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Competence for Elicitation of H_2O_2 in Hypocotyls of Cucumber Is Induced by Breaching the Cuticle and Is Enhanced by Salicylic Acid[1]

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To study H_2O_2 production, the epidermal surfaces of hypocotyl segments from etiolated seedlings of cucumber (*Cucumis sativus* L.) were gently abraded. Freshly abraded segments were not constitutively competent for rapid H_2O_2 elicitation. This capacity developed subsequent to abrasion in a time-dependent process that was greatly enhanced in segments exhibiting an acquired resistance to penetration of their epidermal cell walls by *Colletotrichum lagenarium*, because of root pretreatment of the respective seedlings with 2,6-dichloroisonicotinic acid. When this compound or salicylic acid was applied to abraded segments, it also greatly enhanced the induction of competence for H_2O_2 elicitation. This process was fully inhibited by 5 μM cycloheximide or 200 μM puromycin, suggesting a requirement for translational protein synthesis. Both a crude elicitor preparation and a partially purified oligoglucan mixture from *Phytophthora sojae* also induced, in addition to H_2O_2 production, a refractory state, which explains the transient nature of H_2O_2 elicitation. Taken together, these results suggest that the cucumber hypocotyl epidermis becomes conditioned for competence to produce H_2O_2 in response to elicitors by a stimulus resulting from breaching the cuticle and/or cutting segments. This conditioning process is associated with protein synthesis and is greatly enhanced when substances able to induce systemic acquired resistance are present in the tissue.

The interaction of plant cells with potentially pathogenic microorganisms is associated with rather complex biochemical and physiological events. Experiments on these processes are, therefore, often performed with simplified systems in which the pathogen is reduced to "elicitors" and the plant to a mechanically wounded tissue surface or to cell-suspension cultures. These models have already contributed many insights into the mechanisms of plant responses presumably related to defense but still require refinement to better cover the diverse biological features of host/pathogen interactions. It should be considered, for instance, that several signals may act subsequently in the course of pathogenesis and that the nutritional status, age, or a previous infection of a plant can influence the effectiveness of defense responses.

SAR in cucumber (*Cucumis sativus* L.) plants has long been known to be associated with enhanced papillae formation (for citations, see Stein et al., 1993). This implies a requirement for increased production of various cell-wall materials induced by the challenge infection. We have recently shown that a parsley cell-suspension culture can be used as a model to study at the biochemical level relationships between the systemic defense strategy of plants and local elicitor-mediated defense responses (Kauss et al., 1992, 1994; Kauss and Jeblick, 1995). This suspension culture does not give optimal responses to fungal elicitors when routinely grown. Pretreatment for 1 d with SA or other compounds related to SAR conditions the culture to increased sensitivity and responsiveness to fungal elicitors, resulting in enhanced expression of phenylpropane enzymes, secretion of coumarin derivatives (phytoalexins), deposition of cell-wall phenolics, and H_2O_2 production. These results indicate that, in addition to preformed "pathogenesis-related" proteins such as chitinase and 1,3-β-glucanase, SAR might indeed also imply a type of developmental improvement of diverse local defense reactions, triggered by the invading pathogen.

To verify whether the conditioning effects demonstrated with the parsley cell-suspension model indeed have significance for the resistance of plant tissues against pathogens, we have recently used segments from dark-grown cucumber hypocotyls that can be infected by *Colletotrichum lagenarium* (Siegrist et al., 1994). SAR was induced by growing the seedlings in the presence of DCIA or by pretreatment of the cut segments with SA. In this case, similar to biologically induced SAR in cucumber plants (for citations see Stein et al., 1993), resistance appears to be due mainly to inhibition of fungal penetration into epidermal cells, which is associated with the formation of papillae below fungal appressoria. These papillae contain autofluorescent phenolics in addition to callose. Phenolics are also deposited in the existing cell wall around the attempted site of penetration. In addition, little chitinase is present before infection in the cucumber hypocotyls rendered resistant with DCIA, but this enzyme activity greatly increases concomitantly

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

with the inhibition of fungal penetration (Siegrist et al., 1994). Thus, resistance to fungal attack in the pretreated cucumber hypocotyls is associated with an increased response of several coordinated and biochemically diverse defense reactions. This conclusion is in accordance with the results of elicitor experiments performed with cucumber hypocotyl segments. They are cut and preincubated overnight with DCIA or SA, subsequently split along their axes, and then treated with fungal elicitors (Siegrist et al., 1994). Both the rapid production of activated oxygen species and the long-term incorporation of cell-wall phenolics were enhanced in the resistant segments.

The cucumber hypocotyl segment system as described above has several disadvantages. DCIA can be applied through the roots and has been shown to mimic biologically induced SAR in many respects (Kessmann et al., 1994). Nevertheless, DCIA is a chemically prepared plant protection substance and it appeared desirable to compare it further with SA, an endogenous inducer of SAR (Delaney et al., 1994; Ryals et al., 1994). Previously, SA could be used only with cut cucumber hypocotyl segments and 1 mM was required to induce full resistance (Siegrist et al., 1994). In the present study, we introduced hypocotyl segments that were gently abraded at their cuticular surface to allow diffusion of water-soluble substances, e.g. exogenous SA at low concentrations. This procedure is widely used in experiments on extension growth (Schopfer, 1993) and also overcomes a drawback of the previously used cucumber hypocotyl system, namely elicitation of split segments, in which mainly parenchyma cells were exposed to the elicitors. The latter is remote from the natural situation, in which resistance appears to result mainly from the inhibition of fungal penetration through the outer cell walls of epidermal cells (Siegrist et al., 1994). As in the abraded segments mainly the epidermal cells become accessible to the elicitors, the exogenous inducers now exert their action on those cells in which the defense reactions occur on fungal infection.

Abrasion of the cuticle for its "permeabilization" has been combined in the present study with the determination of elicited active oxygen species. This rapid elicitor response was selected to properly separate the long-term conditioning effects from the elicitation step simply by timing. Most of the active oxygen species, which likely arise from primarily formed superoxide anions, have a very short half-life, whereas H_2O_2 is more stable (Mehdy, 1994; Auh and Murphy, 1995). H_2O_2, therefore, is most likely the only active oxygen species that accumulates to a significant concentration in the buffer used to suspend the abraded hypocotyl segments. In the experiments described, the apoplastic H_2O_2 diffuses out through the abraded cuticle. However, during penetration attempts under a fungal appressorium, H_2O_2 would accumulate in situ and fulfill multiple functions in defense, e.g. as a toxic agent, in the polymerization of cell-wall components, and in the induction of secondary defense reactions (Mehdy, 1994).

We show here that competence for rapid H_2O_2 elicitation is not constitutive but develops subsequent to abrasion of the cucumber hypocotyl cuticle. This process is greatly enhanced when DCIA is systemically present in the tissue or when SA is applied to the abraded segments during the conditioning phase.

MATERIALS AND METHODS

Preparation of Hypocotyl Segments

Seeds of cucumber (*Cucumis sativus* L. cv Mervita) were placed on paper towels wetted with 50 mL of water in closed plastic containers and grown in the dark at 24°C. After 5 d the hypocotyls routinely used were 6 to 8 cm long. To induce SAR in unwounded seedlings, the paper was wetted throughout the growth time with 0.1 mM DCIA in water.

The cotyledons were removed and the hypocotyl surface was abraded as described by Schopfer (1993) for maize coleoptiles. As in this report, we routinely used a Vitex polishing cloth (No. 364 rouge; Vereinigte Schmirgel-und Maschinenfabriken AG, Hannover, Germany) but confirmed some results with two water-resistant polishing papers (P 2000, AC 58 [C.F. Schröder Corp., Düsseldorf, Germany] and [Wetordry Tri-M-ite 3M 737, 3M Corp., Neuss, Germany]). A 5-mm broad strip of polishing cloth was slightly moistened with water from the backside to make it smooth, placed as a loop around the hypocotyl above the root crown, and moved in the apical direction while applying gentle pressure. The loop was placed again at the initial point above the root grown but turned by 90° and moved as above. The Vitex abrasive routinely used left a red stripe on the surface, together with some liquid that had been pressed out of the tissue, allowing confirmation that the major part of the circumference of the hypocotyl segment was abraded. If segments were not sufficiently abraded, the procedure was repeated once or twice on the nonabraded regions. Routinely, two 2-cm segments were cut from 5-d-old abraded hypocotyls, starting about 1 cm above the root crown. The segments were collected in tap water over 1 to 2 h, during which the residue from the abrasive was washed off for the most part and the turgescence recovered.

To induce competence for H_2O_2 elicitation, the abraded segments were placed in 10 mM Mes/KOH buffer, pH 6.5, containing 10 μg/mL each of chloramphenicol, penicillin G, and streptomycin. When indicated, the solution also contained neutralized SA or DCIA used for conditioning of the abraded segments. The segments were then slowly rotated at room temperature in the dark for the indicated time, washed on a funnel with 10 mM Mes/KOH, pH 6.5, and used for H_2O_2 elicitation.

Determination of H_2O_2 and Resistance

Ten of the washed segments were randomly taken and placed in 3 mL of 10 mM Mes/KOH buffer, pH 6.5, in plastic Petri dishes of 3.5 cm diameter, slowly rotated for 15 to 30 min, and then treated with elicitor at various concentrations. After the indicated times, a 100-μL aliquot of the suspension medium was removed, and the H_2O_2 was assayed by ferricyanide-catalyzed oxidation of luminol (Siegrist et al., 1994). A calibration curve was constructed with H_2O_2 in the above buffer.

Resistance of the segments to *Colletotrichum lagenarium* was determined as described by Siegrist et al. (1994). Briefly, the segments were shaken for 8 h in a suspension of spores and then placed on sterile, wet paper for an additional 40 h. Two surface cuts from five segments were observed by phase-contrast microscopy and scored for successful penetrations (infection vesicles or primary hyphae visible under the appressoria in the epidermal cells). Fifty appressoria per cut (100 per segment) were scored; the percentage of penetrations ± SD is given.

Materials

Unless otherwise stated, materials were as described by Kauss et al. (1992, 1994) and Siegrist et al. (1994). All abrasives were purchased in a local auto supply shop. A crude elicitor fraction was prepared by autoclaving cell walls of *Phytophthora sojae* according to the method of Ayers et al. (1976). Glucan oligomer mixtures were liberated from *P. sojae* mycelial cell walls by limited acid hydrolysis (Sharp et al., 1984a, 1984b, 1984c) and eluted in the void volume (DP > 20) or soon after the void volume (DP 10–20) of a Bio-Gel P-2 column (Hahn et al., 1992). The two defined hepta-β-glucosides were chemically synthesized (Fügedi et al., 1987, 1988) and correspond to structures 1 and 5 of Cheong et al. (1991). Compound 1 is the highly active hepta-β-glucoside elicitor originally characterized by Sharp et al. (1984a, 1984b, 1984c), and 5 is almost inactive for glyceollin induction in wounded soybean cotyledons.

RESULTS

The cuticle of plant tissues is impermeable to water-soluble compounds. In plant growth studies this problem is usually overcome by gentle abrasion of the tissue surface. Schopfer (1993) recently published color pictures of abraded maize coleoptiles demonstrating that this technique produces abrasions in the cuticle, allowing an uptake of the vital stain neutral red to the epidermal and subepidermal cell layer within 5 to 10 min. The stain reached the inner tissue only slowly. Control staining with Evan's blue proved that, under standard conditions, only a few epidermal cells were destroyed. We have applied the same abrasion and staining technique to etiolated hypocotyls of cucumber and found very similar results with this tissue (not shown). Less than four tiny streaks staining with Evan's blue were routinely observed per segment, suggesting that most of the surface is not "wounded" but merely breached at the cuticle, allowing the entry of neutral red stain to most of the surface of the abraded tissue within minutes. Because of the short incubation time, the fungal elicitors used in the experiments described here exert their action preferentially on the epidermal cells.

Segments from seedlings grown in the presence of DCIA were previously shown to fully inhibit fungal penetration through their epidermis (Siegrist et al., 1994). Although not documented in detail, we routinely established that this resistance was also evident for the seedlings used in this report. For instance, in an experiment performed similar to that shown in Figure 1, only 1 of the 500 appressoria scored

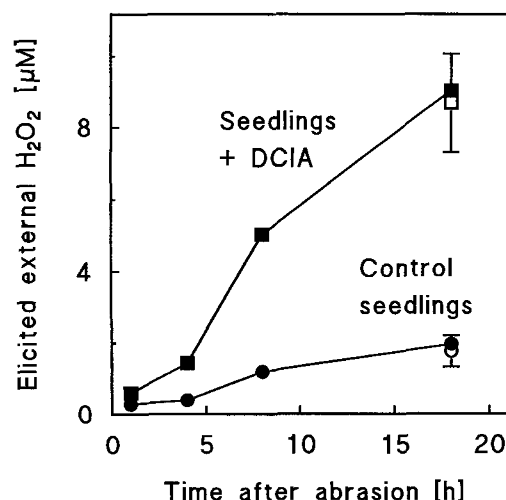

Figure 1. Conditioning for H_2O_2 elicitation in abraded hypocotyl segments from cucumber seedlings grown with DCIA. Seedlings were grown for 5 d on 0.1 mM DCIA or on water as controls. The hypocotyls were than abraded, and segments were cut and collected in water. At each point indicated, 10 segments were removed and supplied with 10 μg/mL crude fungal elicitor, and the extracellular H_2O_2 was determined after 30 min. The H_2O_2 concentration determined before elicitor addition was subtracted. The filled symbols refer to one representative experiment of four performed. The open symbols indicate means ± SD for the 18-h value from these experiments.

on segments from DCIA-treated seedlings was associated with a penetration. This corresponds to a penetration rate of 0.2 ± 0.6%, whereas on control segments 58.8 ± 19.1% penetrations occurred. As autofluorescent cell-wall phenolics could be observed with epifluorescence under and around many of the failed appressoria on DCIA segments (for a picture, see Siegrist et al., 1994), the resistant epidermal cells readily reacted with defense reactions and were expected, therefore, to be rather potent for elicitor responses. Surprisingly, when hypocotyls were abraded and the segments immediately used for elicitation, neither the resistant nor the susceptible control segments considerably produced H_2O_2 (Fig. 1, 1-h point). A few hours after abrasion, the segments started to develop a competence for H_2O_2 elicitation and this was far more pronounced when the seedlings were grown on DCIA. No significant further increase was observed when the conditioning was performed for more than the routinely used 18 h (data not shown).

These results suggested that the presence of DCIA in the hypocotyl alone is not sufficient to induce competence for H_2O_2 elicitation. An additional stimulus generated during preparation of the abraded segments also appears to be required for the conditioning process. One possible source of such components could have been the Vitex polishing cloth routinely used for abrading the hypocotyls. However, polishing papers based on waterproof glues were equally effective for inducing H_2O_2 elicitation competence (data not shown). They were not routinely used, however, because destruction of the epidermis appeared to be more frequent.

Alternatively, the stimulus resulting from abrasion of the cuticle could relate to physical stress on the hypocotyl caused by the abrasion or by wounding during cutting of the segments. It appeared possible, therefore, that the stress hormone methyl jasmonate is involved in the conditioning process, especially since we have previously found that pretreatment of parsley suspension cultures with methyl jasmonate enhanced the subsequent elicitation of H_2O_2 (Kauss et al., 1994). To test this possibility, DCIA-grown cucumber seedlings were treated during their last day of growth with methyl jasmonate (2–7 μM) either as a topical spray or through the roots. None of these treatments induced H_2O_2 elicitation competence in undisturbed hypocotyls when assayed in freshly abraded segments (data not shown).

It is known that root treatment of unwounded seedlings with SA does not induce resistance in the hypocotyls, presumably because SA is not sufficiently taken up or transported into the epidermis (Siegrist et al., 1994). To include SA in the further studies we adapted the experimental protocol. The seedlings were grown on water, and the hypocotyls were abraded and cut. These abraded segments were conditioned for 18 h in the presence of SA or DCIA. Both compounds considerably enhanced the conditioning for subsequent H_2O_2 elicitation (Fig. 2). DCIA at 0.075 or 0.1 mM SA present during the 18-h conditioning phase was sufficient to induce maximally the competence for rapid H_2O_2 elicitation (Fig. 3). The time course of H_2O_2 elicitation was not identical for controls and segments treated with the two compounds (Fig. 2). Furthermore, the rate of decrease in H_2O_2 after reaching the maximum, which indicates the degradation of H_2O_2, varied to some extent in different experiments (data not shown). The time course was verified, therefore, for every experiment performed, and the time at which maximal H_2O_2 concentration was reached is given in the legends. It should be noted that a second, smaller H_2O_2 burst becomes evident about 3 h after addition of the elicitor, especially with SA-conditioned segments (data not shown). This late H_2O_2 elicitation is not further considered in this report even though it was also observed with halved segments (Siegrist et al., 1994).

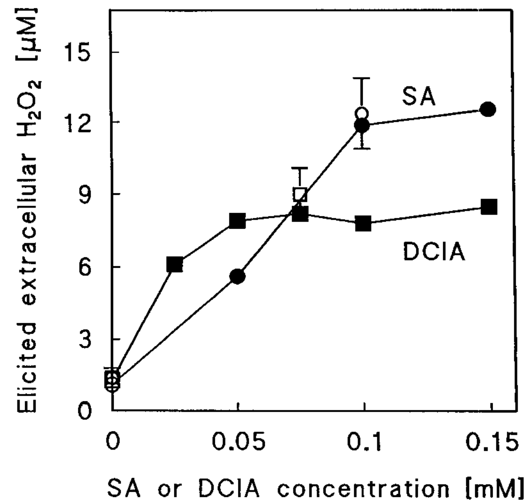

Figure 3. Influence of SA and DCIA concentration on the development of H_2O_2 elicitation competence in abraded cucumber hypocotyl segments. Seedlings were grown and the abraded segments conditioned as in Figure 2 with the indicated concentrations of SA or DCIA. The segments were washed and elicited with 10 μg/mL crude fungal elicitor, and the H_2O_2 was determined after 30 min. The nonelicited H_2O_2 concentrations (time zero of Fig. 2) were subtracted. For technical reasons, the experiments with SA or DCIA were performed independently with two batches of seedlings and are representative results from three similar experiments each (filled symbols). The open symbols indicate the means ± SD from 10 experiments with 0.075 mM DCIA and 17 experiments with 0.1 mM SA performed over 4 months.

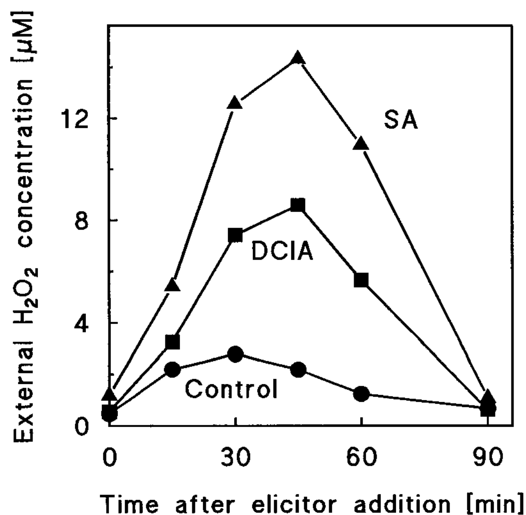

Figure 2. Time course of H_2O_2 elicitation in abraded and conditioned cucumber hypocotyl segments. The seedlings were grown on water and abraded, and the cut segments were conditioned for 18 h either as controls or in the presence of 0.075 mM DCIA or 0.1 mM SA. The segments were washed, crude fungal elicitor (10 μg/mL) was added at zero time, and the external H_2O_2 was determined at the times indicated. See text for a note on variability between the individual experiments. In similar experiments with the three types of conditioned segments, no change in the basal H_2O_2 concentration seen at zero time was observed when water was added instead of elicitor.

A crude fungal elicitor preparation from *P. sojae* was used for the above experiments on H_2O_2 elicitation. This preparation was near saturation at a concentration of 10 μg/mL (Fig. 4). The same result was found for abraded segments conditioned, as for Figure 1, after DCIA application through the roots (data not shown). To elaborate the chemical nature of the molecules active for H_2O_2 elicitation in the abraded cucumber hypocotyl segments, we have also used two partially purified oligoglucan mixtures from the same fungus. The initial time course of H_2O_2 production was similar with the two oligoglucan mixtures and the crude fungal elicitor, but the phase of H_2O_2 decline was reached earlier with the oligoglucans (Fig. 5). Nevertheless, with both oligoglucan preparations saturation was attained at far lower concentrations than with the crude fungal elicitor, although at saturating concentrations of oligoglucans the maximal H_2O_2 values were lower (Fig. 4). Two

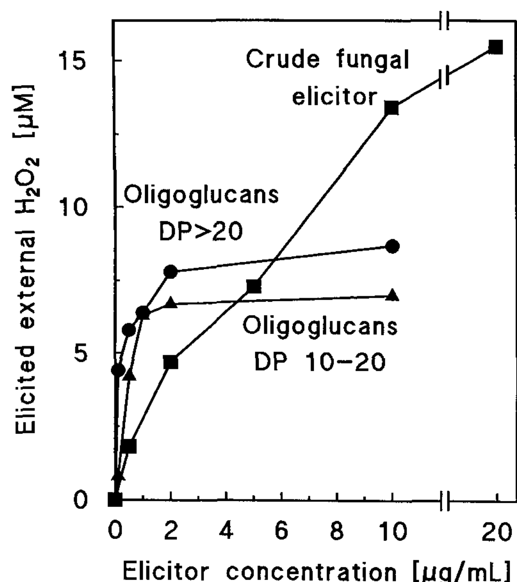

Figure 4. Dose response for various elicitor preparations inducing H_2O_2 in abraded cucumber hypocotyl segments conditioned with SA. The segments were abraded and conditioned with 0.1 mM SA for 18 h, and the indicated concentrations of elicitors were added at time zero. The time course for the DP > 20 oligoglucans was similar to DP 10 to 20 (not shown). The elicited increase in H_2O_2 level reached after 20 min is given for the oligoglucan preparation and after 30 min for the crude fungal elicitor. Means are from three independent experiments. SD was between ±6 and ±20%, values above 5 μM H_2O_2 corresponding to the lower and below 5 μM H_2O_2 corresponding to the upper part of this range. No significant H_2O_2 production was induced by 0.5 μg/mL maltoheptaose, heptapustulan, and two chemically synthesized hepta-β-glucosides that differ in their phytoalexin induction activity in soybean (see "Materials and Methods").

chemically synthesized heptaglucans, one being the most active oligoglucan for glyceollin induction in the soybean cotyledon assay and the other being about 800-fold less active (Cheong et al., 1991), were not able to induce H_2O_2 in the abraded hypocotyls (see legend of Fig. 4). Both with the crude fungal elicitor and with the oligoglucan preparation a second application of the same elicitor preparation did not induce a second H_2O_2 burst (Fig. 5). The same was found when the crude fungal elicitor was followed toward the end of the respective burst by oligoglucans (Fig. 5A). In contrast, when the oligoglucans were followed by crude fungal elicitor, a considerable second burst of H_2O_2 was induced (Fig. 5A). These results suggest that the crude fungal elicitor and the oligoglucans are similar but not fully identical with respect to the chemical structures recognized by the epidermal cells.

It should be noted that conditioning of the abraded segments in the presence of SA also significantly enhanced the spontaneous H_2O_2 production (= nonelicited part, zero time in Figs. 2 and 5B). This effect varied in extent between the experiments performed (cf. Figs. 2 and 5B). These results may indicate that the H_2O_2-producing enzyme system, not the elicitor reception system, becomes improved during conditioning with SA, as suggested for parsley suspension cultures (Kauss and Jeblick, 1995).

The SA-enhanced development of H_2O_2 elicitation competence was very sensitive to cycloheximide and was fully inhibited at 5 μM (Fig. 6). Addition of 1 μM (Fig. 6) or 10 μM (data not shown) cycloheximide after conditioning with SA, but 10 min before the elicitor, did not decrease elicited H_2O_2 production. These results indicate that cycloheximide did not inhibit the H_2O_2-producing enzyme system. Puromycin, present during the SA-enhanced conditioning of abraded hypocotyl segments, acted similarly to cycloheximide, although in this case 200 μM was required to achieve full inhibition (Table I). The conditioning effect found in control segments without exogenous SA (Figs. 1 and 2) was also greatly inhibited with 5 μM cycloheximide and 200 μM puromycin (data not shown), indicating that in this case translational protein synthesis is also required for the conditioning process. Curiously, incubation of abraded segments for 18 h with 0.1 mM SA, together with 20 μM

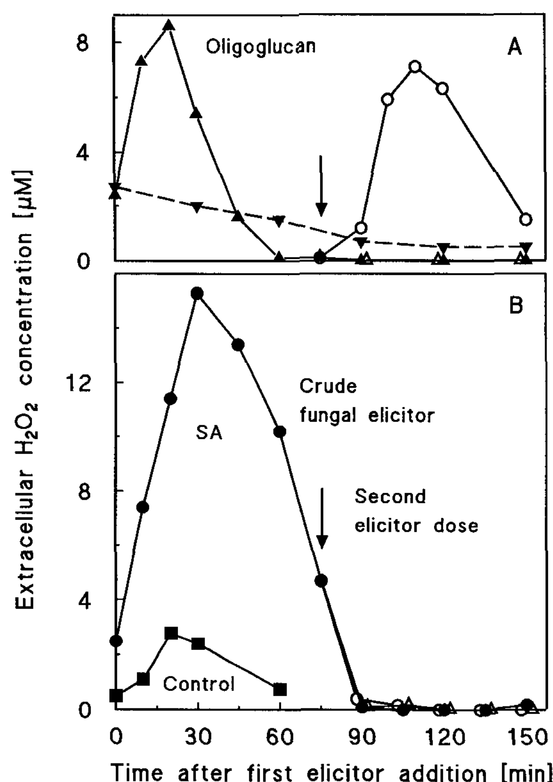

Figure 5. Time course for H_2O_2 production and differences in the refractory state induced with oligoglucans and crude fungal elicitor. Abraded segments conditioned for 18 h with 0.1 mM SA were used for most of the experiment (▲, ●, ▼, O); the response to fungal elicitor of segments conditioned without SA (■) is also shown for comparison. The first dose (time zero) was an oligoglucan mixture of DP 10 to 20 in A (▲, 3 μg/mL) and crude fungal elicitor in B (●, 10 μg/mL). A second dosage of these elicitors (△, O) was applied at the time indicated by the arrows to one of three batches of segments induced in parallel at time zero either with oligoglucans (A) or with crude fungal elicitor (B). The dashed line in A (▼) refers to external H_2O_2 in a sample of SA-conditioned segments, which received no elicitor. Representative of four similarly performed experiments. The time course for oligoglucans of DP > 20 was the same as for oligoglucans with DP 10 to 20 (data not shown).

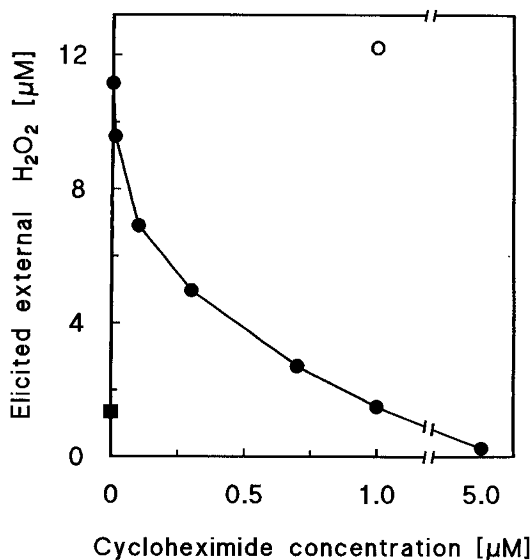

Figure 6. Inhibition by cycloheximide of the induction of H_2O_2 elicitation competence. The abraded hypocotyl segments were conditioned at 0.1 mM SA for 18 h in the presence of the indicated concentrations of cycloheximide, followed by elicitation with 10 µg/mL crude fungal elicitor for 30 min. The filled square indicates a sample conditioned without SA. The open circle refers to a sample that was conditioned with SA, but without cycloheximide, and received the 1 µM cycloheximide after conditioning, 10 min prior to the elicitor. The nonelicited level of H_2O_2 was subtracted. Results are given from one representative experiment of three performed.

α-amanitin, resulted in a 2-fold higher elicitor competence, compared to samples without this inhibitor of RNA synthesis (data not shown). In contrast, addition of 20 µM α-amanitin or 50 µM actinomycin D after the conditioning phase, shortly before the elicitor, greatly decreased the subsequent elicitation of H_2O_2, indicating that these inhibitors are toxic to the H_2O_2-producing system or interfere with regulatory features in an unexpected manner. Thus, studies with inhibitors do not appear to be suitable to decide whether transcription is also involved in competence induction for H_2O_2 elicitation.

DISCUSSION

DCIA applied through the roots is readily distributed through whole plants and can thereby mimic SAR at local infection sites (Kessmann et al., 1994). It was shown previously (Siegrist et al., 1994) and confirmed in the course of the experiments reported here that growing etiolated cucumber seedlings in the presence of low DCIA concentrations results in resistance of the hypocotyl against *C. lagenarium.* This resistance appears to be due mainly to inhibition of fungal penetration through the outer epidermal cell walls by a process including deposition of polymeric cell-wall phenolics, which likely requires apoplastic H_2O_2. However, even the abraded segments from resistant seedlings were not constitutively competent for elicitation of H_2O_2 soon after abrasion (Fig. 1). Thus, the presence of DCIA in the tissue was not sufficient by itself for the induction of elicitor competence. After abrasion of the cuticle, some competence developed over time in segments from control seedlings, and it was this process that was greatly enhanced by DCIA introduced through the root (Fig. 1) or by DCIA and SA supplied exogenously through the abraded cuticle (Figs. 2 and 3). Obviously, these substances exert their action on H_2O_2 elicitation indirectly by enhancing a type of developmental process, which implies the synthesis of proteinaceous competence factor(s) and is initiated by a signal resulting from cuticle abrasion and/or the wounds produced on cutting the segments, even though these wounds occupy only about 5% of the surface (Fig. 7). The nature of this stimulus remains unknown. The negative results of the experiments with methyl jasmonate (data not shown) argue against the implication of this stress hormone in H_2O_2 elicitor competence induction, although other unknown hormone-like signals derived from physical stress could be involved. Alternatively, unknown lipophilic surface wax components or degradation products from cutin may enter the tissue and play a role as a chemical signal in the conditioning process.

The crude fungal elicitor used for most of the experiments contains a glycoprotein active in the elicitation of

Table I. *Inhibition of the development of H_2O_2 elicitation competence by puromycin*

Conditioning Procedure[a]	H_2O_2 Elicitation[b]
	µM
Without SA	0.8
+ SA	11.1
+ SA + puromycin (100 µM)	5.9
+ SA + puromycin (200 µM)	0.7

[a] Abraded segments were conditioned for 18 h with or without 0.1 mM SA, in the presence or absence of the indicated concentration of puromycin. [b] Elicitation and H_2O_2 determination after 45 min as in Figure 2. The nonelicited level of H_2O_2 (zero time in Fig. 2) was subtracted.

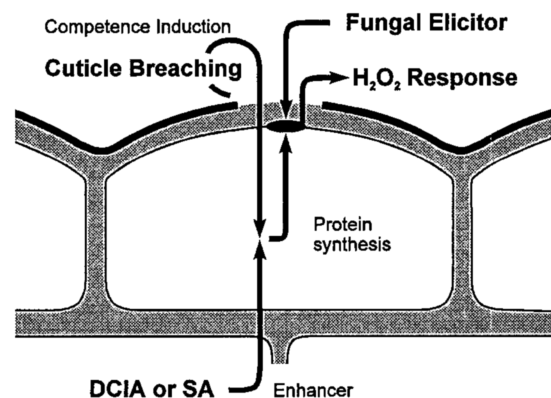

Figure 7. Schematic representation of the cooperation of three different signals leading to enhanced H_2O_2 production in epidermal cells of resistant cucumber hypocotyls. Breaching the cuticle by abrasion induces a process associated with protein synthesis, which leads to competence for the subsequent elicitation of H_2O_2. This conditioning is enhanced by the presence of the systemic signal substances DCIA or SA in the tissue. It is speculated that abrasion of the cuticle simulates its penetration by fungal pathogens, implying that this provides a local signal initiating the H_2O_2 defense response.

phenylpropanoid responses and of H_2O_2 in parsley suspension cultures (Nürnberger et al., 1994) and also a branched β-glucan active for glyceollin induction in suspension cells and various wounded tissues of soybean (Hahn et al., 1992, 1993). Preliminary experiments indicated that treatment of the crude fungal elicitor with trypsin under conditions sufficient to fully destroy the H_2O_2 elicitation activity in parsley cells (Kauss et al., 1994) did not affect the activity of the elicitor preparation for H_2O_2 elicitation in conditioned abraded cucumber hypocotyls (data not shown). This observation suggested that a carbohydrate might be responsible for H_2O_2 elicitation in the cucumber system. This hypothesis is further supported by the data in Figure 4, which indicate that mixtures of oligoglucans exhibit high H_2O_2 elicitation activity at low concentrations. These mixtures thus appear to contain active oligoglucans, which are more abundant in the preparation with higher DP. However, a defined β-heptaglucoside, which is very active for glyceollin induction in soybean cotyledons (Sharp et al., 1984a, 1984b, 1984c; Cheong et al., 1991), was inactive for H_2O_2 elicitation in the conditioned cucumber hypocotyls (see legend of Fig. 4), indicating that the structural requirements for elicitor activity are different in the two plants.

Both with the crude fungal elicitor and the oligoglucan mixtures, the H_2O_2 production is transient and is followed by a decline of H_2O_2 concentration due to degradation by unknown mechanisms (Figs. 2 and 5). Addition of a second dose of either oligoglucosides or crude fungal elicitor to the abraded segments does not lead to a second H_2O_2 burst, suggesting that the transient nature of H_2O_2 production is not likely due to degradation of the elicitor. This was directly shown by replacing, in experiments performed similar to Figure 5, the refractory segments after 75 min by noncommitted ones. This procedure resulted in a second, rapid H_2O_2 burst (data not shown), indicating that at least part of the elicitor is still present in an active form after the first H_2O_2 burst. These results suggest that down-regulation of elicited H_2O_2 production is due to some form of adaptation. In fact, the H_2O_2 concentration in cucumber hypocotyl segments in the refractory state falls even below the level of nonelicited segments (dashed line in Fig. 5A). This result suggests that even the nonelicited level of H_2O_2 production is subject to down-regulation by the elicitor-induced desensitization. Induction of such a desensitized state is well known for various sensory systems in animals and has also been suggested and discussed to occur on elicitation of suspension-cultured cells of pear (Campbell and Labavitch, 1991), soybean (Legendre et al., 1993), tobacco (Mathieu et al., 1991), and tomato (Felix et al., 1993; Granado et al., 1995).

The presumed desensitized state in the cucumber hypocotyls is apparently reached earlier, and the maximal H_2O_2 level remains lower with oligoglucans, compared to the crude elicitor preparation (Fig. 5). Of interest in this respect is the observation that abraded segments that are desensitized by saturating concentrations of oligoglucans still partially respond to the crude elicitor preparation (Fig. 5A), whereas the reverse is not true (Fig. 5B). This might indicate that the oligoglucan mixtures do not contain the full range of active structures present in the crude elicitor preparation.

The present results show that in cucumber hypocotyls the elicitation of H_2O_2 is a rather complex process. DCIA, which was applied through the root to experimentally render the hypocotyl resistant, can act on subsequent H_2O_2 elicitation only in cooperation with a stimulus resulting from cuticle abrasion. In the case of a fungal attack on epidermal cells, this latter event might correspond to breaching the cuticle by the fungus, which subsequently also is the source of the second, local signal, the elicitor. These two cooperative local signals resulting from the invasive presence of the fungus would lead to an enhanced H_2O_2 defense response when the epidermal cells contain endogenous SA that accumulated during SAR caused by a previous infection (Fig. 7). These ideas are admittedly speculative but fit the observation that, in infected cucumber hypocotyl segments exhibiting SAR due to pretreatment with DCIA or SA (Siegrist et al., 1994), the epidermal cells react at the time and the place of attempted penetration with biochemically diverse defense responses, which include polymerization of cell-wall phenolics, a process that appears to require H_2O_2. Further studies will be required to show whether the conditioning reported here for H_2O_2 elicitation also applies to other defense responses.

Some of the results reported here for cucumber hypocotyls are reminiscent of observations with the soybean cotyledon system, in which competence for elicitation of glyceollin and phenolic cell-wall polymers appears to develop only in those cell layers near the cut surface or adjacent to the site of injection of elicitor (Graham and Graham, 1994). In this case, competence induction was attributed to signals arising from wounding of the cotyledon tissue. In contrast, in soybean suspension cultures, competence for elicitation of different defense responses appears to be established without any apparent induction event (for refs. see Hahn et al., 1993; Legendre et al., 1993; Graham and Graham, 1994). A similar observation was made in the parsley cell suspensions in which some competence for H_2O_2 elicitation is observed in routinely grown cultures, although the H_2O_2 response can be further enhanced by pretreatment with DCIA, SA, or methyl jasmonate (Kauss et al., 1992, 1994; Kauss and Jeblick, 1995). DCIA and SA appear to act in a similar manner in abraded cucumber hypocotyl segments with the important difference that in the latter case a further initial stimulus set by abrasion is also required (Fig. 7). It seems possible that the apparent lack of competence induction in cell suspensions relates to the fact that such cultures correspond to permanently wounded and stressed tissues and, therefore, exhibit competence without further experimental treatment.

The studies on soybean cotyledons by Graham and Graham (1994) and our present results on cucumber hypocotyls both illustrate that elicited plant defense responses can be subject to modulation by physiological factors up to now not considered and also emphasize that model systems for physiological and biochemical studies on plant/microbe interactions should be designed to mimic the nat-

ural situation as much as possible to understand the full complexity of plant disease resistance mechanisms.

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