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Identification of a fungal cutinase promoter that is inducible by a plant signal via a phosphorylated trans-acting factor

ASLAM BAJAR, G. KRISHNA PODILA, AND P. E. KOLATTUKUDY*

The Ohio State Biotechnology Center, The Ohio State University, Columbus, OH 43210

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ABSTRACT **Plant cutin monomers trigger, and glucose suppresses, the expression of the cutinase gene of pathogenic fungi. To identify the cutinase promoter region responsible for induction by the unique plant components, a promoter analysis was done with transformants. Plasmids were constructed that contained (*i*) the 5′ flanking region of the cutinase gene or its deletion mutants from *Fusarium solani pisi* fused with a chloramphenicol acetyltransferase (CAT) reporter gene and (*ii*) a constitutive promoter fused with a hygromycin phosphotransferase gene. Hygromycin-resistant transformants of *F. solani pisi* generated by electroporation were assayed for CAT activity inducible by cutin hydrolysate and for glucose repression of this induction. CAT was induced in a glucose-repressible manner when fused with a 360-base-pair (bp), or longer, segment of the 5′ flanking region of the cutinase gene, and deletion of the next 135 bp abolished this induction. Gel retardation assays showed that a protein(s) in nuclear extract from the fungus bound to the 5′ flanking region of cutinase gene, and this binding was also abolished when the same 135-bp segment was deleted. These results show that the −225 to −360 segment of the cutinase gene contains a cis-acting regulatory element that binds trans-acting factor(s) in the nuclei. Treatment of the nuclear extract with immobilized phosphatase abolished binding to the promoter, suggesting that binding required a phosphorylated form of the protein. With isolated nuclei, phosphorylation of a protein occurred only in the presence of both cutin monomer and the fungal protein factor. The presence of protein kinase inhibitor H7 during the preincubation of nuclei with the monomer and protein factor inhibited cutinase gene transcription. These results suggest that cutin monomer causes phosphorylation of a transcription factor that binds to the −225 to −360 segment of the cutinase gene and enhances transcription of this gene.**

Fungi that can infect intact plant organs use cutinase to penetrate through cutin, the major structural component of the plant cuticle (1). Contact with cutin causes induction of cutinase in the spores of highly pathogenic fungi (2). Within minutes after contact with cutin, cutinase transcripts become detectable. Evidence has been presented that the small amount of cutin monomers released by the small amount of cutinase carried by the fungal spore induces cutinase synthesis in the spores of *Fusarium solani pisi* (*Nectria hematococca*). The 10,16-dihydroxy C_{16} acid and 9,10,18-trihydroxy C_{18} acid, the unique monomers of cutin, are the best inducers of cutinase. Nuclear run-off experiments showed that these hydroxy acids enhanced transcription of the cutinase gene in *Fusarium solani pisi* (3). In nuclei isolated from uninduced cultures of the fungus, the hydroxy acids triggered cutinase gene expression when supplemented with a protein factor (4). This selective activation of cutinase gene transcription involved enhancement of transcription initiation and normal termination of transcription. Studies with analogues of cutin monomers indicated that optimal activation of cutinase gene transcription required the structural elements naturally present in the dihydroxy and trihydroxy fatty acid monomers of cutin. How such monomers trigger cutinase gene transcription is not understood. Here we show that a 135-base-pair (bp) 5′ flanking segment of the cutinase gene from *F. solani pisi* is required for induction of cutinase by cutin monomers and for glucose repression of the gene. Nuclear protein binding selectively to the same region of the gene is demonstrated. Evidence is presented that cutin monomer causes phosphorylation of a transcription factor that binds to the bp −225 to −360 segment of the cutinase gene only when phosphorylated and this binding is involved in the activation of transcription of the cutinase gene.

MATERIALS AND METHODS

Materials. Single-spore isolates of *F. solani pisi* field isolate T8 (2) were maintained and cultures were grown on 1% glucose and salts (5). *Escherichia coli* DH5 was used as host for all vector constructs in pBluescript KS. Hygromycin B, driselase, and D-sorbitol were from Sigma. Novozyme 234 was from Novo Industries (Bagsvaerd, Denmark) and labeled nucleotides were from New England Nuclear. All restriction enzymes, T4 DNA ligase, and T4 DNA polymerase were from Pharmacia or from New England Biolabs. Kinase inhibitor H7 was from Seikagaku Kogyo, Tokyo.

Construction of Transformation Vectors. A hygromycin-resistance gene (*hph*) fused to a constitutive promoter from *Cochliobolus heterostrophus* (provided by Olin Yoder, Cornell University) was isolated as a 2.3-kilobase (kb) *Kpn* I–*Bam* III fragment (6), blunt-ended by T4 DNA polymerase, and then ligated into the T4 polymerase-blunted *Sac* I site of pBluescript KS. A transformant with the promoter adjacent to the polylinker region (pBluescript KS Coc Hyg) was selected. A 1.5-kb *Acc* I–*Bgl* I DNA fragment from U5-11 (7), containing the 5′ flanking region of the cutinase gene and 34 bp of the coding region, blunted by T4 DNA polymerase, was ligated into an *Acc* I, T4 DNA polymerase-blunted *Xho* I site of pBluescript KS. The 1.5-kb cutinase gene segment was fused to the coding region of the chloramphenicol acetyltransferase (CAT) gene, which was isolated as an *Xba* I–*Sal* I fragment from pGA583 (8) and ligated into *Xba* I/*Sal* I-digested pBluescript KS to produce pBluescript KS CAT. To obtain the correct translational fusion, the 1.5-kb cutinase gene in pBluescript was digested with *Kpn* I, T4 DNA polymerase-blunted, and then digested with *Xba* I. This fragment was ligated into *Xba* I/*Hpa* I-digested pBluescript KS CAT. The 1.5-kb cutinase promoter–CAT gene fusion was isolated as an *Xba* I–*Sal* I fragment and ligated into *Xba* I/*Sal* I-digested pBluescript KS Coc Hyg. A 465-bp *Pvu* II, T4 DNA polymerase-blunted *Bgl* I deletion of the cutinase gene was ligated into the *Eco*RV site of pBluescript KS. The cutinase gene segment was fused to the CAT coding region as

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Abbreviation: CAT, chloramphenicol acetyltransferase.

*To whom reprint requests should be addressed.

indicated above. An *Xba* I–*Hin*cII fragment containing the cutinase gene was ligated into *Xba* I/*Hpa* I-digested pBluescript KS CAT. The cutinase promoter–CAT gene fusion was ligated into pBluescript KS Coc Hyg. Further deletions were obtained by isolating a *Pvu* I–*Sal* I fragment from the 431-bp deletion fused to the CAT gene in pBluescript. This deletion contains 360 bp of cutinase leader sequence fused to the CAT gene. This fragment was ligated into *Sma* I/*Sal* I-digested pBluescript KS Coc Hyg. An *Aat* II digestion of pBluescript KS containing the 431-bp deletion fragment fused to the CAT gene left 225 bp of cutinase leader sequences. The *Aat* II site was blunted with T4 DNA polymerase and the DNA was cut with *Sal* I. This fragment was then ligated into *Sal* I-cut pBluescript KS Coc Hyg. Similarly, a *Bst*XI digestion left a 94-bp cutinase 5′ segment.

Transformation of Protoplasts. *F. solani pisi* protoplasts were prepared by a modification of a published procedure (9). Conidia (10^6) were shaken for 30–36 hr at 75 rpm in 100 ml of mineral medium (5) containing 2% glucose at room temperature, and mycelia were washed and suspended in 10 mM sodium phosphate (pH 5.8) containing 1.2 M $MgSO_4$, driselase (10 mg/g of tissue), and Novozyme 236 (25 mg/g of tissue). After the suspension was incubated at 30°C for 2 hr with shaking (75 rpm), the protoplasts (10^8) were recovered as described (9) and resuspended in 1.2 M sorbitol/10 mM Tris·HCl, pH 7.5/10 mM $CaCl_2$ (STC buffer). Protoplasts (10^7) in 700 μl of STC buffer were gently mixed with 10 μg of linearized DNA in a 1-ml electroporation cuvette and incubated on ice for 15 min before a 100-msec pulse at 1 kV was applied. The protoplasts were again incubated on ice for 10 min before they were plated at various dilutions onto V-8 medium containing 1.2 M sorbitol, mineral salts (5), and 2% agar. Overlays of 1% agarose containing hygromycin at 200 μg/ml were added 24 hr later. After 7 days of growth, agar plugs containing the fastest-growing colonies were transferred to V-8 plates containing hygromycin at 300 μg/ml. After 7 days, agar plugs from the periphery of each growing transformant were transferred onto V-8 agar containing no hygromycin. After 4 or 5 days of growth, agar plugs from the periphery of each growing transformant were transferred to V-8 agar containing hygromycin at 300 μg/ml. Ten of the fastest-growing colonies were transferred to individual plates containing hygromycin at 150 μg/ml and kept as stock plates. This procedure yielded about 20 transformants per μg of DNA; all of the transformants obtained in the first selection were stable with no abortive false positives; 100–250 stable transformants were isolated for each transformation vector. From each deletion class, 4 transformants that grew well on medium with hygromycin at 300 μg/ml were randomly selected and grown on V-8 plates containing hygromycin at 150 μg/ml for 6–7 days. Conidia from these plates were used to generate protoplasts.

For promoter analysis, an equal number of protoplasts from each transformant were added to 1 ml of T8 medium with or without 2% glucose. For induction, cutin hydrolysate (10), dispersed in STC buffer by sonication, was added at 80 μg/ml. The protoplasts were incubated in the dark at 26°C for 48 hr and centrifuged in an Eppendorf centrifuge; the supernatant was assayed for cutinase spectrophotometrically (11). The pelleted cells were suspended in 300 μl of buffer and disrupted by sonication. To minimize background the cell lysate was incubated at 65°C for 10 min and centrifuged for 5 min in the Eppendorf centrifuge. Equal amounts of protein were assayed for CAT activity at 37°C (12).

Binding of Nuclear Proteins to the Cutinase Gene. After glucose depletion of the medium as determined by glucose oxidase assay, nuclei were prepared (4) and nuclear protein extract was isolated (13). Aliquots of the nuclear extract (1–5 μg of protein) were incubated in 20 μl with ^{32}P-labeled DNA fragment (2–5 ng) and 1 μg of poly(dI-dC) in 25 mM Hepes, pH 7.9/150 mM NaCl/5 mM $MgCl_2$/10 mM dithiothreitol/2 mM EDTA/3 mM phenylmethanesulfonyl fluoride/5% (vol/vol) glycerol for 15 min, the mixture was subjected to electrophoresis, and autoradiograms were prepared. Nuclear extract was incubated for 30 min with or without immobilized calf intestinal mucosal alkaline phosphatase (Sigma) at 22°C and beads were removed by centrifugation prior to incubation with labeled DNA.

Protein Phosphorylation. Isolated nuclei were preincubated with the protein factor and dihydroxy C_{16} acid with or without 1 mM protein kinase inhibitor H7 and assayed for production of cutinase transcripts (4); in the control the inhibitor was added after preincubation. Protein factor used in all experiments was obtained from the supernatant of the fungal homogenate prepared for the isolation of nuclei (4). Upon gel filtration of the supernatant on a Sepharose 6B column, cutinase transcription-activating protein factor (14) emerged at the void volume, and aliquots of this protein were designated "protein factor."

To measure phosphorylation, various combinations of nuclei, protein factor, and monomer (5 μg) were mixed with 60 mM Hepes (pH 7.6), 90 mM NaCl, 0.06% Triton X-100, 6.3 mM $MgCl_2$, 1.5 μg of bovine serum albumin, 10 mM $MnCl_2$, 1 mM phenylmethanesulfonyl fluoride, and 30 μCi of [γ-^{32}P]ATP (1 μCi = 37 kBq) in a total volume of 35 μl and incubated 10 min at 26°C. The reaction was stopped, the protein was subjected to SDS/10% PAGE, and the gel was dried and autoradiographed.

RESULTS

In isolated fungal nuclei the hydroxy acids from the plant, together with a fungal protein factor, promote initiation of cutinase gene transcription (4). To identify the hydroxy acid-inducible promoter of fungal cutinase, we performed a promoter analysis using fungal transformation. The plasmid used for transformation contained a hygromycin-resistance gene fused to a constitutive promoter from *C. heterostrophus* (6) and the CAT gene fused to the 5′ flanking region of the cutinase gene from *F. solani pisi* (7) (Fig. 1). The plasmids were used to transform *F. solani pisi* protoplasts. Protoplasts from the resulting stable transformants were analyzed for inducible expression of CAT activity and repression by glucose. The 1.5-kb 5′ flanking region of the cutinase gene

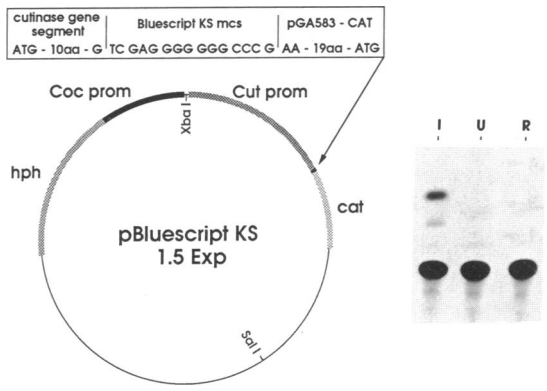

FIG. 1. (*Left*) Plasmid containing a constitutive *Cochliobolus heterostrophus* promoter (Coc prom) with hygromycin phosphotransferase gene (hph) and cutinase 5′ flanking region (Cut prom) with CAT gene. (cat) In cutinase promoter–CAT junction (top): aa, amino acid codons; mcs, multiple cloning site. (*Right*) Thin-layer chromatographic assay for CAT activity of *F. solani pisi* transformed by electroporation with the plasmid. Lanes: I, induced with cutin hydrolysate; U, uninduced; R, induction by cutin hydrolysate repressed with glucose.

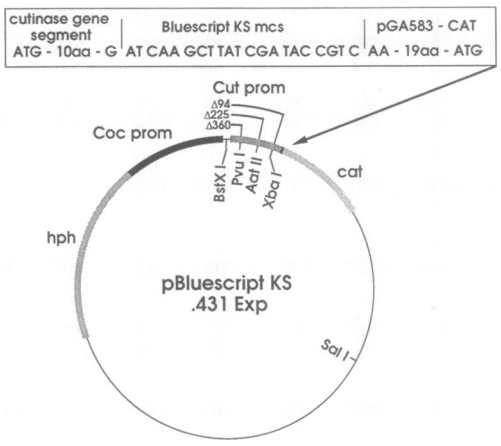

FIG. 2. Transformation vector containing the CAT gene with cutinase promoter deletion (Δ) derivatives (Cut prom) and hygromycin phosphotransferase gene (hph) with a constitutive *C. heterostrophus* promoter (Coc prom). Detailed structure of the fusion region is shown (see Fig. 1 legend for explanation).

conferred inducibility of CAT activity by cutin hydrolysate, and glucose completely repressed this induction (Fig. 1).

To identify the segment of the 5′ flanking region of the fungal cutinase gene responsible for induction by the plant signal, deletion mutants derived from the 5′ flanking region were fused to the CAT gene (Fig. 2), and introduced into protoplasts of *F. solani pisi* by electroporation, and mitotically stable transformants were used for induction studies. To verify that the deletion mutants had integrated into genomic DNA, Southern blot analyses were performed. In all cases, the *Pvu* II fragment of the cutinase gene hybridized with high molecular weight DNA corresponding to the fungal genomic DNA, indicating integration of the plasmid into the fungal genome (data not shown). When Southern blots of *Sst* I-digested DNA were probed with the vector containing no cutinase gene segment (to avoid hybridization to the native cutinase gene), a single band was seen for each transformant, and the differences in size indicated random integration into the fungal genome (Fig. 3 *Left*). The fragment size is consistent with the possibility that the plasmids underwent tandem duplication before integration into the fungal genome. Southern blots of *Xba* I/*Sca* I-digested DNA from each of the transformants showed a band representing the endogenous cutinase gene in the lanes representing all of the transformants when probed with a construct containing a cutinase gene fragment (arrow, Fig. 3 *Right*). This result indicated that the endogenous cutinase gene was not disrupted by the introduced exogenous sequences, suggesting nonhomologous recombination. Consistent with this conclusion, all transformants produced equal amounts of cutinase when induced with cutin hydrolysate (data not shown). The multiple bands observed in the Southern blot suggests random integration of exogenous plasmids into the fungal genome, possibly after tandem duplication or concatemerization, as previously concluded with other filamentous fungi (14–16). This mode of integration is quite suitable for promoter analysis.

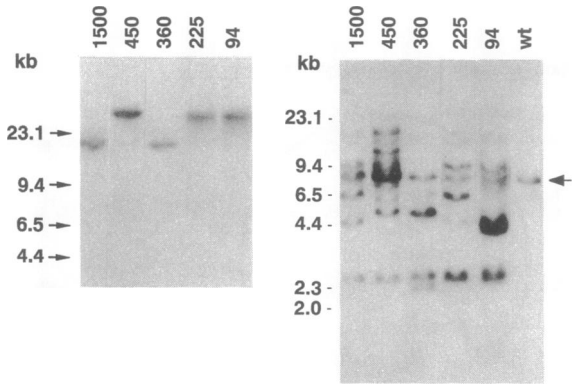

FIG. 3. Southern blots of genomic DNA isolated from some *F. solani pisi* transformants obtained with the vector containing the cutinase flanking region indicated above the lanes. (*Left*) Genomic DNA was digested with *Sst* I and probed with a ^{32}P-labeled DNA containing pBluescript, *hph*, and CAT sequences but no cutinase sequence; electrophoresis was done in 0.5% agarose gel. DNA from untransformed cells showed no hybridization. (*Right*) DNA was digested with *Xba* I and *Sca* I, and the deletion construct containing the *Pvu* II derivative, labeled with ^{32}P, was used as the probe. Arrow, major band from untransformed, wild-type (wt) cells.

To identify the monomer-inducible promoter region of cutinase gene, the transformants containing a *Pvu* II-shortened 5′ flanking region of the cutinase gene fused to the CAT gene (Fig. 2) were tested for inducibility of the marker gene by cutin hydrolysate and for repression by glucose. The 431-bp 5′ flanking portion of the cutinase gene conferred inducibility by cutin hydrolysate on the CAT gene, and the presence of glucose repressed this induction (Fig. 4*A*). When

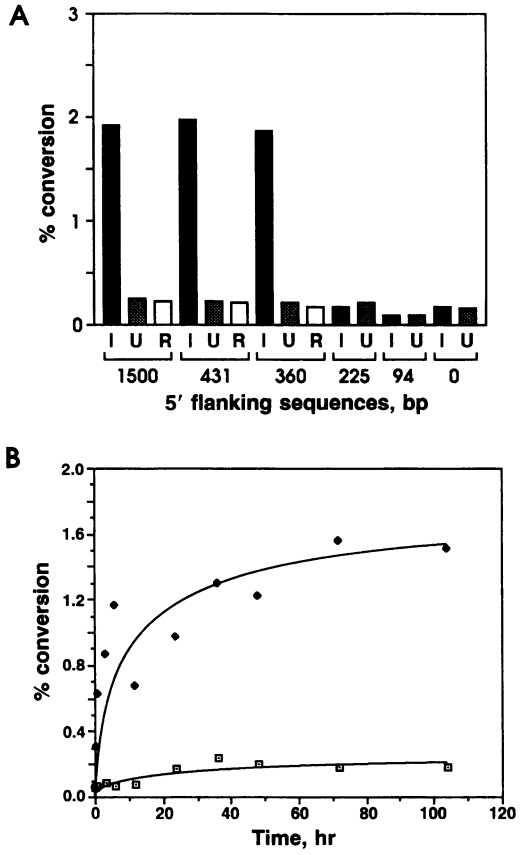

FIG. 4. (*A*) CAT activity of transformants obtained with the vector shown in Fig. 2, containing the indicated segments of the 5′ flanking region of cutinase. Bars: I, induced with cutin hydrolysate; U, uninduced; R, induction by cutin hydrolysate repressed with glucose. Four independent transformants were assayed for each case and the average is given. To obtain accurate rate measurements, percent conversion (acetylation of [^{14}C]chloramphenicol) was kept low. (*B*) Time course of induction of CAT activity in a transformant generated with the vector shown in Fig. 2 containing the 431-bp 5′ flanking region of the cutinase gene. One of the transformants used in *A* was grown in medium with glucose; upon depletion of glucose, cutin hydrolysate was added. After various induction periods, CAT assays were done. ◆, Induced; ⊡, uninduced.

individual transformants were tested, the induction ranged from 6- to 15-fold overall and variation was within ±15% for the four independent transformants used for each construct in each experiment. The time course of CAT induction by cutin hydrolysate and its repression by glucose (Fig. 4*B*) were quite similar to the previously observed time course of cutinase induction by cutin monomers and glucose repression in *F. solani pisi* cultures (10). Thus, the induction of CAT activity appears to be an authentic test for the promoter activity of the cutinase gene. When the 5′ flanking region of the cutinase gene was shortened by *Pvu* I digestion, the resulting 360-bp promoter likewise conferred on the CAT gene inducibility by cutin monomers and repression by glucose. The degree of inducibility was the same with the 1500-bp, 431-bp, or 360-bp cutinase promoter (Fig. 4*A*), and in all cases glucose completely repressed the gene. However, further shortening of the cutinase promoter by digestion with *Aat* II or *Bst*XI abolished inducibility of the CAT gene (Fig. 4*A*). Thus, the −225 to −360 region of the fungal cutinase gene contains cis-acting elements necessary for the induction of this gene by the plant signal. Examination of the 135-bp segment, essential for cutinase induction by the signal generated from the plant cuticle and for glucose repression, showed the presence of a cAMP-responsive element and an Sp1 transcription factor-binding element (17, 18) (Fig. 5).

To test whether nuclear proteins bind to the cutinase promoter region, the 5′ flanking region of the cutinase gene and its derivatives obtained by restriction enzyme digestion were tested for retardation of their electrophoretic mobility after incubation with nuclear extract prepared from *F. solani pisi* grown until glucose was depleted. Incubation of ^{32}P-labeled 360-bp *Pvu* I fragment of the cutinase gene with nuclear extract gave a discrete band indicating retardation of mobility; inclusion of a 100-fold excess of unlabeled 360-bp segment in the incubation mixture eliminated this band (Fig. 6 *Left*), indicating that the retarded band represented binding of nuclear protein specifically to the 360-bp segment of DNA. Deletion of the 135 bp at the 5′ end of this DNA segment by *Aat* II treatment yielded a 225-bp fragment that did not show any indication of binding to nuclear protein(s) (Fig. 6 *Right*). Thus, the same region of the cutinase promoter that is required for induction by cutin monomer and repression by glucose is also involved in binding nuclear protein. Treatment of nuclear protein extract with immobilized phosphatase abolished binding to the promoter, indicating that phosphorylation of the protein is required for DNA binding (Fig. 6 *Right*).

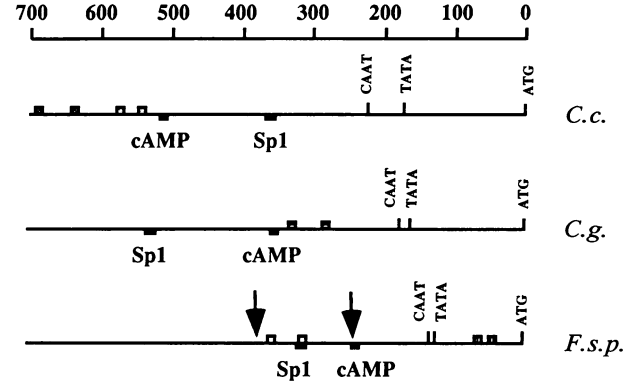

FIG. 5. The 5′ flanking region of three fungal cutinase genes [*C.c., Colletotrichum capsici; C.g., Colletotrichum gloeosporioides; F.s.p., F. solani pisi* (24)]. cAMP-responsive element and Sp1 consensus binding sequence are shown by solid boxes and tandem 11-bp direct repeats are shown by open boxes. Arrows show the promoter region found to be necessary for inducible expression. Scale is in base pairs.

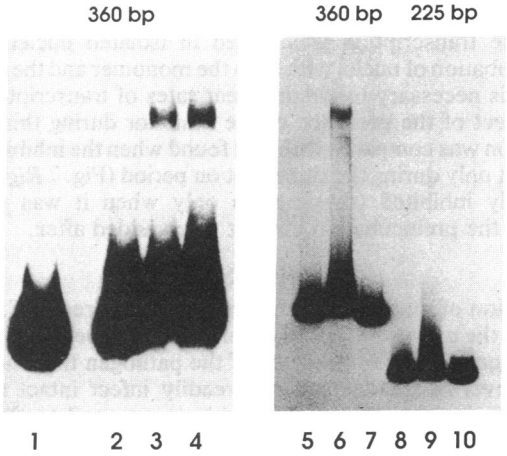

FIG. 6. Gel retardation of 5′ flanking region of cutinase gene by interaction with nuclear proteins from *F. solani pisi* and prevention of this interaction by phosphatase treatment of the nuclear protein. ^{32}P-labeled cutinase gene fragments (indicated above the autoradiograms) were incubated with (lanes 2, 3, 4, 6, 7, 9, and 10) or without (lanes 1, 5, and 8) nuclear proteins from *F. solani pisi* for 15 min and the mixture was subjected to electrophoresis; 1 μg of nuclear protein was used for lane 3, and 5 μg was used for the other lanes. For lane 2, a 100-fold excess of unlabeled 360-bp fragment was added; for lanes 7 and 10, the nuclear protein was treated for 30 min with 5 units of immobilized alkaline phosphatase at 22°C; for lanes 6 and 9, similar incubation was done without alkaline phosphatase before incubation with DNA.

In an attempt to detect phosphorylation of the trans-acting factor(s), isolated nuclei were incubated with [γ-^{32}P]ATP in the presence of the protein factor (from the cell extract) that was found to be required for activation of cutinase transcription in isolated nuclei (4) and/or the cutin monomer (10,16 dihydroxy C_{16} acid), and the products were analyzed by SDS/PAGE (Fig. 7 *Left*). Maximal phosphorylation of a protein of about 52 kDa required both cutin monomer and protein factor, although some phosphorylation was detected when only the monomer was added to the nuclei. To determine whether phosphorylation is relevant to cutinase transcription,

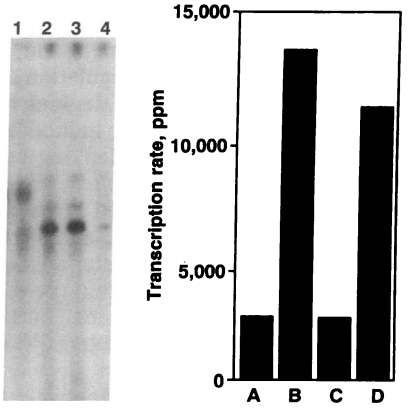

FIG. 7. (*Left*) Protein phosphorylation in nuclei incubated with the protein factor from the supernatant and dihydroxy C_{16} acid and inhibition of phosphorylation by protein kinase inhibitor H7. Incubation was done with [γ-^{32}P]ATP under conditions that activate transcription of the cutinase gene (4). Lanes: 1, nuclei with the supernatant protein factor; 2, nuclei with dihydroxy C_{16} acid; 3, nuclei with the supernatant protein factor and dihydroxy C_{16} acid; 4, same as 3 but with 1 mM H7. (*Right*) Inhibition of cutinase gene transcription in isolated nuclei by protein kinase inhibitor H7. Bars: A, nuclei; B, nuclei plus protein factor plus dihydroxy C_{16} acid; C, same as B but preincubated with 1 mM H7 for 30 min prior to transcription assay; D, same as B but with the addition of 1 mM H7 at the beginning of the transcription assay, after the 30-min preincubation. Transcription rate is expressed as parts per million (4).

the effect of protein kinase inhibitor H7 (19) on activation of cutinase transcription was tested in isolated nuclei. Since preincubation of nuclei with both the monomer and the protein factor is necessary to obtain linear rates of transcription (4), the effect of the presence of the inhibitor during this preincubation was compared with that found when the inhibitor was present only during the transcription period (Fig. 7 *Right*). H7 severely inhibited transcription only when it was present during the preincubation but not when added after.

DISCUSSION

Induction of cutinase in the germinating spores of *F. solani pisi* by the unique cutin monomer appears to be important for pathogenesis (1, 20). Isolates of the pathogen that showed a high level of induction could readily infect intact organs, whereas isolates that were unable to respond to the plant signal could not infect intact pea stem. To identify the cutinase promoter involved in the induction of cutinase by the plant signal, we used a construct that contains constitutive *C. heterostrophus* promoter to drive the hygromycin-resistance gene used for selection of transformants and the cutinase promoter to drive the CAT marker gene. Electroporation yielded ample stable transformants that showed remarkably similar (±15%) degrees of induction, and therefore quantitative comparison could be readily done. With this approach, we demonstrated inducible and repressible expression of the marker gene. The results obtained with the transformants suggest that a 135-bp DNA segment in the 5′ flanking region of the cutinase gene is necessary for induction by cutin hydrolysate and for glucose repression. The results obtained with promoter analysis using transformants agreed with the results of DNA binding of nuclear proteins. The same 135-bp segment of the 5′ flanking region was found to be involved in binding nuclear protein. An examination of the nucleotide sequence of the promoter showed that a cAMP-responsive element is present 224 bp upstream of the ATG initiation codon. This sequence is not present anywhere else in the known 2868-bp sequence of the *Fusarium* cutinase gene. Removal of the region containing the cAMP-responsive element eliminated induction and repression. Furthermore, the 5′ flanking region of the cutinase gene from *Colletotrichum gloeosporioides* and from *Colletotrichum capsici* (21) has one cAMP-responsive element (Fig. 5). These results suggest that the cAMP-responsive element and/or the adjacent regions might be involved in the regulation of the fungal cutinase gene. Another feature of the 5′ flanking region of the cutinase gene found to be essential for induction is the Sp1 site, which is also present in the 5′ flanking region of the cutinase gene from the two *Colletotrichum* species (20). In each of the three cutinase genes, nested 11-bp direct reeats were found in the 5′ flanking region, and in the case of *Fusarium*, the only case in which promoter analysis has been done, the direct repeats are located within the region shown to be essential for induction and repression. Further analysis of the promoter region is necessary to determine whether the direct repeats play a functional role.

Since a nuclear protein also binds selectively to the same 5′ flanking region as that required to confer inducibility on the CAT marker gene, it is most likely that this protein represents a trans-acting factor involved in induction of the cutinase gene. In isolated fungal nuclei, cutinase transcription was induced by cutin monomer and a fungal protein factor from the fungal extract supernatant (4). How the cutin monomer enhances cutinase transcription is not clear. Since the monomer and the protein factor were required for the phosphorylation of a protein in the presence of nuclei, and phosphatase treatment of nuclear protein extract prevented binding to the promoter, it appears that the role of cutin monomer is to cause phosphorylation of a transcription factor that binds to the cutinase promoter (and/or another transcription factor) only when it is phosphorylated, and this binding activates cutinase gene transcription. The present result that protein kinase inhibitor H7 inhibits activation of cutinase transcription by cutin monomer and the protein factor supports this hypothesis. This inhibition of transcription required the presence of H7 during the preincubation of isolated nuclei with the cutin monomer and the protein factor that was found to be essential for eliminating the lag observed in the activation of cutinase gene transcription. Thus, the lag probably represents biochemical reactions required for transcription activation that include phosphorylation of transcription factors. The protein factor (from the fungal extract) that was required to activate cutinase gene transcription was a 100-kDa protein in its native form (21), and the protein that was phosphorylated when the nuclei were incubated in the presence of cutin monomer and the protein factor showed a subunit size that was about half of this native size. Therefore it is tempting to speculate that a dimer of the transcription factor whose phosphorylation is enhanced by the cutin monomer binds to the promoter. Thus, the mechanism of regulation of the cutinase gene, involved in fungal pathogenesis, by the dihydroxy and trihydroxy fatty acids of the host plant cuticle would be analogous to the regulation of gene expression by lipid-type bioregulators such as steroid hormones, thyroxine, and retinoic acid (22, 23), and the transcription factor involved may belong to the superfamily of factors involved in transcription regulation by such regulatory molecules.

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1. Kolattukudy, P. E. (1985) *Annu. Rev. Phytopathol.* **23**, 223–250.
2. Woloshuk, C. P. & Kolattukudy, P. E. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 1704–1708.
3. Podila, G. K., Dickman, M. B., Rogers, L. M. & Kolattukudy, P. E. (1989) in *Molecular Biology of Filamentous Fungi*, eds. Nevalainen, H. & Pentillä, M. (Found. Biotech. Ind. Fermentation Res., Helsinki), pp. 217–226.
4. Podila, G. K., Dickman, M. B. & Kolattukudy, P. E. (1988) *Science* **242**, 922–925.
5. Hankin, L. & Kolattukudy, P. E. (1968) *J. Gen. Microbiol.* **51**, 457–463.
6. Turgeon, B. G., Garber, R. C. & Yoder, O. C. (1987) *Mol. Cell. Biol.* **7**, 3297–3305.
7. Soliday, C. L., Dickman, M. B. & Kolattukudy, P. E. (1989) *J. Bacteriol.* **171**, 1942–1951.
8. An, G. (1986) *Plant Physiol.* **81**, 86–91.
9. Yelton, M. M., Hamer, J. E. & Timberlake, W. E. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 1470–1474.
10. Lin, T. S. & Kolattukudy, P. E. (1978) *J. Bacteriol.* **133**, 942–951.
11. Kolattukudy, P. E., Purdy, R. E. & Maiti, I. B. (1981) *Methods Enzymol.* **71**, 652–664.
12. Gorman, C. M., Moffat, L. F. & Howard, B. H. (1982) *Mol. Cell. Biol.* **2**, 1044–1051.
13. Dignam, J. D., Lebovitz, R. M. & Roeder, R. G. (1983) *Nucleic Acids Res.* **11**, 1475–1489.
14. Fincham, J. R. (1989) *Microbiol. Rev.* **53**, 148–170.
15. Woloshuk, C. P., Seip, E. R., Payne, G. A. & Adkins, C. R. (1990) *Appl. Environ. Microbiol.* **55**, 86–90.
16. Tsuge, T., Nishimura, S. & Kobayashi, H. (1990) *Gene* **90**, 207–214.
17. Briggs, M. R., Kadonaga, J. T., Bell, S. P. & Tjian, R. (1986) *Science* **234**, 47–52.
18. Jones, N. C., Rigby, P. W. J. & Ziff, E. B. (1988) *Genes Dev.* **2**, 267–281.
19. Kawamoto, S. & Hidaka, H. (1984) *Biochem. Biophys. Res. Commun.* **125**, 258–264.
20. Kolattukudy, P. E. (1987) in *The Biochemistry of Plants, Vol. 9. Lipids: Structure and Function*, ed. Stumpf, P. K. (Academic, New York), pp. 291–314.
21. Kolattukudy, P. E. (1991) in *Molecular Signals in Plant–Microbe Communication*, ed. Verma, D. P. S. (CRC, Boca Raton, FL), in press.
22. Beato, M. (1989) *Cell* **56**, 335–344.
23. Evans, R. M. (1988) *Science* **240**, 889–895.
24. Ettinger, W. F., Thukral, S. K. & Kolattukudy, P. E. (1987) *Biochemistry* **26**, 7883–7892.