cascade (4, 5). The SA-binding protein was found to be a catalase, whose enzymatic activity is inhibited by SA and analogues of SA that induce PR gene expression and enhanced disease resistance (6). SA treatment of tobacco plants not only induced PR genes but also elevated endogenous H_2O_2 levels. Moreover, artificially raising the levels of H_2O_2 in tobacco leaves activated expression of PR-1 genes, while antioxidants suppressed PR-1 gene activation by exogenous SA (ref. 6; Z. Chen, U. Conrath, and D.F.K., unpublished results). Recently, Conrath *et al.* (8) demonstrated that the synthetic inducer of PR gene expression and enhanced disease resistance, 2,6-dichloroisonicotinic acid (INA), also blocked catalase activity *in vitro* and *in vivo*, as did SA. Taken together, these results suggest that one mechanism of action of SA is to bind to and inhibit catalase, thereby elevating H_2O_2 levels. The increased H_2O_2 or other reactive oxygen species (ROS) derived from it then may activate plant defense-related genes such as PR-1 (6). This mode of activation of plant defenses has striking parallels to induction of genes associated with vertebrate immune, inflammatory, and acute-phase responses mediated through H_2O_2 (redox) activation of the transcription factor NF-κB (9–11).

In plants, H_2O_2 is continuously produced as a by-product of photorespiration, fatty acid β-oxidation, photosynthesis, and oxidative phosphorylation. Oxidative damage is minimized by the concerted action of powerful antioxidant enzymes. Most important among them are catalases, which are localized to peroxisomes, glyoxysomes, and mitochondria, and ascorbate peroxidase (APX) and glutathione reductase, which are located in both the chloroplasts and the cytoplasm. The latter two form part of the ascorbate–glutathione (Halliwell–Asada) cycle (for reviews, see refs. 12 and 13). In the present report, we demonstrate that SA and INA inhibit APX, as previously reported for catalase. In contrast, the guaiacol-utilizing peroxidases, whose activities are thought to be necessary for defense responses (14, 15), are not affected by SA or INA.

MATERIALS AND METHODS

Materials. SA, SA analogues, *p*-aminophenol, guaiacol, pyrogallol, purpurogallin, hydroquinone, and ascorbic acid (AsA) were purchased from Sigma or Aldrich. Horseradish peroxidase (HRP; EC 1.11.1.7) was from Sigma (type XII, 265 units per mg). Tobacco plants (*Nicotiana tabacum* cv. Xanthi nc) were grown at 22°C in a 14-hr light cycle and used for experimentation at 6–8 weeks.

Protein Extraction. Tissue homogenization, ammonium sulfate precipitation, and buffer exchange were performed at 4°C. Two grams of tobacco leaves was homogenized to a fine powder in a mortar under liquid nitrogen. To obtain APX-containing extracts, the soluble proteins were extracted by grinding the powder in 10 ml of 100 mM potassium phosphate, (pH 7) containing 250 μM AsA and 1% (wt/vol) polyvinylpyrrolidone, with a small amount of quartz sand. The homogenate was centrifuged at 14,000 × *g* for 5 min. After fractionation by ammonium sulfate precipitation (45–85%, centrifugation steps were 14,000 × *g* for 15 min), the final pellet was resuspended in extraction buffer and desalted on a PD-10 column (Pharmacia) equilibrated with 50 mM potassium phosphate, pH 7/100 μM AsA. The extract was used immediately.

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Abbreviations: AsA, ascorbic acid; APX, ascorbate peroxidase; PR, pathogenesis-related; SA, salicylic acid; INA, 2,6-dichloroisonicotinic acid; ROS, reactive oxygen species; HRP, horseradish peroxidase.

*To whom reprint requests should be addressed.

Total guaiacol peroxidase activity was extracted with 100 mM potassium phosphate, pH 6.2/1% polyvinylpyrrolidone. To also extract the ionically bound cell wall isozymes, the extraction buffer was supplemented with 600 mM KCl. Extraction and centrifugation steps were as described for APX. After removal of cell debris and ammonium sulfate precipitation (0–85%), the extract was desalted against 50 mM potassium phosphate, pH 6/25% (vol/vol) glycerol. The extract was stored at −20°C until further use.

HRP (1 mg) was dissolved in 1.5 ml of 200 mM potassium phosphate, pH 6.2/150 mM NaCl. After a 15-min incubation at room temperature, the enzyme solution was centrifuged at 10,000 × *g* for 10 min. After buffer exchange [PD-10; equilibrated with 25 mM potassium phosphate (pH 6.2)], the protein concentration was adjusted to 0.5 mg/ml. Protein concentration was determined by the method of Bradford (16) with the Bio-Rad micro assay and bovine serum albumin as a standard.

Enzyme Assays. APX activity was determined spectrophotometrically as described (17) in 1 ml of a reaction mixture containing 50 mM potassium phosphate (pH 7), 750 μM AsA, and 100 μM H_2O_2. The amount of protein per assay was 30–50 μg. Oxidation of AsA was followed by the decrease in the absorbance at 290 nm (2.8 $mM^{-1}\cdot cm^{-1}$). The reaction rates measured were linear for at least 3 min and were corrected for autooxidation of AsA in presence of H_2O_2.

Guaiacol peroxidase activity was determined by the method of Chance and Maehly (18) with guaiacol as the electron (e^-) donor. The formation of tetraguaiacol (26.6 $mM^{-1}\cdot cm^{-1}$) was monitored at 470 nm. The reaction mixture contained 0.25% (18 mM) guaiacol and 100 mM H_2O_2 in 20 mM potassium phosphate (pH 6.2). The amount of protein in the assay was 50–80 μg/ml. The reaction was measured for 5 min. The assay for HRP was identical, except that only 0.2–0.5 μg of protein per ml was used. Peroxidase activity was also measured by using the traditional substrate, pyrogallol (20 mM). Purpurogallin formation was measured at 430 nm (2.47 $mM^{-1}\cdot cm^{-1}$).

RESULTS

Inhibition of APX by SA and INA. Previously, SA had been shown to inhibit catalase activity in crude extracts from tobacco leaves and in tobacco suspension cultures (6, 8). Interestingly, in the presence of low concentrations of H_2O_2 and a suitable e^- donor (e.g., phenolic compounds such as pyrogallol, resorcinol, or SA), catalase acts as a peroxidase (19). Therefore, the effects of SA, SA analogues, and INA on peroxidases were tested. To determine whether APX or guaiacol-utilizing (guaiacol) peroxidases (including HRP) are sensitive to SA, crude extracts were prepared from tobacco leaves, subjected to differential ammonium sulfate precipitation, and analyzed for peroxidase activity in the absence or presence of SA. Both SA and INA were found to be effective inhibitors of APX (Table 1). Final concentrations of 100 μM SA or INA led to 59% and 54% inhibition, respectively. At 1 mM, almost complete inhibition was achieved (95%) with either compound. In contrast, HRP and other peroxidases from tobacco that utilize guaiacol as a substrate were insensitive to SA or INA. Similar results were obtained after fractionation of tobacco peroxidases into soluble and cell wall-bound isozymes, or when lower concentrations of guaiacol (1 mM) or pyrogallol were used (data not shown).

To gain insight into the mechanism of SA and INA inhibition of APX, their kinetics of inhibition was compared to that of the well-characterized APX inhibitor *p*-aminophenol. *p*-Aminophenol serves as a suicide substrate or mechanism-based inhibitor of APX (20). The reaction intermediate of *p*-aminophenol is a free radical that irreversibly impairs the catalytic center of the enzyme. As anticipated from its mechanism of inhibition, 200 μM *p*-aminophenol inactivated APX activity in tobacco leaf extracts in a time-dependent manner as indicated by its nonlinear substrate (AsA) consumption (Fig. 1). The rate of inactivation was similar to that described for APX II from tea leaves (20). In contrast, inhibition by 100 μM SA or INA was not time-dependent, exhibiting a constant rate of AsA consumption. Preincubation of the extract in the presence of 250 μM *p*-aminophenol and H_2O_2 irreversibly destroyed most of the enzyme activity, while preincubation with 1 mM SA and H_2O_2 did not. Removal of 200 μM SA from an extract exhibiting 80% inhibition of APX activity by gel filtration (PD-10) resulted in 40–50% recovery of enzyme activity (data not shown). Thus, the mechanism of inhibition by SA and INA appears to differ from that of *p*-aminophenol.

To estimate the inhibitor concentration necessary for 50% inhibition (IC_{50}), APX activity was measured in the presence of 750 μM AsA and different concentrations of SA or INA (25–500 μM; Fig. 2). At 500 μM of either compound, there was almost complete inhibition (95%). Double-reciprocal Lineweaver–Burk plots gave substrate-dependent IC_{50} values of 78 μM and 95 μM for SA and INA, respectively (Fig. 2 *Inset*).

Correlation Between Biological Activity of SA Analogues and Inhibition of APX. It has been shown that the ability of SA and its derivatives to bind and inhibit catalase from several plant species correlates with their biological activity—that is, their ability to induce PR-1 gene expression and enhance resistance to tobacco mosaic virus (6, 8, 21). To assess the functional relevance of SA's inhibition of APX, several analogues of SA were compared for their biological activity (7, 22–25) and their ability to inhibit APX activity (Table 2). The biologically active analogues were very effective, with inhibition (at 200 μM) ranging from 58% to 83%. In contrast, biologically inactive analogues in general were much poorer inhibitors. In summary, there is a strong correlation between biological activity and ability to inhibit both catalase and APX.

SA Is a One-Step Reductant of HRP. APXs from various plant species (pea, spinach, tea) exhibit high degrees of specificity for AsA as the e^- donor. Nonetheless, the enzyme

Table 1. Effects of SA and INA on APX, guaiacol peroxidases (POX), and HRP

	APX		POX		HRP	
Substrate	Activity*	Inhib., %	Activity*	Inhib., %	Activity*	Inhib., %
Control†	4.9 ± 0.2	—	2.8 ± 0.5	—	240 ± 2	—
SA						
100 μM	2.0 ± 0.2	59	2.7 ± 0.3	3	251 ± 11	−4
1000 μM	0.24 ± 0.1	95	2.7 ± 0.4	3	237 ± 21	1
INA						
100 μM	2.25 ± 0.4	54	2.8 ± 0.4	0	236 ± 8	1
1000 μM	0.24 ± 0.2	95	2.7 ± 0.4	3	242 ± 16	0

Inhib., inhibition.

*Enzyme activities are given as μmol of oxidized substrate per min per mg of protein. Assays were done in triplicate.

†APX and guaiacol peroxidases (and HRP) were assayed as described in text.

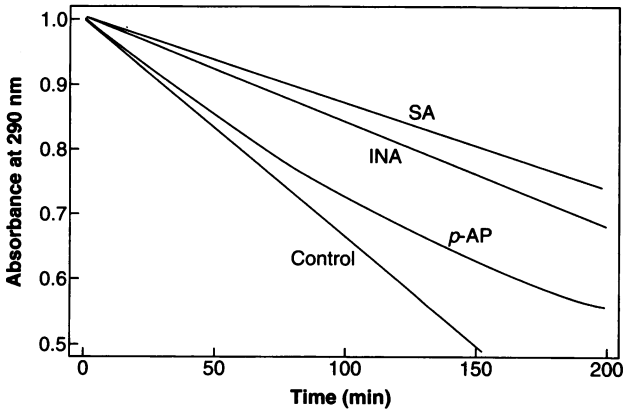

FIG. 1. Inhibition of APX activity by *p*-aminophenol (*p*-AP), SA, and INA. The final concentrations of the inhibitors were 200 μM *p*-aminophenol (10 mM stock in 50 mM potassium phosphate, pH 7), 100 μM SA, and 100 μM INA. The reactions were started by the addition of H_2O_2 (final concentration, 100 μM). The recordings have been overlayed for graphic clarity.

shows a low rate of oxidation of guaiacol and pyrogallol (17, 26, 27). Similarly, guaiacol peroxidases such as HRP can also use AsA as e^- donor but with low efficiency compared with guaiacol (27, 28). Given this partially overlapping specificity for various e^- donors, the striking difference in inhibition by SA (or INA) between APX and guaiacol peroxidases was surprising.

To gain insight into this unexpected difference between APX and guaiacol peroxidases, formation of the enzyme intermediates in the peroxidase reaction cycle were analyzed spectrometrically. These experiments should clarify whether there is no spectrum-perturbing interaction between SA and guaiacol peroxidases or whether any SA-induced effect is suppressed in the presence of alternative e^- donors such as guaiacol. All peroxidases have a similar mechanism of catalysis, as shown by Fig. 3. Basically, the native ferric enzyme first undergoes a $2e^-$ equivalent oxidation by the peroxide molecule (such as H_2O_2) to form the enzyme intermediate compound **I**. Compound **I** next accepts a $1e^-$ equivalent from an e^- donor (e.g., guaiacol or AsA) to form the second intermediate compound **II**. Compound **II** then accepts a second $1e^-$ equivalent from the same or another e^- donor to return to the native enzyme state. The various intermediates of peroxidase

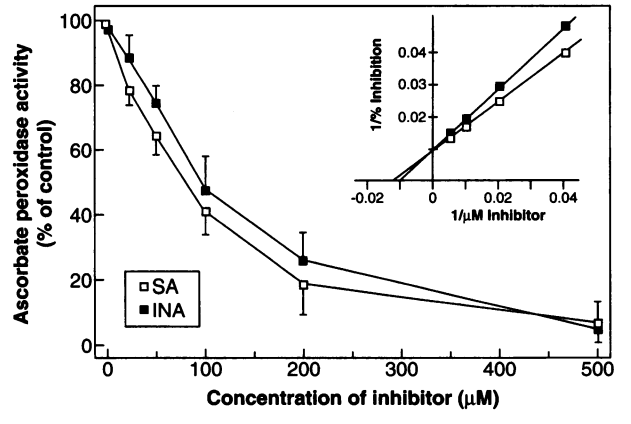

FIG. 2. Concentration-dependent inhibition of APX activity by SA and INA. Values are expressed as percent activity of the control and represent the average of three replicated assays. The specific activity of the control was 5.0 μmol·min^{-1}·mg^{-1}. The inset shows a double-reciprocal plot of the data (1/% inhibition versus 1/concentration of SA or INA, respectively).

Table 2. Comparison of SA and its analogues with respect to biological activity and inhibition of APX from tobacco

SA and analogues	Biological activity*	% Inhibition†
SA	+	83 ± 9
4-Chloro-SA	+	58 ± 4
5-Chloro-SA	+	73 ± 5
3,5-Dichloro-SA	+	59 ± 5
2,6-Dihydroxybenzoic acid	+	72 ± 8
3-Hydroxybenzoic acid	−	28 ± 7
4-Hydroxybenzoic acid	−	0 ± 6
Thio-SA	−	0 ± 2
4-Amino-SA	−	9 ± 5

*Biological activity is based on the results of White (22), Van Loon (23), Abad *et al.* (24), Doherty *et al.* (25), and Conrath *et al.* (8).
†APX was assayed in the presence of 200 μM of the various compounds, which had been added from stock solutions (10 mM in water, adjusted to pH 5.8 with KOH). Values represent results from several independent preparations, each assayed in triplicate. The specific activity of the control was between 4.1 and 5.8 μmol of oxidized AsA per min per mg of protein. Several of the analogues showed significant quenching of autooxidation of AsA in the absence of enzyme. Therefore, all of the % inhibition values presented have been corrected for this quenching.

can be readily distinguished by their characteristic spectra in the Soret region (29, 30).

Purified HRP was analyzed as a prototypic guaiacol peroxidase that is insensitive to SA (Table 1). The ferric enzyme has an absorbance maximum at 403 nm (Fig. 4*A*, trace 1), which upon addition of H_2O_2 and SA was converted over 15 min (traces 2–5) to a form with an absorbance spectrum identical to that of compound **II** (418 nm; ref. 30). Incubation for up to 1 hr did not result in any further change of the spectrum (not shown). The decreased absorbance shortly after addition of H_2O_2 and SA (4 min; trace 2) indicates the presence of the intermediate compound **I** (absorbance maximum at 410 nm) that precedes compound **II** formation (30).

To verify that the SA-induced spectrum resulted from the formation of compound **II**, hydroquinone was added to the sample. Since hydroquinone can serve as e^- donor for compound **II** (30), the resulting spectrum (Fig. 4, trace 6) should be very similar to that seen in the absence of SA (Fig. 4, trace 1). Indeed, the similarity of the two spectra confirm that SA induced the accumulation of compound **II**. Since the reaction products of guaiacol and pyrogallol (tetraguaiacol and purpurogallin) partially interfere with the spectral analysis in the Soret region, only low amounts (2.5 μM) of these e^- donors could be used. This resulted in a small shift in the absorbance spectrum as expected (data not shown).

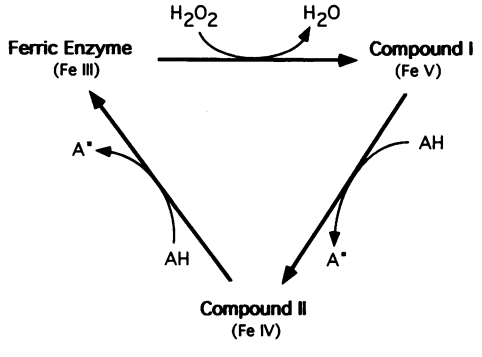

FIG. 3. Scheme for the mechanism of catalysis by peroxidases. Formal oxidation states of iron are shown in parentheses. AH represents an e^- donor (e.g., guaiacol, AsA, pyrogallol), and A· represents the resulting radical formed after donation of an e^-.

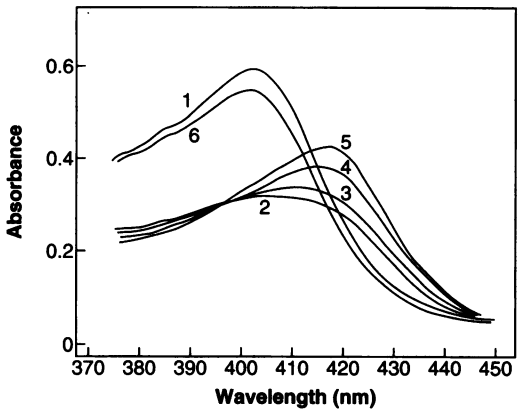

FIG. 4. Formation of the enzyme intermediate compound II of HRP in the presence of SA. The assay contained 0.4 mg of HRP in 50 mM potassium phosphate (pH 6) (trace 1). Addition of 1 μM H_2O_2 resulted in conversion of the ferric enzyme into compound I (trace 2). After the addition of 200 μM SA, spectral changes in the Soret region were measured at 4-min intervals (traces 3–5). After 16 min (i.e., after plotting trace 5), 25 μM hydroquinone (final concentration) was added to the sample resulting in trace 6. Spectra were recorded with a Beckman DU-7 spectrophotometer with 1-ml semimicro black-sidewall quartz cuvettes.

The SA-mediated accumulation of compound II would be expected to block the enzyme activity. A variety of phenolics including SA act as inhibitors of myeloperoxidase, since they can donate an e^- to compound I but not to compound II (31). This results in the accumulation of compound II and inhibition of the enzyme activity, which depends on the continuous cycling of the enzyme through the three states (Fig. 3). To further investigate the effects of SA and hydroquinone on HRP, hydroquinone was added to the sample at various concentrations together with 200 μM SA. Accumulation of compound II (increase in absorption at 418 nm) was significantly reduced even by 0.1 μM hydroquinone, and almost complete suppression was achieved in the presence of 25 μM hydroquinone (data not shown). Thus, hydroquinone both prevented and reversed the accumulation of compound II induced by SA. Since guaiacol, pyrogallol, and hydroquinone compete with each other as e^- donors (30), it is likely that guaiacol and pyrogallol, like hydroquinone, prevent SA inhibition of HRP and other guaiacol peroxidases (Table 1) by effectively serving as e^- donors for compound II, as well as for compound I, even in presence of SA.

DISCUSSION

The discovery that the putative SA receptor from tobacco is a catalase that can be inhibited by SA both *in vitro* and *in vivo* suggests that SA's mechanism of action is to elevate ROS levels by blocking the ability of catalase to degrade H_2O_2 (6). Catalase is a heme-containing enzyme and is the basis of one of the key mechanisms by which aerobic organisms cope with endogenously generated H_2O_2. In addition to degrading H_2O_2 via the catalytic reaction, catalase can utilize H_2O_2 for the peroxidatic oxidation of various organic substrates (see Fig. 3). The latter reaction mechanism is virtually identical with that of heme (protoporphyrin IX)-containing peroxidases (Fig. 3). Indeed, catalase as well as guaiacol peroxidases (e.g., HRP) and APX share a common spectrum of e^- donors. Each of these enzymes can utilize phenolic compounds such as pyrogallol, guaiacol, and AsA as e^- donors, though there are strong substrate preferences (12, 19, 27). Although SA's precise mechanism of inhibition of catalase has not yet been deciphered, preliminary results suggest that it promotes the peroxidative reaction of catalase. Therefore, we examined the effect of SA on guaiacol peroxidases and APX from tobacco.

Inhibition of APX. There are several types of APX in plants. Two isoforms are found in chloroplasts, either in the stroma or bound to thylakoid membranes, while another isozyme is located in the cytosol (12). No effort was made to separate these different isozymes. However, at high concentrations (≥500 μM), both SA and INA showed almost complete inhibition of APX activity. Since the homogenization procedure used should have extracted at least the soluble isozymes, we suspect that the two major isozymes (stromal and cytosolic) are both sensitive to SA and INA.

The IC_{50} values obtained for SA (78 μM) and INA (95 μM) in the presence of 750 μM AsA indicate a moderately high affinity of APX for these inhibitors. With 1.5 mM AsA, the addition of 100 μM SA or INA resulted in 52% and 49% inhibition, respectively (data not shown). These results indicate the potential for substantial inhibition at the physiological levels of SA detected in infected tobacco leaves (7–56 μM; refs. 32–34), even in the presence of high AsA concentrations reported for chloroplasts (35). In contrast, the levels of SA in uninfected leaves of infected plants are considerably lower and may not be sufficient to effectively inhibit APX or catalase. Nonetheless, peroxidases and catalases may play a role in SA-mediated, systemic induction of PR genes. Even at relatively low concentrations, SA and INA induce lipid peroxidation and formation of lipid peroxides (Z. Chen and D.F.K., unpublished results). These lipid peroxides can activate PR genes and are likely formed via a SA (INA) free radical. This radical is generated when SA donates an e^- to catalase or peroxidase during the peroxidative cycle of these enzymes (see Fig. 3). A similar mechanism has been suggested for the bioactivation of phenytoin by thyroid peroxidase or HRP (36).

Concerted Action of SA on APX and Catalase. Plants possess several powerful antioxidant systems to scavenge ROS. The reactivities of the major ROS differ vastly. Highly reactive species such as singlet oxygen or hydroxyl radicals (HO·) react at the site of generation. In contrast, H_2O_2 is relatively stable and readily permeates membranes, allowing for the interaction between different scavenging systems, even those located in separate organelles. For example, while chloroplasts, which eliminate H_2O_2 via the ascorbate–glutathione (Halliwell–Asada) cycle, may have excess capacity to cope with all of the H_2O_2 produced by the cell (37), peroxisomes (sites of catalase) may function as scavengers for H_2O_2 produced by other subcellular compartments (13).

The simultaneous inhibition of APX and catalase by SA may have two functions. First, blocking the two major H_2O_2-degrading pathways in the plant cell will lead to increased levels of endogenous H_2O_2. It is probable that the elevated endogenous H_2O_2 activates PR genes, since treatment of plants with prooxidants activated PR-1 genes, while antioxidants suppressed SA- or UV-induced PR-1 gene expression (refs. 6 and 38; Z. Chen, U. Conrath, and D.F.K., unpublished results). H_2O_2 induction of PR genes is likely to be concentration dependent, as previously demonstrated in soybean for genes associated with protection against the early oxidative burst(s) induced by infection with avirulent strains of *Pseudomonas syringae* (39) and in maize for genes associated with chilling tolerance (40). The effective concentration range in which H_2O_2 can function as an inducer without acting as a cytotoxic compound remains to be determined. Exogenous applied H_2O_2 is rather ineffective at inducing PR-1 genes in tobacco (6). However, in the presence of functional antioxidative enzymes like APX and catalase, H_2O_2 added to chloroplasts or plant cells is rapidly degraded, even when applied in the millimolar range (13, 37, 39). Treatment of tobacco plants with 1 mM SA, the concentration typically used to activate PR genes, induced a 50–60% increase in endogenous H_2O_2 levels. A similar increase was induced by the application of the

catalase inhibitor 3-aminotriazole to tobacco (6) or to maize (40), where it induced PR-1 genes or genes associated with chilling tolerance, respectively. Although the H_2O_2 levels differ substantially [0.15 μmol of H_2O_2 per g (fresh weight) of untreated tobacco versus 1.5 μmol for maize], these data indicate that a moderate increase of H_2O_2 is sufficient to induce defense or stress-related genes. This predicted rise in H_2O_2 resulting from inhibition of APX and catalase would occur much later than the oxidative burst(s) that takes place within a few hours after infection, since increases in SA are not detected until 8–48 hr after infection depending on the plant–pathogen combination (14, 32, 41).

A second function of inhibition of both catalase and the ascorbate–glutathione pathway may be to prevent the cell from depleting its NAD(P)H reserve. While catalase degrades H_2O_2 without consuming reducing equivalents [NAD(P)H], the regeneration of oxidized AsA via the ascorbate–glutathione pathway requires NAD(P)H, since both monodehydro-AsA reductase and glutathione reductase use NAD(P)H as an e^- donor. Although NAD(P)H is probably not limiting in plastids under light (42), prolonged increase of H_2O_2 levels due to complete or even partial inhibition of catalase activity by SA, together with a functional AsA–glutathione pathway, could deplete NAD(P)H. Thus, the coordinated inhibition of catalase and APX not only enhances H_2O_2 levels but also may prevent the cell from consuming of reducing equivalents that may be required for production of antimicrobial compounds and proteins necessary for establishing resistance.

A prolongued increase of the intracellular H_2O_2 level is likely to affect the redox balance of the cell. The activation of defense-related genes like the PR genes may be modulated by the cell's redox state. Perturbations of the glutathione metabolism in plants have been shown to induce the genes for phenylalanine ammonium lyase, chalcone synthase, and a cytosolic copper/zinc superoxide dismutase (43, 44). In mammals, the activities of the NF-κB transcription factor and Bcl-2 (an inhibitor of apoptosis) are modulated by the ratio of reduced to oxidized forms of glutathione (9, 45). The effects of SA on the cell's redox state should be investigated.

Effect of SA on Guaiacol Peroxidases. Neither SA nor INA inhibited guaiacol peroxidases from tobacco or HRP (Table 1). Surprisingly, SA was able to reduce the prototypic guaiacol peroxidase, HRP, to its intermediate compound II (Fig. 4) but had limited, if any, capacity to reduce this intermediate back to the ferric enzyme. Therefore, SA fulfills the essential criteria for a potent inhibitor for peroxidases (31). However, SA is a poor inhibitor of this class of enzymes because it is unable to compete with more suitable substrates of these peroxidases such as guaiacol, pyrogallol, and hydroquinone, which readily reduce compound **II** to the ferric enzyme, thereby completing the peroxidase cycle (see Fig. 3). The ability of anti-inflammatory drugs including aspirin and SA to form compound **II** of the mammalian myeloperoxidase has been previously described. *In vivo* this process is reversed by AsA, which reduces compound **II** back to the ferric enzyme (31). Appropriate combinations of e^- donors (each one able to serve as donor for only one of the two reduction steps in Fig. 3) have been shown not only to maintain peroxidase activity, but have been suggested to be necessary for the oxidation of compounds that are not able to serve as two-step donors (31, 46, 47).

In summary, the demonstration that SA (and INA) blocks APX activity supports a role for elevated H_2O_2 levels or directly coupled perturbations of the cellular redox state in SA-induced defense responses activated at the site of infection. Moreover, the observed specificity of the inhibition of peroxidases by SA and INA is consistent with the roles that different peroxidases play in mediating a defense response. Inhibition of APX (as well as catalase) results in an increased level of H_2O_2 that contributes to defense gene activation and acts as a substrate for SA-insensitive guaiacol peroxidases that are involved in defense responses such as lignification and crosslinking of cell wall proteins (7).

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