Influence of Salicylic Acid on H_2O_2 Production, Oxidative Stress, and H_2O_2-Metabolizing Enzymes[1]

Salicylic Acid-Mediated Oxidative Damage Requires H_2O_2

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We investigated how salicylic acid (SA) enhances H_2O_2 and the relative significance of SA-enhanced H_2O_2 in *Arabidopsis thaliana*. SA treatments enhanced H_2O_2 production, lipid peroxidation, and oxidative damage to proteins, and resulted in the formation of chlorophyll and carotene isomers. SA-enhanced H_2O_2 levels were related to increased activities of Cu,Zn-superoxide dismutase and were independent of changes in catalase and ascorbate peroxidase activities. Prolonging SA treatments inactivated catalase and ascorbate peroxidase and resulted in phytotoxic symptoms, suggesting that inactivation of H_2O_2-degrading enzymes serves as an indicator of hypersensitive cell death. Treatment of leaves with H_2O_2 alone failed to invoke SA-mediated events. Although leaves treated with H_2O_2 accumulated in vivo H_2O_2 by 2-fold compared with leaves treated with SA, the damage to membranes and proteins was significantly less, indicating that SA can cause greater damage than H_2O_2. However, pretreatment of leaves with dimethylthiourea, a trap for H_2O_2, reduced SA-induced lipid peroxidation, indicating that SA requires H_2O_2 to initiate oxidative damage. The relative significance of the interaction among SA, H_2O_2, and H_2O_2-metabolizing enzymes with oxidative damage and cell death is discussed.

Plants respond to necrotizing pathogens and/or to abiotic stresses by altering their cellular metabolism and invoking various defense mechanisms (Enyedi et al., 1992; Mehdy, 1994). Survival under these stressful conditions depends on the plant's ability to perceive the stimulus, generate and transmit signals, and instigate biochemical changes that adjust the metabolism accordingly (Enyedi et al., 1992). Various agents such as Ca^{+2}, ethylene, jasmonic acid, and SA have been proposed as signal transducers (Enyedi et al., 1992; Klessig and Malamy, 1994). SA has

received particular attention because of its pharmaceutical properties and its universal application in both animal and plant systems, especially in plant defense against disease (for review, see Klessig and Malamy, 1994).

Although the mode of action of SA in inducing resistance to pathogens is not known, exogenous application of SA induced PR gene expression and established SAR (Bi et al., 1995; Conrath et al., 1995). The observation that a specific SA-binding activity found in tobacco (Chen et al., 1993a) and in several other plant species (Sanchez-Casas and Klessig, 1994) has CAT activity led to the proposal that SA acts by enhancing AOS such as H_2O_2 (Chen et al., 1993b). Inhibition of CAT activities by SA in vitro (Chen et al., 1993a; Sanchez-Casas and Klessig, 1994) and in tobacco cell cultures (Conrath et al., 1995) provides support for this hypothesis. Leaves treated with SA accumulate H_2O_2 and have enhanced PR gene expression (Chen et al., 1993b), whereas antioxidants suppress the induction of PR genes by SA (Conrath et al., 1995).

In recent years, however, a considerable body of evidence has accumulated suggesting that physiologically relevant concentrations of SA (<1 mM) do not inhibit CAT in tobacco (Willekens et al., 1994; Bi et al., 1995), maize (Guan and Scandalios, 1995), and *Arabidopsis thaliana* (Summermatter et al., 1995). Moreover, SA may not bind specifically to CAT but rather to several Fe-containing enzymes such as CAT, aconitase, lipoxidase, and peroxidase (Ruffer et al., 1995). No major changes in H_2O_2 levels occur during the onset of SAR, indicating that SA-mediated establishment of SAR may not involve H_2O_2 accumulation (Neuenschwander et al., 1995). Furthermore, high concentrations of H_2O_2 have been shown to induce the biosynthesis of SA (Leon et al., 1995; Summermatter et al., 1995), suggesting that H_2O_2 may act upstream of SA in establishing SAR.

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Abbreviations: AOS, active oxygen species; APX, ascorbate peroxidase; CA-POX, coniferyl alcohol peroxidase; CAT, catalase; Chl, chlorophyll; DMTU, dimethylthiourea; GR, glutathione reductase; HR, hypersensitive response; POX, guaiacol-peroxidases; PR, pathogenesis-related; SA, salicylic acid; SABP, salicylic acid-binding protein; SA*, salicylic acid free radical; SAR, systemic acquired resistance; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances.

Although it is not clear how either SA- or H_2O_2-derived signals are perceived and propagated, both SA and H_2O_2 are considered to have a regulatory role in developing resistance to pathogens (Wu et al., 1995; Hammond-Kosack and Jones, 1996). Whether SA acts up- or downstream of H_2O_2 is controversial; to date no single study to our knowledge has critically examined the influence of SA on H_2O_2 production and enzymes capable of metabolizing H_2O_2 in vivo. SA treatments have been shown to have no major influence on foliar CAT activities (Bi et al., 1995; Summermatter et al., 1995), but changes in H_2O_2 and other enzymes capable of metabolizing H_2O_2 were not investigated. On the other hand, SA-mediated inactivation of CAT in vitro has been shown to be dependent on the presence of H_2O_2 fluxes (Durner and Klessig, 1996). Further, no single study has investigated the influence of SA on H_2O_2 production and H_2O_2-metabolizing enzymes in a single species. In view of the existing contradictory results derived from in vitro and in vivo studies, how SA enhances H_2O_2 production in vivo and the relative significance of such SA-enhanced H_2O_2 are unclear.

Our preliminary studies indicated that leaves treated with SA exhibit greater damage to cellular pigments and organelles compared with leaves treated with high concentrations of H_2O_2 alone. Because treatment of leaves with SA is believed to enhance H_2O_2 levels (Kauss and Jeblick, 1994; Fauth et al., 1996), we initiated experiments to examine how SA enhances H_2O_2 and whether SA-mediated oxidative stress is dependent on H_2O_2. The major objectives of the present study were (a) to elucidate the underlying mechanisms by which SA enhances H_2O_2 levels in vivo and (b) to systematically examine the influence of SA and/or H_2O_2 on cellular oxidative stress.

MATERIALS AND METHODS

Plant Culture

Seeds of *Arabidopsis thaliana* genotype Landsberg *erecta* were sown in a synthetic soil medium (Promix-BX, Premier Brands, Red Hill, PA) as described previously (Rao et al., 1996). Plants were grown in growth chambers at 23/18°C (day/night) temperature, 60 to 80% RH, and a PPFD of 130 μmol m^{-2} s^{-1} with a 16-h photoperiod.

Treatment Conditions

Rosette leaves (third and fourth) of 26-d-old plants were cut at their basal end, rinsed briefly in double-distilled water, and incubated in a solution containing varying concentrations of SA (0.1, 0.5, 1, 2, 3, and 5 mM) or 10.0 mM H_2O_2 for 8 h at room temperature. Leaves incubated in double-distilled water were considered controls. To investigate whether SA-induced oxidative damage is due to SA-enhanced H_2O_2, leaves were treated with water or 5 mM DMTU, a trap for H_2O_2 (Levine et al., 1994), for 4 h prior to treatment of leaves with water or 5 mM SA for another 4 h. The long-term influence of physiological concentrations of SA was investigated by incubating leaves with 0.5 mM SA for 48 h as described above. To avoid

imposition of water stress during the incubation period, precautions were taken to make sure that cut ends were in contact with the solution. Immediately after 8 or 48 h of incubation, leaves were rinsed briefly, frozen under liquid N_2, and stored at -80°C for further analysis.

H_2O_2 Measurements

In vivo levels of H_2O_2 were measured by monitoring the A_{415} of the titanium-peroxide complex following the method described by Brennan and Frenkel (1977). Absorbance values were calibrated to a standard graph generated with known concentrations of H_2O_2.

Oxidative Damage

Oxidative damage to membranes and proteins was periodically monitored by estimating TBARS and carbonyl content, respectively. Lipid peroxidation was determined by measuring TBARS following the method of Heath and Packer (1968). Carbonyl content was estimated by reacting protein extracts with 2,4-dinitrophenylhydrazine following the method described by Levine et al. (1990).

HPLC of Photosynthetic Pigments

Fresh leaves were homogenized in 80% (v/v) acetone containing $CaCO_3$, as described by Gilmore and Yamamoto (1991). The extract was centrifuged at 14,000g for 15 min at 4°C. The supernatant was filtered through a 0.22-μm nylon filter (Fisher Scientific) and frozen at -80°C under N_2. All analyses were completed within 48 h of extraction, and no degradation of pigments was observed during this period. A 626 HPLC system (Waters, Milford, MA) was used. Fifty microliters of the extract was injected into a C_{18} reverse-phase column (3.8- × 150-mm cartridge containing 5 mm of Lichrosorb, Novapack, Waters) equilibrated with solvent A containing acetonitrile, methanol, and 100 mM Tris-HCl, pH 8.0 (72:8:3). The pigments were eluted with a flow rate of 2 mL min^{-1} at 25°C isocratically with solvent A from 0 to 3 min, followed by a 3-min linear gradient to 100% solvent B (methanol:hexane, 4:1, v/v). The pigments were further eluted with solvent B for 10 min and the elution was monitored at 440 nm. The column was re-equilibrated for at least 10 min with solvent A between each sample. The program resolved eight major peaks identified by comparison with authentic standards (in order of elution) as: 9'-*cis*-neoxanthin, violaxanthin, antheraxanthin, lutein, zeaxanthin, Chl *b*, Chl *a* and *b*, and β-carotene.

Extraction of Soluble Protein

Frozen leaves (0.2 g) were crushed to fine powder in a mortar under liquid N_2. Soluble proteins were extracted by homogenizing the powder in 1 mL of 100 mM potassium phosphate buffer (pH 7.5) containing 2 mM Na_2-EDTA, 1% (w/v) PVP-40, and 1 mM PMSF. The homogenate was centrifuged at 14,000g for 15 min and the supernatant was passed through a PD-10 column (Pharmacia) equilibrated with 50 mM potassium phosphate buffer, pH 7.5. Protein

extracts for measuring APX activity were prepared as described above, except that extraction buffer was supplemented with 4 mM ascorbate and the PD columns were equilibrated with phosphate buffer containing 2 mM ascorbate.

Antioxidant Enzyme Assays

Activities of SOD, CAT, POX, APX, and GR were measured following the methods described by Rao et al., (1996). SOD (EC 1.15.1.1) activity was determined in 3 mL of a reaction mixture containing 50 mM $Na_2CO_3/NaHCO_3$ buffer, pH 10.2, 0.1 mM Na_2-EDTA, 0.015 mM ferricytochrome c, and 0.05 mM xanthine. CAT (EC 1.11.1.6) activity was determined by following the consumption of H_2O_2 at 240 nm for 5 min in 3 mL of a reaction mixture containing 100 mM potassium phosphate buffer, pH 7, and 10 μL of 30% H_2O_2. POX (EC 1.11.1.7) activity was determined at 470 nm for 5 min in a 3-mL reaction mixture containing 100 mM potassium phosphate buffer, pH 6.5, 16 mM guaiacol or coniferyl alcohol, and 10 μL of 10% H_2O_2. APX (EC 1.11.1.11) activity was determined by following the decrease in A_{290} for 3 min in 1 mL of a reaction mixture containing 100 mM potassium phosphate buffer, pH 7.5, 0.5 mM ascorbate, and 0.2 mM H_2O_2. GR (EC 1.6.4.2) activity was determined by following the oxidation of NADPH at 340 nm for 3 min in 1 mL of an assay mixture containing 100 mM Tris-HCl, pH 7.8, 2 mM Na_2-EDTA, 0.2 mM NADPH, and 0.5 mM GSSH. All enzyme assays were performed at 25°C with equal amounts of protein (100 μg). Protein content was measured following the method of Bradford (1976), with BSA as a standard.

Native PAGE and Activity Staining

Equal amounts of protein from leaves incubated with various concentrations of SA or H_2O_2 were subjected to discontinuous PAGE under nondenaturing, nonreducing conditions (Rao et al., 1996). SOD isoforms were visualized by staining the gels with 2.5 mM nitroblue tetrazolium for 25 min, followed by incubation in 50 mM phosphate buffer, pH 7.8, containing 28 mM riboflavin and 28 mM tetramethylethylene diamine, and illuminating on a light box. Cu,Zn-, Mn-, and Fe-SOD isoforms were differentiated by incubating the gels in 50 mM phosphate buffer, pH 7.0, containing 3 mM KCN or 5 mM H_2O_2 before staining for SOD activity.

CAT isoforms were visualized following the method described by Woodbury et al. (1971). Gels were incubated in 100 mL of double-distilled water containing 20 mL of 50% H_2O_2 for 25 min at room temperature and then briefly rinsed with water. CAT isozymes were visualized by incubating gels in 100 mL of double-distilled water containing 1% ferric chloride and 1% potassium ferricyanide. Color development was continued for 4 min and the reaction was stopped with a brief wash in double-distilled water. Staining of POX isoforms was achieved by incubating the gels in sodium acetate buffer, pH 4.5, containing 2 mM benzidine, and initiating the reaction by the addition of 3 mM H_2O_2. APX isoforms were visualized by submerging the gels in 50

mM phosphate buffer containing 28 mM tetramethylethylene diamine and 2.5 mM nitroblue tetrazolium for 10 min. GR activity was detected by incubating the gels in Tris-HCl buffer, pH 7.5, containing 10 mg of 3-(4,5-dimethylthiazol-2-4)-2,5-diphenyl tetrazolium bromide, 10 mg of 2,6-dichlorophenolindophenol, and 0.5 mM NADPH with or without 3.4 mM GSSH.

In Vitro Treatments

Soluble proteins were extracted from the third and fourth rosette leaves of 26-d-old plants as described above. Equal amounts of protein (1 mg mL^{-1}) were mixed with varying concentrations of SA (final concentration 0.5, 1, 3, and 5 mM) in test tubes and held for 1 h. Aliquots containing equal amounts of protein were sampled periodically at 0, 5, 10, 30, and 60 min, flash-frozen under liquid N_2, and stored at -80°C for further analysis. Activities of antioxidant enzymes such as CAT, POX, and APX were measured following the methods described above.

All experiments described above were repeated twice with at least two to three replicates in each experiment. Significant differences between the mean values were evaluated using statistical software (SigmaStat, Jandel Scientific, San Rafael, CA). At least three of the extracts that were used to measure enzyme activities from each experiment were subjected to native PAGE and stained for enzyme activities. Photographs presented are from one experiment and represent the general trend.

RESULTS

SA Enhances H_2O_2 Levels

Plants treated with SA have previously been shown to accumulate H_2O_2 (Chen et al., 1993b; Fauth et al., 1996); however, to our knowledge, none of these studies has provided detailed information concerning the pattern of H_2O_2 accumulation in plants treated with SA. In the present study SA treatments enhanced H_2O_2 levels in the leaves of A. thaliana in a dose- and time-dependent manner (Fig. 1). Treatment of leaves with ≥ 1 mM SA for 8 h significantly enhanced H_2O_2 levels compared with leaves treated with water (controls; Fig. 1A). Leaves treated with 1 and 5 mM SA for 8 h enhanced H_2O_2 levels by 59 and 194%, respectively, compared with control leaves. SA treatments enhanced H_2O_2 levels significantly within the first 2 h of incubation (Fig. 1B). Incubation of leaves with >5 mM SA resulted in phytotoxic symptoms (data not shown); subsequent experiments were restricted to SA concentrations that did not result in visible symptoms. Treatment of leaves with 10.0 mM H_2O_2 for 8 h enhanced in vivo H_2O_2 levels by 367% compared with control leaves (Table I).

Influence of SA and H_2O_2 on Oxidative Damage

Because SA treatments enhanced H_2O_2 levels in vivo, we investigated the damage to lipids and proteins by monitoring TBARS and carbonyl content. Although damage to membranes and proteins was negligible in leaves treated
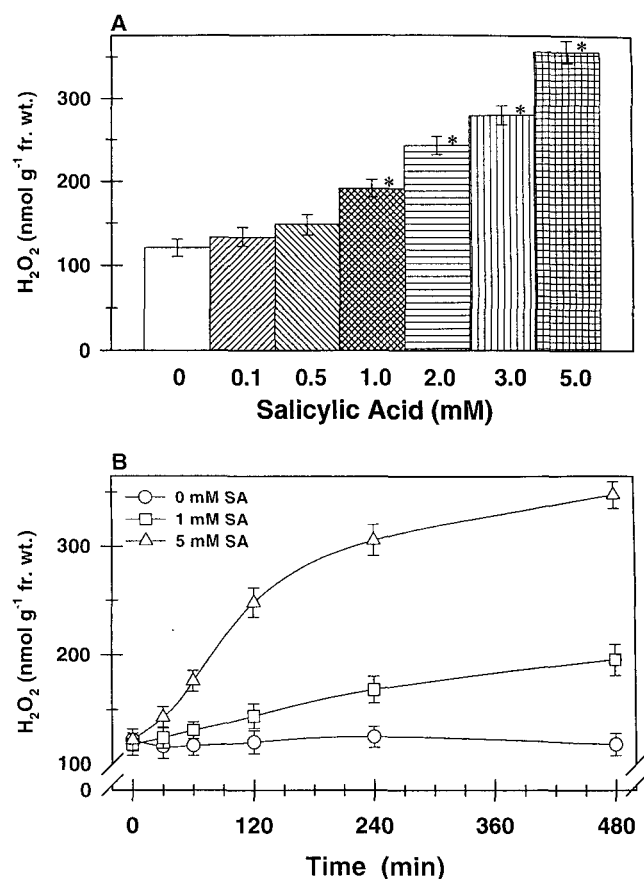
Figure 1. Dose- (A) and time-dependent (B) accumulation of H_2O_2 levels in the leaves of *A. thaliana* treated with SA. Cut ends of rosette leaves were incubated with varying concentrations of SA for 8 h and the H_2O_2 levels were monitored at the indicated intervals. Mean values shown are averages of three different experiments. Error bars represent SEs with $n = 6$. Asterisks indicate that mean values are significantly different compared with control (P < 0.05). fr. wt., Fresh weight.

with <1 mM SA for 8 h, both TBARS and carbonyl content were significantly higher in leaves treated with ≥1 mM SA (Fig. 2). Leaves incubated with 1 mM SA enhanced TBARS and carbonyl groups by 49 and 35%, respectively, compared with control leaves. To investigate whether SA-enhanced H_2O_2 may have inflicted damage to membranes and proteins, we incubated leaves with H_2O_2 (≤10.0 mM) for 8 h and assessed changes in TBARS content and carbonyl groups. Treatment of leaves with H_2O_2 enhanced foliar TBARS content and carbonyl groups by 25 and 23%, respectively, compared with control leaves (Table I). No

major changes were observed in TBARS or carbonyl content in leaves treated with low concentrations of H_2O_2 (<10.0 mM; data not shown).

Influence of SA and H_2O_2 on the Composition of Photosynthetic Pigments

Although photosynthetic pigments are considered to be highly sensitive to AOS, carotenoids (including xanthophylls) are believed to scavenge AOS such as O_2^- and $^1O_2$ (Demmig-Adams and Adams, 1993). Because SA enhanced the production of H_2O_2, we investigated the changes in the composition of photosynthetic pigments by HPLC. Treatment of leaves with 1 mM SA for 8 h enhanced the content of pigments such as violaxanthin, neoxanthin, antheraxanthin, zeaxanthin, and β-carotene but not lutein, Chl *a*, or Chl *b* (Fig. 3). However, 5 mM SA decreased the levels of all major pigments, and additional peaks that co-eluted with Chl *a* and β-carotene, respectively, were observed. Although the co-eluted components were not identified, their absorption spectra resembled those of purified Chl *a* and β-carotene (data not shown); therefore, we assumed that they were isomers (Fig. 3; histograms marked with an asterisk). No major changes were observed in the photosynthetic pigments of *A. thaliana* leaves treated with ≤10.0 mM H_2O_2 for 8 h (data not shown).

Influence of SA and H_2O_2 on H_2O_2-Metabolizing Enzymes

Plant cells presumably regulate H_2O_2 levels by coordinating activities of H_2O_2-generating enzymes such as SOD and H_2O_2-degrading enzymes such as CAT, POX, APX, and GR (Creissen et al., 1994; Van Camp et al., 1994). Therefore, the influence of SA on antioxidant enzymes capable of synthesizing and degrading H_2O_2 was assessed to determine which enzymes are likely responsible for elevated H_2O_2 levels. We also investigated the influence of H_2O_2 on H_2O_2-metabolizing enzymes to determine whether SA-induced changes in enzyme activities are specific to SA.

SA but Not H_2O_2 Enhances in Vivo SOD

H_2O_2 in plant cells is produced by the dismutation of O_2^- by SOD localized in chloroplasts, cytosol, and mitochondria. Since the primary role of SOD is to generate H_2O_2 (Van Camp et al., 1994), we investigated whether SA-enhanced H_2O_2 levels are related to the changes in SOD activity.

Table I. *Changes in H_2O_2 levels, lipid peroxidation (as indicated by TBARS content), oxidative damage to proteins (as indicated by the number of carbonyl [C=O] groups), SOD, CAT, POX, CA-POX, and APX activities in the leaves of A. thaliana treated with 10.0 mM H_2O_2 for 8 h*

Mean values ± SE are shown ($n = 6$). Values in parentheses indicate changes relative to control.

Treatment	H_2O_2	TBARS	C=O groups	SOD	CAT	POX	CA-POX	APX
	$nmol\ g^{-1}$ fresh wt	$\mu mol\ g^{-1}$ fresh wt	$nmol\ mg^{-1}$ protein	$units\ mg^{-1}$ protein	$\mu mol\ min^{-1}\ mg^{-1}$ protein			$nmol\ min^{-1}\ mg^{-1}$ protein
Control	136 ± 10 (100)	17.12 ± 1.22 (100)	12.14 ± 1.12 (100)	5.64 ± 0.26 (100)	1.42 ± 0.06 (100)	4.30 ± 0.22 (100)	1.49 ± 0.08 (100)	5.38 ± 0.21 (100)
H_2O_2	635[a] ± 66 (367)	21.36[a] ± 1.57 (125)	14.98[a] ± 1.1 (123)	5.88 ± 0.24 (104)	1.94[a] ± 0.08 (136)	4.46[b] ± 0.26 (104)	2.38[a] ± 0.18 (160)	7.18[a] ± 0.35 (133)

[a] Mean values are significantly different compared with control values (P < 0.05).   [b] Not significant.
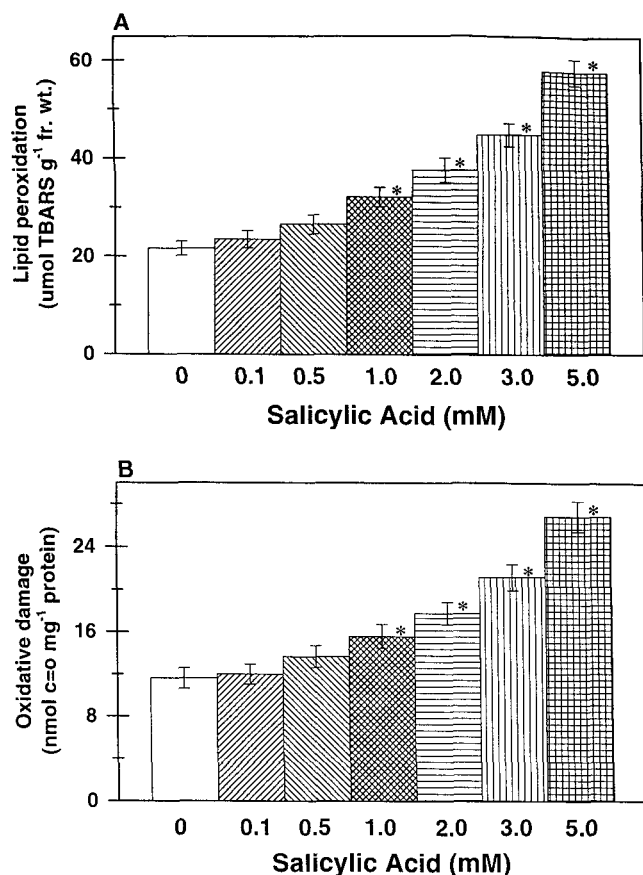
Figure 2. SA-induced changes in lipid peroxidation (A) and oxidative damage (B) in the leaves of *A. thaliana*. Cut ends of rosette leaves were incubated with varying concentrations of SA for 8 h, and TBARS and carbonyl contents were measured as described in "Materials and Methods." Mean values shown are averages of three different experiments. Error bars represent SEs with $n = 6$. Asterisks indicate that mean values are significantly different compared with control ($P <$ 0.05). fr. wt., Fresh weight.

Incubation of leaves with SA enhanced SOD activity in a dose-dependent manner (Fig. 4A), and significant changes occurred within the first 2 h of treatment (Fig. 4B). Treatment of leaves with 1 mM SA enhanced SOD activity by 32% compared with control leaves. Total SOD activity represents the combined action of Cu,Zn-, Mn-, and Fe-SOD, which can be distinguished by their differential sensitivity to KCN and/or H_2O_2 (Van Camp et al., 1994). Gels incubated with KCN and H_2O_2 before staining for SOD activity indicated that SOD-1 was Mn-SOD, whereas isoforms 2 to 5 were Cu,Zn-SOD. No Fe-SOD activity was detected in the extracts (Rao et al., 1996; data not shown). Activities of SOD-3, -4, and -5 isoforms were preferentially enhanced in leaves treated with SA (Fig. 4C), whereas the intensity of SOD-2 decreased with increasing SA concentrations (Fig. 4C). The activity of the SOD-1 (Mn-SOD) isoform remained unaltered with SA treatments. No major changes were observed in the activities of SOD (Table I) or the isoform composition of SOD (Fig. 4C; lane f) of leaves treated with H_2O_2 compared with control leaves.

SA but Not H_2O_2 Inactivates CAT and APX in Vivo

Because SA-enhanced H_2O_2 levels are closely related to increased SOD activities, we investigated whether SA-mediated inactivation of H_2O_2-degrading enzymes may have also contributed to elevated H_2O_2 levels in leaves treated with SA.

No major changes were observed in CAT activities of leaves incubated with ≤ 2 mM SA (Fig. 5A). However, treatment of leaves with 5 mM SA decreased CAT by 33% compared with control leaves. SA-mediated inactivation of CAT appeared to require more than 4 h of incubation (Fig. 5B). CAT is believed to be inactivated by its substrate, H_2O_2 (Durner and Klessig, 1996). Because high concentrations of SA inactivated CAT, we investigated whether H_2O_2 alone would do the same. Treatment of leaves with H_2O_2 enhanced CAT activities by 36% compared with control leaves (Table I).

Concentrations of 0 to 5 mM SA or 10.0 mM H_2O_2 had no major effect on POX (data not shown). However, POX is known to utilize different substrates to metabolize H_2O_2; CA-POX utilizes coniferyl alcohol and H_2O_2 as substrates to initiate lignification chain reactions (Rao et al., 1996). CA-POX was enhanced by 26 and 66%, respectively, in leaves treated with 1 and 5 mM SA for 8 h compared with control leaves (Fig. 6). Similarly, treatment of leaves with 10.0 mM H_2O_2 enhanced CA-POX activities by 60% compared with control leaves (Table I).

Guaiacol serves as a common substrate in estimating the total peroxidase activities; however, plants possess peroxidases with specific affinity with substrates such as ascorbate (Creissen et al., 1994). APX activity remained unaltered in leaves incubated with ≤ 2 mM SA (Fig. 7A). However, leaves treated with 5 mM SA for 8 h had a lower APX activity (33%) compared with control leaves. Determination of the time-dependent effect of 5 mM SA on APX activity revealed the inhibitory effect of SA only after 4 h of treatment (Fig. 7B). To delineate the SA-induced effect from that of H_2O_2 levels enhanced by SA, we investigated the influence of H_2O_2 on APX activity. Incubation of leaves with 10.0 mM H_2O_2 for 8 h enhanced APX activity by 32% compared with control leaves (Table I).

GR regenerates GSH in an NADPH-dependent reaction and plays an important role in maintaining the reduced components of ascorbate and glutathione (Creissen et al., 1994). Because APX and GR are interrelated, changes in APX activity might influence GR activity. However, no major changes were observed in the GR activity of leaves incubated with ≤ 5 mM SA or 10.0 mM H_2O_2 (data not shown).

Influence of SA and H_2O_2 on the Isoform Composition of H_2O_2-Degrading Enzymes

In plant cells CAT, POX, APX, and GR exist as multiple isoforms, and the spectrophotometric analyses indicate only the combined activity of different isoforms. Therefore, we analyzed the isoform composition of different enzymes by native PAGE, and gels stained for CAT revealed three isoforms. Analysis of these isoforms indicated that SA
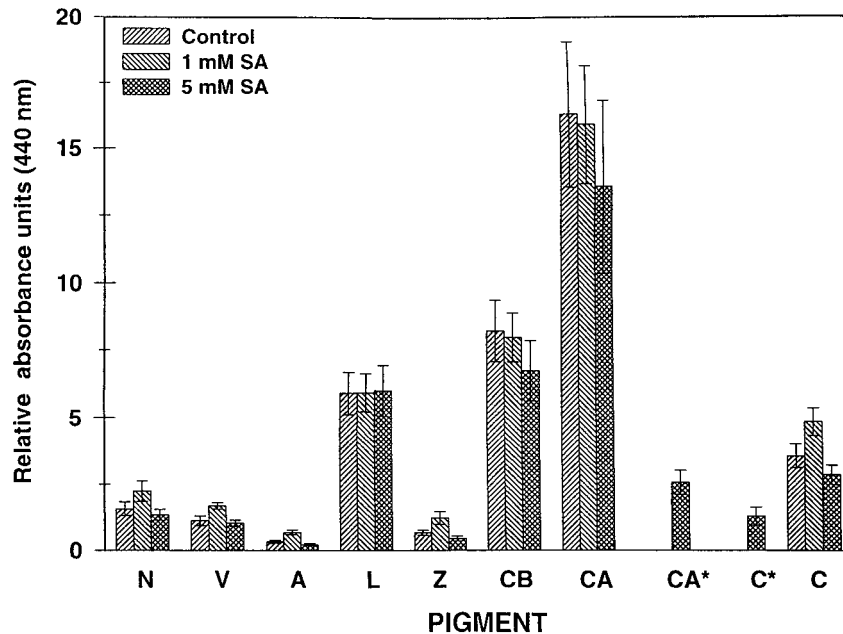
Figure 3. Effects of SA on the composition of carotenoids and Chl pigments (relative units) of *A. thaliana*. Cut ends of rosette leaves were incubated with 1 and 5 mM SA for 8 h, and pigments were analyzed with HPLC as described in "Materials and Methods." Histograms denoted with an asterisk indicate pigments co-eluted with Chl *a* and β-carotene in leaves treated with SA. Mean values shown are averages of two experiments. Error bars indicate SEs; $n = 4$. N, Neoxanthin; V, violaxanthin; A, antheraxanthin; L, lutein; Z, zeaxanthin; CB, Chl *b*; CA, Chl *a*; CA*, Chl *a*-isomer; C*, β-carotene-isomer; and C, β-carotene.

treatment (5 mM) preferentially inhibited the activity of the CAT-3 isoform, whereas no major changes were observed in the activity of CAT-1 and CAT-2 isoforms (Fig. 8A). In contrast, H_2O_2 treatments enhanced the activity of all CAT isoforms compared with control (Fig. 8A; corresponding data shown in Table I). Two isoforms were observed when native gels were stained for POX activity, and no major changes were observed in the activity of either isoform in leaves treated with SA or H_2O_2 (Fig. 8B).

Native gels stained for APX activity revealed two isoforms. SA treatments decreased the activity of both isoforms (Fig. 8C), whereas H_2O_2 enhanced the activities of all APX isoforms (Fig. 8C; corresponding data shown in Table I). Three GSSH-specific GR isoforms were observed when gels were stained for GR activity (Fig. 8D), and no major changes were observed by either SA or H_2O_2 treatments.

SA Inhibits CAT and APX in Vitro

We observed inactivation of CAT/APX only in leaves incubated with high concentrations of SA. Therefore, we investigated the influence of varying concentrations of SA on the in vitro activities of several H_2O_2-degrading enzymes. Activities of CAT, POX, and APX were measured in protein extracts incubated with 0 to 5 mM SA for up to 60 min. SA of 1 mM had no major influence on CAT and APX activities. However, increasing concentrations of SA to 5 mM decreased the activities of both CAT and APX by 52 and 41%, respectively (Fig. 9). No major changes were observed in POX (data not shown).

SA Requires H_2O_2 to Initiate Lipid Peroxidation

Although SA treatments enhanced in vivo H_2O_2, it was not clear whether the observed oxidative damage to cellular organelles was specific to SA or SA-enhanced H_2O_2. Although we have shown that leaves treated with SA exhibit greater damage to lipids and proteins compared with leaves treated with H_2O_2 alone, it is not clear whether SA in the absence of H_2O_2 would cause oxidative damage to cellular organelles. To separate SA-mediated lipid peroxidation from that of SA-enhanced H_2O_2, we pretreated leaves with DMTU, a trap for H_2O_2 (Levine et al., 1994). If SA-induced lipid peroxidation were specific to SA, then leaves treated with DMTU should also exhibit similar damage to lipids compared with leaves treated with SA alone. However, the data presented in Table II suggest that the magnitude of lipid peroxidation was significantly reduced in leaves treated with DMTU and SA compared with leaves treated with SA alone.

Long-Term Influence of SA

The above studies indicated that SA enhances H_2O_2 production and oxidative damage independently of its effect on H_2O_2-degrading enzymes, and only high concentrations of SA (5 mM) that are physiologically irrelevant inactivate H_2O_2-degrading enzymes. To investigate whether the continuous availability of physiologically relevant concentrations of SA (similar to in vivo conditions) would inactivate H_2O_2-degrading enzymes, we incubated leaves with 0.5 mM SA for 48 h and periodically assessed the changes in
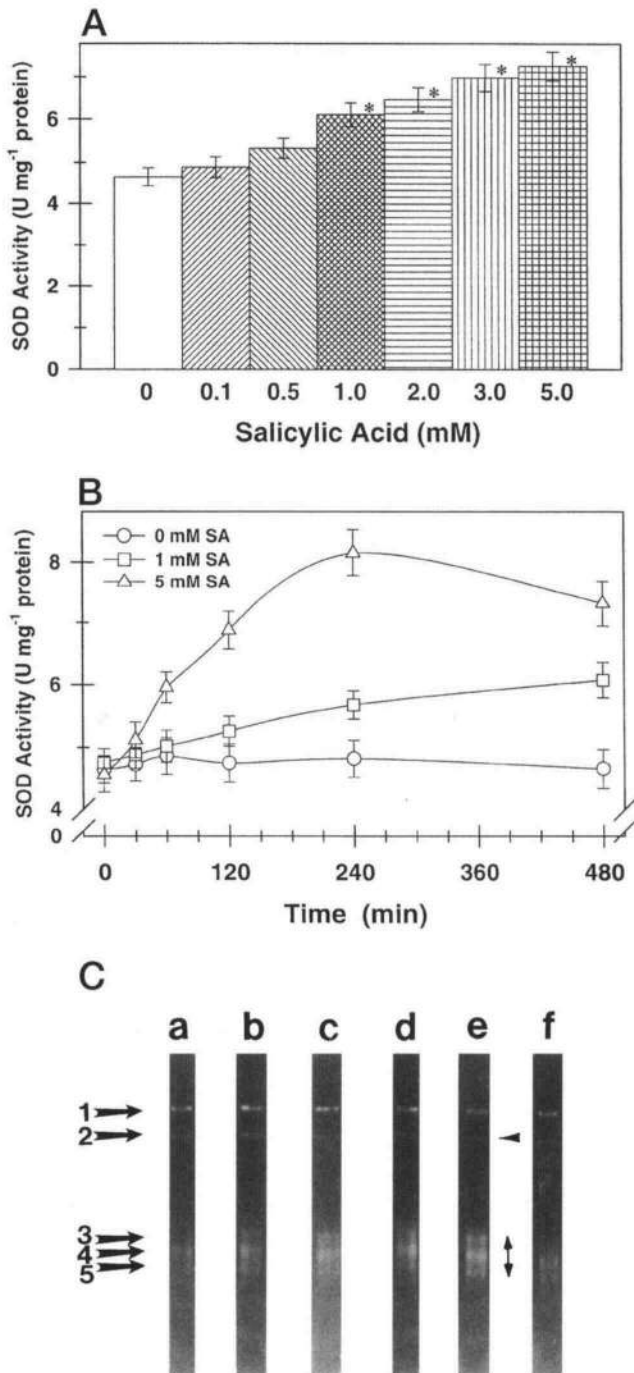
Figure 4. Dose- (A) and time-dependent (B) effect of SA on the in vivo activity and on the isozyme profiles (C) of SOD of *A. thaliana*. Cut ends of rosette leaves were incubated with varying concentrations of SA for 8 h, and SOD activities were measured with equal amounts of protein (100 µg) as described in "Materials and Methods." Mean values shown are averages of three different experiments. Error bars represent SEs; n = 6. Asterisks indicate that mean values are significantly different compared with control (P < 0.05). Equal amounts of protein (100 µg) were subjected to native PAGE as described in "Materials and Methods." Gels stained for SOD activity revealed five isoforms (long arrows). Incubation of gels with KCN or H₂O₂ prior to staining for SOD activity suggested that SOD-1 is Mn-SOD, whereas SOD-2, -3, -4, and -5 are Cu,Zn-SOD (data not shown; Rao et al., 1996). Lane a, Control; lane b, 0.25 mM SA,
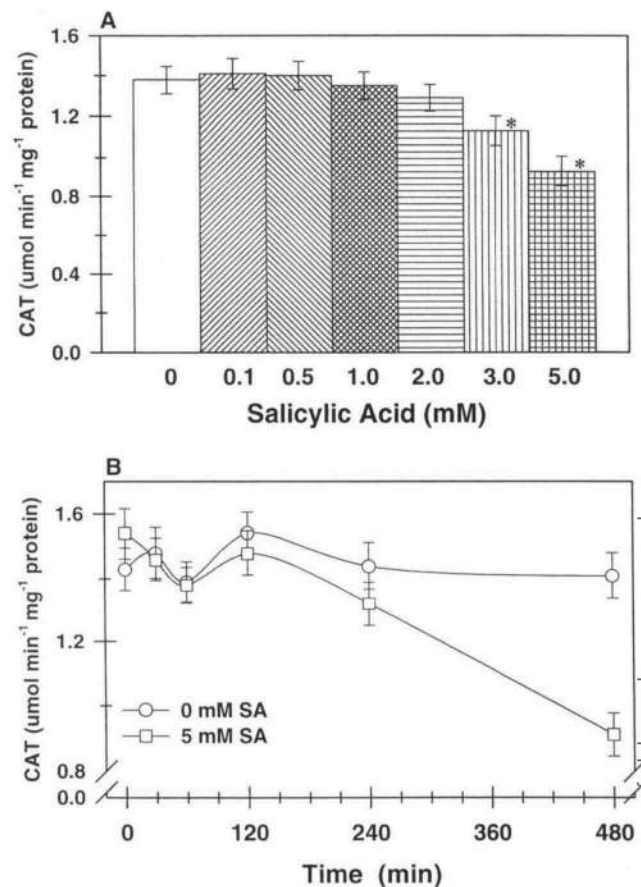
Figure 5. Dose- (A) and time-dependent (B) effect of SA on the in vivo activity of CAT of *A. thaliana*. Cut ends of rosette leaves were incubated with varying concentrations of SA for 8 h, and CAT activities were measured with equal amounts of protein (100 µg) as described in "Materials and Methods." Mean values shown are averages of three different experiments. Error bars represent SEs; n = 6. Asterisks indicate that mean values are significantly different compared with control (P < 0.05).

H₂O₂ levels, oxidative damage, and H₂O₂-metabolizing enzymes.

Treatment of leaves with 0.5 mM SA significantly enhanced H₂O₂, TBARS, and carbonyl content and SOD activities in a time-dependent manner (Table III). No major changes were observed in CAT and APX activities in leaves incubated with 0.5 mM SA for 24 h. However, prolonging the treatments reduced CAT and APX activities by 32 and 30%, respectively, compared with control leaves (Table III). Furthermore, prolonging treatments for longer than 60 h resulted in phytotoxic symptoms (data not shown).

lane c, 0.5 mM SA; lane d, 1 mM SA; lane e, 5 mM SA; and lane f, 10.0 mM H₂O₂. Note decreased intensity of SOD-2 isoform (arrowhead), and the intensities of SOD-3, -4, and -5 were enhanced by SA (double-sided arrow). Electrophoretic analysis was performed separately, with three of the samples used to measure enzyme activities from each experiment; the photograph represents the general trend.
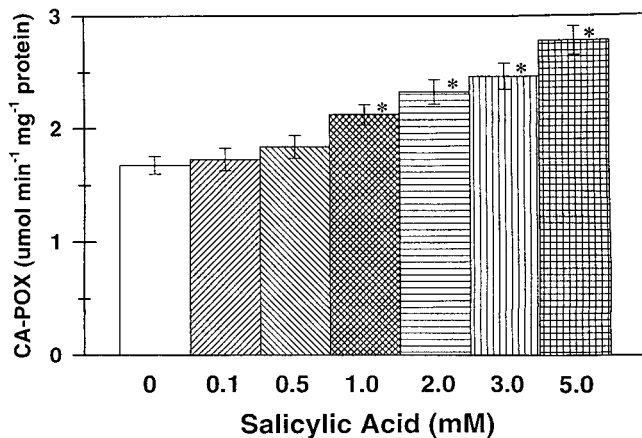
Figure 6. Influence of SA on CA-POX activities of *A. thaliana*. Cut ends of rosette leaves were incubated with varying concentrations of SA for 8 h, and CA-POX activities were measured with equal amounts of protein (100 μg) as described in "Materials and Methods." Mean values shown are averages of two different experiments. Error bars represent SEs; $n = 4$. Asterisks indicate that mean values are significantly different compared with control (P < 0.05).

DISCUSSION

We did not observe phytotoxic symptoms in leaves incubated with 5 mM SA for 8 h or in leaves treated with 0.5 mM SA for 48 h, but longer exposures resulted in visible phytotoxic symptoms (data not shown). A strong relation between SA and H_2O_2 levels and lipid peroxidation and carbonyl groups suggests that SA is capable of generating H_2O_2 in vivo (Fig. 1; Table III) and inflicting oxidative damage to membranes and proteins (Fig. 2; Table III). Furthermore, leaves treated with high concentrations of SA (5 mM) accumulated Chl and carotene isomers (Fig. 3), similar to leaves treated with other compounds capable of generating O_2^- and $^1O_2$ (Young and Britton, 1991).

Increased accumulation of AOS in elicited cell cultures has been shown to enhance membrane damage and cell death (Naton et al., 1996; Rusterucci et al., 1996). Plants challenged with fungal species and/or elicitors have been shown to generate O_2^- by activating plasma membrane-localized NADPH-oxidase (Doke and Ohashi, 1988; Radhika et al., 1996) and/or by activating plasma membrane-localized superoxide synthase (Auh and Murphy, 1995). Our observations that leaves treated with SA have accumulated Chl and carotene isomers (Fig. 3) and enhanced SOD activities (Fig. 4) suggest increased production of O_2^- in SA-treated leaves.

A detailed comparison of the dose- and time-dependent influence of SA on H_2O_2 production (Fig. 1), SOD activities (Fig. 4), and H_2O_2-degrading enzymes such as CAT (Fig. 5) and APX (Fig. 7) suggests that leaves treated with SA exhibit enhanced SOD activities and accumulate H_2O_2 well before the detection of the SA-mediated inactivation of H_2O_2-degrading enzymes. The existing relation between SA and H_2O_2 production clearly suggests that leaves treated with SA may have enhanced H_2O_2 largely by activating enzymes capable of generating H_2O_2. These results are in contrast to the notion that SA enhances H_2O_2 by

inactivating enzymes that are capable of degrading H_2O_2 (Chen et al., 1993a, 1993b; Conrath et al., 1995).

Although in the present study we attempted to relate changes in SOD activity with increased levels of H_2O_2, it is possible that other enzymes that produce H_2O_2 may also have been activated by SA treatments. Plants challenged with pathogens produce H_2O_2 from oxalic acid by inducing a germin-like oxalate oxidase protein (Zhang et al., 1995) and/or by activating cell wall peroxidases that produce H_2O_2 (Bolwell et al., 1995).

Because SA treatment enhanced H_2O_2 levels, it is not clear whether the observed changes in lipid peroxidation and oxidative damage to proteins were due to SA or were related to SA-enhanced H_2O_2. If SA-enhanced oxidative stress is related to SA-enhanced H_2O_2, then the treatment of leaves with H_2O_2 alone should also induce similar damage to cellular organelles. Although leaves treated with 10.0 mM H_2O_2 accumulated in vivo H_2O_2 by 2-fold compared with leaves treated with 5 mM SA (Fig. 1; Table I), the
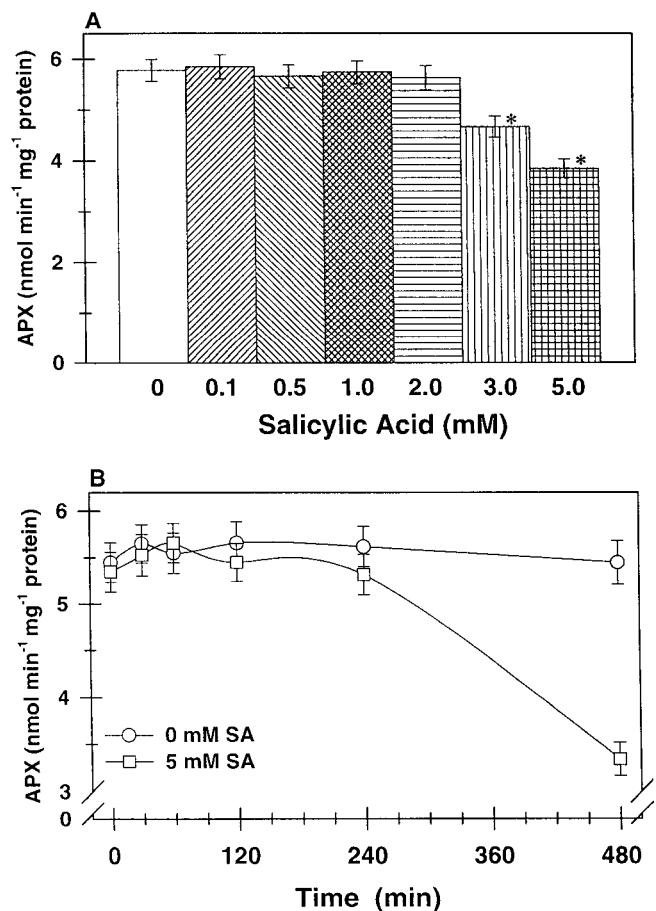

Figure 7. Dose- (A) and time-dependent (B) effect of SA on the in vivo activity of APX of *A. thaliana*. Cut ends of rosette leaves were incubated with varying concentrations of SA for 8 h, and APX activities were measured with equal amounts of protein (100 μg) as described in "Materials and Methods." Mean values shown are averages of two different experiments. Error bars represent SEs; $n = 6$. Asterisks indicate that mean values are significantly different compared with control (P < 0.05).

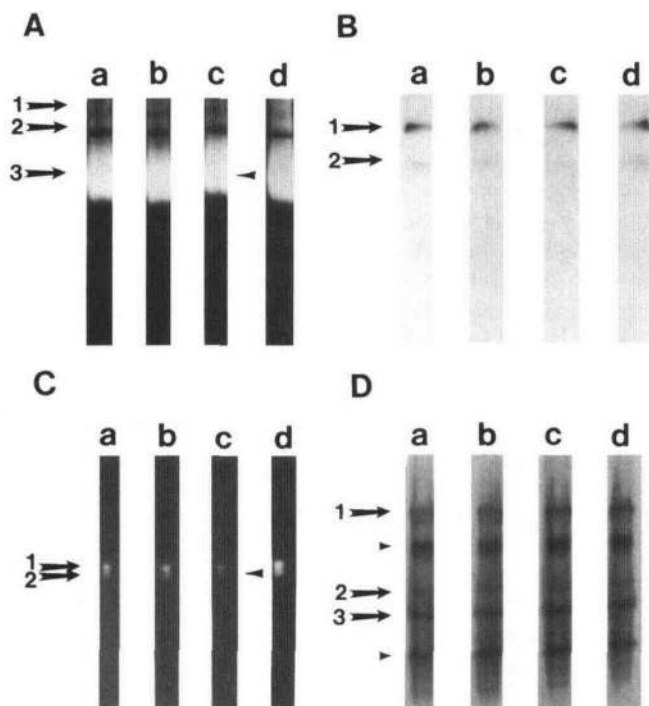**Figure 8.** Influence of SA and H$_2$O$_2$ on the isozyme profiles of CAT (A), POX (B), APX (C), and GR (D) of *A. thaliana*. Cut ends of rosette leaves were incubated with varying concentrations of SA for 8 h, and equal amounts of protein (100 μg for CAT, POX, and GR; 200 μg for APX) were subjected to native PAGE, and gels were stained for CAT, POX, APX, and GR, as described in "Materials and Methods." Lane a, Control; lane b, 1 mм SA; lane c, 5 mм SA; and lane d, 10.0 mм H$_2$O$_2$. A, Gels stained for CAT activity revealed three isoforms (large arrows). Note the preferential inhibition of the CAT-3 isoform (lane c; arrowhead) and enhanced activities of all isoforms in leaves treated with 10.0 mм H$_2$O$_2$ for 8 h (lane d). The CAT-3 isoform was revealed as one isoform when protein concentrations were reduced and stained for CAT activity. B, Gels stained for POX activity revealed two isoforms (large arrows). No major changes were observed in the activity of either isoform of leaves treated with SA or H$_2$O$_2$. C, Gels stained for APX activity revealed two isoforms (large arrows). Note the inhibition of the activities of APX isoforms by SA (lane c; arrowhead) and enhanced activity of all APX isoforms by H$_2$O$_2$ (lane d). D, Gels stained for GR activity revealed three GSSH-specific isoforms (large arrows) and two nonspecific GR isoforms (arrowheads). No major changes were observed in the activities of GR isoforms of leaves treated with SA or H$_2$O$_2$. Electrophoretic analysis was performed separately with three of the samples used to measure enzyme activities from each experiment. Similar trends were observed in all experiments and the photograph represents the general trend.

damage to lipids and proteins was significantly less in leaves treated with H$_2$O$_2$ (Fig. 2; Table I), suggesting that SA-induced oxidative damage is probably not due to enhanced H$_2$O$_2$ levels. However, when DMTU was used as a trap for H$_2$O$_2$, the magnitude of SA-mediated lipid peroxidation was significantly reduced (Table II), suggesting that SA requires H$_2$O$_2$ to initiate oxidative damage.

Plant CATs are heme-Fe-containing enzymes and are peculiar in that they are highly sensitive to their substrate, H$_2$O$_2$. However, we observed enhanced activities of CAT and APX in leaves treated with H$_2$O$_2$ (Table I). In contrast,

treatment of leaves with SA inhibited both CAT and APX, indicating that the inactivation of H$_2$O$_2$-degrading enzymes may be specific to SA. In the presence of H$_2$O$_2$, SA or other phenolic compounds serve as one electron donor for CAT (Durner and Klessig, 1996) or peroxidases (Durner and Klessig, 1995) to convert oxidized intermediate (compound I) into an inactive and partially reduced state (compound II). As SA donates one electron to compound I of CAT or peroxidases, SA is converted into an oxidized form, SA*.

Phenolic free radicals are capable of initiating lipid peroxidation and modifying other macromolecules (Savenkova et al., 1994; Durner and Klessig, 1996). The observation that SA-treated leaves exhibited greater damage to lipids and proteins (Fig. 2; Tables I and III) also suggests that SA* generated because of the interaction of SA and CAT/APX may have enhanced lipid peroxidation and oxidative damage to proteins. Furthermore, the reduction in the magnitude of SA-mediated lipid peroxidation in leaves pretreated with DMTU (Table II) suggests that H$_2$O$_2$ may play a role in SA-mediated lipid peroxidation.
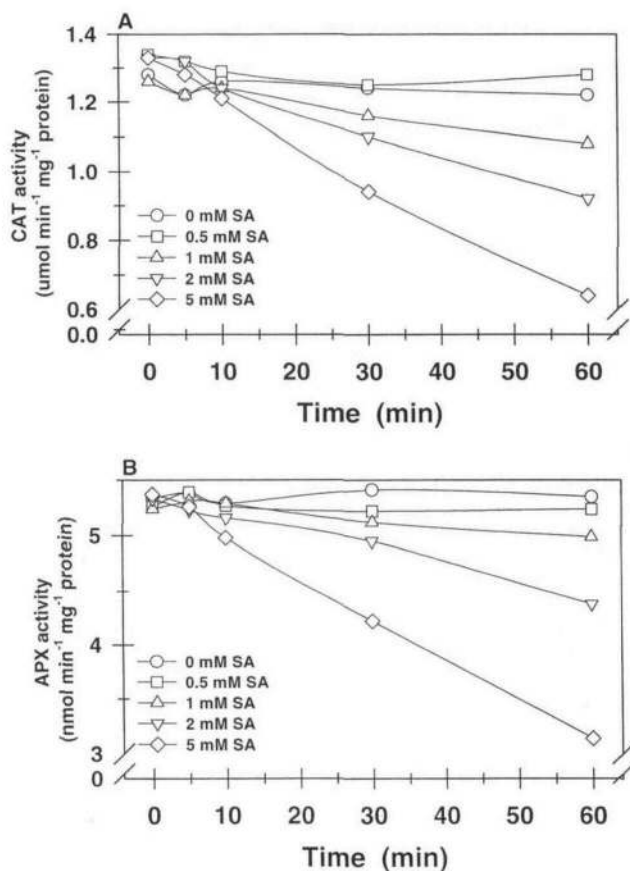

Figure 9. Influence of SA on the in vitro activities of CAT (A) and APX (B). Soluble proteins were treated with varying concentrations of SA (0.1, 0.5, 1, 2, and 5 mм) and the activities of CAT and APX were monitored periodically as described in "Materials and Methods." The experiments were repeated with the same protein extract and the mean values presented are averages of two experiments with $n = 4$. All deviations were always <7% of the mean value.

Table II. *Influence of 5 mM DMTU and/or SA on lipid peroxidation (as indicated by TBARS content) in A. thaliana*

Mean values presented are averages of two different experiments ± SE (n = 4). Values in parentheses indicate changes relative to control.

Treatment	TBARS
	$\mu mol\ g^{-1}\ fresh\ wt$
Control	19.9 ± 2.2 (100)
DMTU	16.9[a] ± 3.1 (86)
SA	41.2[b] ± 3.8 (207)
DMTU + SA	25.8[b] ± 2.8 (145)

[a] Not significant.   [b] Mean values are significantly different compared with control values ($P < 0.05$).

SA is believed to inhibit CAT by the chelation of heme Fe and by causing conformational changes (Ruffer et al., 1995). However, Durner and Klessig (1996) have presented evidence suggesting that SA-mediated inhibition of CAT and APX probably results from peroxidative reactions. In our study SA-mediated inhibition of CAT and APX activities was observed only in leaves exhibiting greater oxidative damage to cellular components, indicating that SA-mediated inactivation of CAT and APX may be due to peroxidative reactions. However, in spite of the inhibitory effect of SA on H_2O_2-degrading enzymes, SA is not believed to influence peroxidases involved in lignin biosynthesis (Hammond-Kosack and Jones, 1996).

Although neither SA nor H_2O_2 treatments influenced POX, CA-POX was enhanced by both treatments (Fig. 6; Table I). The presence of additional H_2O_2 may have activated CA-POX and initiated the lignification chain reaction. Enhanced synthesis of lignin is believed to be a defense response that restricts pathogen growth (Hammond-Kosack and Jones, 1996).

We observed inhibition of CAT and APX in leaves treated with high concentrations of SA (5 mM) for 8 h, as well as in leaves treated with physiological concentrations (0.5 mM) for 48 h. Under both situations the observed reduction in CAT and APX ranged between 25 and 35% of total activity (Figs. 5 and 7; Table III). Although the reduction in H_2O_2-degrading enzymes was small compared with in vitro studies (Fig. 9), even modest effects on the in vivo activity of these two major H_2O_2-degrading enzymes could cause further inactivation by the slow and time-dependent accumulation of H_2O_2. Supporting this notion was the observation that prolonging the incubation period in both

experiments resulted in phytotoxic symptoms, and under such conditions both CAT and APX activities were decreased by 89 and 79%, respectively, compared with control leaves (data not shown). This suggests that SA-mediated inactivation of H_2O_2-degrading enzymes may further enhance H_2O_2 levels, ultimately leading to hypersensitive cell death.

Although SA inhibited all APX isoforms, it appeared to have preferential influence on the major isoform (CAT-3) of *A. thaliana* (Fig. 8). However, the reason SA specifically inactivated the CAT-3 isoform is not known and merits further study. Similar SA-insensitive isoforms of CAT have been observed in rice (Sanchez-Casas and Klessig, 1994) and maize (Guan and Scandalios, 1995).

Typical SA concentrations in infected leaves are estimated to be approximately 100 μM (Durner and Klessig, 1996; Ryals et al., 1996), and in distant leaves SA levels are expected to be 100- to 200-fold lower than in infected tissues (Ryals et al., 1996). Furthermore, SA binds to CAT and APX with a K_D of approximately 14 μM (Chen et al., 1993a) and 78 μM (Durner and Klessig, 1995), respectively. SA-mediated induction of PR genes and SAR is usually observed after 24 to 48 h of treatment (Chen et al., 1993b; Bi et al., 1995; Shah et al., 1997), indicating the possible time period for the SA-derived signal to be generated and perceived and to instigate a biochemical change.

We arbitrarily selected a 48-h incubation on the basis of existing literature (Chen et al., 1993b; Bi et al., 1995; Neuenschwander et al., 1995) and assumed that SA treatment would invoke PR genes. SA-induced lipid peroxidation was significantly higher in leaves treated with 0.5 mM SA for 24 h (Table III), and a significant induction in PR genes was observed in *A. thaliana* within 24 h of SA application (Shah et al., 1997). Naturally occurring lipid peroxides are believed to induce PR genes (unpublished results cited by Durner and Klessig, 1996). However, the influence of SA-mediated lipid peroxides in inducing PR genes remains to be elucidated.

Enhanced production of AOS is often recognized as the first response activated in many incompatible interactions (Mehdy, 1994; Hammond-Kossack and Jones, 1996). The enhanced production of H_2O_2 is believed to invoke several defense responses that ultimately develop resistance to pathogens (Mehdy, 1994). H_2O_2 has been shown to enhance the biosynthesis of SA (Leon et al., 1995; Summermatter et al., 1995), to initiate cross-linking of cell walls (Tenhaken et al., 1995), to coordinate hypersensitive cell

Table III. *Long-term (48 h) influence of low concentrations of SA (0.5 mM) on H_2O_2 production, lipid peroxidation (TBARS content), carbonyl content (C=O groups), and SOD, CAT, and APX activities of A. thaliana*

Mean values presented are averages of two different experiments ± SE (n = 6). Values in parentheses indicate changes relative to control.

Treatment Duration	H_2O_2	TBARS	C=O Groups	SOD	CAT	APX
h	$nmol\ g^{-1}\ fresh\ wt$	$\mu mol\ g^{-1}\ fresh\ wt$	$nmol\ mg^{-1}\ protein$	$units\ mg^{-1}\ protein$	$\mu mol\ min^{-1}\ mg^{-1}\ protein$	$nmol\ min^{-1}\ mg^{-1}\ protein$
0	116 ± 9 (100)	19.68 ± 1.16 (100)	11.68 ± 0.86 (100)	5.12 ± 0.33 (100)	1.30 ± 0.1 (100)	5.62 ± 0.24 (100)
24	195[a] ± 14 (168)	32.86[a] ± 1.45 (167)	17.58[a] ± 1.12 (150)	6.98[a] ± 0.28 (136)	1.12[b] ± 0.12 (86)	5.14[b] ± 0.28 (92)
48	235[a] ± 15 (202)	38.34[a] ± 1.58 (194)	19.89[a] ± 1.36 (170)	7.16[a] ± 0.27 (140)	0.88[a] ± 0.11 (68)	3.94[a] ± 0.18 (70)

[a] Mean values significantly different compared with control values ($P < 0.05$).   [b] Not significant.

death (Levine et al., 1994), and to trigger various defense-related genes (Levine et al., 1994; Mehdy, 1994; Hammond-Kosack and Jones, 1996).

Recently, Wu et al. (1995) provided evidence for enhanced resistance to pathogens in transgenic plants over-expressing an H$_2$O$_2$-generating Glc-oxidase enzyme. However, H$_2$O$_2$ has been shown to remain unaltered in leaves during the onset of SAR (Neuenschwander et al., 1995). In the present study we have shown that SA enhances H$_2$O$_2$ but that SA-mediated events are specific and are not invoked by H$_2$O$_2$ alone. What, then, is the relative significance of enhanced production of H$_2$O$_2$ in SA-treated tissues?

We propose a hypothetical model to illustrate the relative functions of SA and H$_2$O$_2$ and their interdependent involvement in establishing SAR (Fig. 10). In the present study, plants responded to SA in a way similar to the way they respond to pathogen infection, by increasing the production of AOS such as O$_2^-$ that are dismutated to H$_2$O$_2$ by the activation of SOD. Increased production of H$_2$O$_2$ initiates cell wall cross-linking (Tenhaken et al., 1995) and enhances the enzymes involved in the biosynthesis of lignin (present study) and SA (Leon et al., 1995; Summermatter et al., 1995). Increased SA levels generate more H$_2$O$_2$ and, thus, an interaction between H$_2$O$_2$ and SA would result in very high free radical concentrations.

High concentrations of free radicals alter the cellular redox state, resulting in the activation of various defense-related genes (Levine et al., 1994; Mehdy, 1994; Hammond-Kosack and Jones, 1996) and favors the interaction between SA and CAT/APX (Fig. 10; denoted with a dotted arrow).

SA interacts with CAT/APX by donating one electron and converting itself into an oxidized form, SA* (Durner and Klessig, 1996). SA* is believed to initiate lipid peroxidation, and lipid peroxide(s) may invoke the expression of PR genes (unpublished results cited by Durner and Klessig, 1996) and establish SAR. SA-mediated inactivation of H$_2$O$_2$-degrading enzymes further enhance the cellular free radical concentrations (Fig. 10) that invoke hypersensitive cell death.

Consistent with this scheme, we observed enhanced lipid peroxidation, damage to proteins (Fig. 1 and 2; Table III), reduced CAT/APX activities (Figs. 5 and 7; Table III), and enhanced activities of peroxidases involved in lignin biosynthesis (Fig. 6) in leaves treated with SA. Recent studies suggest that SA potentiates the expression of several defense-related genes in leaf tissues exhibiting SAR (Mur et al., 1996). Treatment of plant cells with either methyl jasmonate (Kauss et al., 1994) or SA (Kauss and Jeblick, 1995) increased H$_2$O$_2$ production on secondary elicitation. Furthermore, a combination of SA and methyl jasmonate has been shown to hyperinduce PR1 genes, whereas methyl jasmonate alone had no affect (Xu et al., 1994).

From several studies it is known that plant cells that are wounded, challenged with pathogens, or treated with SA or methyl jasmonate have enhanced H$_2$O$_2$ production and that PR1 genes are responsive to exogenous H$_2$O$_2$ (Bi et al., 1995). More recently, Shirasu et al., (1997) presented evidence for the more potent role of SA in the presence of an appropriate agonist than when tested on naive cells in the absence of pathogen infection. Thus, it is suggested that the presence of SA in the tissue is not sufficient for potentia-
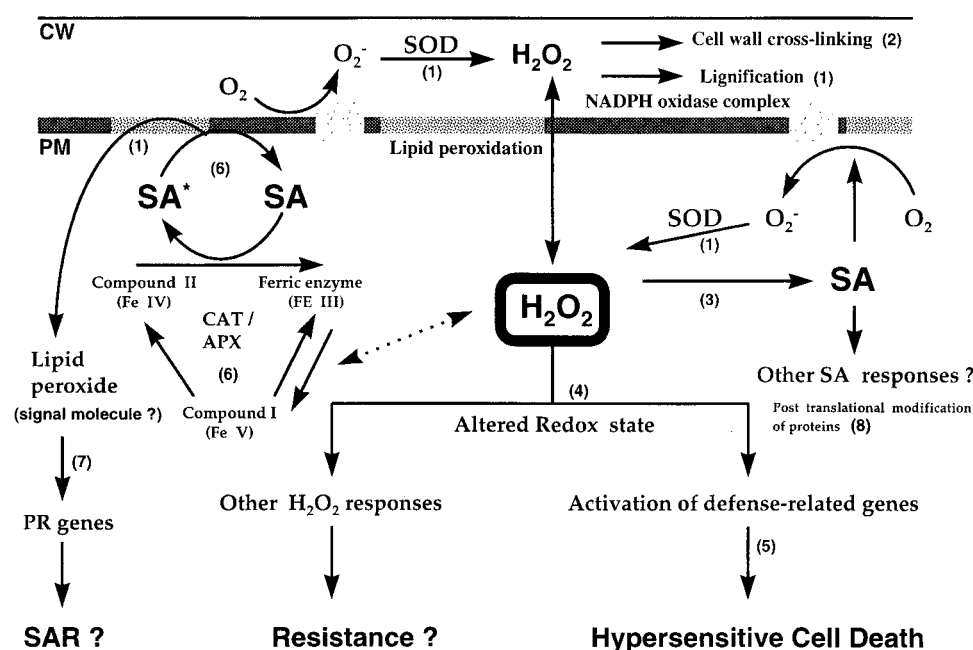
Figure 10. Schematic diagram illustrating the interdependent and independent functions of SA and H$_2$O$_2$ in establishing resistance to pathogens. CW, Cell wall; PM, plasma membrane. Numbers in parentheses indicate the following references: 1, Present study; 2, Tenhaken et al. (1995); 3, Leon et al. (1995); Summermatter et al. (1995); 4, Hammond-Kosack and Jones (1996); 5, Levine et al. (1994); Mehdy (1994); 6, Durner and Klessig (1995 and 1996); 7, unpublished results cited by Durner and Klessig (1996); 8, M.V. Rao, G. Paliyath, D.P. Ormrod, and D.P. Murr, unpublished results.

tion; the induction of competence factors is also required (Fauth et al., 1996; Kauss and Jeblick, 1996; Mur et al., 1996; Shirasu et al., 1997).

The ability of SA to convert to SA* is dependent on H_2O_2 fluxes and altered cellular redox state (Durner and Klessig, 1996). Therefore, it is possible that SA enhances H_2O_2 production by influencing H_2O_2-generating enzymes (Fig. 4) to alter the cellular redox state and to provide an atmosphere that favors the generation of SA* to initiate lipid peroxidation (Table II), hypersensitive cell death, and the activation of several other defense mechanisms. This may explain why H_2O_2 requires SA to induce PR genes (Neuenschwander et al., 1995). From our results it appears that SA requires H_2O_2 to potentiate lipid peroxidation, induce PR genes, and establish SAR.

Because SA-induced events are not invoked by even high concentrations of H_2O_2, it is therefore likely that H_2O_2 acts upstream of SA in a signal transduction mechanism leading to SAR. Thus, in the context of plant-pathogen interactions, it appears that the enhanced production of AOS such as H_2O_2 plays a central role in invoking several defense mechanisms that ultimately develop SAR. One such mechanism is enhanced biosynthesis of SA. Supporting this assumption, O_3, which is believed to act by generating AOS, enhances the biosynthesis of SA and induced SAR in *A. thaliana* (Sharma et al., 1996).

Because SA has been shown to be essential for the establishment of SAR but is not the translocated signal (Vernooij et al., 1994), it may be possible that a specific, SA-generated lipid peroxide is the translocated signal that invokes PR genes. We have other evidence to suggest that SA modulates the phosphorylation of several proteins and that such action is independent of H_2O_2 requirement (M.V. Rao, G. Paliyath, D.P. Ormrod, and D.P. Murr, unpublished results). Identification of such an SA-generated signal molecule or SA-specific event will help dissect the molecular-genetic basis of SA-mediated SAR.

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