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Influence of Salicylic Acid on the Induction of Competence for H_2O_2 Elicitation[1]

Comparison of Ergosterol with Other Elicitors

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Hypocotyls from etiolated cucumber (*Cucumis sativus* L.) seedlings were gently abraded at their epidermal surface, and cut segments were used to study the rapid and transient elicitation of H_2O_2 by ergosterol, chitosan, mastoparan, and a polymeric fungal elicitor. Freshly abraded segments were only barely competent for any H_2O_2 production, but they developed this competence subsequent to abrasion. This process was enhanced by 2,6-dichloroisonicotinic acid and salicylic acid, which induced acquired resistance to fungal penetration in the epidermal cells. Enhancement of competence induction by salicylic acid was also evident for spontaneous H_2O_2 production and differed in degree for the various elicitors, indicating that mainly the enzyme complex producing H_2O_2, but also other components of the elicitation system, improved. Ergosterol, chitosan, and fungal elicitor also rendered the segments refractory to a second stimulation by the same compound, whereas mastoparan was inactive in this respect. The four elicitors also differed markedly in their ability to diminish or enhance H_2O_2 production by a second treatment with a different elicitor, indicating that several sites of the H_2O_2 elicitation system are subject to short-term regulation.

Infection of plants by fungal pathogens is in many cases initiated by the formation of appressoria at the epidermal surface. Differentiation of these fungal infection structures appears to require chemical and/or mechanical signals derived from the plant surface (Kolattukudy et al., 1995). The cuticle and outer epidermal cell wall beneath the appressorium are subsequently penetrated. This process can evoke two different syndromes of biochemical responses that represent a first line of plant defense at the level of the infected cell. The epidermal cell may either undergo a hypersensitive response associated with programed cell death or the cell wall can be reinforced at the site of attempted penetration. The latter process is associated with the arrest of penetrating fungal hyphae and, thus, survival of the attacked cell. This local reinforcement of the cell wall is called papillae formation. Papillae are microscopically visible as appositions at the cytoplasmic side of the cell wall and contain a variety of materials (callose, proteins, phenolic polymers, silicon). Papillae formation also includes the incorporation of autofluorescent lignin-like polymers into the existing cell wall around the appressoria (for refs., see Aist et al., 1988; Kauss, 1990; Nicholson and Hammerschmidt, 1992; Stein et al., 1993).

The degree of hypersensitive reaction development or papillae formation appears to be dependent on plant species, genetic background, and type of tissue. For example, in barley cultivars that are susceptible to powdery mildew infection, DCIA-induced SAR correlated with an increase in hypersensitive reactions of epidermal leaf cells, mimicking resistance that is governed by the barley *Mlg* resistance gene (Kogel et al., 1994). In contrast, in epidermal cells of barley coleoptiles carrying *mlo* genes, papillae formation appears to be the major defense reaction (Aist et al., 1988). It has been suggested for potato leaves that have been infected with *Phytophthora infestans* that papillae formation and the hypersensitive response are parts of a multistage process, since only those epidermal cells that failed to produce successful papillae appeared to subsequently undergo a hypersensitive response at a time when the fungal haustorium becomes visible (Freytag et al., 1994).

Many details concerning the biochemistry and regulation of plant defense reactions are known from elicitor studies with suspension cultures and wounded tissues (Dixon et al., 1994; Boller, 1995). Coordination of defense responses leading to the syndrome of hypersensitive response appears to involve reactive oxygen species (Levine et al., 1994; Tenhaken et al., 1995). However, little is known about the coordination of the induced defense responses leading to papillae formation, which includes callose deposition by the plasma membrane-localized 1,3-β-glucan synthase (Kauss, 1990) and the secretion of precursors of lignin-like phenolic polymers. For these two processes, the exact location could be correlated with the supply of fungal constituents at the site of attempted penetration (Kauss, 1990). However, the polymerization of cell-wall phenolics probably also requires the production of reactive oxygen species in a well-dosed manner to avoid hypersensitive cell death.

In cucumber (*Cucumis sativus* L.) plants infected by *Colletotrichum lagenarium*, the formation of successful papillae in epidermal cells of cotyledons, petioles, or true leaves is greatly increased in plants exhibiting SAR that has been

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Abbreviations: DCIA, 2,6-dichloroisonicotinic acid; SA, salicylic acid; SAR, systemically acquired resistance.

induced by previous infection (for refs., see Nicholson and Hammerschmidt, 1992; Stein et al., 1993; Siegrist et al., 1994). Formation of successful papillae involves a more rapid and increased deposition of various materials. This increased sensitivity of SAR plants is long known (Kuc, 1995) and might relate to an increase in sensitivity to fungal elicitors shown for suspension cultures pretreated with SA or DCIA (Kauss et al., 1992; Kauss and Jeblick, 1995). These substances are involved in the establishment of SAR in various plant species, although their mode of action appears to be more complex as hitherto assumed (Raskin, 1992; Klessig and Malamy, 1994; Ryals et al., 1994; Kuc, 1995).

We have recently used etiolated cucumber hypocotyls to correlate biochemically oriented classical elicitor experiments with the resistance of epidermal cells against fungal infection. It was found that this tissue acquires resistance to *C. lagenarium* when DCIA is applied to the roots of unwounded seedlings or when cut hypocotyl segments were preincubated with SA (Siegrist et al., 1994). As in cucumber plants exhibiting biologically induced SAR, we found in this case that resistance is associated with the inhibition of penetration into epidermal cells by the formation of papillae. In addition, little chitinase activity is present in the resistant hypocotyls but becomes induced during infection and thus possibly is a contributor to a complementary mechanism of resistance. Hypersensitive reactions of epidermal cells are occasionally observed but appear to play a minor role in the overall resistance of the etiolated cucumber hypocotyls.

Deposition of autofluorescent polymerized lignin-like phenolics in and above papillae very likely depends on H_2O_2 production. Therefore, it was expected that the resistant epidermal cells should be competent for elicitor-induced defense responses. Surprisingly, when hypocotyls were surface abraded to allow entrance of fungal elicitor, segments were barely able to produce H_2O_2 immediately after surface abrasion and cutting (Fauth et al., 1996). Instead, this capacity developed subsequent to abrasion and was greatly enhanced in segments that were previously supplied with DCIA systemically through the roots or with SA applied through the abraded surface. The induction of competence for H_2O_2 elicitation was inhibited by cycloheximide or puromycin, indicating a requirement for translational protein synthesis. Taken together, these results indicated that induction of competence for H_2O_2 elicitation requires a signal not previously considered, which is derived from abrasion-mediated cuticle breaching. In addition, the segment ends represent wounds that may also contribute to signaling, even though the cut surface represents only about 5% of the total segment surface and the elicited H_2O_2 is presumably released mainly through the abraded cuticle.

In the above studies (Fauth et al., 1996), we used a crude polymeric fungal elicitor preparation from *Phytophthora sojae*. Oligoglucans derived from the same fungus were also active as H_2O_2 elicitors, and it has been shown that the polymeric fungal elicitor also rendered the segments refractory against these oligoglucans. These results suggested that a polymeric glucan is the active component in the crude fungal elicitor preparation. For a further characterization of the competence induction process, we screened for other elicitors of H_2O_2 production. Our attention was focused on ergosterol, which has been shown by Granado et al. (1995) to cause a highly specific alkalinization response in suspension-cultured tomato cells and also rendered the cells refractory toward a second stimulation by ergosterol. These results suggested that plant cells can perceive ergosterol, the main sterol of most higher fungi.

In the present report we demonstrate that ergosterol represents a specific and potent elicitor of H_2O_2 production in abraded and conditioned cucumber hypocotyl segments. Comparison of ergosterol with a variety of other elicitors also shows that the induction of competence for H_2O_2 elicitation and its enhancement by DCIA and SA are processes common to a variety of elicitors, even though there are some differences with respect to the refractory state.

MATERIALS AND METHODS

Cucumber seedlings (*Cucumis sativus* L. cv Mervita) were grown in the dark for 4 to 5 d, and the hypocotyl was abraded with a polishing cloth, cut into segments of 2 cm, and washed as described by Fauth et al. (1996). These segments were used for experimentation either immediately (within about 1 h of collection) or after conditioning for 18 h in buffer containing antibiotics (Fauth et al., 1996). When supplied to the roots, DCIA was at a concentration of 100 μM. For conditioning the abraded segments, DCIA or SA was added to the buffer at a final concentration of 75 or 100 μM, respectively. These concentrations were shown previously to be saturating (Fauth et al., 1996).

The segments were washed in 10 mM Mes/KOH buffer at pH 6.5, and batches of 10 segments were floated in Petri dishes (3.5 cm in diameter) containing 3.0 mL of the same buffer. After the segments had adapted for about 1 h, elicitors were added and at the indicated times 100 μL of suspension buffer were immediately used to determine H_2O_2 by the luminol method (Fauth et al., 1996). When samples were taken at one time only (tables), the means of four parallel aliquots from one batch of segments are given. The SD between these four values was less than $\pm$5%. To calculate spontaneous H_2O_2 production, a blank performed with the suspension buffer was subtracted. For time-course studies (figures), a single aliquot was used per time point.

Unless otherwise stated, materials were as described by Siegrist et al. (1994), Kauss and Jeblick (1995), or Fauth et al. (1996), including a polymeric fraction prepared from cell walls of *P. sojae*, which is designed throughout this report as "fungal elicitor." All sterols and chitosan (degree of polymerization 3420, 22.3% *N*-acetylation; Kauss et al., 1989) were purchased from Sigma. Mastoparan, a synthetic peptide, was purchased from Bachem BioSciences (King of Prussia, PA), and the inactive analog Mas17 was obtained from American Peptide (Sunnyvale, CA). DCIA was kindly synthesized and checked for purity by GC-MS and elemental analysis by H.W. Breiner (our department). Xylanase was a commercial preparation from *Trichoderma viridae* (Fluka).

RESULTS

Ergosterol was found to be a potent elicitor of H_2O_2 synthesis in abraded cucumber hypocotyl segments that had been conditioned for 18 h in the presence of SA (Fig. 1). Induction was saturated at 1 nM, whereas some analogs exhibited no activity even at a 1000-fold higher concentration. Dehydroergosterol at 1 μM induced about 80% of the H_2O_2 burst when compared to the same concentration of ergosterol (data not shown). Since dehydroergosterol is chemically produced from ergosterol, induction by the compound may be due to an impurity of ergosterol in the commercial preparation. Therefore, we have not investigated further the H_2O_2 elicitation by dehydroergosterol.

The increase in extracellular H_2O_2 concentration was maximal at about 30 min after ergosterol application and then decreased rapidly thereafter (Fig. 2). A second dose of ergosterol, given at the 90-min point, did not evoke a further H_2O_2 burst, whereas segments pretreated with ergosterol were still fully reactive to fungal elicitor (Fig. 2) as well as to chitosan and mastoparan (data not shown). When the second dose of ergosterol was added 2 or 4 h after the first dose, a slight second H_2O_2 burst was induced but was retarded in time (Table I) when compared to the first one. Since it is not known to which extent the ergosterol that was added first was bound by the segments in the type of experiment shown in Figure 2, the second dose possibly caused only a step up in concentration. Thus, we have also washed the elicited segments with fresh buffer 90 min after the first ergosterol dose (Table I). The second dose of 10 nM ergosterol was then added 2 or 4 h after the first dose to these resuspended segments. Again, only a minor H_2O_2 peak was observed with ergosterol, but the segments still responded strongly to the fungal elicitor. These results confirmed that ergosterol rendered the segments refractory to a second stimulation by ergosterol and

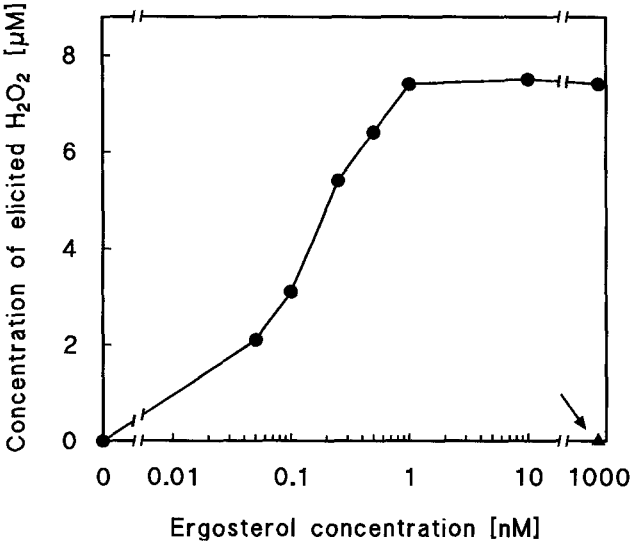

Figure 1. Dose-response curve and specificity of ergosterol for elicitation of H_2O_2 production. Abraded hypocotyl segments were conditioned for 18 h in the presence of 0.1 mM SA. After washing, batches of 10 segments were supplied with the indicated concentrations of ergosterol, diluted from a 1 mM stock in methanol (final solvent concentration 0.1%, v/v). The increase in H_2O_2 level after 30 min was determined by the luminol assay. The arrow pointing to ▲ indicates the values determined when ergosterol was replaced by either sitosterol, stigmasterol, lanosterol, or cholesterol. A representative of four similar experiments is given.

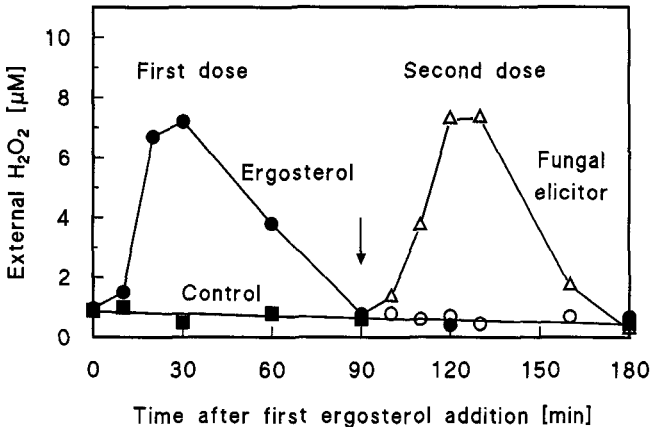

Figure 2. Time course for the increase in extracellular H_2O_2 elicited by ergosterol, which also renders the segments refractory to further treatment with ergosterol but not with fungal elicitor. Abraded segments were conditioned with SA as in Figure 1. One batch remained unelicited (■, control). Three batches of segments were supplied with 10 nM ergosterol (first dose, ●). Two of these samples that were elicited with ergosterol received, at the time indicated by the arrow, a second dose of elicitor, either 10 nM ergosterol (○) or 10 μg/mL fungal elicitior (△). The curve for fungal elicitor given as a first dose was similar in shape and height to that of the second dose.

Table I. *Induction and partial reversion of a refractory state for ergosterol-induced H_2O_2 production*

Ergosterol Treatment of Segments	H_2O_2 Burst Induced by			
	Ergosterol		Fungal elicitor	
	Conc.[a]	Max[b]	Conc.[a]	Max[b]
	μM	min	μM	min
Standard assay[c]				
First dose	10.6	30	14.1	30
Second dose, 2 h	0.5	50	13.4	30
Second dose, 4 h	0.3	40	12.1	30
Washing assay[d]				
First dose	14.7	30	—[e]	—[e]
Second dose, 2 h	1.1	40	8.5	30
Second dose, 4 h	1.4	40	6.5	30

[a] Concentration of H_2O_2 at the peak maximum. [b] Time at which the H_2O_2 burst reached its maximum. [c] Same standard experimental procedure as in Figure 2, except that the second dose of elicitors was given 2 or 4 h, respectively, after the first dose of ergosterol. [d] A 4-fold standard assay was elicited with 10 nM ergosterol as in Figure 2. The segments were then washed 90 min after elicitation on a funnel with 50 mL of suspension buffer, suspended in 12 mL of buffer, and then resuspended in four standard assays that were elicited by either 10 nM ergosterol or 10 μg/mL fungal elicitor 2 or 4 h, respectively, after addition of the first ergosterol dose. A representative of three experiments with similar results is shown. [e] —, Not determined, but presumably similar to standard assay.

also showed that the cells can obviously partly escape from the refractory state with time.

H_2O_2 production induced by mastoparan at an apparently saturating concentration has also been shown to be transient (Fig. 3). For this compound, however, a second dose of mastoparan readily caused another burst of about equal intensity. The segments also did not become refractory toward fungal elicitor treatment after mastoparan addition (Fig. 3). In contrast, the H_2O_2 burst induced by ergosterol given 2 h after mastoparan was retarded and less pronounced when compared to the burst induced when ergosterol was first given (Fig. 3).

Partly acetylated chitosan of high molecular weight was found to be saturating at 15 μg/mL as an elicitor of H_2O_2 production in the conditioned hypocotyl segments (data not shown). At this concentration, chitosan induced an H_2O_2 burst and also rendered the segments refractory to chitosan (Fig. 4). The response to fungal elicitor, given as a second dose after chitosan, was decreased. In this case, the response to ergosterol was slightly enhanced and that to mastoparan was strikingly enhanced when compared to when these compounds were applied as a first dose (Fig. 4).

The transient nature of H_2O_2 elicitation (Figs. 2–4) suggests that the hypocotyl segments not only produce but at the same time also degrade H_2O_2. Degradation rates were directly determined by adding exogenous H_2O_2 in the concentration range that was maximally reached by elicitation of the same segments (Fig. 5). With conditioned segments a similar initial decrease in H_2O_2 concentration was observed regardless of whether SA was present or

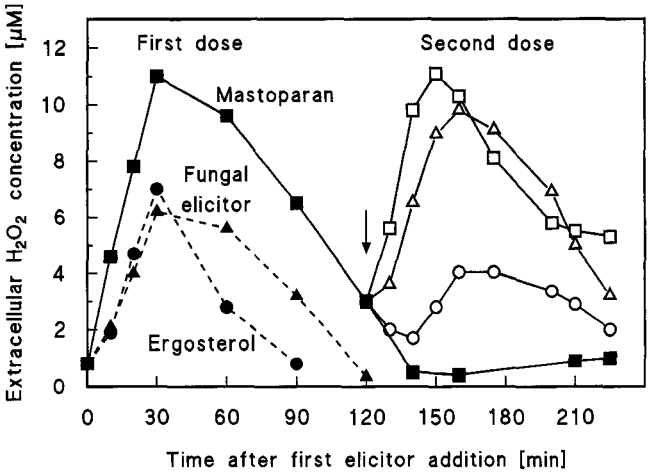

Figure 3. Time course for elicitation of H_2O_2 by the peptide mastoparan, followed by a second addition of elicitor, either mastoparan, ergosterol, or fungal elicitor. The same experimental procedure was used as for Figure 2. Four batches of segments were supplied with 1 μM mastoparan at time 0 (■), followed at the arrow in three of these batches by a second elicitor dose, 1 μM mastoparan (□), 10 nM ergosterol (○), or 10 μg/mL fungal elicitor (△). For comparison (dashed lines), two other batches of segments received the same concentrations of ergosterol (●) or fungal elicitor (▲) as a first dose only. The concentration of mastoparan used (1 μM) was optimal, since in the same batch of segments 0.5 μM mastoparan induced a H_2O_2 burst of 8.0 μM H_2O_2, whereas 2 μM of the compound caused a burst of 6.8 μM H_2O_2 with the same shape of the curves as for 1 μM.

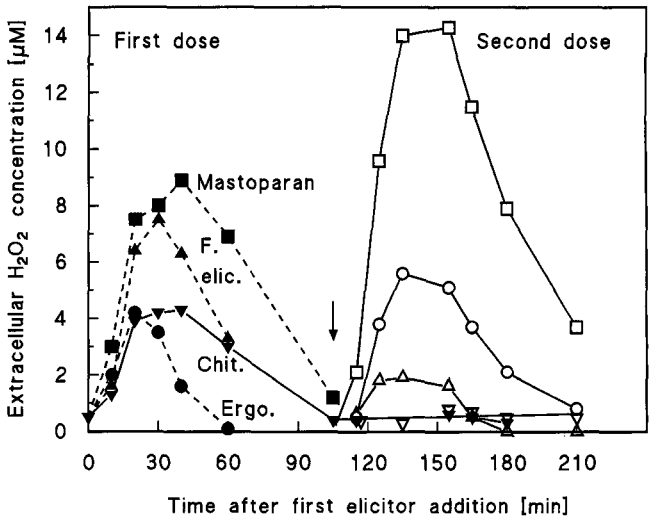

Figure 4. Time course for elicitation of H_2O_2 production by chitosan and differences in the induction of the corresponding refractory state for various elicitors. The same experimental procedure was used as for Figure 2. Batches of 10 segments were induced either by a first dose of chitosan (▼, 15 μg/mL) or for comparison (dashed lines) by ergosterol (●, 10 nM), mastoparan (■, 1 μM), or fungal elicitor (▲, 10 μg/mL). Four batches that were first induced by chitosan received a second dose of elicitor, chitosan (▽), ergosterol (○), mastoparan (□), or fungal elicitor (△) at the time indicated by the arrow. Compare Figures 2 and 3 and see text for variability of the efficiency of various elicitors among individual experiments.

absent during conditioning (Fig. 5B). This indicates that the approximately 3-fold higher H_2O_2 burst subsequently elicited by ergosterol does not result from a difference in degradation. The experiment also allowed us to estimate H_2O_2 production rates by assuming that at the culmination point degradation must equal production. Thus, the production rate can be estimated by providing the degradation rates (Fig. 5B, numbers in boxes) with a positive sign. It should be noted, however, that the actual production rate must be even higher at the ascending flank of the curves to result in a net increase in H_2O_2 concentration. Nevertheless, an approximately 2.5-fold higher rate of H_2O_2 evolution was found in this way in SA-conditioned segments when compared to segments without SA. The same experimental protocol was also applied to corresponding freshly abraded segments to demonstrate that despite a considerable capacity for H_2O_2 degradation, our assays also monitor a minor H_2O_2 burst observed in some batches of freshly abraded segments (Fig. 5A). This is mainly due to H_2O_2 degradation being faint in the low concentration range. Comparison of Figure 5A with Figure 5B elucidates the huge impact of the conditioning procedure. The H_2O_2 burst elicited with ergosterol was about 19- or 57-fold increased by conditioning the abraded segments with or without SA, respectively. The increase in the maximally elicited H_2O_2 concentration due to conditioning of the abraded segments is reflected by the great increases in the roughly estimated production rates (Fig. 5, numbers in boxes provided with positive signs).

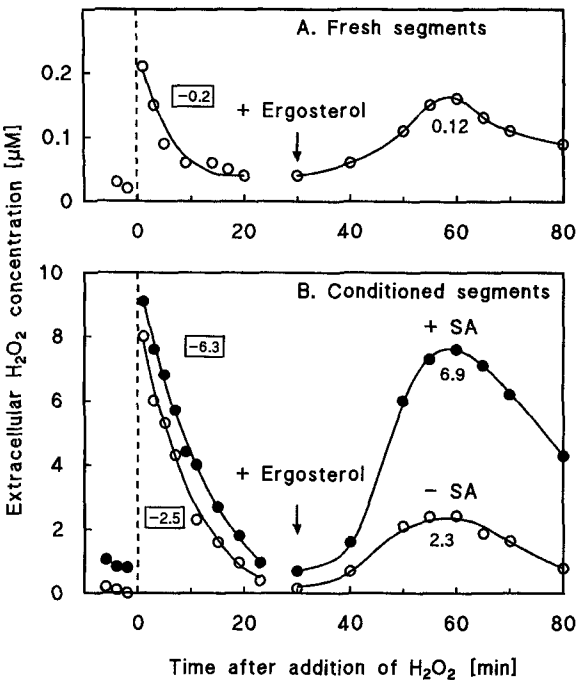

Figure 5. Degradation of exogenous H_2O_2 and subsequent elicitation by ergosterol of H_2O_2 evolution in freshly abraded hypocotyl segments (A) and in corresponding abraded segments that had been conditioned for 18 h (B) in the absence (− SA) or presence (+ SA) of 0.1 mM SA. Note the different scales of the ordinates. At time 0 (dashed line), H_2O_2 was added to an external concentration of either 0.22 μM (A) or 8.5 μM (B). After the H_2O_2 concentration returned to basal levels (about 20 min), the incubation volume was adjusted to 3.0 mL and ergosterol was added to 10 nM at time 30 min (arrow). The negative numbers in boxes indicate the approximate decrease in H_2O_2 (μM) per 10 min. This decrease was estimated from the slope of H_2O_2 degradation at the maximal H_2O_2 concentration that was subsequently elicited by ergosterol addition. At this culmination point, H_2O_2 degradation equals H_2O_2 production. Consequently, H_2O_2 evolution is represented by the numbers given in the boxes but with a positive sign. The numbers below the climax of the H_2O_2 curves indicate the increase in H_2O_2 concentration (μM) due to elicitation by ergosterol. The experiment shown represents the average of H_2O_2 elicitation (0.13 ± 0.15 μM; mean ± SD) from 20 determinations with freshly abraded segments from five different batches of seedlings. One of these batches was grown in the presence of 0.1 mM DCIA.

To compare the various elicitors within the same batch of segments, we had to change the experimental protocol. The values for the H_2O_2 concentration reached in elicited segments were corrected by values determined in parallel with a batch of nonelicited segments (Tables II and III). In this case, the elicited freshly abraded segments sometimes exhibited even slightly lower values than the nonelicited parallels, resulting in negative values for H_2O_2 elicitation. Therefore, in Tables II and III the means for H_2O_2 elicitation in freshly abraded segments are close to 0 and the SD masks the minor H_2O_2 production also observed in some of the experiments performed with this protocol, similar to the time-course studies reported in Figure 5A. When the abraded segments were conditioned for 18 h, they developed the ability for H_2O_2 elicitation by fungal elicitor, chitosan, ergosterol, and mastoparan (Table II). However, near-saturating concentrations of the four elicitors resulted in different levels of H_2O_2. Fungal elicitor and chitosan exhibited similar activity for H_2O_2 elicitation (Table II), whereas ergosterol was more active than fungal elicitor (Tables II and III). Mastoparan was the most potent elicitor under these experimental conditions (Table II). When SA was present during conditioning, the spontaneous H_2O_2 production as well as the induction of elicitor competence was greatly increased (Tables II and III). Under this condition, the four elicitors induced an H_2O_2 burst of comparable extent, with the consequence that the effect of SA was most pronounced for fungal elicitor and comparatively low for mastoparan. It should be noted that the variation between individual experiments (Tables II and III) mainly results from differences in the overall level of H_2O_2 production. These may be caused by differences in abrasion intensity and/or age of the seedlings (4 or 5 d) and by the fact that measurements at one time did not necessarily match the maximum of the burst and also did not account for peak broadness. However, the overall tendency for efficiency of SA expressed as an x-fold increase as in Table II was similar among individual experiments, although in some cases differences were found between the maximal level of H_2O_2 induced by different elicitors in SA-conditioned segments. For instance, in Figure 3 (first dose) fungal elicitor and ergosterol induced a comparable peak height, whereas in Figure 4 ergosterol was less active than fungal elicitor.

When DCIA was systemically present in the hypocotyl segments because of a prior application to the roots of 0.1 mM DCIA, a concentration sufficient to cause resistance to fungal penetration (Siegrist et al., 1994), the freshly abraded segments were not significantly competent for H_2O_2 elicitation by ergosterol (Table III). A similar result was shown previously for the fungal elicitor (Fauth et al., 1996) and is confirmed in Table III. The competence induced by conditioning was, however, enhanced in the segments from seedlings pretreated with DCIA. Similar results were found when chitosan or mastoparan were used as elicitors (data not shown). When DCIA was present in the buffer used for conditioning the abraded segments, it also enhanced the competence induction for fungal elicitor and ergosterol (Table III). Root pretreatment with 0.1 mM DCIA was, however, less effective than application of the saturating concentration of 0.075 mM DCIA to the abraded segments (Table III). This might be due to a lower local concentration in the epidermal cells when the substance is systemically distributed over the whole seedling.

In addition to the above elicitors, some additional compounds were tested for the ability to elicit H_2O_2 synthesis in abraded segments conditioned with SA. Mas17 did not induce considerable H_2O_2 production (Table II). Amphotericin B (20 μM), an inducer of an H_2O_2 burst in parsley suspension cultures (Kauss and Jeblick, 1995), increased the H_2O_2 level in the cucumber system within 30 min only by 3 to 4% of the value obtained with the fungal elicitor and then steadily increased the extracellular H_2O_2 concentra-

Table II. *Elicitation of H_2O_2 production in hypocotyl segments immediately after surface abrasion and in segments that had been conditioned for 18 h in the absence (−) or presence (+) of 0.1 mM SA*

Elicitor Used (concentration)	H_2O_2 Concentration Resulting from Segments			
	Freshly abraded	After conditioning for 18 h		
		−SA	+SA	(-fold; n)[a]
		μM[b]		
None (spontaneous)	0.0 ± 0.1	0.4 ± 0.2	1.8 ± 0.7	(×4.5; 9)
Fungal elicitor (10 μg/mL)	0.0 ± 0.1	1.7 ± 0.7[d,e]	8.7 ± 5.1	(×5.1; 12)
Chitosan (10 μg/mL)	0.1 ± 0.2	2.2 ± 1.1	7.1 ± 4.2	(×3.2; 8)
Ergosterol (10 nM)	−0.1 ± 0.1	3.4 ± 1.6[d]	7.3 ± 2.2	(×2.3; 9)
Mastoparan (1 μM)	0.0 ± 0.2	4.9 ± 2.5[e]	9.0 ± 2.6	(×1.7; 12)
Mas 17[c] (1 μM)	0.0 ± 0.1	0.0 ± 0.1	0.2 ± 0.2	(n = 5)

[a] Increase in elicited H_2O_2 levels by the presence of SA during conditioning; n = number of independent experiments performed with conditioned segments. [b] Concentration of H_2O_2 reached 30 min after elicitor addition. The spontaneously adjusted level of H_2O_2 was determined in parallel samples without elicitor. This value was subtracted to result in elicited H_2O_2 (means ± SD). For freshly abraded segments n was 5; see text for a remark on variability. [c] Inactive analog of mastoparan in which Leu^6 is replaced by Lys, and Ile^{13} is replaced by Leu. [d] Values significantly different at the P = 0.05 level. [e] Values significantly different at the P = 0.01 level.

tion over at least 4 h (data not shown). This substance was not further used since it did not induce a rapid and transient H_2O_2 burst to be compared to the four elicitors investigated in more detail. A crude xylanase preparation (100 μg/mL) induced at about the same H_2O_2 burst as fungal elicitor (data not shown). Xylanase was, however, not further explored in the cucumber hypocotyl system because the chemical nature of the active fraction in the crude xylanase elicitor preparation is hitherto unknown.

DISCUSSION

H_2O_2 elicitation by fungal elicitor in segments of freshly abraded cucumber hypocotyls was found previously to be nearly zero (Fauth et al., 1996), even when the seedlings were rendered systemically resistant to infection by pretreatment with DCIA. Instead, elicitor competence developed subsequent to segment preparation. This observation was confirmed and extended in the present paper to three chemically different elicitors (Fig. 5; Tables II and III). The results suggest that the requirement of a stimulus derived from cuticle abrasion and a subsequent induction phase for H_2O_2 elicitation competence is not unique for the fungal elicitor but represents a more general process.

Table III. *Influence of DCIA and SA on the induction of competence for H_2O_2 elicitation in abraded hypocotyl segments*

Abraded Segments	H_2O_2 Concentration Elicitated with	
	Fungal elicitor	Ergosterol
	μM[a]	
Shortly after abrasion[b]		
Control seedlings grown on water	0.0 ± 0.1	−0.1 ± 0.1
Seedlings grown on 0.1 mM DCIA	0.0 ± 0.1	0.0 ± 0.0
Conditioned for 18 h[b]		
Control seedlings	1.6 ± 0.4[c]	2.6 ± 0.8[c]
Control + 0.1 mM SA	6.9 ± 1.5	6.6 ± 1.4
Control + 0.075 mM DCIA	5.7 ± 2.5	5.4 ± 1.2
Seedlings grown on 0.1 mM DCIA	3.9 ± 1.7	4.7 ± 2.7

[a] Elicitation, determination, and calculation of elicited H_2O_2 as in Table II. [b] For experiments shortly after abrasion, n = 4; for conditioned segments, n = 6. [c] Values differ significantly at the P = 0.05 level.

With any of the four elicitors, induction of competence for H_2O_2 elicitation is further enhanced by the presence of SA during conditioning (Fig. 5B; Tables II and III). In tobacco, the two major cellular H_2O_2-scavenging enzymes, catalase (Chen and Klessig, 1991; Conrath et al., 1995) and ascorbate peroxidase (Durner and Klessig, 1995), can be inhibited by SA and DCIA. Thus, it appeared possible that the enhancement of the H_2O_2 burst in conditioned cucumber segments is due to inhibition of H_2O_2 degradation by traces of SA or DCIA, which might be present in the tissue from the previous treatment. The degradation rates for H_2O_2, however, are very similar for segments that have been conditioned either in the absence or in the presence of SA (Fig. 5B). This fact and the increase in the rates of H_2O_2 production in SA-pretreated segments (Fig. 5B, numbers in boxes provided with a positive sign) clearly show that in cucumber hypocotyls enhancement by SA of H_2O_2 elicitation is due to increased production competence rather than to inhibition of H_2O_2-degrading enzyme activities. In fact, inhibition by SA and DCIA of catalase appears not to be a general mode of action of these two compounds among different plant species since they did not inhibit catalase activity in an in vivo assay with cultured parsley cells (U. Conrath, personal communication). The concept that SA acts in pathogen defense by inhibiting H_2O_2-scavenging enzymes has also been challenged by the observation that at the high concentrations often used SA appears to be a general inhibitor of iron-containing enzymes (Rüffer et al., 1995).

Based on pH measurements with tomato cell suspensions, Granado et al. (1995) suggested that plants may have a sensory system for the recognition of ergosterol, which is a characteristic component of many fungi. Our observation that in the conditioned cucumber hypocotyl segments ergosterol represents a potent and specific elicitor for H_2O_2 production further supports this suggestion and links the ergosterol response to plant defense reactions against

pathogens. Tomato cell suspensions treated with ergosterol also became refractory to a second stimulation by ergosterol (Granado et al., 1995). We have made a similar observation for the elicitation of H_2O_2 in cucumber (Fig. 2), supporting the assumption of a regulated ergosterol signal perception system by which plant cells might recognize invading fungi.

Ergosterol appears to be recognized by a specific complementary receptor. For instance, stigmasterol is fully inactive (Fig. 1), although it differs from ergosterol only by one methyl group and one double bond (for structural formulas, see Granado et al., 1995). The involvement of specific receptors is also evident from the observation that the refractory state induced by ergosterol still allows elicitation with the fungal elicitor (Fig. 2). It has previously been shown that the refractory state induced by the fungal elicitor also rendered oligoglucans derived from the same fungal cell walls inactive (Fauth et al., 1996). This result suggests that both compounds are sensed by a common specific receptor. The crude fungal elicitor preparation, therefore, appears to contain a polymeric glucan as the active compound (Fig. 6). Specific receptors possibly also exist for chitosan (Fig. 4, see discussion below). In contrast, mastoparan is considered to mimic the activated side of receptors and thus causes GDP/GTP exchange at heterotrimeric G proteins (Ross and Higashijima, 1994). These authors postulated that experiments solely based on the use of mastoparan indicate but do not necessarily prove that heterotrimeric G proteins may be involved in a given physiological response. Nevertheless, the negative result with Mas17 (Table II) indicates that induction of the H_2O_2 burst by mastoparan in the conditioned hypocotyl segments is specific for a peptide sequence. Mastoparan is a venom component of the wasp *Vespula lewisii*, which is able to induce an H_2O_2 burst in various plant cells (for refs., see Kauss and Jeblick, 1995) even though mastoparan is not likely to occur in plant pathogens. The action of mastoparan is, therefore, presumably independent of specific plant receptors. This assumption is in accordance with our observation that mastoparan does not render cucumber segments refractory to mastoparan (Fig. 3). The induction of competence for mastoparan-induced H_2O_2 production (Table II) thus at least indicates that components downstream of specific receptors become upgraded (Fig. 6). It appears possible that this applies to constituents of the plasma membrane NAD(P)H oxidase complex that is assumed to be responsible for production of H_2O_2 (Mehdy, 1994). It has been proposed from experiments with parsley suspension cultures and the protein kinase inhibitor K-252a and La^{3+} that this enzyme is under the control of an autoregulatory system involving protein phosphorylation and Ca^{2+} (Kauss and Jeblick, 1995). Upon conditioning of cucumber segments, elicitor-independent spontaneous H_2O_2 production also becomes evident and is further enhanced by the presence of SA (Fig. 5; Table II). These results also support the assumption that the competence induction process includes the H_2O_2-producing oxidase complex. However, it is very likely that this is not the only switch at which SA modulates the H_2O_2 elicitation system. After conditioning without SA (Table II), H_2O_2 elicitation by fungal elicitor, chitosan, and ergosterol was low when compared to that of mastoparan, indicating that reactions associated with presumed receptors might be rate limiting under this condition and become preferentially improved in the presence of SA (Table II).

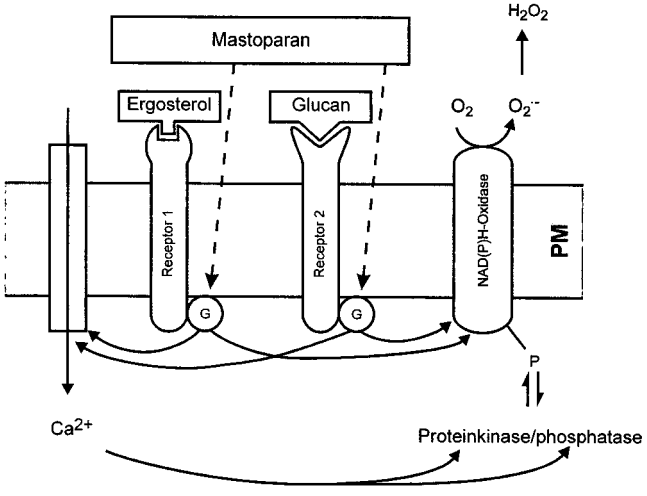

Figure 6. Scheme of the H_2O_2 elicitation system that is induced upon conditioning of surface-abraded cucumber hypocotyl segments. The development of spontaneous H_2O_2 production and the high elicitor activity of the presumed G protein-binding peptide mastoparan indicate that part of competence induction is related to the enzyme system reducing O_2 at the plasma membrane (PM) surface. This enzyme complex presumably is a superoxide/H_2O_2-forming NAD(P)H oxidase (Mehdy, 1994), which appears to be under self-regulation involving steady protein phosphorylation/dephosphorylation and Ca^{2+} (Kauss and Jeblick, 1995). Conditioning in the presence of SA or DCIA further tunes up this enzyme and also reactions associated with the perception of specific elicitors, including ergosterol and fungal glucan for which respective receptors are shown here. Additional specific receptors might exist for chitosan. A complicated pattern of refractory states is induced by the different elicitors, indicating mutual regulatory influences at both the receptor and oxidase levels. These details are not depicted in the scheme but are discussed in the text.

The present report also adds a new aspect to the action of chitosan as an elicitor. This compound originates from partial deacetylation of chitin and is a component of many fungal cell walls. Chitosan has been used for elicitation of a variety of presumed plant-defense responses but little is known about the interaction with its target plant cells (for refs., see Kauss, 1990; Hadwiger et al., 1994). For the induction of callose in *Catharanthus* cell suspensions (Kauss et al., 1989) and of callose and furanocoumarins in parsley suspension cells (Conrath et al., 1989), activity of chitosan increased with molecular weight and decreased with the degree of acetylation. It has been suggested, therefore, that ionic interference of chitosan with general membrane constituents may initiate these responses. Elicitation of H_2O_2 by chitosan in the conditioned hypocotyl segments appears to be more specific as chitosan-treated segments become refractory to a second dose of chitosan and exhibit altered sensitivity to mastoparan as well as to the pre-

sumed receptor-specific fungal elicitor (Fig. 4). These results encourage further studies with the aim of identifying complementary chitosan membrane receptors.

Induction of a desensitized state by particular elicitors has been observed in various suspension cultures (for refs., see Fauth et al., 1996). Comparison of the refractory states induced by several elicitors in the same system, namely the conditioned hypocotyl segments used in the present report, allows some conclusions about the mechanisms involved in short-term regulation of H_2O_2 elicitation. With fungal elicitor (Fauth et al., 1996), ergosterol (Fig. 2), and chitosan (Fig. 4), the cells became refractory to a second stimulation by the same but not by other elicitors, suggesting a mechanism that is selective at the level of the presumed individual receptors. This assumption fits with the observation that mastoparan does not render the segments refractory to the inducing agent (Fig. 3). The transient nature of the mastoparan-induced burst in turn indicates an additional regulatory point downstream of receptors.

A similar conclusion can be drawn from the observation that the H_2O_2 burst induced by mastoparan can be further enhanced by a previous treatment with chitosan (Fig. 4). Obviously, an inhibitory group, possibly at the level of the superoxide-forming oxidase complex (Mehdy, 1994), is partly released by chitosan action and thereby tunes up the reaction to subsequently applied mastoparan. However, this latter regulatory feature also decreases elicitation by ergosterol when given after mastoparan but not elicitation by fungal elicitor (Fig. 3). Thus, the receptor-associated regulatory sides differ between the two elicitors with regard to a cross connection with the side governing down-regulation of the mastoparan-induced H_2O_2 burst. Chitosan given as a first dose similarly decreases the H_2O_2 elicitation by fungal elicitor but increases the response to mastoparan (Fig. 4), making the story even more complicated. Thus, down-regulation of H_2O_2 elicitation by the various compounds appears to occur at several sites that cooperate in multiple ways. The physiological significance of this complex regulatory pattern may be that in situ elicitation of H_2O_2 by various fungal constituents presumably has to occur in a well-balanced manner to allow polymerization of cell-wall phenolics but simultaneously has to avoid the hypersensitive response, a situation observed in epidermal cells of the cucumber hypocotyl that were rendered resistant to fungal penetration by root treatment with DCIA (Siegrist et al., 1994).

When introduced systemically into the hypocotyls, DCIA is by itself not sufficient to induce competence of the epidermal cells for fungal elicitor and ergosterol (Table III), chitosan, or mastoparan (data not shown). In contrast, an additional stimulus resulting from surface abrasion and/or cutting of the segments is required, which induces the competence for subsequent elicitation of H_2O_2 by the four elicitors, a process upgraded in combination with DCIA or SA (Fig. 5; Tables II and III). DCIA can mimic and SA can act as an endogenous inducer of SAR in many plant species (Raskin, 1992; Klessig and Malamy, 1994; Ryals et al., 1994). In segments of cucumber hypocotyls, DCIA or SA also induce SAR, which in the case of infection with *C. lagenarium*, is associated with the formation of papillae (Siegrist et al., 1994). Thus, the enhancement of competence for the H_2O_2 defense response elicited by various fungal compounds in epidermal cells of cucumber hypocotyls correlates with the development of acquired resistance to fungal penetration. It has been speculated recently (Fauth et al., 1996) that abrasion of the epidermal surface may mimic its local breakdown during fungal penetration. If the latter occurs at a cell containing DCIA supplied through the roots or SA endogenously present because of biologically induced SAR, improved competence induction would give the epidermal cell a better chance to react promptly to the production of H_2O_2, which presumably is one of the materials required to deposit successful papillae, thereby assisting penetration resistance. Further studies should reveal whether competence induction shown here for H_2O_2 elicitation also affects elicitation of other responses presumably contributing to the resistance of the cucumber hypocotyl epidermis.

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