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Cucumber Hypocotyls Respond to Cutin Monomers via Both an Inducible and a Constitutive H_2O_2-Generating System[1]

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Hypocotyls from etiolated cucumber (*Cucumis sativa* L.) seedlings were gently abraded at their surface to allow permeation of elicitors. Segments from freshly abraded hypocotyls were only barely competent for H_2O_2 elicitation with fungal elicitor or hydroxy fatty acids (classical cutin monomers). However, elicitation competence developed subsequent to abrasion, reaching an optimum after about 4 h. This process was potentiated in seedlings displaying acquired resistance to *Colletotrichum lagenarium* due to root pretreatment with 2,6-dichloroisonicotinic acid or a benzothiadiazole. Induction of competence depended on protein synthesis and could be effected not only by surface abrasion, but also by fungal spore germination on the epidermal surface or by rotating the seedlings in buffer. Inhibitor studies indicated that the inducible mechanism for H_2O_2 production involves protein phosphorylation, Ca^{2+} influx, and NAD(P)H oxidase. In contrast, a novel cucumber cutin monomer, dodecan-1-ol, also elicited H_2O_2 in freshly abraded hypocotyls without previous competence induction. This finding suggests the presence of an additional H_2O_2-generating system that is constitutive. It is insensitive to inhibitors and has, in addition, a different specificity for alkanols. Thus, dodecan-1-ol might initiate defense before the inducible H_2O_2-generating system becomes effective.

When plant cells interact with potential pathogens, they often produce active oxygen species. The biochemical basis for this rapid defense response has been elucidated mainly by applying elicitors derived from pathogens to plant cell suspension cultures. The major source for active oxygen species appears to be an NAD(P)H-oxidase system that is associated with the plasma membrane (Baker et al., 1997; Lamb and Dixon, 1997; Alvarez et al., 1998; Blumwald et al., 1998). This enzyme complex is directly linked to the elicitor signaling cascade and reduces molecular oxygen to $O_2^{\cdot -}$, which is rapidly dismutated to the more stable H_2O_2.

To investigate whether the features elaborated with cell culture models are of significance for the resistance of whole-plant tissues against pathogens, we have used etiolated cucumber (*Cucumis sativa* L.) seedlings that can be infected by *Colletotrichum lagenarium* (Siegrist et al., 1994). SAR is induced in the hypocotyl by root pretreatment with INA. By doing so, the SAR inducer initially does not come into direct contact with the pathogen attacking from the epidermal surface. Salicylic acid could not be used to induce SAR via the roots of entire cucumber seedlings because the millimolar concentrations required caused phytotoxic effects (Kästner et al., 1998).

SAR in etiolated cucumber hypocotyls is manifested as an inhibition of fungal penetration through the outer epidermal cell wall (Siegrist et al., 1994). Hypersensitive reactions are very rare in this tissue; essentially all attacked epidermal cells remain alive. We have described up to now two locally triggered defense complexes associated with SAR in the hypocotyls. One is the formation of papillae, which includes a very localized deposition of lignin-like phenolics into the plant cell wall around the fungal appressoria (Siegrist et al., 1994). Phenolic deposition is already evident prior to penetration of the epidermal cell wall, indicating that epidermal cucumber cells exhibiting SAR are able to perceive, at very early time points, one or more signals derived from fungal attack. This cytological observation has recently been confirmed at the molecular level (Kästner et al., 1998). Systemic-resistant cucumber hypocotyls contain only low amounts of chitinase mRNA prior to infection. However, chitinase transcript levels are greatly enhanced upon infection with *C. lagenarium*. Induction of mRNA occurs before appressorium formation and is also observed with a melanin-deficient mutant fungus that can barely penetrate the epidermal cell walls. It has been shown with antibodies that the apoplastic chitinase is indeed produced prior to penetration (Kästner et al., 1998). Thus, the timely induction of chitinase beneath the outgrowing fungal spores appears to be the second defense response contributing to arrest of fungal penetration into the systemic-resistant epidermal cells.

Cucumber hypocotyls represent a convenient material for comparing elicited defense responses in susceptible and systemic-resistant epidermal cells. For the application of elicitors, we had to gently abrade the cuticle to make it permeable. Segments cut from freshly abraded tissues are, however, only barely competent for rapid H_2O_2 elicitation with FE, ergosterol, chitosan, or chitin oligomers (Fauth et al., 1996; Kauss and Jeblick, 1996; Kauss et al., 1997). However, competence develops once the abraded cut segments

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Abbreviations: BTH, benzo-(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester; DDO, dodecan-1-ol; DHSA, threo-9,10-dihydroxystearic acid; DPI, diphenylene iodonium; FE, fungal elicitor preparation from cell walls of *Phytophthora sojae*; HPA, 16-hydroxypalmitic acid; INA, 2,6-dichloroisonicotinic acid; SAR, systemic acquired resistance.

are rotated in buffer for a certain time period. We refer to this procedure as "conditioning." Elicitation competence in cucumber hypocotyls thus is not constitutive, but requires physiological changes induced by surface abrasion. Requirement for conditioning subsequent to surface abrasion was also shown for etiolated hypocotyls or epicotyls from another six plant species using partially acetylated chitosan as an universal H_2O_2 elicitor (Kauss et al., 1997).

Induction of competence for the H_2O_2 response is reminiscent of observations with soybean cotyledons, which develop competence for elicitation of glyceollin and phenolic cell wall polymers only in cell layers close to the cut surface or adjacent to the site of elicitor injection (Graham and Graham, 1994, 1996). In the abraded cucumber hypocotyls, it is the conditioning process that is enhanced by systemically supplied INA or by salicylic acid applied to the abraded segments (Fauth et al., 1996; Kauss and Jeblick, 1996). We and others (Mur et al., 1996; Thulke and Conrath, 1998) refer to such an enhancement of locally triggered defense responses in cells primed by SAR inducers as "potentiation."

Induction of elicitor competence is suppressed by the presence of cycloheximide and puromycin during the conditioning period and, thus, appears to require protein synthesis (Fauth et al., 1996). Taken together, our results showed that intact plant tissues require an additional stimulus derived from surface abrasion as a prerequisite to develop a functional H_2O_2 elicitation system by a process potentiated under SAR conditions.

The early response of resistant epidermal cells to germinating fungal spores (Kästner et al., 1998) suggested, as a working hypothesis, that the cuticle might play a role in signaling. This idea was sustained by the observation that alkaline hydrolysates from cutin of either cucumber hypocotyls or leaves can elicit H_2O_2 production in abraded and conditioned hypocotyl segments (Fauth et al., 1998). The cutin hydrolysates are rich in DDO and also contain some hydroxy fatty acids, which, due to their small amounts, have not been identified. We used the hypocotyl segment system to screen a large collection of authentic hydrocarbons for H_2O_2 elicitation. Hydroxy groups, epoxy groups, and double bonds are important features for the H_2O_2 elicitation potency of fatty acids (Fauth et al., 1998). Short-chain alkanols, including the novel cucumber cutin monomer DDO, are also active at H_2O_2 induction. In addition, the cucumber surface wax and certain alkanols and hydroxy fatty acids also enhance the activity of H_2O_2 elicitors of fungal origin (FE, ergosterol, and chitosan; Fauth et al., 1998). Thus, the epidermal cells of cucumber hypocotyls can perceive and respond to monomers derived from their cutin and/or components of their surface wax layer.

In the present report we show that upon conditioning of entire cucumber seedlings after hypocotyl abrasion, induction of competence for elicitation with FE and hydroxy fatty acids is much more rapid compared with conditioning of cut segments. With this improved conditioning protocol, we could show that the induction of elicitor competence occurs not only after surface abrasion but also when fungal spores germinate on the epidermal surface. The inducible H_2O_2-generating system is potentiated under SAR conditions and exhibits properties similar to the NAD(P)H-oxidase system characterized in detail in suspension culture models. In addition, cucumber hypocotyls can generate H_2O_2 by another pathway that is constitutively present but only responds to the cucumber cutin monomer DDO.

MATERIALS AND METHODS

Cucumber (*Cucumis sativus*) seedlings were grown in closed plastic boxes in the dark for 4 to 5 d (Fauth et al., 1996). The cultivar Mervita was used routinely, and the cultivars Mepram and Bimbo were used for comparison. Abrasion of the hypocotyls was performed with a slurry of SiC (Fauth et al., 1998; Kästner et al., 1998) and the seedlings were conditioned on a rotary shaker (80 rpm) in 10 mM Mes/KOH buffer, pH 6.5. The volume varied with the amount of seedlings. For example, for 20 to 30 seedlings, 50 mL of buffer was used in a beaker of 8.5 cm in diameter. Under these conditions, the seedlings showed little motion even though the buffer rotated. The seedlings were briefly washed under running tap water. Segments (3 cm) were subsequently cut from the seedlings, starting about 1 cm above the root crown. Five segments were used for the H_2O_2 assay in 3.5 cm Petri dishes containing 3 mL of the above buffer. The dishes were rotated (140 rpm) and elicitors were added after a 45 min adaptation period which was necessary as shortly after cutting an extra H_2O_2 burst is evident especially in INA-pretreated seedlings. This burst is generated by segment handling. DHSA, HPA, and DDO were added from a DMSO stock solution (final solvent concentration 0.2%, v/v). In this case, the controls without elicitor which were run in parallel contained DMSO at the same concentration. At the indicated times, 100 μL of the buffer was removed and H_2O_2 was determined by ferricyanide-catalyzed oxidation of luminol as described previously (Fauth et al., 1996). For determination of H_2O_2 degradation, exogenous H_2O_2 (10 μM) was added in the absence of the elicitor and the initial rate of decrease in concentration was determined (Kauss and Jeblick, 1996).

For SAR induction, the cucumber seeds were germinated on paper towels wetted with suspensions of formulated INA or BTH at an inducer concentration of 100 μM unless stated otherwise. Infection experiments and scoring of penetration by *C. lagenarium* were performed as described by Siegrist et al. (1994). Induction of elicitor competence with the *C. lagenarium* melanin-deficient mutant was as described by Kästner et al. (1998) for induction of chitinase mRNA. For unknown reasons, the spores of the mutant strain in some periods formed clumps, causing uneven and scarce germination. Such experiments were not included in this paper.

The various elicitors were bought or prepared as described by Fauth et al. (1998). Formulated INA and BTH (under the trade name Bion) were kindly supplied by Novartis (Basel, Switzerland). Seeds were from a local store. Cycloheximide and anisomycin were from Sigma. The latter was applied from a methanolic stock solution that was freshly prepared every day because it appeared to lose its activity within a week. The final methanol con-

centration in the sample and respective controls was 0.1% (v/v).

RESULTS

An Inducible H_2O_2-Generating System Develops after Surface Abrasion and Responds to FE and Hydroxy Fatty Acids

Hypocotyl segments from freshly abraded cucumber seedlings cannot respond to FE with H_2O_2 production (Fig. 1, 0 time point). In contrast, when the entire hypocotyl-abraded seedlings were rotated for some time in buffer, the subsequently cut segments responded to FE with H_2O_2 generation, exhibiting a burst maximum at 30 to 45 min after elicitor addition (Fig. 1). This induction of competence for H_2O_2 elicitation with FE was complete within 3 to 4 h, whereas in the previously used cut segments, at least 10 h were required for an optimal effect (Fauth et al., 1996). Although the absolute level of H_2O_2 elicitation varied between individual experiments, the H_2O_2 burst was further elevated in all experiments performed when the seedlings were pretreated at the roots with either INA or BTH (see legend of Fig. 1). The induced elicitor competence was found to be transient. In the example shown in Figure 1,

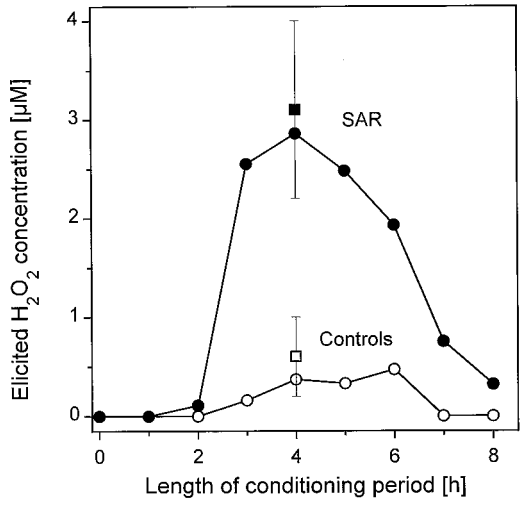

Figure 1. Surface abrasion and a subsequent conditioning period are required for H_2O_2 elicitation by FE. Cucumber seedlings were grown either on water (control; white symbols) or in the presence of INA or BTH (SAR; black symbols). Their hypocotyl surface was gently abraded and segments were either cut immediately (0 time) or after rotating the whole abraded seedlings in buffer for the indicated time periods (conditioning). The segments were adapted for 45 min in the H_2O_2 elicitation assay before the addition of 20 μg mL^{-1} FE. The H_2O_2 concentration at the burst maximum (45 min after elicitation) was corrected against values determined in a parallel batch of segments without elicitor. The curves (○ and ●) refer to one representative experiment in which SAR was induced by root pretreatment with INA. For the 4-h time point, means ± SD are given from 15 experiments with controls (□) and 13 experiments with seedlings rendered systemic resistant by root pretreatment with BTH (■). For further notes on the variability in the time course of elicitation competence induction, see text.

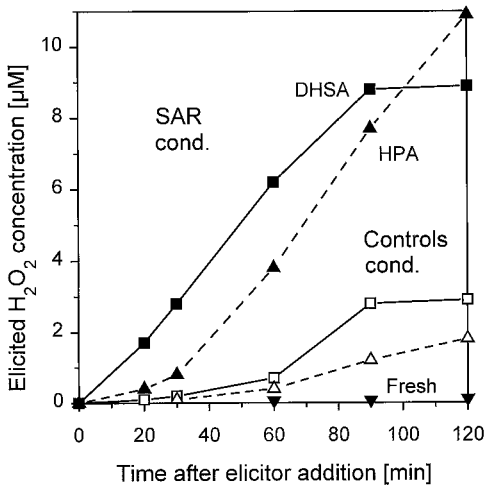

Figure 2. Influence of a conditioning period on the time course of H_2O_2 elicitation by the hydroxy fatty acids DHSA and HPA. Seedlings were either grown on water (controls; □ and △) or on INA (SAR; ■, ▲, and ▼). Segments were cut from hypocotyls of freshly abraded seedlings (▼) or from seedlings conditioned for 4 h subsequent to abrasion (■ and ▲). DHSA (□ and ■) or HPA (△ and ▲) were used as elicitors at 50 μM. In freshly abraded segments only the values from SAR seedlings elicited with DHSA are shown (▼), but those from control segments elicited with any of the two elicitors were similarly low. One representative experiment is given. In additional experiments, the absolute level of the H_2O_2 burst maximum (120 min) differed for susceptible (control) segments between 1 and 4 μM, and for systemic resistant segments between 3 and 10 μM. An increase in the elicited H_2O_2 burst due to SAR induction was seen in any individual experiment performed. For instance, upon elicitation with DHSA in four independent experiments with INA this increase was 3.2 ± 1.2-fold, and in seven experiments with BTH it was 2.4 ± 0.8-fold. For further notes on the variability between experiments, see text.

elicitor competence in INA-treated seedlings remained near maximal for about 2 h, and this result was similar in a total of five experiments performed.

In two additional experiments the elicitor competence was maximal only for about 1 h, and in another experiment it took 6 h to reach the maximum (data not shown). As we routinely had to restrict conditioning to one competence induction time (namely 4 h), it appears possible that in individual experiments the maximum of elicitor competence was either not yet reached or no longer evident. This fact may at least in part explain the apparently great variability in the level of H_2O_2 elicitation between various experiments (Fig. 1). Variability between experiments also likely relates to a further observation. The rate of degradation of exogenous H_2O_2, for unknown reasons, can differ by a factor of up to 2 between different batches of seedlings (data not shown). As H_2O_2 degradation occurs concomitantly with H_2O_2 generation, the absolute level of H_2O_2 concentration reached at the burst maximum only partly reflects actual H_2O_2 generation.

Hydroxy fatty acids also barely elicited H_2O_2 in freshly abraded segments, as exemplarily shown for DHSA and HPA in Figure 2. With both of these hydroxy fatty acids as the elicitor, a 4-h conditioning period induced elicitor com-

petence, with a further potentiation in SAR hypocotyls (Fig. 2). As in the example shown in Figure 2, a major H_2O_2 burst maximum was reached in all experiments at 2 to 2.5 h post elicitation. In some of the experiments we observed an additional minor peak or shoulder at about 30 min, which was not considered further in this report. DHSA and HPA were used throughout this report as commercially available models for classical cutin hydroxy fatty acids. From the other oxygenated fatty acids found active under previously used conditions (Fauth et al., 1998) we confirmed H_2O_2 elicitor activity under the new conditioning protocol for 12-epoxylinoleic acid and 13-hydroxy-9Z,11E-octadecadienoic acid (data not shown).

In the presence of cycloheximide during conditioning, subsequent H_2O_2 elicitation by FE was fully suppressed (Table I). This observation confirms our previous results with conditioning of cut segments (Fauth et al., 1996). Full inhibition of the process leading to competence for FE was also found for anisomycin, another inhibitor of translational protein synthesis, and for the competence to elicit H_2O_2 generation with DHSA (Table I). These results indicate that the induction of competence for H_2O_2 elicitation both by FE and DHSA requires protein synthesis.

Similar to INA, root pretreatment with BTH also potentiated induction of H_2O_2 elicitation competence at the hypocotyls of cucumber seedlings (Figs. 1 and 2). It was previously shown that this tissue becomes resistant to *C. lagenarium* upon root application of INA (Siegrist et al., 1994). We investigated, therefore, whether BTH is also effective at SAR induction in the etiolated cucumber hypocotyls. *C. lagenarium* penetrated the outer epidermal cell walls of water-grown seedlings beneath 61.2% ± 11.4% of the appressoria formed, whereas in BTH-grown seedlings the penetration rate was reduced to 1.0% ± 1.6% ($n = 5$). In the same experiments, papillae were observed in water-grown controls only beneath 3.9% ± 4.0% of the appressoria, whereas in BTH-grown seedlings 55.8% ± 10.2% of the appressoria were associated with papillae. Thus, systemically provided BTH primed the epidermal cells of cucumber hypocotyls for successful formation of papillae. That BTH can induce SAR is known for various other plants and pathogens (Görlach et al., 1996; Sticher et al., 1997).

Table I. *Influence of protein synthesis inhibitors on conditioning for subsequent elicitation of H_2O_2*

Protein synthesis inhibitors were present during the 4-h conditioning period of hypocotyl-abraded seedlings that were root pretreated with BTH to induce SAR. Subsequently, hypocotyl segments were cut and elicited with either 20 μg mL^{-1} FE or 50 μM DHSA. The H_2O_2 concentrations reached burst maxima at 45 min with FE and at 2 h with DHSA. Means ± SD from five independent experiments are given relative to controls (100%).

Inhibitor Used	H_2O_2 Elicitor Used	
	FE	DHSA
	%	
Cycloheximide (10 μM)	−0.3 ± 1.6	2.9 ± 3.0
Anisomycin (25 μM)	0.0 ± 1.6	9.0 ± 5.2
Anisomycin (50 μM)	–[a]	0.2 ± 1.7

[a] –, Not determined.

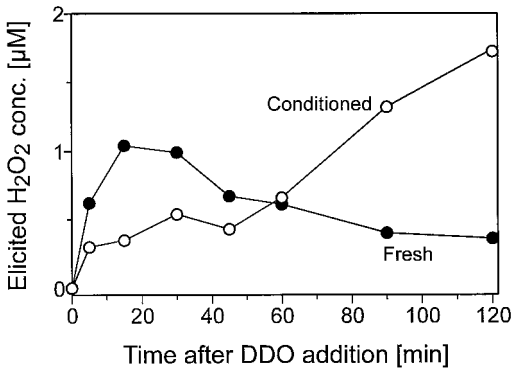

Figure 3. Time course for H_2O_2 elicitation by the novel cucumber cutin monomer DDO. Hypocotyl segments were from either freshly abraded susceptible seedlings grown on water (●) or from seedlings grown on INA and conditioned for 4 h subsequent to abrasion (○). One experiment is given as an example. The variability between experiments is given in Table II.

DDO Elicits H_2O_2 Also by a Constitutive Mechanism

Cucumber cutin hydrolysate contains a high proportion of DDO (Fauth et al., 1998). This novel cucumber cutin monomer elicited H_2O_2 in cucumber hypocotyl segments that had been conditioned for 18 h (Fauth et al., 1998). Figure 3 documents that this also holds true for segments from abraded seedlings that were conditioned for 4 h according to the new protocol. In contrast to FE (Fig. 1) and hydroxy fatty acids (Fig. 2), DDO was also active when added to freshly abraded segments, reaching maximal H_2O_2 levels after only 15 to 20 min (Fig. 3). These results indicate the existence of another H_2O_2-generating system that is constitutive and only responds to stimulation with DDO.

The H_2O_2 level induced by DDO in freshly abraded systemic-resistant seedlings (pretreated with INA) reached only 71% ± 16% ($n = 6$) of the peak maximum observed with water-grown control seedlings (data not shown). When different cucumber cultivars were compared, the routinely used cv Mervita reached a maximum of 1.0 ± 0.3 μM ($n = 5$) whereas the cv Mepram reached 1.9 ± 0.1 μM ($n = 3$) and cv Bimbo came up to 2.4 ± 0.1 μM ($n = 3$). Interestingly, the latter two cultivars are F_1 hybrids, claimed by the breeders to be tolerant or resistant against several fungal pathogens. No considerable H_2O_2 production was induced by DDO in freshly abraded hypocotyl segments of squash (cv custard white), melon (cv Bastion), bean (cv Dufix), sunflower (cv unknown), or freshly abraded pea epicotyl segments (cv Rheinländerperle, data not shown).

The specificity of various alkanols for H_2O_2 elicitation in abraded cucumber hypocotyls is shown in Table II. The most interesting findings are that dodecan-1,2-diol exhibited no activity in freshly abraded segments, whereas the C_8 and C_6 mono-1-ols were about as active as DDO. In contrast, in segments from conditioned seedlings, dodecan-1,2-diol was about twice as active as DDO, whereas the C_8 and C_6 mono-1-ols had significantly weaker activity than DDO (Table II). Thus, the constitutive H_2O_2-generating

Table II. *Specificity of various fatty alcohols for H_2O_2 elicitation in segments from freshly abraded or conditioned cucumber hypocotyls*

Segments cut from freshly abraded hypocotyls of water-grown seedlings were incubated in the presence of 50 μM fatty alcohols and the H_2O_2 concentration was determined after 30 min (see Fig. 3). The number of carbon atoms of the alcohols is given in parentheses. Means ± SD from three independent experiments are given. Ethanol had no considerable activity. Seedlings grown on BTH were abraded at the hypocotyl and the entire seedlings were conditioned for 4 h. Segments were subsequently cut and treated as above. The H_2O_2 concentration was determined after 90 min. Means ± SD from five independent experiments are given.

Fatty Alcohol		H_2O_2 Production in Abraded Segments	
		Fresh	Conditioned
		μM	
Tetradecan-1-ol	(14)	0.3 ± 0.1	0.1 ± 0.1
DDO	(12)	1.4 ± 0.6	3.0 ± 1.2
Decan-1-ol	(10)	1.3 ± 0.4	2.0 ± 0.6
Octan-1-ol	(8)	1.6 ± 0.5	0.4 ± 0.3
Hexan-1-ol	(6)	1.2 ± 0.3	0.1 ± 0.1
Butan-1-ol	(4)	0.2 ± 0.0	–[a]
Dodecan-1,2-diol	(12)	0.0 ± 0.0	6.2 ± 1.4
Dodecan-1,12-diol	(12)	1.2 ± 0.2	2.3 ± 0.4
8,10-Dodecadien-1-ol	(12)	1.4 ± 0.4	1.6 ± 0.4

[a] –, Not determined.

system differs from the inducible mechanism with respect to elicitor specificity.

The Inducible and the Constitutive H_2O_2-Generating Mechanism Differ in Inhibitor Sensitivity

To further characterize the two H_2O_2 elicitation mechanisms in the cucumber hypocotyl physiologically, we took advantage of inhibitors with known effects in suspension culture systems. For elicitation with FE (Fig. 1) and hydroxy fatty acids (Fig. 2), the abraded seedlings required a conditioning time of 4 h to develop a functional H_2O_2-generating system. This inducible pathway for H_2O_2 generation was fully inhibited by 10 μM DPI, 1 μM K-252a, and 0.8 mM La^{3+} (Fig. 4). In contrast, H_2O_2 elicitation by DDO in freshly abraded segments was not affected by these inhibitors (Fig. 4), indicating that the constitutive system consists of enzymes that are not involved in the inducible H_2O_2 system.

The level of H_2O_2 generated with DDO in freshly abraded segments was increased by NaN_3 and KCN (Table III). These compounds inhibited the degradation of exogenously supplied H_2O_2, indicating the participation of peroxidase and/or catalase in H_2O_2 degradation. Thus, the increase in the level of H_2O_2 due to the presence of KCN and NaN_3 likely results from the inhibition of H_2O_2 degradation, which occurs concomitantly with H_2O_2 production.

In conditioned segments, the action of DDO was only partly inhibited by DPI and La^{3+} (Fig. 4). These inhibitors fully suppress the inducible mechanism, which is evident from their effect on elicitation with FE and DHSA. Thus,

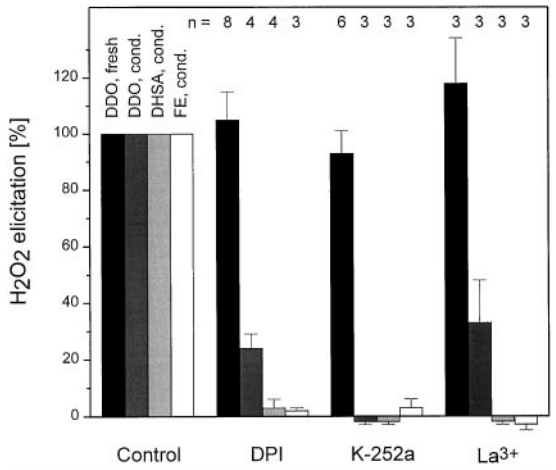

Figure 4. H_2O_2 elicitation with DHSA, DDO, and FE is differently inhibited in freshly abraded and conditioned hypocotyls. Segments from either freshly abraded control hypocotyls or from systemic-resistant (INA) abraded seedlings that had been conditioned for 4 h were used. DPI (10 μM), K-252a (1 μM), or La^{3+} (0.8 mM) were applied 15 min prior to elicitation in assays performed as for Figures 1 to 3. With freshly cut segments, H_2O_2 elicitation was determined 20 min after DDO addition. In conditioned seedlings, H_2O_2 elicitation was determined 2.5, 2, or 1 h after addition of DHSA, DDO, or FE, respectively. The number (n) of individual experiments performed is given in the top. Negative values indicate that in addition to a full inhibition of the elicited H_2O_2 production, the slight non-elicited H_2O_2 production was also affected by the respective inhibitor.

the observation that a part of DDO-elicited H_2O_2 is resistant to DPI and La^{3+} indicates that in conditioned segments both the inducible and the constitutive pathway contribute to H_2O_2 production by DDO. Interestingly, H_2O_2 generation by DDO in conditioned segments was fully suppressed by the protein kinase inhibitor K-252a, whereas in fresh segments this inhibitor was inactive (Fig. 4). Thus, conditioned hypocotyls differ from freshly abraded segments in their sensitivity to K-252a.

Induction of Elicitor Competence by Germinating Fungal Spores

With the new conditioning protocol, the competence for elicitation of H_2O_2 with FE and DHSA was induced within

Table III. *Influence of KCN and NaN_3 on the generation of H_2O_2 with DDO and on the degradation of exogenously supplied H_2O_2 in freshly abraded segments from water-grown seedlings*

Segments were incubated for 30 min with 50 μM DDO (see Fig. 3). The inhibitors were added 15 min prior to DDO. Means from five independent experiments ± SD are given in relation to controls. In absence of DDO, 10 μM H_2O_2 was added into the assay and the H_2O_2 concentration determined in 1 min intervals.

Inhibitor	H_2O_2	
	Generation	Degradation
	%	
None (controls)	100	100
KCN (0.1 mM)	228 ± 51	41 ± 5
NaN_3 (0.1 mM)	293 ± 94	31 ± 10

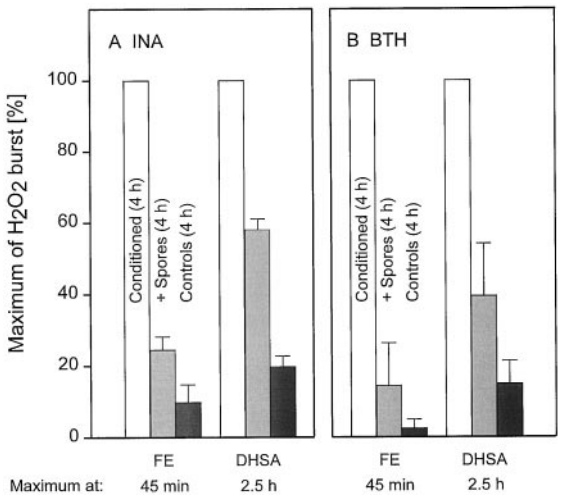

Figure 5. Germinating spores of a melanin-deficient *C. lagenarium* mutant and rotating the seedlings in buffer can partially replace surface abrasion with regard to the stimulus needed for induction of elicitor competence. SAR was induced in the seedlings by root pretreatment with either 100 μM INA (A) or 40 μM BTH (B). As in Figure 1, the hypocotyls of one batch of seedlings were abraded and the seedlings conditioned for 4 h. The other part of the seedlings was rotated in a suspension of spores that adhere to the surface and germinate during the 4-h period. A third part of seedlings (controls) was rotated for 4 h in buffer only. Both the samples with spores and the controls were abraded after rotating for 4 h. Segments from all three batches were then cut and elicited with either FE (20 μg mL^{-1}) or DHSA (100 μM). Means ± SD from three (A) or five (B) independent experiments are given. Note that in contrast to the freshly abraded segments used in Figures 1 and 2, the controls also exhibited considerable elicitor competence. In four independent experiments similar to B, we confirmed 6 months later that the stress presumably caused by rotating the nonabraded seedlings in buffer can indeed induce some elicitor competence. In these experiments, freshly abraded hypocotyls directly from the growth box exhibited 1.0% ± 0.7%, whereas the buffer-treated hypocotyls had 8.7% ± 3.2% competence for elicitation with FE compared with abraded and conditioned seedlings (100%).

about 4 h of conditioning in entire SAR cucumber seedlings abraded at their hypocotyls (Fig. 1). Within the same time period, outgrowing spores of a melanin-deficient mutant strain of *C. lagenarium* induces expression of chitinase in systemic-resistant epidermal cells, indicating an early plant/microbe interaction (Kästner et al., 1998). We therefore determined whether germinating spores might also induce H_2O_2 elicitor competence in nonabraded seedlings. Figure 5 shows that, indeed, within 4 h after spore application the competence for H_2O_2 elicitation with FE and DHSA was significantly higher than in controls without spores. It is especially noteworthy that in these controls, in which the nonabraded seedlings were rotated in buffer only, the H_2O_2 elicitor competence was also significantly higher compared with freshly abraded seedlings taken directly from the growth box (legend of Fig. 5).

DISCUSSION

When cucumber hypocotyls were abraded at their surface to allow permeation of elicitors, freshly cut segments barely exhibited H_2O_2 generation with FE (Fig. 1) and hydroxy fatty acids (Fig. 2). Elicitor competence rapidly developed subsequent to abrasion in a time-dependent process that was fully suppressed by established inhibitors of translational protein synthesis (Table I). These results indicate that the induction of H_2O_2 elicitor competence involves synthesis of as yet unknown proteins that are rate-limiting in native epidermal cells.

The H_2O_2-generating mechanism induced in the hypocotyls during conditioning of abraded seedlings was characterized using a pharmacological approach (Fig. 4). H_2O_2 elicitation by FE and DHSA was completely inhibited by La^{3+}, K-252a, and DPI, indicating that influx of Ca^{2+} and protein phosphorylation are involved in signal transmission and that the plasma membrane-located NAD(P)-oxidase complex likely produced $O_2^{\cdot -}$ as an intermediate for the evolving H_2O_2. The above features are all hallmarks of the elicitation of reactive oxygen species in cell suspension cultures (Hammond-Kosack and Jones, 1996; Baker et al., 1997; Lamb and Dixon, 1997; Blumwald et al., 1998; Keller et al., 1998). Thus, the H_2O_2 elicitation system that responds to FE and hydroxy fatty acids and is induced subsequently to surface abrasion of cucumber hypocotyls appears to be similar to the system reported in suspension cultures.

For the H_2O_2-generating system induced on conditioning, FE is active at a rather low concentration, especially since a glucan present only in minor amounts is the active fraction (Fauth et al., 1996). In contrast, elicitation of H_2O_2 with DHSA and HPA was just evident at 2 μM and was saturated at about 25 μM (data not shown). Therefore, a hydroxy fatty acid concentration of 50 μM that may appear quite high was routinely used (Fig. 2). However, these compounds form micelles that likely enter the scratches produced in the hypocotyl cuticle poorly and/or can hardly diffuse through the apoplast. In addition, a large amount of the lipid material is likely bound to the hydrophobic segment surface. Thus, the nominal concentration of the lipids probably does not reflect the actual concentration at the plasma membrane.

The induction of competence for H_2O_2 elicitation during conditioning was even further enhanced in hypocotyls containing systemically supplied INA or BTH (Figs. 1 and 2). The inhibition of fungal penetration of the outer epidermal cell wall of these systemic-resistant cucumber seedlings involves formation of papillae that contain lignin-like polymerized phenolics. Phenolic polymers are also incorporated into the existing plant cell wall, visible as a "halo" around the appressoria (Siegrist et al., 1994; Fauth et al., 1996). The polymerization of cell wall phenolics likely requires the production of H_2O_2. In fact, the production of reactive oxygen species beneath fungal appressoria has recently been shown in barley (Thordal-Christensen et al., 1997; Hückelhoven and Kogel, 1998). Thus, the physiological relevance of the observation that INA and BTH potentiate the process leading to H_2O_2 elicitation competence (Figs. 1 and 2) correlates with the potency of systemic-resistant epidermal cells to readily react with the formation of effective papillae.

The development of H_2O_2 elicitation competence in the cucumber hypocotyl is initiated by a stimulus created upon surface abrasion (Fauth et al., 1996; Figs. 1 and 2). In the present report we have optimized the conditioning protocol to avoid any "wounding" in the classical sense. Essentially no epidermal cells become stainable with Evan's blue when abrasion is gently performed (Kästner et al., 1998), indicating that no cells have been destroyed. Nevertheless, breaching the cuticle with the abrasive also impairs the integrity of the epidermal cells, even though a severe damage of the protoplast does not occur. However, it appears possible that abrasion might provide some type of mechanical stimulation, followed by the production of as yet unknown signal compounds. The results in Figure 5 provide a first hint that the inductive abrasion can be replaced to a certain extent by rotating the seedlings for 4 h in buffer. It is possible that the sheering forces resulting from rotating the buffer constantly over the epidermal surface might be sufficient to create some stimulus. It is of interest in this context that a local mechanical stimulation imposed by placing a needle to a suspension-cultured parsley cell can induce defense responses including production of reactive oxygen species (Gus-Mayer et al., 1998).

The induction of H_2O_2 elicitor competence in cucumber hypocotyls was further enhanced when spores of the melanin-deficient *C. lagenarium* mutant adhered and germinated on the epidermal surface (Fig. 5). The induction efficiency was not as pronounced as that after abrasion, likely because only a smaller part of the cell surface was engaged compared with abrasion, which likely disturbs a larger part of the cuticle. Nevertheless, the adhesion of spores and the outgrowth of the germ tubes on the surface in situ might be recognized by the attacked epidermal cell, and thus induce competence for H_2O_2 elicitation concomitantly with the first availability of elicitors derived from either the fungus or from degradation of the plant's cuticle by esterases. The fact that the epidermal cells of cucumber recognize fungal attack at this early time point is evident from the induction of chitinase mRNA (Kästner et al., 1998). Induction of plant defense responses in the absence of penetration has recently also been observed with a nonpathogenic mutant of *Magnaporthe grisea* (Xu et al., 1998).

In contrast to FE and hydroxy fatty acids, the novel cucumber cutin monomer DDO elicited H_2O_2 synthesis in freshly abraded hypocotyl segments (Fig. 3; Table II). This H_2O_2 production was not inhibited by DPI, K-252a, or La^{3+} (Fig. 4), indicating a constitutive mechanism that clearly differs from the NAD(P)H-oxidase pathway requiring induction by conditioning. The enhanced level of H_2O_2 generated with DDO in the presence of KCN and NaN_3 is to be expected because of the inhibition of H_2O_2 degradation under the same conditions (Table III), and argues against the participation of peroxidase in H_2O_2 generation from DDO, one of the most discussed possibilities distinct from the NAD(P)-oxidase system (Hammond-Kosack and Jones, 1996).

The inducible mechanism responded to dodecan-1,2-diol, whereas this alkanol was inactive in freshly abraded hypocotyls (Table II). Thus, the additional hydroxyl group added at the second C atom of DDO fully prevents H_2O_2 production by the constitutive mechanism, suggesting that DDO may directly serve as a substrate for a H_2O_2-producing enzyme. The wax storage vacuoles in jojoba beans contain a fatty acid ester of DDO, as well as an enzyme that can use O_2 and DDO to produce the respective aldehyde with a stoichiometry that suggests that the other product of the reaction may be H_2O_2 (Moreau and Huang, 1979). If a similar enzyme were responsible for H_2O_2 production from DDO in cucumber, in the conditioned seedlings it must have the remarkable property of being under regulation by Ca^{2+}-independent protein phosphorylation. This can be concluded from the full inhibition by K-252a but not by La^{3+} of the DPI-resistant part of the H_2O_2 elicited by DDO in conditioned hypocotyls (Fig. 5). The fate of further products eventually arising from DDO remains unclear. Nevertheless, the constitutive H_2O_2-generating system may operate in attacked epidermal cells of cucumber before the inducible mechanism discussed above becomes functional. The H_2O_2 produced from the cucumber cutin monomer DDO may act as a systemic signal, as is increasingly discussed for other systems (e.g. Alvarez et al., 1998; Chamnongpol et al., 1998). Additional pathways for H_2O_2 production distinct from the NAD(P)H-oxidase system have also been discussed as possibly playing a role in plant/microbe interactions, e.g. the apoplastic oxalate oxidase (Lane, 1994), peroxidase (Hammond-Kosack and Jones, 1996), and amine oxidase (Rea et al., 1998; Tipping and McPherson, 1995).

Taken together, our results show that the mechanism of H_2O_2 elicitation in plant tissues is more complex than was hitherto assumed from studies with suspension cultures. A further stimulus is required to render the NAD(P)H-oxidase system functional by a type of short-term developmental process potentiated under SAR conditions. The successful defense of pathogens obviously requires coordination of rather complex and diverse responses. In cucumber these events may involve an additional constitutive system for H_2O_2 production from the cutin monomer DDO, which possibly represents an early product from the plant/pathogen interface and may cover the time range before the inducible H_2O_2 generation system becomes functional.

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