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POSSIBLE INVOLVEMENT OF LIPID PEROXIDATION IN SALICYLIC ACID-MEDIATED INDUCTION OF *PR*-1 GENE EXPRESSION

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Key Word Index—disease resistance; lipid peroxidation; pathogenesis-related proteins; salicylic acid; tobacco mosaic virus.

Abstract—Salicylic acid (SA) is a natural signal molecule which plays an important role in plant defense responses against pathogen infection. Previous work has demonstrated that catalase may be involved in the mechanism of SA action. Recently, SA has been shown to inhibit catalase by serving as a one electron-donating substrate for its peroxidative reaction. One of the likely by-products of this interaction is a SA free radical. Since free radicals are potent inducers of lipid peroxidation, SA and its analogues were tested for their ability to induce lipid peroxidation in tobacco cell cultures. SA and its biologically active analogues, which increase *PR* gene expression, enhance disease resistance and also inhibit catalase, induced accumulation of lipid peroxidation products. Inactive analogues did not. Exogenous application of lipid peroxides induced *PR*-1 gene expression. Furthermore, the induction of *PR*-1 genes by SA was inhibited by diethyldithiocarbamic acid, a compound that converts lipid peroxides into their hydroxyl derivatives. We suggest that the induction of lipid peroxidation by a SA radical may be involved in the action of SA and that one or more of the lipid peroxidation products may mediate signal transduction leading to *PR*-1 expression.

INTRODUCTION

The mechanisms by which plants detect pathogen infection and initiate a defense response are incompletely understood but are the subject of intensive investigation. During an incompatible plant–pathogen interaction, many plants activate a localized hypersensitive response (HR) in which necrotic lesions form and the multiplication and spread of the pathogen is restricted [1]. Additionally, resistance mechanisms are activated in uninfected tissues (systemic acquired resistance, SAR) which render the plant more resistant to secondary infection by the same or unrelated pathogens [2–4]. Both HR and SAR are associated with the increased expression of a wide array of defense genes, among which are those encoding various classes of pathogenesis-related (PR) proteins [5, 6]. The functions of some of these proteins during the resistance response are known, such as the antimicrobial activity of the β-1,3-glucanases (PR-2) and chitinases (PR-3). In contrast, the functions of other PR proteins are not well understood, although they are thought to be integral components of disease resistance. Due to the strict correlation between *PR* gene induction and the development of disease resistance, the *PR* genes are commonly-used molecular markers for the defense response.

In the signal transduction pathway(s) leading to expression of *PR* genes and disease resistance, there is increasing evidence that salicylic acid (SA) plays an important role [2–4, 7, 8]. For example, endogenous SA levels are elevated at least 20-fold in infected tobacco leaves following infection by tobacco mosaic virus (TMV) and 5-fold in uninfected leaves in a manner that parallels accumulation of *PR*-1 mRNA [9]. Similarly, local and systemic elevation of SA has been observed to correlate with the development of disease resistance in a variety of plant-pathogen systems [10–14] and in response to abiotic stress [15, 16], illustrating the widespread involvement of SA in defense responses.

The importance of SA as a component of the resistance response has further been demonstrated using tobacco and *Arabidopsis* plants expressing the bac-

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terial salicylate hydroxylase-encoding gene (*nahG*), which destroys the SA signal [17, 18]. NahG plants challenged with avirulent pathogens develop larger lesions on their infected leaves than wild-type plants and fail to develop SAR in their uninfected leaves. Thus, SA appears to play a role in the development of both the HR and SAR. In addition, several mutants of *Arabidopsis* have been identified that are defective in SA signal transduction (e.g. *npr*, *nim*, *eds*, *sai*) and exhibit enhanced susceptibility to pathogens [19–22]. Conversely, several *Arabidopsis* mutants (e.g. *cpr*, *lsd*, *acd*, *cep*), which constitutively express *PR* genes and show enhanced resistance, also have elevated levels of SA [3, 23, 24; Silva and Klessig, unpublished]. There also is evidence suggesting that SA or its derivative, methyl salicylate, may be one of the translocated signals responsible for systemic induction of defense responses in uninfected leaves [10, 25, 26], although this is the subject of ongoing debate [11, 27, 28].

In an effort to identify the molecular target(s) of SA in the signal transduction pathway(s), we [29] identified a soluble SA-binding protein that was later demonstrated to be catalase [30]. Further analysis demonstrated that SA inhibited the activity of partially purified catalase and caused a $\approx 50\%$ rise in the level of endogenous H_2O_2 in treated tobacco leaves. In addition, exposure to H_2O_2, or compounds that cause elevation of endogenous H_2O_2, caused the induction of *PR*-1 expression in tobacco leaves. These results led us to propose that one likely mode of action for SA is to inhibit catalase, thereby elevating H_2O_2 levels. H_2O_2, or other compounds arising from this increasingly oxidized environment, might then serve as signaling components downstream of SA in the induction of PR proteins, possibly via a reactive oxygen species (ROS) or redox-activated transcription factor analogous to NF-κB in humans [31, 32] or the products of the *oxyR* and *soxR* genes in bacteria [33]. Subsequent work has shown a correlation between the ability of various analogues of SA and 2,6-dichloroisonicotinic acid (INA; a synthetic inducer of *PR* genes) to induce *PR*-1 expression and enhance disease resistance and their capacity to inhibit catalase *in vivo* [34]. In addition, another synthetic inducer of defense responses, benzothiadiazole (BTH), was found to also inhibit catalase [35]. Moreover, it was found that various antioxidants can suppress SA-, INA-, and BTH-induced *PR*-1 expression [34, 35]. Furthermore, SA, its biologically active analogues, INA and BTH, have been shown to inhibit ascorbate peroxidase, the other major H_2O_2-scavenging enzyme in plants [35, 36]. These results lend support to a model in which SA acts as a prooxidant in the induction of *PR* genes.

Recently, Durner and Klessig [37] demonstrated that SA can act as a one-electron donor for the peroxidative cycle of catalase (Fig. 1). Under normal physiological conditions, where the level of H_2O_2 is low to moderate, SA is able to inhibit catalase by siphoning the enzyme intermediate, compound I, from the extremely rapid catalatic cycle into the slow peroxidative cycle (at least 1000 times slower) [38]. This is accomplished by providing a single electron from SA to compound I, thereby converting it to compound II. In contrast, when H_2O_2 is present at extremely high levels, such as may occur during the oxidative burst following attack by an avirulent pathogen, SA protects catalase from inactivation by donating electrons to intermediates of the peroxidative cycle. Through this process, ferric catalase is regenerated and two SA radicals are formed (Fig. 1). Thus, elevation of SA may serve two roles following pathogen attack. At the site of infection, H_2O_2 levels are high and the increased amount of SA might prevent inactivation of catalase. In this scenario, SA may help contain the oxidative burst. In tissues further removed from the infection site, H_2O_2 levels will be lower and SA may inhibit catalase, as has been observed in soybean [39]. Levine *et al.* [40] have suggested that H_2O_2 diffusing away from tissues experiencing an oxidative burst induces cellular defenses in the adjacent tissue. The SA-mediated inhibition of catalase might facilitate this process.

The involvement of SA-mediated catalase inhibition and elevated H_2O_2 levels in the activation of defense responses is currently a subject of considerable debate. Based on evidence from a variety of studies [41–46] H_2O_2 appears to act upstream of SA in the signaling pathway in addition to, or rather than, functioning downstream of SA. While the inhibition of catalase by SA may contribute to the resistance response, perhaps a more significant consequence of

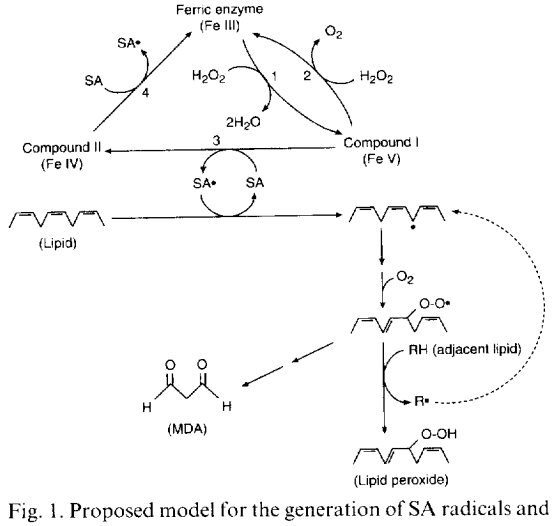

Fig. 1. Proposed model for the generation of SA radicals and the initiation of the lipid peroxidation chain reaction. The catalatic cycle of catalase is represented by reactions 1 and 2. SA can act as an electron donor in either the one-electron reduction of compound I to compound II (reaction 3), or the regeneration of the ferric enzyme from compound II (reaction 4) [37]. The resulting SA radical can withdraw an electron from an unsaturated lipid, initiating the lipid peroxidation chain reaction (diagram reproduced in modified form from Buege and Aust) [99]. Lipid peroxides and MDA are major products of lipid peroxidation.

SA's interaction with catalase is the production of SA radicals, formed as SA donates a single electron to enzyme intermediates of the peroxidative cycle. One of the primary targets of free radical attack (including phenolic free radicals) [47] is membrane lipids, which results in the initiation of lipid peroxidation [48, 49]. Thus, the SA radicals generated during the peroxidative cycle may induce lipid peroxidation and the products of this reaction may then activate defense responses. In this work, we examine the possibilities that SA can cause lipid peroxidation and that the resulting products participate in *PR*-1 gene induction.

RESULTS

Induction of lipid peroxidation

The possibility that SA-induced increases in *PR*-1 gene expression was mediated by lipid peroxidation was initially investigated by monitoring lipid peroxidation in response to the addition of SA to tobacco cell culture. Lipid peroxidation was measured by the accumulation of malondialdehyde (MDA), a major product of lipid peroxidation. Treatment of cells with various concentrations of SA for 1 h caused a dose-dependent increase in lipid peroxidation (Fig. 2). With 500 μM SA there was a 60–70% rise in accumulated lipid peroxidation products; this increased to $\approx$2.5-fold at 1 mM SA.

Lipid peroxidation is most readily observed when cells are subjected to a stress severe enough to cause significant membrane damage. Therefore, we needed to consider the possibility that detectable accumulation of lipid peroxidation products was occurring only at cytotoxic levels of SA. To assess the effect of SA on cell viability, cell death was monitored periodically over a 24 hr period after treatment with various concentrations of SA in the absence of sucrose (Fig. 3). Note that it was necessary to remove sucrose from the media before addition of SA since sucrose led to artificially high levels of MDA during the lipid peroxidation assay (see Experimental). The absence of increased cell death in the SA-free controls indicated that exposure of cells to media devoid of a carbon source for periods of 24 h or less was not lethal. SA concentrations of less than 500 μM had no effect on cell viability. Similarly, no change in viability was observed up through 8 h of exposure to 500 μM SA. In contrast, 1 mM SA caused significant mortality at exposure times of 6 h and longer. Thus, SA was cytotoxic only if applied at a high concentration for extended periods. Examination of lipid peroxidation during longer term exposure (Fig. 4) revealed that 1 mM SA caused a 2- to 3-fold increase in accumulated lipid peroxidation products that was maintained for at least 8 h. On the other hand, treatments with 500 μM (and perhaps 200 μM) SA resulted in lower accumulation of lipid peroxidation products, which peaked at 2–4 h and then declined. Thus, prolonged high levels of lipid peroxidation products were associated with cytotoxicity while exposure of cells to sub-lethal doses of SA resulted in a transient accumulation of lipid peroxidation products.

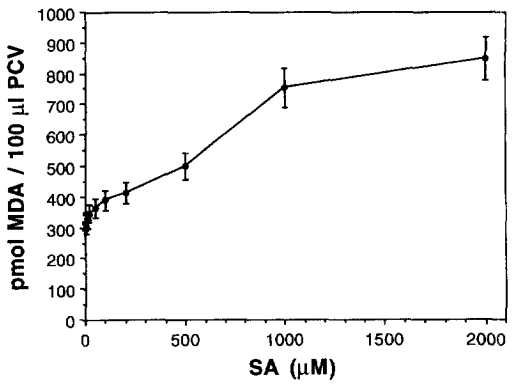

Fig. 2. Lipid peroxidation in tobacco cell culture in response to various SA concentrations. Tobacco cells were exposed to 0, 5, 10, 20, 50, 100, 200, 500, 1000, or 2000 μM SA for 1 h in darkness and the extent of lipid peroxidation was determined by measuring the levels of malondialdehyde (MDA), a major product of lipid peroxidation. MDA was expressed on a packed cell volume (PCV) basis.

Induction of PR-1 *gene expression*

SA is able to induce *PR* gene expression and enhanced resistance in tobacco leaves. As a first step to evaluating the physiological relevance of lipid peroxidation induced by SA, the induction of *PR*-1 genes in tobacco cell culture was examined by monitoring the synthesis and accumulation of PR-1 proteins after 24 h of treatment (Fig. 5A). Concentrations of SA from 50 to 200 μM resulted in high levels of induction while 500 μM and 1 mM SA caused lower levels of PR-1 protein accumulation. The poorer induction of PR-1 protein accumulation at higher SA levels may reflect the cellular stress imposed by the lethal or near-lethal concentrations of SA.

Lipid peroxidation produces a variety of compounds with profound biological activity. In animals, peroxidation of arachidonic acid-containing lipids leads to generation of a group of compounds with prostaglandin-like functions [50]. 4-Hydroxynonenal, a common product of lipid peroxidation, has been shown to influence gene expression and affect important proteins involved in signal transduction (e.g. phospholipase C) [51]. To explore the possible signaling function(s) of lipid peroxidation in the action of SA, we examined whether exogenously added lipid peroxidation products could induce *PR*-1 genes. Two representative lipid peroxides, 13(S)-hydroperoxy-linoleic acid (HPODE) and 13(S)-hydroperoxy-linolenic acid (HPOTrE), effectively activated *PR*-1 genes (Fig. 5C). For both oxidized lipid molecules, activation of *PR*-1 genes occurred at concentrations of 50-200 μM. The failure of the higher concentration of these lipid peroxides (400 μM) to effectively induce

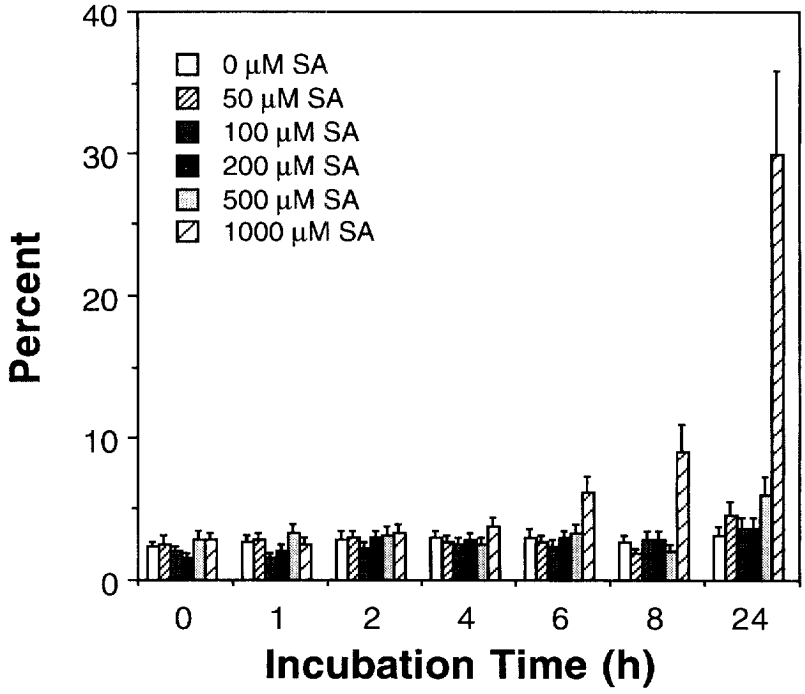

Fig. 3. Mortality of tobacco cells after exposure to various SA concentrations. Tobacco cells were maintained in the dark and exposed to various SA concentrations. Cell death was monitored periodically for 24 h. Cell death was determined by counting live and dead cells after treatment with fluorescein diacetate.

PR-1 protein synthesis and accumulation may again reflect cellular stress as cell damage was visibly evident at this concentration. In contrast to the lipid peroxides, H_2O_2 in the range of 0.5-2 mM gave only low levels of *PR*-1 gene induction. This result supports the notion that lipid peroxides may be the primary mediator of *PR*-1 induction rather than H_2O_2, the other potential product of SA's interaction with catalase.

To further analyze the role of lipid peroxides in the defense responses induced by SA, we utilized diethyldithiocarbamic acid (DIECA), a compound that converts lipid peroxides to their hydroxyl derivatives [52]. This property of DIECA provides a useful tool for examining whether the lipid peroxides generated from SA-induced lipid peroxidation are involved in the induction of *PR* gene expression. For this purpose, DIECA was added to tobacco cell cultures 30 min prior to SA treatment and PR-1 protein levels were analyzed 24 h later. DIECA inhibited SA-induced PR-1 protein accumulation in a dose-dependent manner (Fig. 5D), suggesting that lipid peroxidation is downstream of SA in the signal transduction pathway leading to *PR*-1 gene expression.

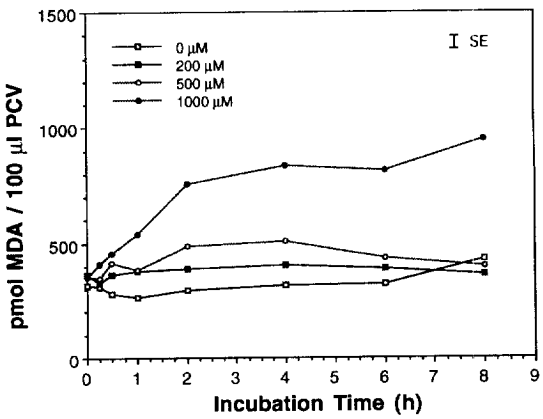

Fig. 4. Kinetics of lipid peroxidation in SA-treated tobacco cells. Cells were treated with 0, 200, 500, or 1000 μM SA for various durations and the extent of lipid peroxidation was determined. Standard error (SE) was determined as described in Materials and Methods.

Lipid peroxidation in response to SA analogues

The biological relevance of SA-induced lipid peroxidation was further examined by monitoring the ability of SA analogues to promote accumulation of lipid peroxidation products and *PR*-1 gene expression. Three chlorinated derivatives of SA (100 μM) induced *PR*-1 in cell culture (Fig. 5B), as has been reported previously in tobacco plants [34]. As with SA, higher concentrations of these analogues promoted accumulation of lipid peroxidation products (Table 1) and inhibited catalase (as previously reported by Conrath *et al.*) [34]. In contrast, the biologically inactive analogues, 3-hydroxybenzoic acid and 4-hydroxybenzoic acid, failed to induce *PR*-1 gene activation (Fig. 5B)

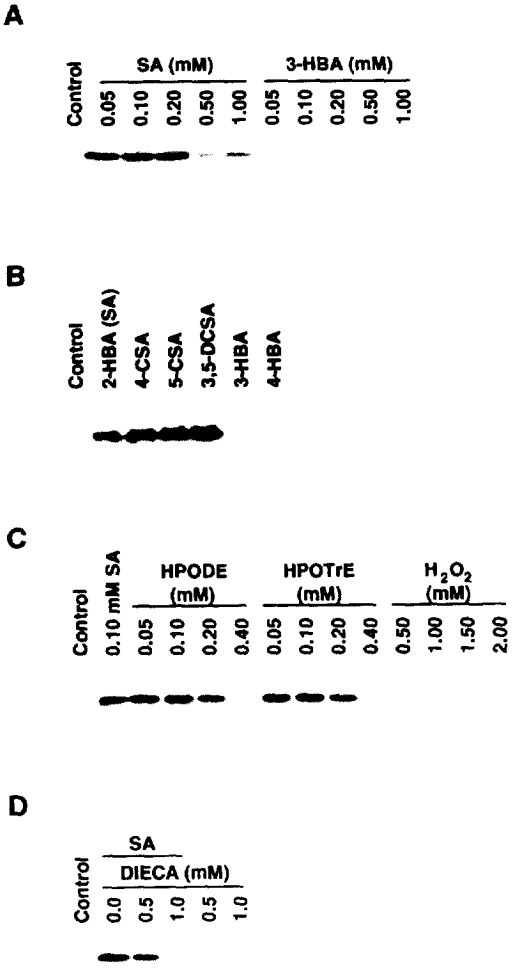

Fig. 5. Synthesis and accumulation of PR-1 proteins in tobacco cells. Cells were exposed to: **A.** several concentrations of SA or 3-HBA, a structural analog of SA, **B.** various SA analogues at 0.1 mM concentrations, **C.** various concentrations of H_2O_2 and two commercially available lipid peroxides, 13(S)-hydroperoxylinoleic acid (HpODE) and 13(S)hydroperoxylinolenic acid (HpOTrE), and **D.** several concentrations of diethyldithiocarbamic acid (DIECA), in the presence or absence of 0.1 mM SA. Cells were treated for 30 min with DIECA prior to addition of SA. In all of the experiments, cells were treated for 24 h in darkness and PR-1 protein was monitored using a PR-1 specific monoclonal antibody according to Chen *et al.* [97]. Untreated cells served as controls.

and lipid peroxidation (Table 1) or to inhibit catalase (Table 1) [34].

Involvement of H_2O_2, *catalase, and lipoxygenase*

Lipid peroxidation is commonly attributed to the action of free radicals (including reactive oxygen species) and the activity of lipoxygenases [49]. The fact that only SA and its analogues, which inhibited catalase activity, were able to induce lipid peroxidation prompted us to examine whether the elevated H_2O_2 levels resulting from catalase inhibition were directly responsible for the observed lipid peroxidation. Lipid peroxidation products did not significantly accumulate in response to exogenous addition of 1 mM H_2O_2 while *t*-butylhydroperoxide, a peroxide that may not be metabolized *in vivo* as quickly as H_2O_2 caused a small increase in lipid peroxidation products (Fig. 6). Furthermore, artificially elevating endogenous H_2O_2 using the catalase inhibitor, 3-amino-1,2,5-triazole (3AT) did not promote accumulation of lipid peroxidation products. Taken together, these results suggest that SA-induced lipid peroxidation does not arise as a result of elevated H_2O_2 levels.

The possibility that SA induced lipid peroxidation via activation of lipoxygenases was examined using lipoxygenase inhibitors. Pretreatment of cells with three different lipoxygenase inhibitors, salicylhydroxamic acid (SHAM), ibuprofen, and mefenamic acid (MFAA), had no significant effect on the ability of SA to induce lipid peroxidation (Fig. 6). On the other hand, pretreatment of cells with 3AT did cause a significant reduction in the accumulation of SA-induced lipid peroxidation products, suggesting that catalase was involved in one mechanism of SA action. This is consistent with the model presented in Fig. 1 whereby inhibition of catalase by 3AT (whose mechanism of inhibition differs from that of SA) would reduce the production of the SA radical and consequently, reduce the level of lipid peroxidation. The failure of 3AT to reduce lipid peroxidation to control levels may be due to incomplete inhibition of catalase

Table 1. *PR*-1 gene activation, inhibition of catalase, and induction of lipid peroxidation by SA analogues in tobacco cell culture

Treatment	*PR*-1 induction	Catalase inhibition (%)	Lipid peroxidation (pmol MDA/ 100 μl PCV)
Control	–	0	274 (+16)
SA	+	86	547 (+32)
4-CSA	+	89	650 (+37)
5-CSA	+	83	660 (+38)
3,5-DCSA	+	91	527 (+31)
3-HBA	–	7	233 (+14)
4-HBA	–	10	271(+16)

Tobacco cells were exposed to various SA analogues at a concentration of 1 mM for 1 h in the dark and the extent of lipid peroxidation was determined. In an independent experiment, *in vivo* catalase activity was determined according to Conrath *et al.* [34]. *PR*-1 gene induction is from Fig. 5B. MDA values are means with one standard error above the mean in parentheses. Note that due to the log transformation, the magnitude of one standard error below the mean is slightly less than the value in parentheses. For catalase inhibition, the arcsine square root transformation for percentage data did not improve the variance estimation; thus, the analysis was conducted on untransformed data. The standard error was 2.9%.

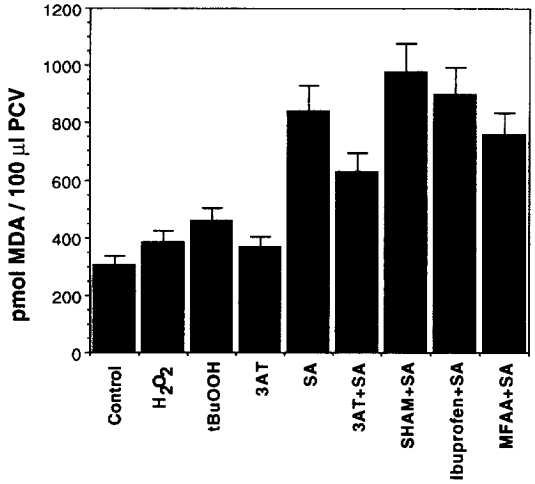

Fig. 6. Lack of involvement of H_2O_2 or lipoxygenase in SA-induced lipid peroxidation. Tobacco cells were treated with 1 mM H_2O_2, 1 mM t-butylhydroperoxide (tBuOOH), 5 mM 3-amino-1,2,5-triazole (3AT), or 1 mM SA for 1 h in darkness and the extent of lipid peroxidation was determined. Cells were also pretreated for 30 min with 5 mM 3AT or one of three lipoxygenase inhibitors, 100 μM salicylhydroxamic acid (SHAM), 100 μM ibuprofen, or 100 μM mefenamic acid (MFAA) before treatment with 1 mM SA for 1 h. Untreated cells served as controls.

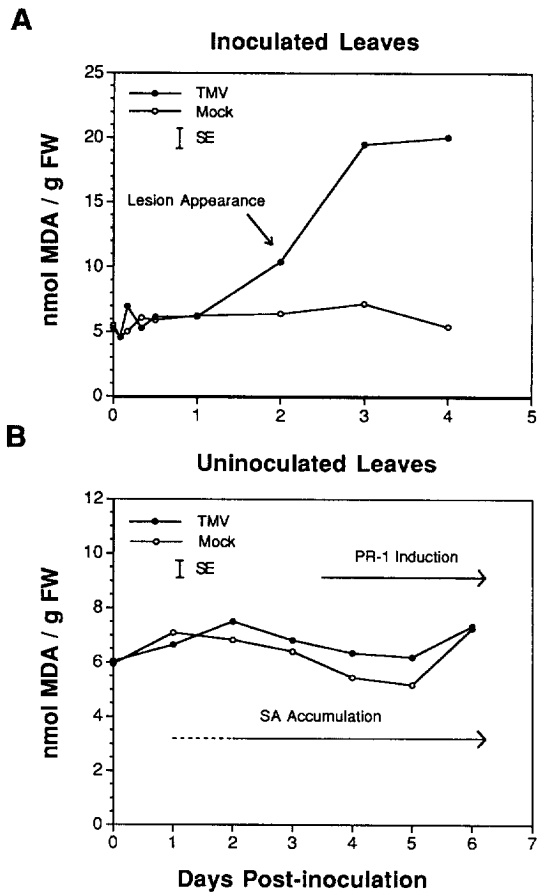

Fig. 7. Lipid peroxidation in tobacco leaves inoculated with TMV. Eight week-old tobacco plants were inoculated on three lower leaves with TMV. Leaves were first dusted with carborundum and TMV was applied using 8 layers of cheese cloth saturated with 5 ml of 5 μg/ml TMV in 50 mM phosphate buffer, pH 7.2. Buffer alone was applied to mock-inoculated controls. **A.** At the indicated times, leaf tissue was sampled by taking two 11 mm leaf disks from random locations (avoiding vasculature) of each of the three inoculated leaves of two individual plants (12 disks / sample) and immediately freezing them in liquid nitrogen. Subsequent samples during the time course were taken from the same leaves. Samples were stored at - 80 C until all samples were collected and lipid peroxidation was measured. Lesions were first observed at two days post-inoculation (dpi). **B.** To determine whether lipid peroxidation was detectable in uninoculated leaves, the three leaves immediately above the inoculated leaves were also sampled. The occurrence of increased SA accumulation (beginning between 1 and 2 dpi) and *PR*-1 gene induction [9; A. Guo and D. F. Klessig, unpublished] are indicated for comparison.

(5 mM 3AT inhibited *in vivo* catalase activity by $\approx$40%, data not shown).

Lipid peroxidation in response to TMV infection

To further explore the physiological relevance of lipid peroxidation during SA-mediated defense responses, this process was monitored in TMV-infected resistant tobacco plants (Fig. 7). In inoculated leaves, a small peak in lipid peroxidation was observed at 4 h, possibly corresponding to the oxidative burst associated with the early response of resistant plants to avirulent pathogens [1, 40, 53]. Subsequently, accumulation of lipid peroxidation products was dramatically elevated as lesions developed. In uninoculated leaves, the slightly higher accumulation of lipid peroxidation products in response to TMV was variable (elevated 2-thiobarbituric acid [TBA]-reactive substances were observed in two of four replicates) and may not be significant. At present, it is unclear whether lipid peroxidation is truly elevated in uninfected leaves and, if so, whether the rate of lipid peroxidation is high enough to cause accumulation of lipid peroxidation products to detectable levels.

DISCUSSION

Mechanism of SA-induced lipid peroxidation

SA has been previously shown to inhibit catalase both *in vitro* and *in vivo* [30, 34]. This observation led to the working hypothesis that the inhibition of catalase by SA would result in elevated levels of endogenous H_2O_2, which might mediate the induction of defense responses by SA. However, a more detailed investigation of the interaction between SA and catalase [37] suggested another possibility. Spectral analysis of the various catalase redox states revealed that incubation of catalase with H_2O_2 and SA resulted in the formation of compound II, an enzyme inter-

mediate in catalase's peroxidative cycle (Fig. 1). In addition, SA also allowed the recovery of ferricatalase from compound II. These results demonstrated that SA is able to participate as an electron-donating substrate for reactions 3 and 4. A consequence of SA's participation as a peroxidative substrate is the generation of SA free radicals. Since the peroxidative cycle of catalase is at least 1000 times slower than the catalatic cycle [38], engagement of the peroxidative cycle results in a decline in the catalatic activity. Thus, SA's interaction with catalase results in two potential signals, H_2O_2 and SA radicals.

There is considerable precedence for the generation of organic free radicals by catalases and peroxidases. For example, mammalian prostaglandin H synthase catalyzes the generation of substrate-derived radicals through a peroxidative reaction when a variety of anti-inflammatory drugs, such as acetaminophen, *p*-aminophenol, aminopyrine, benzidine and phenidone, are provided as substrates [54–59]. Human lactoperoxidase and thyroid peroxidase catalyze generation of organic radicals in their interaction with resorcinol derivatives [60]. In addition, the antituberculosis drug isoniazid interacts with catalase from *Mycobacterium tuberculosis*, the causal agent of tuberculosis. Deletion of the catalase gene from *Mycobacterium tuberculosis* renders the pathogen resistant to isoniazid [61]. Biochemical studies have recently revealed that the sensitivity of the pathogen to isoniazid conferred by its own catalase is likely due to the production of reactive isoniazid metabolites or radicals generated through the peroxidase activity of this catalase [62]. Furthermore, generation of free radicals from isoniazid and phenolic compounds by horseradish peroxidase has also been demonstrated [63, 64].

One of the primary targets of free radicals is polyunsaturated lipids present in membranes. Extraction of a hydrogen atom from polyunsaturated lipids by $OH^{\cdot}$, $O_2^{\cdot}-$, or an organic free radical such as $SA^{\cdot}$ and the repeated reaction of molecular oxygen with the resulting lipid radicals, leads to a lipid peroxidation chain reaction (Fig. 1). Interestingly, phenolic radicals generated by myeloperoxidase have been shown to induce lipid peroxidation [47].

The lipid peroxides formed by lipid peroxidation rapidly undergo secondary oxidation to produce a wide variety of oxidation products including cyclic epidioxides, alkanes, epoxides, alcohols, and aldehydes [65–68]. The lipid peroxide and/or aldehyde products of lipid peroxidation are also known to react with proteins to form carbonyl moieties on a number of amino acid residues [69, 70]. While such oxidized proteins are usually thought of as damage products subject to rapid proteolysis, it is possible that certain oxidized proteins are utilized by plants to signal an oxidizing environment. Thus, generation of SA radicals could lead to membrane attack, initiation of a lipid peroxidation chain reaction, and the production of a large and diverse group of products. Some of these compounds could then potentially serve to mediate SA-induced defense responses.

Consistent with the model proposed above, lipid peroxidation was induced by SA in a dose-dependent manner (Fig. 2). However, there are several alternative mechanisms by which SA could induce lipid peroxidation. For example, in addition to the generation of SA radicals, SA's interaction with catalase can lead to elevated H_2O_2 levels. Despite the fact that H_2O_2 is believed to be incapable of directly initiating lipid peroxidation, its elevation might lead to generation of the $OH^{\cdot}$ (via a Fenton-type reaction or Haber-Weiss reaction) which is a very effective inducer of lipid peroxidation [49]. Alternatively, H_2O_2 may induce lipid peroxidation indirectly by serving as a substrate for the peroxidase-mediated formation of free radicals in the presence of appropriate electron-donating peroxidase substrates (Fig. 1). However, exogenous addition of H_2O_2 failed to promote accumulation of lipid peroxidation products. Furthermore, elevation of endogenous H_2O_2 levels by inhibiting catalase with 3AT, which inactivates catalase by covalently binding to the enzyme intermediate compound I without concomitant generation of free radicals [71, 72], did not cause accumulation of lipid peroxidation products (Fig. 6). These results suggest that lipid peroxidation is not dependent on elevated H_2O_2 levels resulting from inhibition of catalase. In contrast, preincubation of tobacco cells with 3AT partially inhibited SA-induced lipid peroxidation. Presumably, in the presence of fewer active catalase molecules, less SA was converted to its radical form.

The inability of 3AT to induce lipid peroxidation in tobacco cell cultures contrasts with its capacity to activate *PR*-1 gene expression in tobacco plants [30]. A likely explanation for this apparent discrepancy is that 3AT not only inhibits catalases, but also blocks the biosynthesis of chloroplast carotenoid pigments [73]. The primary function of carotenoids is to protect chlorophyll molecules from light-induced bleaching by quenching the chlorophyll triplet states produced through overexcitation [74, 75]. If carotenoid biosynthesis, which is particularly active in young expanding leaves, is inhibited by 3AT, chlorophyll molecules in the triplet state react with O_2 to form either the singlet oxygen or the superoxide anion [74]. Both of these forms of oxygen can cause lipid peroxidation, in addition to other damaging effects in chloroplasts. Thus, 3AT likely induces lipid peroxidation, particularly in the young expanding leaves grown under high light intensity. However, the mechanism through which 3AT induces lipid peroxidation is unrelated to that utilized by SA. Consistent with these suppositions is the observation that 3AT's effectiveness for activating *PR* gene expression is dependent both on leaf age and growth conditions (i.e. light intensity; unpublished results). In contrast, 3AT was unable to induce lipid peroxidation in heterotrophic tobacco cell cultures grown in the dark.

Another mechanism through which SA might

induce lipid peroxidation is by activating lipoxygenases. It has been previously suggested that lipoxygenase-catalyzed lipid peroxidation might produce signal molecules involved in disease resistance [76]. However, inhibitors of lipoxygenases had no effect on SA-induced lipid peroxidation (Fig. 6). Thus, it appears that lipoxygenases are not involved in this process.

Is lipid peroxidation a signaling process?

Lipid peroxidation is a process of oxidative damage to membranes that arises under conditions of oxidative stress and can potentially be devastating to membrane function. Under normal conditions, plants possess protective systems that prevent excessive lipid peroxidation. For example, α-tocopherol is a hydrophobic antioxidant that is present among membrane phospholipids and it serves to break the lipid peroxidation chain reaction [49]. In addition, cells possess a wide array of scavenging systems that remove ROS and/or organic free radicals and thereby minimize the initiation of lipid peroxidation [77]. All of these protective systems serve to prevent lipid peroxidation from exceeding the rate of membrane repair. Nevertheless, under some conditions, the generation of ROS and/or organic free radicals exceeds the capacity of the scavenging systems and pronounced lipid peroxidation occurs. For example, lipid peroxidation has been observed in plants experiencing oxidative stress such as occurs during exposure to low temperature [78] or drought [79] and following pathogen infection [8, 39, 80, 81]. In none of these examples did the levels of lipid peroxidation (as measured by TBA-reactive substances) increase more than 3-fold above controls.

Since the lipid peroxidation levels we observed in response to SA were of similar magnitude to those associated with various stresses, we attempted to establish the level of SA that caused excessive cell damage. Prolonged exposure of tobacco cells to 1 mM SA was cytotoxic, with detectable increases in cell death evident by 6 h (Fig. 3). Under the same conditions, accumulation of lipid peroxidation products began to plateau within 2 h, reaching levels 2- to 3- fold above controls (Fig. 4). In contrast, application of 200 μM or 500 μM SA resulted in smaller increases in the amount of lipid peroxidation products, and these peaked at 2 h and declined by 6 h. Thus, while prolonged and pronounced lipid peroxidation appeared to be associated with cell death, more transient and modest increases were not. However, at some of these lower levels (e.g. 500 μM) SA may still be causing cellular stress, as suggested by the poorer induction of PR-1 protein synthesis and accumulation in the presence of 500 μM compared to 50-200 μM SA (Fig. 5A).

If lipid peroxidation is a component of the signal transduction pathway downstream of SA, two important questions need to be addressed. 1) Why does SA induce *PR*-1 genes at low to moderate concentrations (50-100 μM) but require higher concentrations to induce detectable increases in lipid peroxidation? 2) Do high concentrations of SA induce lipid peroxidation directly, or are elevated levels of lipid peroxidation merely a consequence of SA cytotoxicity? It should be noted that many of the immediate products of lipid peroxidation are toxic [51]. Therefore, accumulation of these compounds is not likely to mediate the signaling process. In fact, one function of glutathione transferases is to rapidly detoxify lipid peroxidation products [82]. Therefore, high levels of SA are probably required to obtain detectable increases in the accumulation of lipid peroxidation products because of the rapid turnover of the initial products of lipid peroxidation. A more innocuous downstream lipid peroxidation product, such as an oxidized protein, is a more likely candidate for a signal.

Since a 2- to 3-fold increase in lipid peroxidation has been associated with cell death in this and other studies [39, 80, 81], the overall increase in cellular lipid peroxidation must be well below this level if the resultant products play a signaling role. Otherwise, the response of the cell to the signal would be severely compromised. Furthermore, the actual signal may be a flux of one or more lipid peroxidation products. Therefore, the net accumulation of lipid peroxidation products may be small. In light of these considerations, perhaps it is not surprising that we were unable to detect significant accumulation of TBA-reactive substances in cell cultures exposed to low concentrations of SA (e.g. 50 μM) which induce *PR*-1 gene expression (Figures 2 and 5A) or in the uninoculated leaves of TMV-infected tobacco (Fig. 7B), where SA levels are low (range 0.5–9 μM SA) [9, 27, 83]. Nonetheless, we have established that SA can induce lipid peroxidation in a concentration-dependent manner and this may involve a SA radical.

In response to the second question, it is evident that measurements of accumulated lipid peroxidation products alone do not allow us to determine whether accumulation of these products is part of a SA signaling pathway or a by-product of SA's cytotoxicity. Clearly, high concentrations of SA are cytotoxic. However, the following findings collectively suggest that lipid peroxidation is a component of the signaling process downstream of SA. First, lipid peroxides induce *PR*-1 (Fig. 5C) and *PR*-2 genes (C. Langebartels and H. Sanderman, Jr., personal communication). Second, destruction of lipid peroxides with DIECA prevented SA-induced *PR*-1 expression (Fig. 5D), although this latter result requires a cautious interpretation because DIECA also has antioxidant properties [84]. It should also be noted that DIECA has been reported to be cytotoxic [85]; therefore, it is possible that the observed inhibition of *PR*-1 expression may be due, in part, to a general reduction in gene expression. However, there was no evidence, using the Evans Blue exclusion method [40], for reduced cell viability in the presence of DIECA.

Third, only biologically active analogues of SA, which induce *PR*-1 gene expression, enhance disease resistance and inhibit catalase, stimulated lipid peroxidation (Table 1). Perhaps the SA-induced lipid peroxidation associated with cytotoxicity (possibly even cell death) and its proposed association with signaling are the same process, which differ only in magnitude. For example, high levels of SA (range 3-150 μM SA) [9, 27, 83, 86] and the resultant lipid peroxidation products may participate in host cell death at the site of infection during the HR, while lower levels of both may serve as signals for induction of defense-related genes. A similar concentration-dependent, dual role has been proposed for H_2O_2 [40].

Interestingly, in TMV-inoculated tobacco leaves, a pronounced increase in lipid peroxidation paralleled the appearance of HR lesions (Fig. 7A). Keppler and Baker [80] observed a similar increase in lipid peroxidation in association with a bacteria-induced hypersensitive reaction in tobacco cell cultures. This dramatic increase in lipid peroxidation is similar in magnitude to that observed after treatment of suspension cells with cytotoxic levels of SA (Fig. 2) and is likely associated with cell death. In the uninoculated leaves of TMV-infected plants, a slight rise in lipid peroxidation was also detected (Fig. 7B). This increase appears to parallel the modest rise in SA levels and precede induction of *PR*-1 expression. However, this latter result must be interpreted with caution, as elevated lipid peroxidation levels in un-inoculated leaves were seen in only two of four experiments, resulting in a high variance and an inability to establish a significant difference between TMV- and mock-inoculated plants.

It is currently unclear how small increases in SA levels lead to *PR* gene activation and enhanced disease resistance in uninoculated systemic tissue. One possible explanation is that SA radicals, formed by the interaction between SA and catalase (or peroxidases) [37], induce lipid peroxidation. The resultant peroxidation products may then play a signaling role, as suggested by the study described here. Once lipid peroxidation has been initiated, it is a self-perpetuating chain reaction (Fig. 1); a single initiation event can result in conversion of hundreds of polyunsaturated fatty acid side chains into lipid peroxides [49]. Therefore, a small amount of SA radical could result in the formation of an effective lipid peroxide signal, without a readily discernible inhibition of catalase or rise in H_2O_2 levels. Alternatively, the SA signal might be transmitted via the recently identified soluble $\approx$25 kDa protein whose affinity for SA is $\approx$150-fold higher than that of catalase [87].

Lipid peroxidation products, such as lipid peroxides, may also play a part in the ability of SA to potentiate certain responses. There is increasing evidence that pre- or co-treatment with SA can positively influence the magnitude (and kinetics) of several defense responses (e.g. H_2O_2 production, gene activation, and cell death) induced by a variety of elicitors. These elicitors include infection [88–90], fungal cell wall fragments, chitosan, ergosterol, mastoparan [91], wounding [88, 92], and H_2O_2 [90]. These effects of SA have been termed potentiation and they can often be seen with low to moderate levels of SA (50–200 μM), which by themselves are insufficient to induce these defense responses.

The mechanism(s) by which SA induces various defense responses is currently unresolved. There is considerable evidence against, as well as in favor of, the model in which SA functions, in part, by inhibiting catalase (and ascorbate peroxidase) and thereby elevating H_2O_2 levels. Here we present results consistent with a model in which SA acts, in part, through catalase to stimulate lipid peroxidation; one or more products of lipid peroxidation then serve as a signal to activate certain defenses.

EXPERIMENTAL

Plant material. Cell suspension cultures of tobacco (*Nicotiana tabacum* cv. Xanthi) were grown at room temperature in the dark in MS medium supplemented with 1 mg L^{-1} α-naphthaleneacetic acid, 0.1 mg L^{-1} 2,4-dichlorophenoxyacetic acid and 0.1 mg L^{-1} benzyladenine. Cells were maintained by 10-fold dilution with fresh medium every 6 to 7 days. Tobacco plants (*Nicotiana tabacum* cv. Xanthi nc) were grown at 22°C in growth chambers with a photoperiod of 14/10 (L/D) and a light intensity of $\approx$250 μmol m^{-2} s^{-1}.

Catalase and lipid peroxidation assays. Determination of *in vivo* catalase activity of tobacco cell cultures was performed as previously described [34]. Lipid peroxidation was determined by measurement of MDA after reaction with TBA (procedure modified from Bird *et al.*) [93]. Sucrose, a major component of the cell culture media, is known to react with certain aldehydes or radicals under the acid-heating conditions of the TBA assay to produce authentic MDA [94, 95]. Consequently, tobacco cells (3 days after dilution) were washed twice with a 10 × vol. (relative to cell volume) of sucrose-free media prior to treatment. To maintain consistency among experiments, cells were always diluted to a 4 × treatment density (culture vol. relative to cell vol.) in sucrose-free media.

After cell treatment, 0.4 ml cells from a given treatment were dispensed into tubes containing 0.4 ml 20% TCA/10 mM EDTA and 16 mg polyvinylpolypyrrolidone (PVPP), mixed by inversion, and placed on ice. Cells were sonicated for 15 pulses of $\approx$1 sec each with 60% pulse duration at 10% output power (Heat Systems–Ultrasonics Inc., Model W-375) and solid material was pelleted in a microfuge for 10 min. An equal vol. of 0.5% TBA was added to 0.4 ml of supernatant and the mixture was boiled for 30 min. After cooling, an equal vol.of 0.15 N NaOH in 50% MeOH was added to neutralize the pH and each sample was filtered using a 0.2 μm pore acrodisc (Gelman Sciences). The TBA_2-MDA complex was separated by HPLC using an ODS column (Dynamax, 25 cm × 4.6

mm ID, 8 μm irregular particle size and 60 Å pore size) with a 50 μl injection vol., a flow-rate of 1 ml min^{-1} and detection at 532 nm. The solvent program was 15–50% MeOH over 15 min with a 20 min column wash using 100% MeOH and a 20 min re-equilibration with 15% MeOH. Since a small loss of the TBA_2-MDA complex occurs over time, a control sample containing 4.8 μl of 1 mM MDA (prepared from 1,1,3,3-tetramethoxypropane according to Yoden and Iio [96] as an internal standard was repeatedly injected every 4–6 samples to establish a correction factor. MDA quantification was based on the peak area of varying concns of the MDA standard.

For lipid peroxidation in leaf tissue, 12 frozen leaf disks of a given treatment were weighed, ground in liquid N_2, and extracted with 0.6 ml 20% TCA/10 mM EDTA and 40 mg PVPP. Subsequent measurement of lipid peroxidation was the same as for cell culture except the washing and re-equilibration steps of the HPLC separation were extended from 20 min to 30 min. With the cell culture, addition of an equal vol. of 0.15 N NaOH in 50% MeOH consistently neutralized samples to pH 6–7. However, some leaf tissue samples required further pH adjustment to maintain a consistent pH 6–7 among samples.

Cell death. Determination of cell death resulting from SA exposure utilized the vital stain fluorescein diacetate, which is rendered fluorescent upon reaction with esterases in live cells but remains non-fluorescent in dead cells. Tobacco cells were washed with sucrose-free medium followed by SA treatment as described above to reproduce the conditions under which lipid peroxidation was measured. At the indicated times, 0.25 ml of cells was removed from incubation, diluted 3-fold with sucrose-free medium, and applied as a single drop to a microscope slide. An equal vol. of 0.01% fluorescein diacetate (prepared fresh by adding 20 μl 5 mg/ml fluorescein diacetate (in Me_2CO) to 980 μl sucrose-free medium) was added and allowed to react 10 min. Live and dead cells from four defined viewing fields were counted using a fluorescence microscope (Zeiss axioskop).

Analysis of PR-1 protein accumulation. For determining induction of *PR-1* gene expression, 1 ml of cells aliquoted from a cell culture 2 days after the culture was diluted, was incubated with various inducers at indicated concns for 24 h at room temp. in the dark on a rotary shaker (100 rpm). Cells were harvested by brief centrifugation in a microfuge. The cell pellets were then sonicated in 200 μl of a buffer containing 50 mM Tris pH 8.0, 1 mM EDTA, 12 mM β-mercaptoethanol, 10 mg/ml phenylmethylsulphonyl fluoride. After clarification by centrifugation in a microfuge, an aliquot of the homogenate was fractionated by SDS/PAGE and the separated proteins were electrophoretically transferred to a nitrocellulose filter and immunoblotted with the mouse monoclonal antibody 33G1 which specifically recognizes PR-1 proteins [97]. Protein content was determined according to Bradford [98].

Commercially purchased lipid peroxides were obtained and stored in 100% EtOH. To eliminate possible effects of the solvent on tobacco cells, EtOH was removed before the compounds were added to the cell cultures. Lipid peroxides in EtOH (10–40 μl) were added to 200 μl of fresh culture medium in the wells of a 24-well plate. EtOH (95%) without lipid peroxides was used as a control. The plate was then placed inside a desiccator on a shaker; a mild vacuum was applied for 1.5 to 2 hr. The vol. of the culture medium after vacuum treatment was restored to 200 μl with sterilized H_2O. 200 μl of cell suspension cultures, aliquoted 2 days after the culture was diluted, were added to the wells and allowed to incubate on a shaker for another 24 h before analysis for PR-1 protein levels as described above.

Statistical analyses. All lipid peroxidation and cell death experiments using cell cultures were replicated ×3 and were set up and analysed as a randomized complete block design. The lipid peroxidation study in tobacco plants responding to TMV infection (Fig. 7) was replicated ×4. Since this experiment involved repeated measures, it was analysed as a split plot with treatment as whole plots and days post inoculation as subplots. Data from inoculated leaves were analysed separately from uninoculated leaves. In many of the experiments (Table 1 and Figs. 2, 3, and 6), the variance was proportional to the means and thus, a natural log transformation was used to stabilize the variance. In these cases, standard errors are reported for each mean. For those experiments where transformation was unnecessary (Figs. 4 and 7), a single experimental standard error is reported.

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