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Defense Responses in Infected and Elicited Cucumber (*Cucumis sativus* L.) Hypocotyl Segments Exhibiting Acquired Resistance[1]

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Segments from dark-grown cucumber (*Cucumis sativus* L.) hypocotyls were used to study defense reactions occurring upon fungal infection and induced by elicitors in the same tissue. The segments were rendered resistant to infection by *Colletotrichum lagenarium* either by growing the seedlings in the presence of dichloroisonicotinic acid (DCIA) or by preincubation of the cut segments with DCIA, salicylic acid (SA), or 5-chlorosalicylic acid (5CSA). This resistance appears to be due mainly to inhibition of fungal penetration into epidermal cells. In the resistant hypocotyl segments, the fungus induced, at the time of attempted penetration, an increased deposition of phenolics, which were visualized by autofluorescence. These phenolics were located mainly in the epidermal cell wall around and in the emerging papillae below appressoria and were quantified either as lignin-like polymers by the thioglycolic acid method or as 4-OH-benzaldehyde, 4-OH-benzoic, or 4-coumaric acid liberated upon treatment with alkali at room temperature. Pretreatment with DCIA, SA, and 5CSA induced little chitinase activity, but this activity greatly increased in resistant tissues upon subsequent infection. These observations indicate that resistance is associated with an improved perception of the pathogen stimulus resulting in the enhanced induction of diverse defense reactions. When the cut segments were pretreated with DCIA, SA, or 5CSA and then split and incubated with chitosan fragments, the deposition of cell wall phenolics was also enhanced. These pretreated and split segments also exhibited an increase in the rapid production of activated oxygen species induced by an elicitor preparation from *Phytophthora megasperma* f. sp. *Glya*. Pretreatment of the segments with methyl jasmonate neither induced resistance nor enhanced induction of cell wall phenolics upon fungal infection, although we observed in the corresponding split segments some increase in chitosan-induced cell wall phenolics and in elicitor-induced rapid production of activated oxygen species.

Localized infection of certain plants by pathogens can result in the development of resistance either near the area of first inoculation or spreading systemically to other plant organs (for citations, see Rasmussen et al., 1991). This SAR is nonspecific with regard to the first, inducing pathogen as well as to the second, rejected pathogen. These observations make the participation of several defense mechanisms likely. Accordingly, various "pathogenesis-related proteins" are systemically induced in the resistant tissue. These proteins are likely related to defense mechanisms. However, until now only some have been identified (e.g. as isoforms of chitinase and 1,3-β-glucanase in tobacco; Ward et al., 1991). These enzymes can degrade fungal cell walls and thereby act directly against pathogens (for citations, see Arlorio et al., 1992). Callose synthase was also found to exhibit an increased specific activity in plasma membranes from resistant cucumber (*Cucumis sativus* L.) leaves (Schmele and Kauss, 1990), representing another example of an enzyme that might be involved in SAR. In this case, however, in addition to a preformed increase in enzyme activity, a pathogen stimulus is also required for initiation of the defense reaction (Kauss, 1990).

The pioneering work of Kuc and co-workers has shown that plants exhibiting SAR are also "sensitized" to better reduce fungal penetration into epidermal cells (for citations, see Richmond et al., 1979). This process is associated in cucumber with papilla formation and an increase in the localized deposition of phenolic material exhibiting some properties of lignin (Hammerschmidt and Kuc, 1982; Stumm and Gessler, 1986; Xuei et al., 1988; Stein et al., 1993). Nonhost resistance in cucumber is also associated with deposition of a similar material from which cupric oxide degradation preferentially liberated 4-coumaryl units (Hammerschmidt et al., 1985). In split cucumber hypocotyls elicited with α-1,4-oligogalacturonides, a similar polymeric material appears to be deposited, but the occurrence of considerable amounts of 4-coumaric acid (Robertsen and Svalheim, 1990) suggests that the phenolic material in cucumber may be of a complicated structure.

There is increasing evidence that lignin in nonwoody plants contains considerable amounts of etherified ferulic acid, most of which is also ester linked to other unknown cell wall material, suggesting that the phenolic acid may serve as an anchoring point during polymerization (Iiyama et al., 1994). Presumably esterified 4-coumaric and/or ferulic acid also occur among the cell wall phenolics deposited during pathogenesis (Niemann et al., 1991), elicitation (Graham and Graham, 1991; Beimen et al., 1992; Kauss et al., 1993), or wounding (Borg-Olivier and Monties, 1993). In addition, substantial amounts of *p*-OH-benzaldehyde and/or vanillin

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Abbreviations: 5CSA, 5-chlorosalicylic acid; DCIA, 2,6-dichloroisonicotinic acid; JAME, jasmonic acid methyl ester; Pmg, *Phytophthora megasperma* f.sp. *Glya*; SA, salicylic acid; SAR, systemically acquired resistance.

are also liberated by treatment with alkali at room temperature. These aldehydes possibly arise from unknown polymers in which phenylpropane units bear hydroxyl groups at carbon-3 (Matern and Grimmig, 1994). Taken together, these results suggest that cell wall phenolics deposited as defense reactions are, in general, even more complex than classical lignin. Nevertheless, SAR-enhanced deposition of cell wall phenolics upon fungal infection of cucumber epidermal cells (Hammerschmidt and Kuc, 1982) implies an enhancement of phenylpropanoid defense reactions that presumably are induced locally by elicitors. The mechanism causing this enhancement effect remains unclear.

Development of SAR implies that signals move systemically from the primarily infected tissue to the target tissue. SA appears to be involved in this process (Malamy and Klessig, 1992; Raskin, 1992; Gaffney et al., 1993), although it is not likely the primary systemic signal in cucumber (Rasmussen et al., 1991). The nonfungitoxic plant protection substance DCIA can be applied to unwounded plants to induce SAR (Métraux et al., 1991; Ward et al., 1991). DCIA, SA, and the related 5CSA were recently used to develop a suspension cell model that allows the investigation of links between SAR and elicited phenylpropanoid defense reactions. Parsley suspension cultures were preincubated for 1 d with these substances and then challenged with Pmg elicitor. The preincubation greatly enhanced the elicited secretion of coumarin derivatives and deposition of cell wall phenolics (Kauss et al., 1992b, 1993). More recently, these studies were extended using JAME for preincubation of the suspension cultures (Kauss et al., 1992a, 1994). This pretreatment also enhanced elicited coumarin secretion and deposition of cell wall phenolics as well as elicited production of activated oxygen species. The effects of preincubation with the above substances were especially pronounced at low elicitor concentrations and included accumulation of mRNAs encoding phenylpropanoid enzymes. It was concluded, therefore, that the treatments increased the sensitivity of the elicitor signal perception/transmission system of the parsley suspension cells. Because the conditioning effects in suspension cultures are reminiscent of the development of SAR in whole plants, we also speculated that such an improvement in the warning system of the cells might result in a timely and, therefore, more successful recognition of pathogens.

To verify these assumptions, it was necessary to select a plant tissue that can be rendered easily resistant to pathogens and is also suited for microscopic observation of the infection process. In addition, a low level of constitutive phenolics would ensure a low background for a quantitative determination of those cell wall phenolics deposited in the few cells actually infected. Dark-grown cucumber hypocotyls appear to fulfill these requirements and can also be split to allow elicitor experiments (Robertsen, 1986). The present report attempts to combine all of these views to demonstrate links between SAR and some elicited defense reactions.

MATERIALS AND METHODS

Growth, Resistance Induction, and Infection of Hypocotyl Segments

Seeds of cucumber (*Cucumis sativus* L. cv Mervita) were placed on wet paper towels in plastic containers sealed with aluminum foil and grown in the dark for 4 d at 25°C. Hypocotyls at that age were about 3 to 4 cm long; 2-cm segments were excised, starting a few millimeters above the root crown.

To induce resistance in seedlings, the towels were wetted with a suspension of formulated DCIA (25% [w/w] DCIA and 75% [w/w] powdered silicates plus detergents), which was 80 μM in terms of DCIA. Controls were treated with the same amount of formulation without DCIA. To induce resistance in cut segments, 50 to 100 segments were placed in 30 mL of a solution of pure DCIA, SA, 5CSA, or JAME at the indicated concentrations. These solutions also contained 10 μg/mL each of chloramphenicol, penicillin G, and streptomycin. JAME was used from a 300-mM ethanolic stock solution. In this case, the controls received the same final concentration of ethanol (0.033% [v/v]). The segments were slowly rotated in the dark for 24 h in the above solutions, followed by 2 h in deionized water.

The cucumber pathogen *Colletotrichum lagenarium* was grown on green bean agar (Dhingra and Sinclair, 1985), and the spores were washed from the surface with water, filtered through cheesecloth, washed twice with water, and adjusted to 3×10^6 spores/mL. For inoculation the hypocotyl segments were added to this spore suspension and rotated at 60 rpm for 8 h. The segments were then placed on sterile, wet filter paper in regular Petri dishes (9 cm) for the indicated time. Uninfected segments were treated in parallel but with water only.

Microscopy and Evaluation of Resistance

Thin, hand-cut surface strips of the segments were examined by phase contrast microscopy to follow the infection progress. Autofluorescence of cell wall phenolics was faint yellow when observed with the Zeiss filter set 18 (excitation 390–420 nm, color splitter 425 nm, cutoff filter 450 nm).

Surface cuts from five segments were also used 48 h after infection to evaluate the degree of resistance by counting under phase contrast the successful penetrations (infection vesicles or primary hyphae visible in the epidermal cells). Penetration is expressed as a percentage of the appressoria observed and rated as follows: +++, more than 40% penetration; ++, 15 to 40% penetration; +, 5 to 15% penetration; −, less than 5% penetration. Resistance based on visual appearance of the infected segments after 8 d was rated as follows: ++, tissue fully turgescent, no visible dark spots; +, as for ++ but about 1 mm of macerated and brown tissue toward the edge of the segments: ±, most of the tissue turgescent but some small dark spots from seta and acervuli visible, about 4 mm macerated and brown toward the ends; −, tissue full of the dark spots, fully macerated, without any turgescence.

Determination of Cell Wall Phenolics

Cell walls were prepared according to the method of Graham and Graham (1991) from 10 segments (200–300 mg, exact weight determined) from which 1 mm was freshly removed at each end to exclude wounding-induced phenolics. The segments were homogenized in a ground-glass

Potter homogenizer in 1 mL of 0.1 M sodium phosphate buffer, pH 6.8, rolled for 15 min, and centrifuged (10 min, 3000*g*). The pellet was resuspended in 1 mL of 80% (v/v) ethanol by sonification with the small tip of a Branson sonifier, type 250, and extracted by rolling for 30 min at room temperature. After the sample was centrifuged as above, the cell wall pellet was resuspended and washed twice in methanol and finally dried in a stream of N_2.

The cell walls were treated at room temperature under N_2 with 1 mL of 1 N NaOH, acidified, and extracted twice with 5 mL of ethylacetate, as described by Kauss et al. (1993). The organic phases were evaporated and solubilized in 30 μL of methanol. An aliquot from this solution was subjected to analytical HPLC separation (Kauss et al., 1993). The UV spectra of the peaks were monitored for identification using a Hitachi-Merck (Darmstadt) diode array detector.

After alkali treatment, the cell walls were washed three times with water, and the lignin-like polymers were determined by the thioglycolic acid method (Graham and Graham, 1991). The final precipitate was solubilized in 1 mL of 0.5 N NaOH and quantified at 290 nm, where the absorption spectrum exhibited a shoulder.

Elicitation of Cell Wall Phenolics and Activated Oxygen Species

Pretreated segments were split into halves along their axes with a razor blade and collected in water for about 2 h. From the randomized sample 15 half-segments were placed in Petri dishes (3.5 cm) in 3 mL of sterile water containing the antibiotics used during the pretreatment, and chitosan was added at the concentrations indicated. The dishes were slowly rotated for 16 h to induce cell wall phenolics.

For induction of activated oxygen species, 15 half-segments were suspended in 3 mL of a solution containing 2% (w/v) Suc, 1 mM NaH_2PO_4, 1 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4$ 1 mM $CaCl_2$, 25 mM KNO_3, 10 μg/mL of chloramphenicol, and 10 mM Mes, adjusted with NaOH to a pH of 6.6 (Schwacke and Hager, 1992). This suspension was rotated as above in the dark and supplied with Pmg elicitor. At the indicated times, 100-μL aliquots of the solution were removed and assayed for activated oxygen species by ferricyanide-catalyzed oxidation of luminol (Kauss et al., 1994) in a Bioorbit luminometer (model 1253; Colora, Lorch, Germany). A calibration curve was constructed with H_2O_2 in the solution used for suspension of the segments. The activated oxygen species are given, therefore, in H_2O_2 equivalents (μM).

Enzyme Activities

For peroxidase determination, 10 segments were homogenized as for the cell wall preparation but in 1 mL of 10 mM sodium phosphate buffer, pH 6.0. The samples were centrifuged (12,000*g*, 20 min), and the supernatant was assayed with guaiacol as a substrate for peroxidase, as described by Smith and Hammerschmidt (1988).

For chitinase determination, the homogenization was performed as above but in 20 mM Tes/NaOH, pH 7.4. The enzyme activity was determined using [^{3}H]acetylchitin as a substrate (Siegrist and Kauss, 1990). An aliquot (150 μL) of the enzyme extract was incubated with 100 μL of a suspension of substrate (1 mg, about 5 kBq) for 1 h at 30°C, and the reaction was terminated by addition of 235 μL of 0.2 N HCl and 15 μL of 1 N acetic acid. The mixture was centrifuged (10,000*g*, 10 min), and 200 μL of the supernatant containing the solubilized chitin oligomers were counted in a scintillation counter. Blanks from assays in which the acids were added before the substrate were subtracted.

Protein in the enzyme extract was determined with Coomassie brilliant blue G 250 according to the method of Bradford (1976) using BSA as a standard.

Materials

Most of the materials are as described by Kauss et al. (1992a, 1992b, 1993). Cucumber seeds were purchased locally. Formulated DCIA, the formulation mixture, and *C. lagenarium* were kindly provided by H. Kessmann (Ciba, Basel, Switzerland). Chitosan fragments were prepared by partial acid hydrolysis of crab shell chitosan (Sigma) according to the method of Robertsen (1986).

RESULTS

Shaking the hypocotyl segments for several hours in a suspension of *C. lagenarium* spores resulted in adhesion of many spores over the entire surface of the segment and resulted in more widespread infection of epidermal cells than with the method usually used, application of several small infection droplets. About 12 h after the onset of infection, an increasing number of hyaline appressoria became visible; most of them were melanized (Fig. 1) and, thus, presumably mature by about 18 h postinfection. No difference was observed in this respect between resistant and susceptible segments. In susceptible control segments, successful penetrations below the appressoria became evident between 24 and 30 h after the appearance in the epidermal cells of small fungal infection vesicles, which thereafter steadily increased in size. From these vesicles primary hyphae spread subsequently through the epidermal cells and finally caused maceration of the tissue (Fig. 2, middle).

Thus, within the limits of phase-contrast microscopy, the penetration process occurs under the fungal appressoria in the susceptible epidermal cells between 18 and 30 h. Accordingly, in DCIA-treated segments, as a first visible indication of resistance, autofluorescence presumably due to the deposition of phenolic material becomes visible at 18 h in the epidermal cell walls in the area around and in small papillae below appressoria. Autofluorescence of cell walls and papillae increases steadily in intensity (Fig. 1). Staining with phloroglucinol-HCl at this stage results in a reddish color in the plant cell walls located mainly concentrically around papillae, similar in size to the autofluorescent "halo" shown in Figure 1 (bottom), indicating lignin-like material (for a picture, see Hammerschmidt et al., 1985). Fully autofluorescent cells indicating a hypersensitive reaction were rarely observed. In addition to autofluorescent phenolics the papillae were shown with the aniline blue technique (Kauss, 1989) to contain callose as well (data not shown). Taken together, a microscopic examination of the infection progress

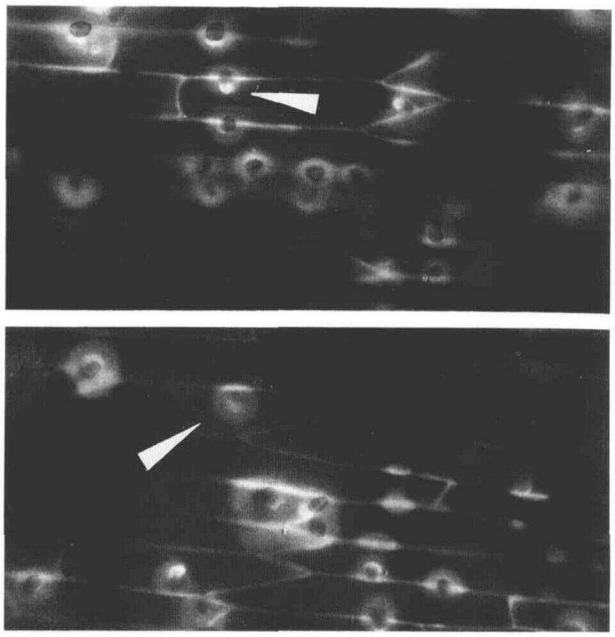

Figure 1. Autofluorescence indicating the deposition of cell wall phenolics in resistant cucumber hypocotyl segments heavily infected with *C. lagenarium*. Two photographs with focus on different details are shown. The white arrowheads indicate a papilla seen in profile (top) and a concentric "halo" around an appressorium (bottom). Note also that autofluorescence occurs mainly near appressoria but sometimes also at cell walls apart from obvious infection sides. For details of the infection procedure see Figure 3. The photographs were taken 48 h after infection of segments from seedlings grown in the presence of DCIA. The epidermal cells are about 20 μm wide. In corresponding susceptible control segments, few and apparently smaller papillae were observed, whereas autofluorescence of most cell walls was evenly faint, similar to those parts in the photograph where no appressoria occur (e.g. in the left lower corner of the top panel).

suggests that the critical time during which attack and penetration occur in susceptible epidermal cells and a successful defense takes place in resistant cells is between 12 and 30 h after the segments are placed in the spore suspension.

For quantification of the cell wall phenolics we used a scheme allowing easy monitoring of several parameters from the same tissue sample. The cell walls were first subjected to treatment with alkali at room temperature and the liberated monomers analyzed by HPLC. A pattern of monomers similar to that from cell walls of parsley suspension cells treated with Pmg elicitor (Kauss et al., 1993) was also observed from the infected hypocotyl segments. The three major peaks were identified by retention time and UV spectrum as 4-OH-benzaldehyde, 4-OH benzoic, and 4-coumaric acid; some other unidentified peaks were not further considered. The polymeric phenolics remaining in the cell walls after alkaline treatment were quantified as thioglycolic acid derivatives.

The time course for the deposition of phenolic compounds into the cell walls is shown in Figure 3. In infected susceptible

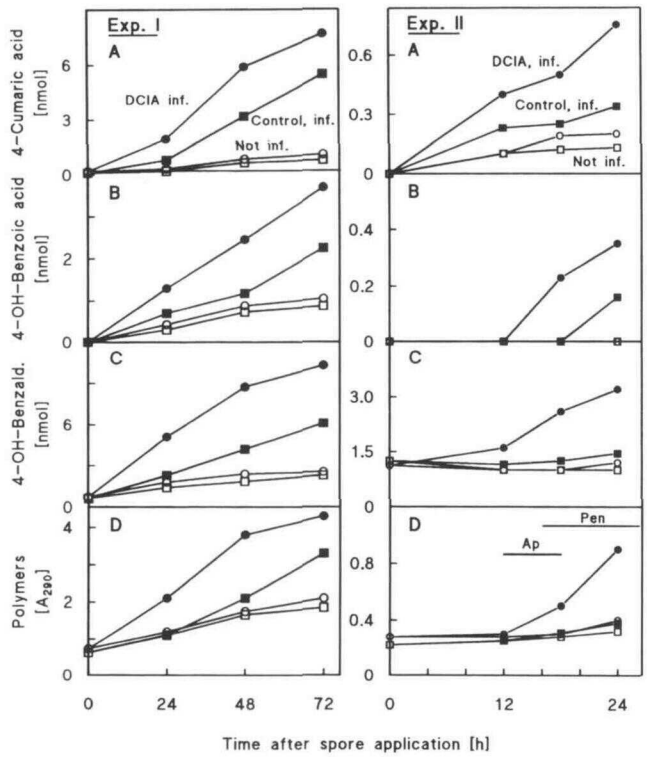

Figure 3. Time course for the incorporation of cell wall phenolics induced by infection of hypocotyl segments from cucumber seedlings made resistant by growth in the presence of DCIA. Two independent experiments covering different time ranges are shown. The segments were placed at time zero into a suspension of spores from *C. lagenarium*, removed after 8 h, and placed in a moist chamber for the indicated times. For each time, 10 segments were homogenized, and the monomeric derivatives of cell wall phenolics (A, 4-Coumaric acid; B, 4-OH-benzoic acid; C, 4-OH-benzaldehyde) were liberated by alkali and analyzed by HPLC, followed by determination of the remaining lignin-like polymers using the thioglycolic acid method (D). Means are given from two HPLC runs per time. All values were calculated per gram fresh weight. Five experiments similar to experiment I were performed and another five similar to experiment II. The results from a representative experiment are given in each case. For an explanation of the variability between individual experiments and of the course of the infection process, see text (experiment II, D: AP, formation of appressoria; Pen, penetration in control segments). Segments from the DCIA-treated seedlings were fully resistant to *C. lagenarium* and those from control plants were susceptible, as shown in Figure 2 and rated in Figure 4.

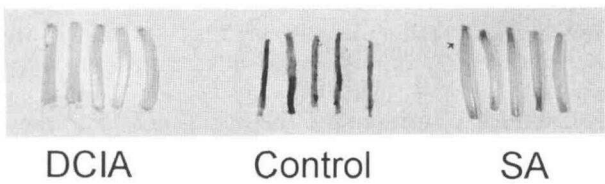

Figure 2. Visual appearance of infected resistant and susceptible cucumber hypocotyl segments. Segments were either left untreated as controls (middle) or treated for 24 h with solutions of DCIA (0.1 mM, left) or SA (1 mM, right), followed by infection with *C. lagenarium*. The photograph was taken 8 d after infection.

control segments, all four parameters monitored remained approximately the same as those of uninfected controls, whereas in the resistant segments from DCIA-treated seedlings all four parameters increased within 24 h as a result of fungal infection (Fig. 3, experiment I). The 4-coumaric acid and 4-OH-benzaldehyde are increased as early as 12 h post-infection, when the first appressoria appear (Fig. 3, experiment II). The lignin-like polymer and 4-OH-benzoic acid lag behind but are also deposited to a greater extent in the resistant DCIA-treated segments. These differences were observed in all experiments performed, even though the absolute values in the individual experiments varied considerably (compare, for example, the 24-h values of experiments I and II in Fig. 3). Accordingly, we qualitatively observed on resistant segments a variable number of appressoria without penetration but also without the autofluorescence response. This could be related to differences in the viability of the spore batches, since a substantial number of morphologically intact appressoria without visible penetration were always observed even on susceptible segments. Because of this, a segment was defined as "susceptible" when penetration was observed under more than 40% of the appressoria, even though we observed up to 75% penetration in some experiments. This indicates an "exhaustion" of a variable part of the appressoria. Alternatively, another unknown defense mechanism may inhibit penetration before cell wall phenolics become deposited, as suggested by Stumm and Gessner (1986).

DCIA can be applied to unwounded seedlings, because this substance permeates into and spreads through the tissues (Métraux et al., 1991). In contrast to DCIA, pretreatment of unwounded seedlings with SA and 5CSA caused no signs of resistance (data not shown). To compare these compounds with DCIA, segments were first cut and then preincubated. The cell wall phenolics deposited in infected segments pretreated with different compounds are compared in Figure 4. DCIA at 0.1 mM rendered the segments fully resistant, whereas SA and 5CSA at this concentration caused only a partial resistance. Even at 1 mM SA caused a full resistance only in the central region of the segment (cf. also Fig. 2). The increase in phenolic derivatives caused by infection was again highest with pretreatment with DCIA and correlates with the degree of resistance; this was also true for the other compounds used for pretreatment.

Of the defense enzymes presumably involved in SAR, we have studied in some detail the activity of chitinase. When the seedlings were pretreated with DCIA, no chitinase activity was found at the initiation of infection in controls and almost none was found in DCIA-treated seedlings (Fig. 5, left, black bases missing). The further increase during the next 24 h in segments not infected was very low in controls and only slightly higher in segments from DCIA-treated plants. Upon infection of control segments, the chitinase activity became considerably increased, and this effect is still more striking in the resistant DCIA-treated segments (Fig. 5, left). When the data from Figure 5 were calculated per gram fresh weight, a still greater effect of DCIA on chitinase induction was found, because the total soluble protein in DCIA-treated seedlings was considerably increased (data not shown).

During the preincubation time necessary for cut segments, some chitinase activity was induced in the controls (Fig. 5,

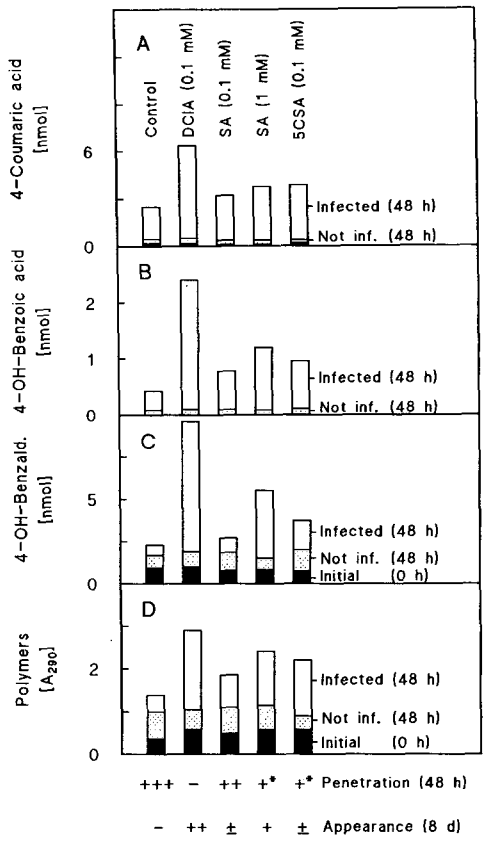

Figure 4. Cell wall phenolics induced by infection of cucumber hypocotyl segments rendered resistant by preincubation with DCIA, SA, or 5CSA. The segments were cut from 4-d-old seedlings and than pretreated for 24 h in solutions of the substances at the indicated concentrations. Infection and determination of cell wall phenolics 48 h after onset of infection were performed as in Figure 3 with the same total number of experiments performed. The values determined for the infected segments correspond to the full height of the columns and include the values determined at the onset of infection (0 h, black bars) and from segments incubated for 48 h without infection (stippled bars). The initial values (0 h, black bars) were below the detection limit for 4-OH-benzoic acid and very low for 4-coumaric acid. Resistance (shown below the panels) was evaluated by rating successful penetrations after 48 h and the visual appearance of the segments after 8 d (see "Materials and Methods"). The asterisk (*) indicates that the penetrations observed were found mainly near the ends of the segments (see also Fig. 2, right, for the consequences).

right, black columns). This process was slightly enhanced in the presence of the various pretreatment compounds, especially DCIA. Some further enhancement of chitinase activity by the DCIA pretreatment became evident in uninfected segments during the next 24 h (Fig. 5, right, stippled bars). Nevertheless, infection of the pretreated segments again caused a further increase in chitinase activity that was enhanced in segments pretreated with SA, 5CSA, and, especially prominently, DCIA (Fig. 5, open bars). Interestingly, the degree of resistance was similar with 0.1 mM DCIA and 0.5 mM 5CSA, but the absolute values for infection-induced chitinase activity were not the same. Differences similar to

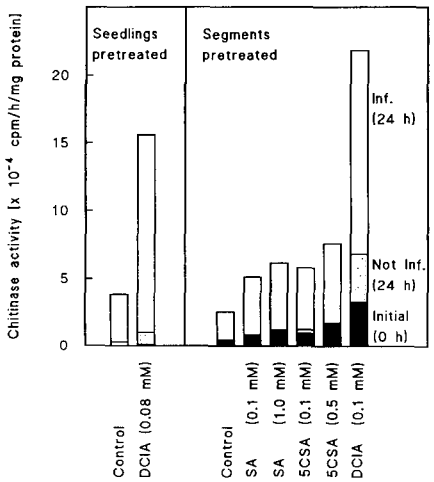

Figure 5. Infection-induced chitinase activity in cucumber hypocotyl segments that were rendered resistant either by growth of the seedlings in the presence of DCIA (left) or by preincubation of the segments with DCIA, SA, or 5CSA (right). For pretreatment and infection of the segments, see Figure 4. Resistance induced by most of the treatments was as in Figure 4; the additional pretreatment with 0.5 mM 5CSA resulted in a degree of resistance similar to that observed with 0.1 mM DCIA in Figure 4. Note that the segments from pretreated seedlings were used for infection at d 4, whereas the pretreated segments were 1 d older at the onset of infection. Different batches of seedlings were used for the two experiments shown, which are, however, representative of all five experiments performed for each condition. Means from two determinations per extract from 10 pooled segments are given.

those shown for 24 h after onset of infection in Figure 5 were also observed at 48 h, although all total activities were greater by a factor of about 1.5 to 1.7. Thus, little chitinase activity is preformed in resistant segments, but it becomes induced during fungal attack of the epidermal cell wall, similarly to the induction of cell wall phenolics (Fig. 3).

Peroxidase activity in unwounded seedlings was slightly increased by pretreatment with DCIA but greatly enhanced after the cut segments were left uninfected for another 24 h (Table I). The further increase caused by infection during this 24 h was comparatively low. When the segments were first cut and then pretreated, peroxidase activity was again mainly induced by the wounding stimulus. Thus, peroxidase induction is not correlated with resistance in the cucumber hypocotyl system, although it is slightly enhanced by DCIA.

When JAME was used at 1 to 100 μM concentrations to pretreat the segments in a way similar to those in Figure 4, we observed an increase neither in the degree of resistance nor in the infection-induced deposition of cell wall phenolics (data not shown). With 100 μM JAME we even observed an apparent enhancement of the infection process, because the segments were already macerated after 5 d to a degree normally found in controls after 8 d (Fig. 2, middle). Similarly, upon application of JAME solutions to unwounded seedlings or of air-borne JAME (for methods, see Farmer and Ryan, 1990) to 3- to 4-week-old cucumber plants, no resistance was detectable (data not shown). In the latter case, resistance was rated according to Schmele and Kauss (1990). These results suggest that JAME cannot induce the overall phenomenon of resistance in cucumber.

When hypocotyl segments were pretreated with JAME at 1 μM (Fig. 6, experiment II) or 2 μM (data not shown) and then split into halves, a subsequent treatment with chitosan for 16 h induced more cell wall phenolics in the pretreated segments. This effect was not found at higher JAME concentrations (10 μM, Fig. 6, experiment II; 100 μM, data not shown). In contrast, pretreatment with DCIA, SA, or 5CSA increased all parameters used to determine the chitosan-elicited phenolic derivatives in cell walls (Fig. 6, experiment I). Staining of the chitosan-treated split segments with phloroglucinol-HCl, a procedure thought to be indicative of lignin, resulted in a reddish color of the cell walls and of the cytoplasmic content of various types of individual cells. Typically, these cells were scattered over the cut surface, but sometimes there were several cells arranged in a group as observed by Robertsen (1986). The chitosan obviously induced a hypersensitive-like reaction in certain cells. We could, however, not yet determine whether pretreatment with DCIA, SA, 5CSA, or JAME, which increased the amount of chitosan-elicited phenolics (Fig. 6), increased either the total number of stained cells or the staining intensity of those cells that had reacted.

The above-mentioned chitosan-induced incorporation of cell wall phenolics requires a relatively long incubation time and presumably involves gene expression and a complex induction mechanism. We were searching, therefore, for a simpler, more rapid elicitor response. When we applied Pmg elicitor to the pretreated split hypocotyl segments, we observed an induction of activated oxygen species proceeding in two phases, a rapid phase completed in about 2 h after elicitor addition (Fig. 7A), and a subsequent smaller response occurring between 3 and 7 h after elicitation (data not shown). The latter phase was not further investigated, but the early oxidative burst was explored in more detail. Elicitation of activated oxygen species was maximal after about 1 h and was greatly enhanced in segments that were pretreated for 24 h with 0.1 mM DCIA or 1.0 mM SA (Fig. 7A). The elicitation was observed with rather low doses of Pmg elicitor, with a

Table I. *Peroxidase activity in infected cucumber hypocotyl segments rendered resistant by pretreatment of the seedlings or of the cut segments with DCIA*

Pretreated Material[a]	Peroxidase Activity in Segments		
	Initial (0 h)	Not infected (24 h)	Infected (24 h)
	$\Delta A_{470}\ min^{-1}\ mg^{-1}$ protein		
Seedlings			
Controls	9	38	82
+DCIA	19	67	94
Segments			
Controls	43	69	87
+DCIA	62	83	97

[a] Same experiments as in Figure 5. Means from two determinations per extract from 10 pooled segments are given.

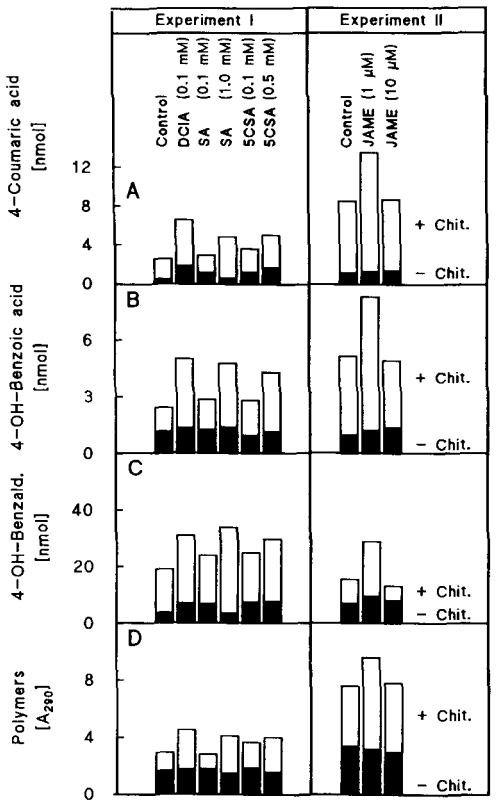

Figure 6. Cell wall phenolics induced by chitosan (Chit.) fragments in split cucumber hypocotyl segments pretreated with DCIA, SA, or 5CSA (experiment I) or JAME (experiment II). Pretreatment of the segments was performed as in Figure 4 and was followed by splitting the segments in half along their axes. Chitosan fragments at 0.75 or 1.0 mg/mL were used for experiments I and II, respectively. Determination of cell wall phenolics was performed after 16 h as in Figure 3. Means are given from two determinations from a representative experiment of five experiments performed.

similar degree of DCIA enhancement at all Pmg elicitor concentrations used (Fig. 7B). Pretreatment with 0.5 mM 5CSA was less efficient than 0.1 mM DCIA (Fig. 7B), although they caused a similar degree of pathogen resistance (Fig. 5). Pretreatment with 2 µM JAME also enhanced the elicitation of active oxygen to about the same degree as did 0.1 mM SA. The effect of JAME pretreatment was comparatively low in the experiment shown in Figure 7A, but it was consistently observed in all six experiments performed. In other experiments not shown, JAME increased the elicitation of active oxygen up to 2-fold compared to control segments.

DISCUSSION

The microscopic examination of the infection process in dark-grown cucumber hypocotyl segments inoculated with *C. lagenarium* suggests that resistance induced by pretreatment with DCIA is due mainly to inhibition of fungal penetration into epidermal cells (Fig. 1). This is in agreement with previous results from studies in which the same pathogen was observed on epidermal strips from cucumber petioles (Hammerschmidt and Kuc, 1982) or on cucumber leaves (Stumm and Gessler, 1986; Kováts et al., 1991) from studies in which the plants were rendered resistant by a previous infection. Under the conditions used in the present report, the infection process in the population of appressoria was not synchronized. It can be estimated, nevertheless, from the microscopic observations and the early quantified response (Fig. 3, experiment II) that fungal attack of the epidermal cells occurred between 12 and 18 h after onset of spore application. Within this time the epidermal cells of resistant segments must be able to prevent or slow down penetration.

DCIA was reported to induce chitinase activity in cucumber leaves, suggesting that synthesis of this enzyme prior to infection represents one of the causes of resistance (Métraux et al., 1991). In contrast, in unwounded cucumber hypocotyls DCIA induced only a very slight increase in chitinase activity (Fig. 5, left, black bars). This slight increase in chitinase and the peroxidase activity induced by DCIA in unwounded

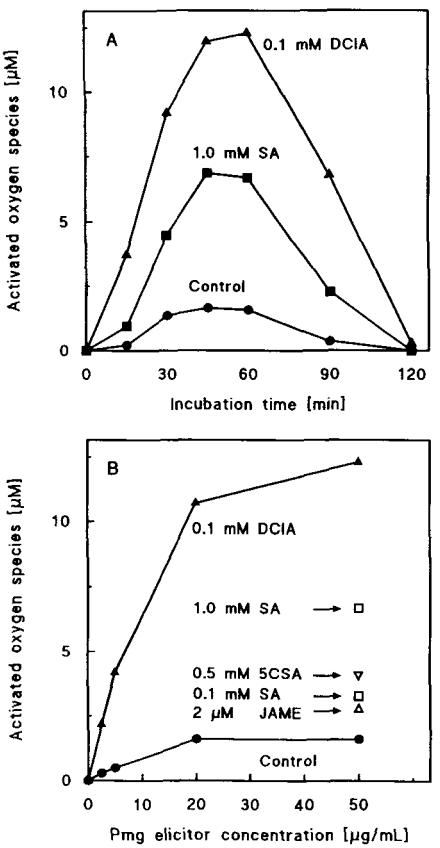

Figure 7. Activated oxygen production induced by Pmg elicitor in split cucumber hypocotyl segments. A, Time course with control segments and segments pretreated as in Figure 4 for 24 h with 0.1 mM DCIA or 1.0 mM SA. Pmg elicitor (50 µg/mL) was added at time 0. B, Pmg elicitor dose response for the oxidative burst in control and DCIA-pretreated segments. The activated oxygen species were determined 60 min after addition of Pmg elicitor. The open symbols indicate the active oxygen production in segments pretreated as in A but with 2 µM JAME, 0.1 mM SA, or 0.5 mM 5CSA. Results in A and B are from the same batch of segments. Results are given from one representative of four experiments performed.

seedlings (Table I) are likely not sufficient to cause resistance, as concluded from the observation that no detectable resistance is associated with the significant increase of chitinase activity (Fig. 5, right, black bars) and peroxidase activity (Table I) in cut control segments. Also, during the pretreatment of unwounded seedlings with DCIA none of the parameters associated with the phenylpropanoid pathway were increased (Fig. 3, time 0). Thus, none of the parameters investigated in the DCIA-treated hypocotyls was sufficiently elevated prior to infection.

In contrast, fungal infection of resistant segments caused a very strong induction of papillae formation (Fig. 1), deposition of cell wall phenolics (Figs. 3 and 4), and chitinase activity (Fig. 5) in resistant segments. The time course for 4-coumaric acid and 4-OH-benzaldehyde (Fig. 3) indicates that this increased response of epidermal cells is already evident at a time when the first, still hyaline fungal appressoria appear. Resistance in the cucumber hypocotyl, therefore, appears to be associated with an enhanced ability of the plant cells to respond to the invader. A similar conclusion based mainly on histological studies was drawn before for other cucumber tissues (Richmond et al., 1979; Hammerschmidt and Kuc, 1982).

Concerted regulation is required for initiating the diverse biochemical and cytological reactions involved in the above-mentioned defense complex. This suggests that SAR in the hypocotyl is associated with improvement of a superimposed regulatory feature. Interestingly, a similar conclusion was drawn from the exact microscopic timing of the penetration process and papillae formation in individual epidermal cells of barley attacked by powdery mildew (Gold et al., 1986). In this case, resistance was genetically determined, whereas SAR in the cucumber hypocotyl segments was due to pretreatment with DCIA.

The enhanced responsiveness of pretreated cucumber hypocotyls for pathogen-induced defense responses is reflected in the results of the elicitor experiments. Pretreatment of the hypocotyl segments with 0.1 mM DCIA, 1.0 mM SA, or 0.5 mM 5CSA induced pathogen resistance (Fig. 5) and also rendered the split segments more responsive to chitosan-elicited deposition of cell wall phenolics (Fig. 6). Similarly, pretreatment with 0.1 mM DCIA and 1.0 mM SA also increased the rapid oxidative burst induced by Pmg elicitor (Fig. 7). The increased ability of the segments to respond to fungal attack with defense responses, therefore, correlates with an increased sensitivity of the tissue to elicitors. This is in accordance with the general assumption throughout the literature that plant cells recognize potential pathogens by elicitor molecules, resulting in defense responses. As first speculated from results with the parsley suspension model (Kauss et al., 1992a, 1992b, 1993, 1994) and now evident for cucumber hypocotyl segments, the degree of elicitor responses can be positively modulated by substances related to the induction of SAR, leading to a timely switching of pathogen-induced defense reactions.

The major open question regarding the cucumber hypocotyl system is whether the enhancement of elicitor responses shown here for the cut surface exposing mainly parenchymatous cells also applies for epidermal cells, which serve in situ as a first line of defense against the invasion by *C. lagenarium* (Fig. 1). In addition, future experiments have to show whether the same results are found with hypocotyl segments exhibiting SAR due to previous infection. It also remains unclear why 0.5 mM 5CSA greatly induced resistance (Fig. 5, legend) and increased the elicitation of cell wall phenolics (Fig. 6, experiment I) but elicited activated oxygen species to a lesser extent (Fig. 7B). The mechanism behind the effect of cutting and aging of the segments on the subsequent induction of chitinase (Fig. 5) and peroxidase activity (Table I) also remains unclear.

The phenolic cell wall material deposited in the course of pathogenesis is ill defined (see introduction). The thioglycolic acid method quantitatively precipitates the lignin-like polymeric portion of the phenolics that are deposited during the defense of cucumber hypocotyls against *C. lagenarium* (Figs. 3 and 4) and that are also elicited by chitosan (Fig. 6). We have also subjected this polymeric material remaining in the cell wall after treatment with alkali to cupric oxide degradation and confirmed the results of Hammerschmidt et al. (1985), who found that in cucurbits the 4-coumaryl residues predominate (data not shown). Consistently, all other identified phenolic monomers liberated simply by alkali bear the same hydroxyl pattern (Fig. 3). The 4-coumaric acid presumably results from an esterified form. Its deposition appears to precede the formation of polymer (Fig. 3, experiment II), suggesting that the polymer might also contain 4-coumaric acid incorporated during subsequent polymerization. The 4-OH-benzaldehyde liberated from unknown insoluble cell wall phenolics upon alkaline treatment appears to be of special interest because this aldehyde is also slowly liberated at neutral pH values from elicited parsley cell walls (Matern and Grimmig, 1994). This suggests a potential role of the phenolic polymers as exactly positioned "phytoalexin precursors," possibly involved in preventing penetration, as discussed for cell wall phenolics in general by Nicholson and Hammerschmidt (1992).

Split hypocotyl segments were introduced as a system for elicitor experiments by Robertsen (1986) and were recently also used for the elicitation of H_2O_2 production with oligo-1,4-α-D-galacturonides and an oligo-β-glucan preparation from *Phytophthora megasperma* f. sp. *Glya* (Svalheim and Robertsen, 1993). These authors used a phenol red method for determination of H_2O_2, which allowed measurement only every 2 h. With the luminol method used in the present report, we could monitor a more rapid elicitation of activated oxygen species. This process is greatly enhanced in pretreated resistant segments (Fig. 7), suggesting that elicited production of active oxygen may possibly contribute to SAR in cucumber. Active oxygen may, however, play multiple roles in pathogen defense, either allowing polymerization of cell wall components or acting as an antibiotic (for citations, see Svalheim and Robertsen, 1993). The pretreatment effect of SA (and DCIA) on elicited active oxygen production reported here is different from the results described for SA by Chen et al. (1993) in tobacco. These authors characterized an SA-binding catalase and suggested that inhibition of this enzyme activity by SA might increase the H_2O_2-mediated activation of genes encoding pathogenesis-related proteins. The cucumber hypocotyl system described here appears to differ from the parsley suspension model in the specificity of the enhance-

ment of the oxidative burst. In cucumber, DCIA was very effective and JAME only slightly effective (Fig. 7B). In contrast, JAME caused a dramatic increase in parsley, whereas DCIA and 5CSA were less active (Kauss et al., 1994). Whether these results reflect differences between various plants or between tissues and suspension cultures remains unclear.

Pretreatment with JAME induced no detectable resistance in the cucumber hypocotyls (see "Results"). This might relate to the observation that JAME enhanced elicitation of chitosan-induced cell wall phenolics only at low, but not elevated, concentrations (Fig. 6, experiment II). Elevated JAME concentrations even accelerated the decay of infected tissues (described in "Results"), reminiscent of the induction of senescence in other plant tissues by high JAME concentrations (Sembdner and Parthier, 1993). It appears possible, therefore, that JAME at the suitable low concentration cannot sufficiently enhance the diversely induced defense responses interacting to cause SAR of the cucumber hypocotyls. The results reported here for cucumber are in accordance with those of Schweizer et al. (1993) for barley and Cohen et al. (1993) for potato and tomato. These authors observed an apparent protection only at relatively high JAME concentrations that were either inhibitory for the pathogens or damaged the plants.

Taken together, the results reported here show that etiolated cucumber hypocotyls made resistant by exogenous application of certain chemicals exhibit a multicomponent defense complex similar to other cucumber tissues protected by biologically induced SAR. The response of resistant tissues to infection includes enhanced papillae formation and localized deposition of phenolics into epidermal cell walls. Surprisingly, infection of resistant segments also resulted in enhanced induction of chitinase activity, an enzyme hitherto discussed only as part of the primary systemic response (Métraux et al., 1991; Ward et al., 1991). In cucumber leaves, pathogens and DCIA induce an acidic class III chitinase, which is also under developmental control but not expressed in uninfected, young, light-grown cucumber stems (Lawton et al., 1994). It appears possible that expression of this enzyme upon infection of the resistant etiolated hypocotyls might be potentiated as a consequence of DCIA pretreatment (Fig. 5), similar to elicited phenylpropanoid enzymes in DCIA-conditioned parsley suspension cells (Kauss et al., 1992b). Because the cucumber hypocotyls also respond to elicitors, this system might greatly facilitate further biochemical studies of the multiple mechanisms involved in SAR.

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