Pretreatment of Parsley Suspension Cultures with Salicylic Acid Enhances Spontaneous and Elicited Production of \( \text{H}_2\text{O}_2 \)^{1}

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Suspension-cultured cells of parsley (Petroselinum crispum L.) were used to study the regulation of extracellular \( \text{H}_2\text{O}_2 \). After resuspension, the washed cells regulated the \( \text{H}_2\text{O}_2 \) concentration spontaneously to a constant level that was greatly increased when the cultures were pretreated for 1 d with salicylic acid (SA). The \( \text{H}_2\text{O}_2 \) level was further increased on addition of a fungal elicitor preparation, macromolecular chitosan, the sterol-binding polyene macrofide amphoterin B, the G protein-activating peptide mastoparan, or La^{3+}. In all cases, this induced enhanced in cell suspensions pretreated with SA. Both the spontaneous and the induced \( \text{H}_2\text{O}_2 \) production were decreased by the protein kinase inhibitor K-252a. It is suggested that production of extracellular \( \text{H}_2\text{O}_2 \) occurs by an endogenously controlled plasma membrane enzyme complex that requires continuous phosphorylation for function and whose activity is increased by pretreatment of the cells with SA. This system can also receive various external stimuli, including those resulting from binding of fungal elicitor. SA can induce acquired resistance against pathogens. The conditioning of the parsley suspension culture by SA represents, therefore, a model for the long-term regulation of apoplastic \( \text{H}_2\text{O}_2 \) concentration by this signal substance, as suggested previously for the wound hormone methyl jasmonate.

Active oxygen species, which are produced intracellularly from several unavoidable side reactions associated with metabolism, are generally considered to be harmful and eliminated, therefore, by antioxidants or detoxifying enzymes (Elsner and Osswald, 1994). In contrast, extracellular active oxygen species may fulfill multiple physiological functions. For instance they may act directly as toxic defense agents against pathogens (Mehdy, 1994) or they may serve as inducers of various defense reactions associated with SAR (Sánchez-Casas and Klessig, 1994) and the hypersensitive response (Levine et al., 1994) or for the peroxidase-catalyzed cross-linking of cell wall polymers such as lignin, proteins, or pectins (Brisson et al., 1994; Iiyama et al., 1994). Extracellular activated oxygen species presumably arise by transfer of electrons through the plasma membrane, resulting in the extracellular reduction of molecular oxygen (Mehdy, 1994). Most of the activated oxygen species have a very short half-life, whereas \( \text{H}_2\text{O}_2 \) is stable enough to build up considerable apoplastic concentrations (Sutherland, 1991).

Even though recent research and discussions have focused on the possible role of extracellular active oxygen in plant defense against pathogens (Mehdy, 1994), the occurrence of apoplastic \( \text{H}_2\text{O}_2 \) in healthy tissue has long been known. The recent development of a histochemical assay for \( \text{H}_2\text{O}_2 \) (Olson and Varner, 1993) and the further development of a more sensitive tissue-printing method for the rapid detection and localization of \( \text{H}_2\text{O}_2 \) allowed direct demonstration of \( \text{H}_2\text{O}_2 \) at relatively high concentrations in the apoplast of many plant tissues (Schopfer, 1994). These experiments also showed that apoplastic \( \text{H}_2\text{O}_2 \) concentration appears to be under developmental regulation by factors such as hormones, light, and wounding.

Suspension-cultured parsley (Petroselinum crispum) cells and the fungal elicitor used in the present report have been extensively investigated as a model system for pathogen defense reactions. The molecular species active in the crude fungal elicitor preparation is a glycoprotein, inducing both the secretion of coumarin derivatives (phytoalexins) and \( \text{H}_2\text{O}_2 \) burst (Nürnberger et al., 1994). Alternatively, macromolecular chitosan can be used to induce coumarin secretion (Conrath et al., 1989) and the \( \text{H}_2\text{O}_2 \) burst (Kauss et al., 1994). We have also used the parsley culture to construct a model in which to study relationships between local elicitor-mediated defense reactions and the SAR defense strategy of plants (Kauss et al., 1992, 1993). It was found that routinely grown cultures do not give optimal responses to fungal elicitors but that pretreatment of the cultures with SA and other substances involved in SAR greatly enhanced the sensitivity and effectiveness of the cells for response to the fungal elicitor. This pretreatment resulted in enhanced secretion of coumarin derivatives and incorporation of phenolics into the cell wall.

These observations were recently extended to the elicitation of \( \text{H}_2\text{O}_2 \). Pretreatment of the parsley culture with the wound-derived signal substance methyl jasmonate greatly enhanced the elicited \( \text{H}_2\text{O}_2 \) burst (Kauss et al., 1994). During these experiments we also observed that these conditioned cells produced more \( \text{H}_2\text{O}_2 \) even without elicitor. In addition, pretreatment of the cultures with 20 \( \mu \text{M} \) 5-chlorosalicylic acid, a compound able to induce pathogen resistance in cucumber hypocotyls (Siegrist et al., 1994), also slightly enhanced the elicited and nonelicited \( \text{H}_2\text{O}_2 \) production.

In the present report we describe these latter effects in more detail using SA for pretreatment of the parsley cul-

1 This work was supported by Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie.

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Abbreviations: SA, salicylic acid; SAR, systemically acquired resistance; SOD, superoxide dismutase.
tured. SA likely does not represent the primary systemic signal, but it is clearly involved as a natural inducer of SAR and appears to be essential for the expression of multiple modes of plant disease resistance (Delaney et al., 1994; Ryals et al., 1994). Our results show that spontaneous \( \text{H}_2\text{O}_2 \) production is enhanced by SA pretreatment and results from a well-regulated process that can also deliver a \( \text{H}_2\text{O}_2 \) burst once stimulated by a fungal elicitor or by certain other substances able to interact with plant plasma membranes.

**MATERIALS AND METHODS**

**Parsley Cell Culture and Its Handling**

The origin and growth conditions for the dark-grown parsley (*Petroselinum crispum*) culture were as described by Kauss et al. (1994). A 50 mm stock solution of SA was prepared by titration with sodium hydroxide to a pH value slightly greater than 7. This solution was sterilized by filtration, and aliquots were added for conditioning of the cultures 3 d after cell inoculation. Conditioning was routinely performed with 170-mL batches of cell culture; exceptions are indicated in the figure legends. The cultures were routinely washed 1 d after SA addition on a filter paper funnel with a solution containing 2 mM bis-Tris/Mes other substances able to interact with plant plasma membranes.

**RESULTS**

For the experiments reported, the parsley suspension cells were routinely washed and resuspended in fresh growth medium, which was diluted for this purpose mainly because the luminol assay used was about 3-fold more sensitive under this condition. In the course of the 1st h after cell resuspension, the extracellular \( \text{H}_2\text{O}_2 \) concentration in suspensions of SA-pretreated cells underwent some oscillation (Fig. 1), reaching a steady-state level that was maintained through the next 1 to 2 h and undergoing only slight changes thereafter (Fig. 2). Control cells exhibited similar changes but at a far lower level (data not shown in

![Figure 1. Adjustment of extracellular \( \text{H}_2\text{O}_2 \) concentration after resuspension of parsley cells pretreated with SA. The cell-suspension culture was pretreated with 0.5 mM SA for 1 d, and the cells were washed and resuspended in fresh diluted growth medium (●). At the times indicated, aliquots were centrifuged, and \( \text{H}_2\text{O}_2 \) was determined in the supernatant using the luminol method. Protein kinase inhibitor K-252a (0.2 μM final concentration, ○) was added in DMSO (final concentration 0.02%, v/v) at the arrows to parallel cell samples. In similar experiments, DMSO of the same final concentration did not influence the \( \text{H}_2\text{O}_2 \) levels. Resuspended control cells exhibited a similar adjustment of \( \text{H}_2\text{O}_2 \) at a lower level and reached the value indicated by the white arrow (■).](#)
In control and SA-treated cells, K-252a immediately inhibited further elicitation of H₂O₂ production even when the inhibitor was given 10 min after the elicitor, a time when H₂O₂ production proceeded at an apparently constant rate (Fig. 2). Addition of 100 μM La³⁺, the approximate concentration of Ca²⁺ in the suspension medium, also inhibited the overall elicited H₂O₂ production in SA-treated cells (Fig. 3). In the experiment shown, inhibition became evident about 2.5 min after La³⁺ addition. In two of the five experiments performed, the H₂O₂ production by elicited SA-treated cells was even enhanced for 2.5 to 5 min after La³⁺ addition, resulting in a transient time course similar to that seen in elicited control cells. Accordingly, the spontaneous H₂O₂ production was always increased by La³⁺, with a far higher increase in SA-treated cells as compared to control cells (Fig. 3).

The conditioning effect was evident at 20 μM SA and maximal at about 0.5 mM SA for both the spontaneous and elicited H₂O₂ formation (Fig. 4) and was similar in degree at any fungal elicitor concentration used (Fig. 5). Conditioning was maximal after 24 h of preincubation with SA and not further increased by 48 h of preincubation time (data not shown).

H₂O₂ production above the spontaneously adjusted level can also be induced by chitosan and mastoparan (Fig. 6). Although the time course with these substances was far more rapid when compared to the fungal elicitor (Fig. 2), the maximal values were in all cases higher for SA-treated cells when compared to control cells. One interesting feature of the mastoparan-induced H₂O₂ burst was the sharp optimum between 0.4 and 0.5 μM mastoparan observed for SA-treated cells (Fig. 7).

The experiments reported in the above figures were performed with different batches of cell suspensions, which was necessary to document many of the details and the time courses of the responses. To allow a quantitative comparison of the diverse inducers used, some important features are summarized for one cell batch in Figure 8, which also contains results from H₂O₂ induction with am-

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**Table 1. Comparison of the routinely used luminol assay and the scopoletin method for determination of extracellular H₂O₂ in parsley cell suspensions and the influence of exogenous SOD on extracellular H₂O₂ level**

<table>
<thead>
<tr>
<th>Treatment of Cell Suspension</th>
<th>H₂O₂ in the Supernatant Assayed with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Luminol</td>
</tr>
<tr>
<td>Routine cell suspension</td>
<td></td>
</tr>
<tr>
<td>Spontaneous</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>Elicited</td>
<td>26.3 ± 0.8</td>
</tr>
<tr>
<td>Cell suspension + SOD</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>Spontaneous</td>
<td>29.6 ± 0.5</td>
</tr>
<tr>
<td>Elicited</td>
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*a Determined 30 min after elicitation of the parallel aliquot.

*b 20 μg/mL fungal elicitor for 30 min.

*c Cell suspension was supplied with 25 μg/mL SOD prior to elicitor addition.
photerin B. Although the time necessary and the maximal values of \( \text{H}_2\text{O}_2 \) concentration achieved differed considerably, the SA-treated cells exhibited a higher \( \text{H}_2\text{O}_2 \) level in all cases. Figure 8 also documents the action of protein kinase inhibitor K-252a when added at the same time as the inducing substances. Inhibition was more drastic with fungal elicitor and amphotericin B, substances that require a comparatively long induction time. In contrast, with the more rapidly inducing chitosan and mastoparan, the inhibition was less pronounced. It is of interest that the \( \text{H}_2\text{O}_2 \)-inducing potency of the amphotericin B stock solution increased considerably when it was stored for several weeks at \( 4^\circ \text{C} \) (data not shown). Because a yellow color appeared in parallel, the increase might have been due to unknown decomposition products.

In many of the experiments shown, the induced \( \text{H}_2\text{O}_2 \) concentration exhibited a maximum after a certain time, the subsequent decrease indicating that the cell suspensions were able to degrade \( \text{H}_2\text{O}_2 \). This became more evident when exogenous \( \text{H}_2\text{O}_2 \) in the concentration range observed endogenously within this report was added to the cell suspensions (Fig. 9). As far as the luminol method allowed the determination of the initial rate of \( \text{H}_2\text{O}_2 \) degradation, there was no considerable difference between control and SA-treated cells, either elicited or not elicited. The slightly smaller potency for degradation of \( \text{H}_2\text{O}_2 \) by

Figure 3. Influence of La\(^{3+} \) on the time course of spontaneous (●, ○) and elicited (▲, △) \( \text{H}_2\text{O}_2 \) level in control cells and cells pretreated with 0.5 mM SA for 1 d. The same conditions and cell batch were used as in Figure 2. LaCl\(_3\) (0.1 mM, open symbols) was added at the arrows. See text for a note on the variability of the early time course after La\(^{3+} \) addition to elicited SA-treated cells.

Figure 4. Dose-response curves for SA pretreatment on the level of spontaneous and elicited \( \text{H}_2\text{O}_2 \) concentrations. Aliquots (20 mL) from a cell culture were pretreated for 24 h with the indicated concentrations of SA. The level of spontaneously formed \( \text{H}_2\text{O}_2 \) was determined 1 h after resuspension of the cells (see Fig. 1). Fungal elicitor (20 μg/mL) was then added, and \( \text{H}_2\text{O}_2 \) was determined again 30 min later. The values given for elicited \( \text{H}_2\text{O}_2 \) represent the increase over the spontaneously adjusted level.

Figure 5. Dose-response curves for the elicited increase in extracellular \( \text{H}_2\text{O}_2 \) in suspensions of control cells and cells pretreated for 1 d with 0.5 mM SA. The increase in the \( \text{H}_2\text{O}_2 \) level induced by the indicated concentrations of fungal elicitor was determined 30 min after elicitor addition and corrected as for Figure 4. The numbers at the time points indicate the fold increase in SA-treated cells over control cells.
Salicylic Acid and the Regulation of Extracellular H$_2$O$_2$

SA-treated cell suspensions was evident in all experiments performed but was always within 10% of the control value. Thus, the increase in spontaneous and elicited extracellular H$_2$O$_2$ concentration caused by SA pretreatment appears not to be due to a decrease in H$_2$O$_2$ degradation by unknown enzymes but to an increased production. This H$_2$O$_2$

formation, however, must actually be far higher than reflected by the determined increase in concentration because it also has to counteract the concomitant H$_2$O$_2$ degradation. Because of the obvious concentration dependence of the degradation rate (Fig. 9), this notion becomes especially important after prolonged induction, when H$_2$O$_2$ concentrations in the range of 20 to 40 μM are reached.

DISCUSSION

Recently it was suggested that production of activated oxygen species in the course of plant defense reactions is likely to occur by electron transfer through the plasma membrane, resulting in extracellular reduction of oxygen (Mehdy, 1994). This view is based mainly on work with animal phagocytes that use a similar mechanism to produce primarily superoxide anions, which rapidly disproportionate to form the more stable H$_2$O$_2$ (Morel et al., 1991). In our elicitor experiments, superoxide appears not to be present in the routinely used supernatant of the cell suspension (compare the luminol and scopoletin methods in Table I). However, when exogenous SOD is present during spontaneous production or elicitation, the final concentration of H$_2$O$_2$ is slightly increased (Table I), presumably because some of the initially arising superoxide radicals normally undergo side reactions (e.g. with Fe$^{3+}$; Mehdy, 1994) but are trapped by the added SOD and thus stabilized as H$_2$O$_2$. This might indicate that at least a part of the H$_2$O$_2$ found in the parsley system arises from superoxide.
All substances used to induce an H$_2$O$_2$ burst were found to be more effective when the cells were pretreated with SA (summarized in Fig. 8). Mastoparan is a peptide that activates G proteins (Ross and Higashijima, 1994), which have been envisaged as a link between elicitor receptors and the H$_2$O$_2$-producing plant plasma membrane redox complex (Legendre et al., 1992, 1993; Morré et al., 1993; Mehdy, 1994). Chitosan presumably acts not by binding to complementary receptors but by disturbing general membrane properties, causing Ca$^{2+}$ influx and K$^+$ efflux, followed by callose and coumarin synthesis (Kauss, 1990). Amphotericin B can bind to plasma membrane sterols, which indicate the decrease in H$_2$O$_2$ concentration per 1 min for the corresponding intervals. In the case of the elicited cells, the numbers given were corrected by the elicited increase per min, given as numbers with positive signs.

Figure 9. Fate of exogenous H$_2$O$_2$ in elicited and nonelicited suspensions of control cells and cells pretreated for 1 d with 0.5 mM SA. At the dashed line, H$_2$O$_2$ was added to result in an increase of about 27 $\mu$M. Elicitation was performed at zero time with 20 $\mu$g/mL fungal elicitor. The numbers with a negative sign at the curves indicate the decrease in H$_2$O$_2$ concentration per 1 min for the corresponding intervals. In the case of the elicited cells, the numbers given were corrected by the elicited increase per min, given as numbers with positive signs.

be followed in the parsley suspension cells by Ca$^{2+}$-dependent callose formation (Kauss, 1990) and the synthesis and secretion of coumarin derivatives (Conrath, 1992). La$^{3+}$ mimics Ca$^{2+}$ binding to ion transport proteins and thereby disturbs ion transport at the plasma membrane. Under the conditions used, the mechanism of La$^{3+}$ action on elicited H$_2$O$_2$ production appears to be indirect, since it inhibits only after 2.5 min (Fig. 3, top) and can even transiently accelerate elicited H$_2$O$_2$ production, e.g. in control cells (Fig. 3). Taken together, the stimuli sent by all of the above compounds enter the signal transduction network at sites different from the fungal elicitor. In addition, SA pretreatment also enhances spontaneous H$_2$O$_2$ production regulated without an obvious exogenous stimulus (Figs. 1, 2, 6, and 8). The SA-induced increase in H$_2$O$_2$ production must, therefore, occur by action at a point in the signal network that is common to spontaneous regulation and induction by all of the exogenous inducers, probably at regulatory or catalytic peptides of the terminal enzyme complex that is responsible for H$_2$O$_2$ production. A similar conclusion can be drawn from the observation that the improvement caused by SA pretreatment is equally effective at low and at high elicitor concentrations (Fig. 5). In contrast, the increase in elicited secretion of coumarin derivatives and incorporation of cell wall phenolics was most pronounced at low elicitor concentrations and was associated with an enhanced synthesis of mRNA encoding certain phenylpropanoid enzymes (Kauss et al., 1992, 1993). In this case, it appears that rate-limiting steps (e.g. at the secretion process) exist behind the presumably improved transcription, whereas in the case of H$_2$O$_2$ elicitation, the increase appears to involve terminal reactions.

Protein kinase inhibitor K-252a added to parsley cell suspensions was active on spontaneous H$_2$O$_2$ production (Figs. 1, 2, and 8) as well as on H$_2$O$_2$ production resulting from the simultaneous addition of an inducer (Fig. 8) and was active even when added subsequent to fungal elicitor (Fig. 2). Taken together, these observations suggest that at least one critical protein involved requires steady phosphorylation for H$_2$O$_2$ production and is located not in the elicitor perception/transduction path but at the enzyme complex actually responsible for H$_2$O$_2$ production. This assumption is in accordance with the situation in phagocytes in which the plasma membrane NAD(P)H oxidase activity requires the binding of a phosphorylated 47-kD regulatory protein together with other cytosolic components, especially the small G protein Rac2 (Morel et al., 1991; Quinn et al., 1993). Our observation that rapid H$_2$O$_2$ induction by mastoparan and chitosan is only partly inhibited by the K-252a concentration used whereas the slower induction by fungal elicitor and amphotericin B is greatly inhibited (Fig. 8) may relate to the fact that in the former case K-252a was present for only a short time and, consequently, a regulatory protein that requires steady reprophorylation was down-regulated to a lesser extent.

We have used mastoparan mainly as a tool to show that conditioning by SA is associated with a reaction different from fungal elicitor perception (Figs. 6 and 8). These exp-
periments have brought one surprising additional result, namely that an optimal mastoparan dose is required, whereas elevated doses are apparently inhibitory (Fig. 7). Mastoparan was also used in vivo by Legendre et al. (1992, 1993) to induce activated oxygen species in soybean suspension cultures. In this case even the highest doses used were not inhibitory. It should be considered, however, that these authors used a different assay system in which the $\text{H}_2\text{O}_2$ produced was immediately trapped by endogenous cell wall peroxidases, most likely without building up considerable external $\text{H}_2\text{O}_2$ concentrations. The apparently inhibitory effect of elevated mastoparan concentrations in the parsley system may indicate the involvement of several processes mediated by $\text{G}$ proteins in regulation of the $\text{H}_2\text{O}_2$ level. However, experiments based solely on the use of mastoparan in vivo must be interpreted with caution (Ross and Higashijima, 1994).

SA is well known as an inducer of SAR and also appears to play a role in the establishment of general disease resistance in various plants (Delaney et al., 1994; Ryals et al., 1994). In addition to the systemic induction of certain pathogenesis-related proteins that seem causally linked to resistance, an enhancement of reactions caused locally by the pathogen stimulus has also been implicated in SAR (Siegrist et al., 1994). Siegrist et al. (1994) showed that in cucumber hypocotyls a pretreatment with SA or other SAR-inducing compounds leads, on subsequent infection, to an enhanced deposition of polymeric phenolics in the plant cell wall below the fungal appressorium and in the emerging papillae, a process likely requiring apoplastic $\text{H}_2\text{O}_2$ (Iiyama et al., 1994). It was indeed shown that halved cucumber hypocotyl segments rendered resistant by pretreatment with SA exhibit an enhanced elicited $\text{H}_2\text{O}_2$ production (Siegrist et al., 1994). These results imply that SA accumulated in a systemic manner in leaves of previously infected plants may similarly improve the $\text{H}_2\text{O}_2$ response during a subsequent challenge infection.

An enhancement of the $\text{H}_2\text{O}_2$ production is, however, not unique to SA. It has been shown that pretreatment of the parsley suspension culture with methyl jasmonate also enhances both spontaneous and elicited $\text{H}_2\text{O}_2$ production (Kauss et al., 1994). In this case also the $\text{H}_2\text{O}_2$ production on addition of mastoparan, chitosan, amphotericin B, and $\text{La}^{3+}$ is enhanced and can be inhibited by K-252a (data not shown), as in cultures pretreated with SA (Fig. 8). Methyl jasmonate can induce multiple developmental steps related to wounding and stress (Sembdner and Parthier, 1993; Reinbothe et al., 1994) but not the multicomponent defense complex of SAR (for citations, see Kogel et al., 1994; Siegrist et al., 1994). Nevertheless, SAR and wound-healing overlap in certain reactions, e.g. the deposition of polymeric cell wall phenolics. The parsley cell suspension appears to be a suitable model for future biochemical work on long-term regulation of apoplastic $\text{H}_2\text{O}_2$ production that is likely involved in both processes.