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Mechanism by which contact with plant cuticle triggers cutinase gene expression in the spores of *Fusarium solani* f. sp. *pisi*

(cutin/fungal penetration of cuticle/cutinase mRNA/hydroxy fatty acids)

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ABSTRACT **Spores of the phytopathogenic fungus *Fusarium solani* f. sp. *pisi* were shown to produce the extracellular enzyme, cutinase, only when cutin or cutin hydrolysate was added to the spore suspension. Dihydroxy-C_{16} acid and trihydroxy-C_{18} acid, which are unique cutin monomers, showed the greatest cutinase-inducing activity. Experiments with several compounds structurally related to these fatty acids suggested that both a ω-hydroxyl and a midchain hydroxyl are required for cutinase-inducing activity. Cutinase appeared in the medium 30–45 min after the addition of the inducers to the spore suspension, and the activity level increased for 6 hr. Addition of cycloheximide (5 μg/ml) completely inhibited cutinase production, suggesting that protein synthesis was involved in the increase of cutinase activity. Immunoblot analysis with rabbit antibodies prepared against cutinase showed that cutinase protein increased in parallel with the increase in enzyme activity. Measurement of cutinase-specific RNA levels by dot-blot hybridization with ^{32}P-labeled cutinase cDNA showed that the cutinase gene transcripts could be detected within 15 min after addition of the inducers. Addition of exogenous cutinase greatly enhanced the level of cutinase gene transcripts induced by cutin. These results strongly suggest that the fungal spore senses that it is in contact with the plant by the production of small amounts of cutin monomers catalyzed by the low level of cutinase carried by the spore and that these monomers induce the synthesis of cutinase needed for penetration of the fungus into the plant.**

Cutin, a polyester composed of hydroxy and hydroxy epoxy fatty acids containing 16 and 18 carbon atoms, is the major structural component of the protective barrier covering the surface of the aerial parts of plants (1, 2). Phytopathogenic fungi use an extracellular enzyme, cutinase, to disrupt the cuticular barrier to gain entry into the plant during infection (3). Experimental results suggest that the level of cutinase can determine the capacity of the fungus to penetrate the host cuticle and cause disease. For example, strains of *Fusarium solani* f. sp. *pisi* capable of penetrating intact cuticular barrier produced much larger amounts of cutinase than strains that required a wound or exogenous cutinase for penetration (4). *Mycosphaerella* sp., a wound pathogen, could infect intact papaya fruit only if exogenous cutinase was added to the inoculum (5). Although the level of cutinase associated with spores of some strains of *F. solani* f. sp. *pisi* correlated with their ability to penetrate pea epicotyls, the amount of cutinase present in the spore appeared to be too low to effectively penetrate the cuticular barrier (4). Whether the germinating spores synthesize cutinase during the early phase of pathogenesis is not known and, if so, how the spores sense the contact with the plant is also not known. In the present paper, we demonstrate that cutinase production by the spores of *F. solani* f. sp. *pisi* is triggered by the presence of cutin or the most characteristic cutin monomers. With cDNA probes for cutinase, we show that cutinase gene transcripts can be detected within 15 min after the addition of the inducers into the medium.

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MATERIALS AND METHODS

Inoculum and Culture Conditions. *F. solani* f. sp. *pisi* strain T-8 was obtained from H. D. Van Etten (Cornell University, Ithaca, NY) and was maintained on V-8 juice agar. Conidia used in all experiments were obtained by washing 5- to 10-day-old cultures with 0.1 M potassium phosphate buffer (pH 7.6). The spores were filtered through glass wool, washed three times, and suspended in the buffer at the desired concentrations. Apple cutin was added as a 60-mesh powder (6). Cutin hydrolysate and all lipids were added as aqueous dispersions prepared as reported (7), and the suspensions were incubated on a rotary shaker at 30°C.

Cutinase Assays. Cutinase activity in the extracellular medium was measured spectrophotometrically (8) by using the model substrate *p*-nitrophenyl butyrate. Enzymatic hydrolysis of tritiated cutin was determined as described (8). Immunological measurement of cutinase was done by a protein dot blot method (9), using rabbit antibodies prepared against the enzymes (10) and ^{125}I-labeled protein A.

Effect of Cycloheximide. To determine the effect of cycloheximide on the increase in cutinase activity, the inhibitor was added to spore suspensions as a methanolic solution 30 min prior to the addition of cutin powder. The methanol concentration did not exceed 1% and an equivalent concentration of solvent was added to the control.

Preparation of Cutin Hydrolysate and Monomers. Cutin hydrolysate was prepared as described (7). The hydrolysate was chromatographed on 1-mm silica gel type G-60 thin-layer plates (20 × 20 cm) with ethyl ether/acetone/formic acid (95:5:1, vol/vol/vol) as the developing solvent. Components were visualized under UV light after spraying the plates with a 0.1% solution of 2',7'-dichlorofluorescein or by spraying plates with 9 M sulfuric acid and charring at 200°C. Bands corresponding to 9,10,18-trihydroxy-C_{18} acid (R_f, 0.34), 10,16-dihydroxy-C_{16} acid (R_f, 0.51), and ω-hydroxy-C_{16} and -C_{18} acids (R_f, 0.81) were eluted from the silica gel with chloroform/methanol (2:1, vol/vol). All fractions were rechromatographed in the same solvent system except the ω-hydroxy acid fraction, which was rechromatographed in ethyl ether/hexane/formic acid (70:30:1, vol/vol/vol; R_f, 0.35). Purified fractions were treated with *N,O*-bis(trimethylsilyl)acetamide (Sigma) for 10 min at 90°C and the resulting trimethylsilyl derivatives were analyzed by combined gas–liquid chromatography/mass spectrometry (11).

RNA Isolation, Purification, and Analysis. Spore suspensions (10 ml) were centrifuged and the pelleted spores were

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resuspended in a homogenizing buffer (pH 8.0) that contained 4 M guanidine·HCl, 10 mM Tris·HCl, 10 mM EDTA, 0.5% lauroyl sarcosine, and 0.1% 2-mercaptoethanol. The spores were homogenized by mixing in a Vortex for 3 min with 10 g of glass beads (5 g of 300-μm beads and 5 g of 500-μm beads; Sigma) (12). The homogenate was centrifuged, supernatant was extracted with chloroform and phenol, and the nucleic acids were precipitated with ethanol (13). The resulting pellet was dried, dissolved in 100 μl of sterile water, and incubated for 30 min at room temperature with 10 μg of DNase I per ml (RNase-free) in the presence of 10 mM $MgCl_2$. The reaction was terminated by adding 5 μl of 5 M glyoxal and 5 μl of 0.4 M $NaPO_4$ (pH 7), and the reaction mixture was incubated for 1 hr at 50°C. The glyoxalated RNA was then ethanol-precipitated and the dried pellet was dissolved in 20 μl of sterile water. RNA (2 μl or 4 μl) was blotted to nitrocellulose paper (14) and the blots were hybridized with a ^{32}P-labeled probe prepared from a cutinase cDNA by nick-translation (15). The relative amount of RNA was determined by scanning the autoradiograms with a Beckman DU-8 spectrophotometer. In all cases, experiments were repeated at least five times and typical results are shown.

Effect of Cutinase on Cutin-Induced mRNA Levels. Cutin (10 mg) suspended in 10 ml of 0.1 M potassium phosphate buffer (pH 7.6) was incubated with cutinase (0.1 unit/ml) for 30 min prior to the addition of 3×10^8 spores and the mixture was incubated for 45 min. RNA was then isolated and the level of cutinase mRNA was determined with the cDNA probe. Controls were run without the addition of cutinase.

RESULTS

Induction of Cutinase in Spores of *F. solani* f. sp. *pisi*. When the fungal spores were suspended in a buffer containing cutin powder, *p*-nitrophenyl butyrate hydrolase activity appeared in the medium in a time-dependent manner (Fig. 1). In the absence of cutin, this hydrolase activity was not detectable during the 6-hr experimental period. Since *p*-nitrophenyl butyrate hydrolysis is known to be catalyzed by cutinase, this result suggested that the presence of cutin induced the synthesis of cutinase in the spores. That the extracellular hydrolase activity was due to cutinase was shown by the observation that rabbit antibodies prepared against cutinase isolated from *F. solani* f. sp. *pisi* (10) almost completely (98%) inhibited the *p*-nitrophenyl butyrate hydrolase activity. To confirm that the esterase activity observed with the model substrate reflected cutinase activity, [G-^3H]cutin (8) was used as a substrate, and the release of label from the insoluble polymer correlated with the observed esterase activity. These results clearly show that the spores either synthesized cutinase or released preformed cutinase. To distinguish between these two possibilities the effect of cycloheximide on the appearance of cutinase in the medium was determined. The presence of increasing concentrations of cycloheximide in the medium caused progressively severe inhibition of cutinase appearance (Fig. 1); cycloheximide at 0.5 μg/ml caused 50% inhibition, and at 5 μg/ml it completely prevented appearance of cutinase in the medium. These results suggested that protein synthesis was involved in the cutin-induced appearance of the enzyme in the medium.

The appearance of the enzyme in the medium did not depend on spore germination, although the amount of cutinase found in the medium increased with the number of spores used. In fact, at the level of $2–6 \times 10^7$ spores per ml used in the present studies, germ tube formation was completely suppressed for the duration of our experiments. Variations in the ratio of macroconidia to microconidia obtained from different spore harvests made it impossible to standardize the proportion of the spore type and number between experiments. However, the cutinase inducibility of the spore suspensions did not depend on the proportion of the two types of conidia.

The amount of cutinase that appeared in the medium increased with increasing amounts of cutin, especially when 3- to 6-hr induction periods were used (Fig. 2). During the early period of induction, the amount of enzyme produced showed much less dependence on the amount of cutin than that observed at later periods. Irrespective of the duration of induction, the level of enzyme generated tended to level off at 20 mg of cutin per ml of medium.

Cutinase Induction by Cutin Monomers. Since cutin is an insoluble polymer, it appeared likely that some component(s) generated from it would be the real inducer. To test this possibility, cutin hydrolysate was dispersed in the spore medium and the time course of appearance of cutinase was determined (Fig. 3). Cutinase induction was detectable with as little as 10 μg of hydrolysate per ml. The dependence of induction level on the amount of hydrolysate added varied with the induction period used. With the shorter periods,

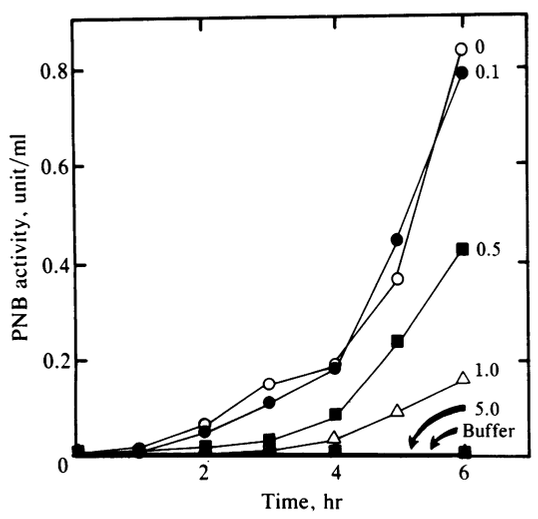

FIG. 1. Effect of cycloheximide on the induction of cutinase activity [measured with *p*-nitrophenyl butyrate as the substrate (PNB activity)] in spores of *F. solani* f. sp. *pisi* by cutin. Cycloheximide concentrations (in μg/ml) are indicated on each line and cutin (0.5 mg/ml) was added to the spore suspensions at time 0. Spores were incubated for 30 min with the inhibitor before cutin was added. Buffer indicates control with no cutin or inhibitor.

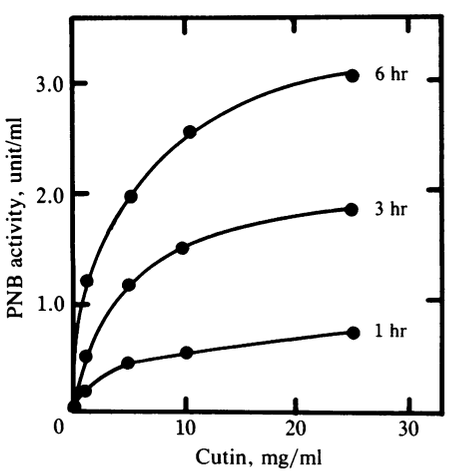

FIG. 2. Effect of cutin addition on cutinase production by the spores of *F. solani* f. sp. *pisi*. Cutinase activity was measured spectrophotometrically with *p*-nitrophenyl butyrate as the substrate (PNB activity).

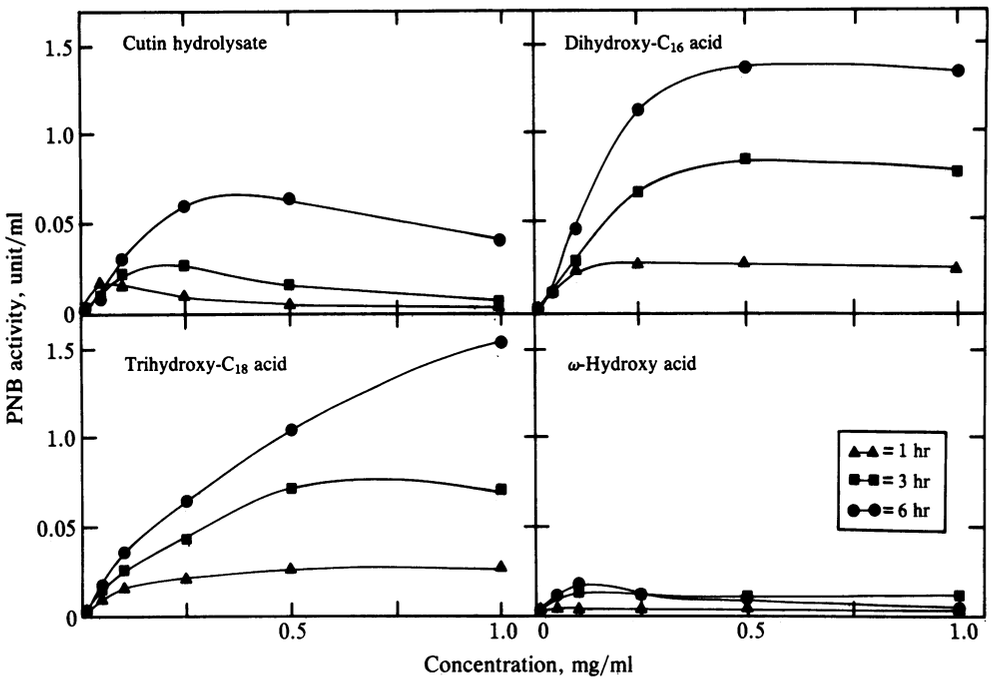

FIG. 3. Induction of cutinase in spore suspensions of *F. solani* f. sp. *pisi* by cutin hydrolysate and cutin monomers. Cutinase activity was measured spectrophotometrically with *p*-nitrophenyl butyrate as the substrate (PNB activity).

such as 1 hr, induction was maximal at 50 μg of hydrolysate per ml, and higher concentrations gave less induction. On the other hand, a higher maximum was observed with 250 μg of hydrolysate per ml when 3-hr induction periods were used, and at the same level of hydrolysate a higher maximum was obtained for a 6-hr induction period. It was verified that the cutin hydrolysate had no direct effect on the enzyme activity. These results suggested that some component(s) present in the hydrolysate suppressed the induction, and removal of this material by catabolism relieved this inhibitory effect in the later periods.

To determine the nature of the inducing component(s) present in the hydrolysate, thin-layer chromatographic techniques were used to fractionate the hydrolysate and the fractions were analyzed by combined gas–liquid chromatography and mass spectrometry. When the materials recovered from the various regions of the thin-layer chromatogram were tested for their ability to induce cutinase in the spore suspensions, the inducing activity coincided with the recognizable cutin monomer fractions. No other region of the chromatogram gave cutinase induction except the origin, which gave a slight induction.

The ω-hydroxy-acid fraction, containing a mixture of saturated C_{16} and mono- and diunsaturated C_{18} ω-hydroxy acids (12), gave maximal induction at 100 μg/ml (Fig. 3). However, the level of induction was much lower than that obtained with the total cutin hydrolysate. The more polar cutin monomers containing midchain hydroxyl group(s) gave much higher levels of induction. With increasing amounts of dihydroxy-C_{16} acid, containing both 9,16- and 10,16- positional isomers, increasing levels of cutinase induction were observed; the maximal level of the enzyme activity reached by the increasing amounts of the monomer depended on the time, with higher maxima at longer periods (Fig. 3). Similar results were obtained with 9,10,18-trihydroxy-C_{18} acid fraction, which contained saturated and Δ^{12} monounsaturated components (12). The maximal levels of induction obtained with the two polar cutin monomers were quite similar and reached approximately twice the level obtained with the total cutin hydrolysate. To test whether cutinase induction by the monomers showed synergistic effects, a variety of mixtures of monomers were tested. The induction levels were always equal to the sum of the levels obtained with the individual components used and revealed no synergism (data not shown).

Structural Features of Inducers. To determine the structural features required to induce cutinase in spore suspensions, a variety of compounds were tested for their ability to induce cutinase. The following compounds at the indicated concentration ranges failed to induce any level of cutinase: 18-hydroxyoctadecanoic acid, 175–250 μg/ml; ricinoleic acid (12-hydroxy-9-octadecenoic acid), 100–500 μg/ml; 9,10-dihydroxyoctadecanoic acid, 10–500 μg/ml; hexadecanoic acid, 10–1000 μg/ml; 16-hydroxyhexadecanoic acid, 10–1000 μg/ml; hexadecanol, 10–1000 μg/ml; phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine, 100–500 μg/ml. 9,10,16-Trihydroxyhexadecanoic acid (aleuritic acid) induced cutinase in the spore suspensions and at 1 mg/ml this acid gave about half the enzyme level obtained with cutin at 10 mg/ml. A thin-layer chromatographic fraction consisting of a mixture of 9,18-dihydroxy-10-ethoxyoctadecanoic acid and 10,18-dihydroxy-9-ethoxyoctadecanoic acid containing their Δ^{12} unsaturated analogues, derived from the 18-hydroxy-9,10-epoxy-C_{18} acids present in apple cutin, induced cutinase to the same degree as the 9,10,18-trihydroxy-C_{18} acid fraction.

Immunological Measurement of Cutinase Production by Fungal Spores. Since the increase in the hydrolase activity levels may not represent actual changes in cutinase level, we used an immunological method to directly measure the enzyme level. Rabbit antibodies prepared against cutinase produced upon saprophytic growth of the fungus on cutin were used because this enzyme was shown to be immunologically quite similar to the cutinase of the spores (4). With this method, it was found that cutinase in the medium became measurable 30–45 min after the spores were added to cutin hydrolysate or the dihydroxy-C_{16} acid was isolated from it, and subsequently a linear increase in cutinase level was observed up to 3 hr (Fig. 4). In the absence of the inducers, cutinase production could not be detected. The isolated dihydroxy-C_{16} acid was more effective than the total hydrolysate in inducing the cutinase production. The esterase

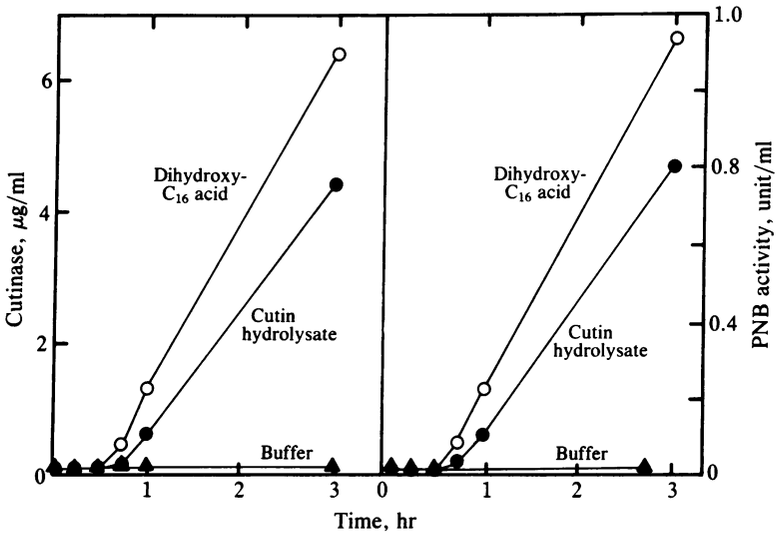

FIG. 4. Time course of induction of cutinase activity (*Right*) and cutinase protein (*Left*) by cutin hydrolysate and dihydroxy-C_{16} acid in spore suspensions of *F. solani* f. sp. *pisi*. Both inducers were used at 0.4 mg/ml. Enzymatic activity was measured spectrophotometrically with *p*-nitrophenyl butyrate as the substrate (PNB activity) and cutinase protein level was measured by an immunoblot assay, as indicated in the text.

activity levels, as measured with *p*-nitrophenyl butyrate as the substrate, showed identical changes.

Measurement of Cutinase Gene Transcripts. To determine whether induction of cutinase synthesis in the spores by cutin and its monomers involved pretranslational control, the level of RNA that hybridized with cutinase cDNA was measured. Since the amount of RNA involved was too low for isolation of poly(A)$^+$ RNA, total RNA was used in these hybridization experiments. Complications caused by hybridization of the labeled probe with the spore DNA could be avoided by treatment of the samples with DNase prior to the blotting. When the samples were treated with RNase, no hybridization with labeled probe could be detected. Dot-blot analysis gave linear responses with increasing amounts of RNA (Fig. 5). Thus, the method appeared to be appropriate for measuring RNA that hybridized specifically with cutinase cDNA probe (cutinase gene transcripts). With this technique, cutinase gene transcripts could be detected 15–30 min after addition of cutin hydrolysate or the dihydroxy-C_{16} acid isolated from it (Fig. 6). The level of the cutinase-specific RNA increased up to a maximum in ≈1 hr and subsequently decreased. This time course of appearance of cutinase gene transcripts is consistent with the observation that cutinase activity became detectable in 30–45 min. When cutin was added to the medium, the cutinase gene transcript began to appear in 15–30 min at detectable levels, and this level did not reach a maximum even after several hours (Fig. 5). The levels of the transcript obtained with cutin in the early periods were much smaller than those obtained with cutin hydrolysate, although eventually the level obtained with the polymer exceeded that obtained with the hydrolysate (data not shown).

To test whether the hydrolysate generated by the small amount of cutinase carried by the spores was the real inducer, the effect of exogenous cutinase on the early phase of appearance of cutinase gene transcript was tested. Addition of cutinase did, in fact, greatly stimulate the production of cutinase gene transcripts. In a 45-min period, cutinase addition with cutin caused the production of ≈5 times as much transcript as that obtained without cutinase supplementation. The decrease in the cutinase gene transcript level observed when the dihydroxy-C_{16} acid was used as the inducer suggested that the inducer might be degraded by the spore and that continued presence of the inducer was necessary for maintaining the transcript level. In fact, addition of another

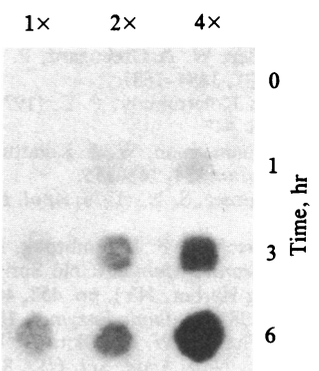

FIG. 5. Dot blots of RNA from the spores of *F. solani* f. sp. *pisi* with ^{32}P-labeled cutinase cDNA. Three levels of RNA obtained from spores incubated with cutin for the indicated periods were blotted, and densitometry showed a linear increase with increasing amounts of RNA.

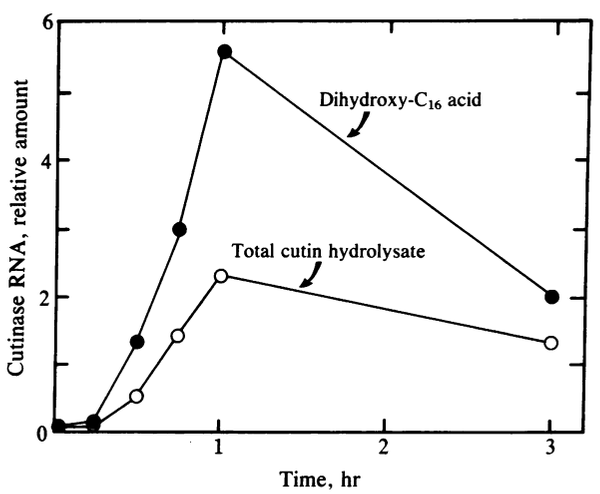

FIG. 6. Time course of induction of cutinase-specific RNA in spore suspensions of *F. solani* f. sp. *pisi* by cutin hydrolysate and dihydroxy-C_{16} acid. The RNA levels were quantitated (area) by densitometer scanning of autoradiograph from dot-blot hybridization with cutinase cDNA.

dose of dihydroxy-C_{16} acid after the mRNA level began to decrease caused a further increase in cutinase mRNA level and in cutinase activity level (data not shown). That the inducer was, in fact, degraded by the spores was demonstrated by the observations that exogenous ^{14}C-labeled dihydroxy-C_{16} acid and 9,10,18-trihydroxy-C_{18} acid were almost completely converted to respiratory CO_2 and other metabolites within a few hours by the spore suspension (data not shown).

DISCUSSION

The results presented in this paper show that cutinase is induced in the spores of *F. solani* f. sp. *pisi* soon after contact with cutin. The results obtained from the enzymatic activity measurements with the model substrate, *p*-nitrophenyl butyrate, or with cutin, agreed with the direct measurement of the enzyme level by using an immunological method. Therefore, it is clear that a contact with cutin or its monomers caused a true increase in the enzyme level rather than some kind of activation. Inhibition of appearance of the enzyme by cycloheximide suggested that protein synthesis was involved in the observed increase of cutinase. Such an increase in translation could be due to a pretranslational level control, such as increased levels of transcription of the cutinase gene. In fact, the level of total RNA that hybridized with labeled cutinase cDNA increased as a result of exposure of the spores to cutin or its monomers. Since mRNA and nuclear RNA were measured together in the hybridization experiments, it is not possible to determine whether the inducers affected processing of RNA. Whether the inducers enhanced actual transcription rates or somehow protected the primary transcripts and/or mRNA from degradation is not known. Irrespective of the precise nature of the mechanism, the fact that cutin or its monomers can induce the production of cutinase by fungal spores could have physiological significance in host–pathogen interaction.

From the limited amount of structure–activity studies conducted on the inducers, it appears that the essential structural feature of the inducer is that the alkyl chain of the acid has hydroxyl groups both in the ω-position and in the middle of the chain. Analogues that contain hydroxyl groups only at the ω-position or in the middle showed no inducing activity. Hydroxy acids most characteristic of cutin, dihydroxy-C_{16} and trihydroxy-C_{18} acids, were the most active inducers. These two hydroxy acids constitute the major components of cutin in most plants (1, 2), and such components are unique to the protective barriers of plants and are not found anywhere else in nature. Therefore, their use as the specific trigger for the induction of the enzyme used in fungal penetration of the plant cuticle would give the fungus a fail-safe method of sensing that it is in contact with the plant.

How cutin, an insoluble polymer, induces the synthesis of cutinase is not clear. The observation that cutin hydrolysate or monomers isolated from it can also induce enzyme synthesis suggests that the low levels of cutinase that might be present in the spore walls when they come into contact with the polymer might trigger the induction process. Consistent with this hypothesis was the observation that exogenous cutinase stimulated the induction of cutinase in spores during the initial periods. This hypothesis also explains the observation that, during the initial period, the level of cutinase induction was almost independent of the amount of cutin added. Even the low amount of cutin added was adequate for maximal production of the inducing monomers by the very low level of cutinase present in the spore.

If cutinase is indeed used by germinating fungal spores to gain entry into plants, induction of this enzyme must occur during the early periods of plant–pathogen interaction. It would also be advantageous for the fungus to trigger this induction only when it is in contact with the plant surface. Our results showing that the cutin monomers generated by the small amount of cutinase carried by the spore can induce cutinase synthesis in the spores reveal a possible mechanism by which the fungal spore can sense the presence of the plant. The small amount of monomers generated by the enzyme induces the production of more cutinase, which assists the germinating spore to penetrate the cuticular barrier. This conclusion is consistent with the previous finding that the level of cutinase found in the spores of different isolates of *F. solani* f. sp. *pisi* correlated with their ability to penetrate the intact cuticular barrier of pea stem (4). Once the fungus penetrates and grows past the cuticular barrier, the cutin monomer level would decrease and induction of cutinase would cease if the continued presence of the monomer is necessary for maintaining induction, as we have observed in the present study. Thus, the fungus spore can use cutin monomers to initiate the production of cutinase upon contact with plant surface and to turn off cutinase production after the penetration process. If this process is essential for pathogenesis, analogues of the inducers could be useful in interfering with the penetration process to protect plants against fungal attacks.

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