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Structure of Cutinase Gene, cDNA, and the Derived Amino Acid Sequence from Phytopathogenic Fungi†

William F. Ettinger, Sushil K. Thukral, and Pappachan E. Kolattukudy*
Institute of Biological Chemistry and Program in Plant Physiology, Washington State University, Pullman, Washington 99164, and Ohio State Biotechnology Center, The Ohio State University, Columbus, Ohio 43210
Received May 26, 1987; Revised Manuscript Received August 3, 1987

ABSTRACT: Cutinase is an extracellular fungal enzyme that allows pathogenic fungi to penetrate through the cuticular barrier into the host plant during the initial stages of the fungal infection. mRNA isolated from glucose-grown *Colletotrichum capsici*, induced to produce cutinase by the addition of cutin hydrolysate, was used to prepare cDNA which was cloned in the expression vector λ gt11. The primary structure of the cutinase from *C. capsici* was deduced from the nucleotide sequence of the cloned cutinase cDNA. Amino acid sequences of two tryptic peptides isolated from cutinase produced by *C. capsici* completely matched with two segments of the amino acid sequence deduced from the nucleotide sequence, strongly suggesting that the cloned cDNA was authentic cutinase cDNA. The cDNA clone was used as a probe to screen *C. capsici* and *Colletotrichum gloeosporioides* genomic libraries constructed in Charon 35 and EMBL 3, respectively. The nucleotide sequences of the cutinase structural genes from *C. capsici* and *C. gloeosporioides* were also determined. S1 mapping was used to reveal the transcriptional start sites and polyadenylation site of the primary transcript from *C. capsici*. The primary sequences and gene structure of the enzymes from the *Colletotrichum* species were compared with the primary structure and gene structure of a cutinase from *Fusarium solani* f. sp. *pisi*. A comparison of the deduced primary structures of the enzymes showed that residues involved in the catalytic triad and disulfide cross-linking of cutinase are strongly conserved. Yet, only 43% of the residues are conserved between all three enzymes. A comparison of the structure of the three genes revealed the location of the single intron has been conserved. The transcriptional start site of the *C. capsici* gene was centered on the sequence TCCAGACCA, the core of which (CAGAC) is found repeated after 21 nucleotides. The same core sequence, repeated after 11 nucleotides, was also identified in the 5′ nontranslated regions of the *C. gloeosporioides* and *F. solani* genes.

Aerial plant organs are protected by a cuticle that is composed of an insoluble polymeric structural component called cutin and associated soluble lipids collectively called waxes (Kolattukudy, 1980a). Cutin is a polyester composed of hydroxy and hydroxyepoxy fatty acids (Kolattukudy, 1980b, 1981). Germinating fungal spores of phytopathogens secrete cutinase to assist in the penetration through the cuticular barrier, and inhibition of this enzyme prevents infection through the intact cuticular barrier (Kolattukudy, 1985). Cutinase is induced in the spores of virulent pathogenic fungi soon after contact with cutin (Woloshuk & Kolattukudy, 1986). Evidence was presented that the unique cutin monomers released by the small amounts of cutinase present on the spores are the true inducers. Although such monomers were shown to cause a large and rapid increase in the level of cutinase transcripts, the mechanism of regulation of expression of this gene has not been elucidated.

An understanding of how hydroxy fatty acids regulate cutinase gene expression may lead to the design of novel methods

†Supported by Grant DMB-8306835 from the National Science Foundation.
*Address correspondence to this author at The Ohio State University, Columbus, OH 43210.

0006-2960/87/0426-7883$01.50/0

of plant protection. Such information may also be useful in designing convenient methods for triggering the expression of engineered genes introduced into fungi for the production and secretion of foreign proteins. Investigations of the mechanisms by which cutin monomers induce the expression of the cutinase gene in fungi would be aided by the knowledge of the structure of the cutinase gene and its flanking regions. The only structural information so far available is the nucleotide sequence of the cloned cDNA for cutinase from *Fusarium solani* f. sp. *pisi* strain T-8 (Soliday et al., 1984). Although the nucleotide sequence of one cutinase gene from *F. solani* f. sp. *pisi* has been determined, elucidation of the mechanism of regulation of this gene is complicated by the presence of two cutinase genes (C. L. Soliday and P. E. Kolattukudy, unpublished data). In this paper, we report the cloning and sequencing of a cDNA for cutinase from *Colletotrichum capsici*, the nucleotide sequences of the single cutinase genes present in *Colletotrichum capsici* and *Colletotrichum gloeosporioides*, and the transcription start site and polyadenylation site of the gene in *C. capsici*. A comparison of the amino acid sequences deduced from the nucleotide sequences reveals that the primary structure around the amino acid residues representing the catalytic triad and the two disulfide bridges are highly conserved but other regions of the enzyme can have considerable divergence.

Materials and Methods

Materials. λ gt11, EMBL 3, and Gigapack in vitro λ packaging kit were purchased from Stratagene Cloning Systems. M13mp19, pUC19, synthetic *Eco*RI linkers, and restriction enzymes were purchased from Pharmacia. Oligo(dT)–cellulose (type 3) was purchased from Collaborative Research. ^{32}P- and ^{35}S-labeled nucleotides, ^{125}I-labeled protein A, [^{35}S]methionine, rabbit reticulocyte lysate translation system, S1 nuclease, [^{3}H]diisopropyl fluorophosphate, [^{14}C]iodoacetamide, and En^{3}Hance were purchased from New England Nuclear. Dideoxynucleotides, sequencing primers, T-4 DNA polymerase, and DNA polymerase I Klenow fragment were purchased from New England Biolabs. Cetyltrimethylammonium bromide, *S*-adenosylmethionine, guanidine hydrochloride, LiCl, and protein A–Sepharose were purchased from Sigma. Nitrocellulose filters (BA 85) were purchased from Schleicher & Schuell. Packagene in vitro λ packaging kit was purchased from Promega. The RD20 oligomer was synthesized in our laboratory on an Applied Biosystems Model 380A DNA synthesizer.

Strains. Colletotrichum capsici (ATCC 48574), an isolate from pepper, and *Colletotrichum graminicola* were kindly provided by Professor Ralph Nicholson, Purdue University. *Colletotrichum gloeosporioides*, an isolate from papaya, was kindly provided by Professor Suresh Patil, University of Hawaii. *Colletotrichum lindemuthianum* was kindly provided by Professor Sally Leong, University of Wisconsin, Madison. For mRNA isolation, spores were inoculated into Roux culture bottles containing 100 mL of a mineral medium (Hankin & Kolattukudy, 1968) supplemented with 0.25% glucose and incubated for 4 days at 28 °C. Subsequently, cutinase induction was initiated by the addition of cutin hydrolysate (20 mg/100 mL) as previously done with *Fusarium solani pisi* (Lin & Kolattukudy, 1978). Mycelia, harvested by filtration through Whatman No. 1 filter paper 24 h after induction, were frozen in liquid nitrogen and lyophilized (Flurkey & Kolattukudy, 1981). For genomic DNA isolation, spores were inoculated into 2.8-L Fernbach flasks containing 500 mL of the mineral medium supplemented with 0.25% yeast extract and 1.0% glucose. The mycelia, harvested by filtration after 4–7 days, were frozen in liquid nitrogen and lyophilized. *Escherichia coli* strains used were as follows: KH802 for λ Charon 35 propagation; Y1088, Y1089, and Y1090 for λ gt11 propagation; JM101 for the propagation of pUC plasmids and M13 phage derivatives; Q359 and Q358 for the propagation of EMBL 3.

Nucleic Acid Isolation. Total cellular RNA was isolated by extraction with guanidine hydrochloride and precipitated with LiCl (Cathala et al., 1983). Poly(A+) mRNA isolated with oligo(dT)–cellulose was subjected to sucrose density gradient centrifugation in an SW 55 rotor at 55 000 rpm for 20 h (Mozer, 1980). To isolate fungal genomic DNA, 2–3 g of the lyophilized mycelia was ground with a mortar and pestle with liquid nitrogen and 100 mg of 500-μm glass beads. Nucleic acids were extracted from the powdered mycelia with cetyltrimethylammonium bromide (20 mL of 1× extraction buffer/g dry weight) (Murray & Thompson, 1980). Plasmid and double-stranded M13 DNA were isolated by an alkaline lysis procedure (Birnboim & Doly, 1979). Bacteriophage was isolated by precipitation with poly(ethylene glycol) (Yamamoto et al., 1970). DNA was liberated from the phage by proteinase K treatment (Maniatis et al., 1982). Genomic, plasmid, and phage λ DNA were all further purified on a CsCl–ethidium bromide step gradient (Garger et al., 1983).

Restriction Analysis of Genomic DNA. Purified DNA was digested with an excess of the restriction enzyme; 10-μg aliquots were electrophoresed and subjected to Southern transfer (Southern, 1975). Cutinase sequences were detected by hybridization to the 838-bp *C. capsici* cutinase cDNA, which had been labeled with [α-^{32}P]dATP by nick translation (Maniatis et al., 1975).

Translation of mRNA. Poly(A+) mRNA from cutin hydrolysate induced cultures of *C. capsici* was translated, and the products were examined as described (Flurkey & Kolattukudy, 1981). Hybrid select translation was performed as described (Parnes et al., 1981), except nitrocellulose disks (0.4-cm diameter) were saturated with denatured cDNA insert (20 μg) by vacuum filtration, prior to washing and hybridization.

Peptide Isolation. C. capsici cutinase (40 mg) was treated with [1,3-^{3}H]diisopropyl fluorophosphate ([1,3-^{3}H]DFP) (7.3 Ci/mol), reduced and carboxyamidomethylated by treatment with [^{14}C]iodoacetamide (0.89 Ci/mol), and digested with trypsin as described (Soliday & Kolattukudy, 1983). The tryptic peptides in 2 M urea were initially fractionated by HPLC on a NOVA-PAC C_{18} RADIAL-PAC column (Waters) with a linear gradient of 0–70% CH_3CN in water containing 0.1% trifluoroacetic acid (TFA), at a flow rate of 2 mL/min in 1 h on a Waters HLPC system. Subsequent fractionation of peptide I on the C_{18} column was performed with a 0–15% gradient of a 2:1 mixture of CH_3CN and 2-propanol in water containing 0.1% TFA in 40 min and a 0–15% gradient of CH_3CN in water containing 0.1% H_3PO_4 in 40 min. Final desalting of peptide I on the C_{18} column was performed with a 0–15% gradient of CH_3CN in water containing 0.1% TFA in 40 min. Subsequent fractionation of peptide II on the C_{18} column was performed with a 0–30% gradient of CH_3CN in water containing 0.1% TFA in 10 min followed by a 30–50% gradient in 40 min. Final desalting of peptide II was performed on a Synchropak C_1 column with 0.1% TFA and 0.165% triethylamine in 10 min, followed by a 30–50% CH_3CN gradient in 40 min. Absorbance of the eluants was monitored at 230 nm on a Waters Model 480 variable-wavelength detector, and the radioactivity was monitored on a FLO-ONE Model HS radioactive flow de-

tector (Radiometric Instruments & Chemical Co.). The amino acid composition of the purified peptides was determined after hydrolysis in 6 N HCl (Pierce) under nitrogen for 16 h at 110 °C. The peptides were sequenced with a Beckman Model 890 C sequencer at the University of California, Davis, by Dr. Alan J. Smith.

Library Construction and Screening. A cDNA library from cutin hydrolysate induced *C. capsici* mRNA was constructed in the expression vector λ gt11 with ^{125}I-labeled protein A for detection (Huynh et al., 1985).

The *C. capsici* and *C. gloeosporioides* genomic libraries were constructed in the phage λ replacement vectors Charon 35 (Loenen & Blattner, 1983) and EMBL 3 (Frischauf et al., 1983), respectively. For insert preparation, 270 μg of *C. capsici* DNA was digested for 30 min with 0.004 units of *Sau*3A/μg of DNA, and 200 μg of *C. gloeosporioides* DNA was digested for 30 min with 0.003 unit of *Sau*3A/μg of DNA; these conditions optimized production of 15–20-kb fragments. The 15–20-kb fragments were enriched by centrifugation through a 5–20% NaCl sedimentation velocity gradient in an SW27 rotor at 26000 rpm for 20 h.

C. capsici insert DNA was annealed to purified Charon 35 arms, and the ligated DNA was packaged into phage coats with a Packagene in vitro packaging kit. Recombinant phage were titered, amplified, and screened on a lawn of KH802 on NZCYM plates. EMBL 3 arms and *C. gloeosporioides* insert DNA were annealed, ligated, and packaged into phage coats with a Gigapack Plus in vitro packaging kit. Recombinant phage were titered and screened on a lawn of Q359 on LB plates. The genomic libraries were screened with the 838-bp *C. capsici* cDNA probe labeled with [α-^{32}P]dATP by nick translation.

DNA Sequencing. The *C. capsici* cDNA clone was sequenced by the chemical degradation method (Maxam & Gilbert, 1980). Part of the sequence was confirmed by dideoxy chain termination sequencing of the alkali-denatured pUC19 clone (Chen & Seeburg, 1985) with a reverse sequencing primer. The *C. capsici* genomic clone (1.3-kbp *Pst*1 and 5.1-kbp *Pst*1 fragments) and the *C. gloeosporioides* genomic clone (2.2-kbp *Sph*1 fragment) were subcloned into the vector M13mp19. Subclones containing complementary strands were identified by the C-test method (Messing, 1983). Serial deletions of the complementary strands of the *C. capsici* genomic clone 1.3-kbp *Pst*1 fragment and the *C. gloeosporioides* genomic clone 2.2-kbp *Sph*1 fragment were created by the unidirectional exonuclease activity of Exo III (Henikoff, 1984). The *C. capsici* genomic clone 5.1-kbp *Pst*1 single-stranded recombinant phage DNA was hybridized to the synthetic oligomer RD 20 and cut with *Eco*RI. Serial deletions were created by the exonuclease activity of T-4 polymerase (Dale et al., 1985). The resulting deleted M13 subclones were sequenced by the dideoxy chain termination method (Sanger et al., 1977). Part of the *C. capsici* genomic clone 3.1-kbp *Pst*1 sequence was confirmed by chemical degradation sequencing. All sequencing reaction mixtures were electrophoresed through 4% or 6% acrylamide–7 M urea gels.

S1 Nuclease Mapping. The transcriptional initiation site and polyadenylation site of the *C. capsici* cutinase mRNA were identified by S1 nuclease mapping (Berk & Sharp, 1978). Double-stranded DNA spanning regions of initiation and termination were labeled at the 5′ end with [γ-^{32}P]dATP and polynucleotide kinase or at the 3′ end with [α-^{32}P]dGTP and DNA polymerase (Maxam & Gilbert, 1980). An aliquot containing the labeled DNA (4×10^5 dpm) was hybridized to 5 μg of poly(A+) mRNA isolated from mycelia induced

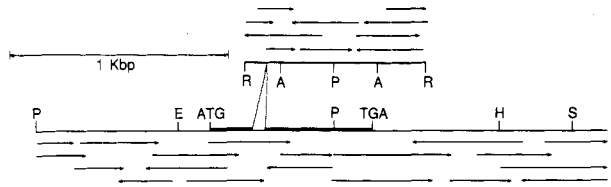

FIGURE 1: Sequencing strategy for the cloned cutinase cDNA (top) and cloned gene (bottom) from *C. capsici*. Arrows indicate the direction and length of each sequence. The gap in the open reading frame of the genomic DNA indicates the position of the single intron. Restriction sites are *Eco*RI (R), *Pst*1 (P), *Ava*II (A), *Hin*dIII (H), *Sal*1 (S), and *Eco*RV (E). Translation start and stop sites are indicated as ATG and TGA.

for 24 h with cutin hydrolysate or yeast tRNA in 13 μL of 40 mM 1,4-piperazinediethanesulfonic acid (PIPES), pH 6.4, containing 1 mM ethylenediaminetetraacetic acid (EDTA), 0.4 M NaCl, and 80% formamide, for 16 h at 52 °C. The hybrids were diluted with 400 μL of 50 mM sodium acetate, pH 4.5, containing 0.28 M NaCl, 4.5 mM $ZnCl_2$, 20 μg/mL denatured salmon sperm DNA, and 30 or 60 units of S1 nuclease. The digests were incubated for 30 min at 20 or 37 °C, reactions were stopped by phenol/chloroform extraction, and DNA was precipitated with ethanol. The products were subsequently electrophoresed on a 6% acrylamide–7 M urea sequencing gel with a dideoxy sequencing reaction as a size standard.

RESULTS

Sequence of the C. capsici Cutinase cDNA. The cDNA library prepared with mRNA from cutin hydrolysate induced *C. capsici* contained 22000 recombinant λ gt11 phage. The library was screened for the ability of IPTG to induce the expression of *C. capsici* cutinase antigens. Upon screening the library, only one plaque produced antigen on duplicate filters and also proved positive upon rescreening. DNA isolated from this clone contained two cDNA inserts of 360 and 500 bp. The 360-bp clone contained cutinase coding regions as shown by hybrid select translation. The hybrid-selected mRNA, when translated by a rabbit reticulocyte lysate system, produced a single peptide of approximately 24 kDa that was immunoprecipitated with rabbit antiserum prepared against *C. capsici* cutinase. The mRNA selected by the 500-bp clone translated into a single polypeptide of approximately 70 kDa, which was not immunoprecipitated. The purified 360-bp cutinase cDNA was ^{32}P-labeled by nick translation and used to rescreen the library for additional cDNA clones. From 30000 recombinant phage screened, three additional cutinase clones were identified and purified. The longest of these, containing 838 bp, was subcloned into pUC19 and sequenced (Figure 1).

The primary structure of cutinase deduced from the DNA sequence of the 838-bp cutinase cDNA contained two segments that matched exactly with the sequence of two tryptic peptides isolated from *C. capsici* cutinase (Figure 2). One tryptic peptide that was sequenced was a tridecapeptide spanning from asparagine-39 to lysine-51. This peptide contained a carboxyamidomethylated cysteine residue corresponding to cysteine-49 (Figure 2). The other peptide sequenced contained 35 amino acid residues from threonine-187 to arginine-221. This peptide contained two carboxyamidomethylated cysteine residues corresponding to cysteines-191 and -198. This cDNA did not contain the entire coding region for cutinase. The first methionine in the sequence was methionine-66, which could not be the initiation codon, since residues 39–51 showed complete match with a cutinase peptide

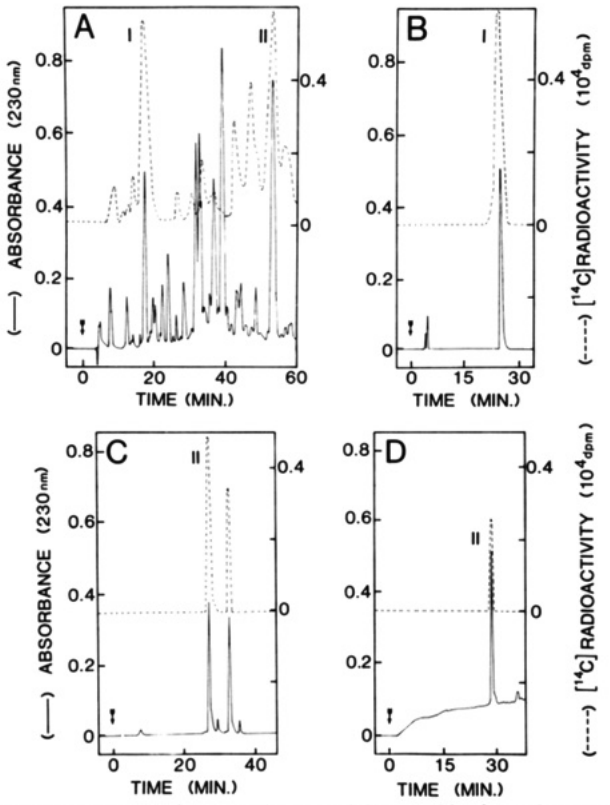

FIGURE 2: Isolation of the cysteine-containing peptides from *C. capsici* cutinase by HPLC. The arrows indicate the injection times. Chromatograms are representative of several injections for each step in the purification. (A) HPLC fractionation of the total tryptic digest of labeled *C. capsici* cutinase on a C_{18} column. Eluant fractions representing peaks marked I and II were collected and further purified. (B) Final desalting of peak I on a C_{18} column. (C) Peak II fractionated into two radioactive peptides upon rechromatography on the C_{18} column. The first peak, containing greater than 50% of the total radioactivity, was collected and desalted on a C_1 column. (D) Final purification and desalting of peak II on a C_1 column. The presence of ^{14}C-carboxyamidomethylated cysteine residues in the peptides was indicated by the coincidence of radioactivity and UV absorbance. Details of the methods are described in the text.

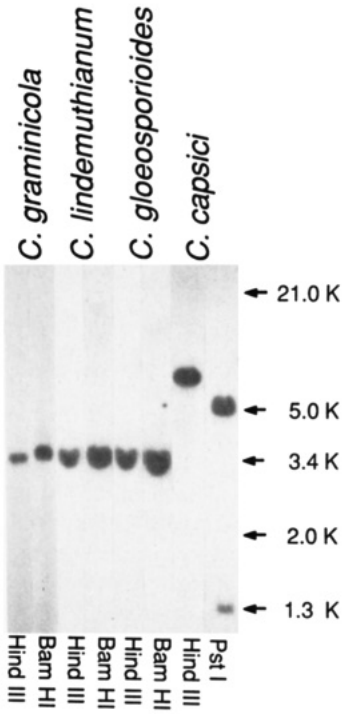

FIGURE 3: Southern blot analysis of genomic DNA isolated from several representative species of the genus *Colletotrichum*. Cutinase coding regions were detected by hybridization with ^{32}P-labeled *C. capsici* cutinase cDNA. Selected size standards from an *Eco*RI–*Hin*dIII digest of λ DNA are indicated in kilobase pairs in the right side.

isolated from the mature protein. As determined later, from the sequence of the genomic DNA (Figure 4), nucleotides coding for the N-terminal 37 amino acids were missing from the cDNA sequence. Therefore, the complete primary structure of *C. capsici* cutinase was deduced from the combined sequences of the cDNA and genomic DNA. The cDNA sequence contained a large open reading frame coding for 190 of the 228 amino acids of *C. capsici* precutinase. The open reading frame was terminated by a 260-bp nontranslated region which did not contain a polyadenylation site.

Presence of Cutinase Genes in the Genus Colletotrichum. Total genomic DNA from a number of phytopathogenic species of the genus *Colletotrichum* was digested with *Bam*H1, *Hin*dIII, or *Pst*1, and the resulting DNA fragments were analyzed for the ability to hybridize with *C. capsici* cutinase cDNA. These results indicated that not all species of the genus have DNA sequences that hybridize with the *C. capsici* cutinase cDNA. However, in addition to *C. capsici*, *C. gloeosporioides*, *C. graminicola*, and *C. lindemuthianum* showed sequences that hybridized to the cDNA. All of these species appeared to contain a single cutinase gene, as shown by a lack of multiple bands of hybridization on Southern blots (Figure 3).

Sequence of the C. capsici Cutinase Gene. The *C. capsici* genomic library in λ Charon 35 consisted of 9×10^5 recombinant phage, before amplification. On the basis of the haploid genome content of 4.3×10^7 bp as in *Neurospora* (Fincham et al., 1979), a library of 11 300 recombinant phage of average length of 17.5 kbp should have a 99% probability of containing the cutinase gene (Clarke & Carbon, 1976). To screen the library, approximately 12 000 pfu were plated onto each of six 150-mm Petri plates. The plaques were transferred to nitrocellulose and hybridized with the 838-bp *C. capsici* cutinase cDNA, which had been labeled with [α-^{32}P]dATP by nick translation. The primary screening of 72 000 recombinant λ Charon 35 clones yielded 23 plaques that hybridized to the cDNA. Of the six clones, randomly selected for purification and analysis by Southern blotting, four yielded 5.1- and 1.3-kbp *Pst*1 fragments, matching a Southern analysis of *C. capsici* genomic DNA. By restriction mapping, the 5.1-kbp *Pst*1 fragment corresponded to the 3′ end of the gene, while the 1.3-kbp *Pst*1 fragment corresponded to the 5′ end of the gene. The 1.3- and 5.1-kbp *Pst*1 fragments were isolated from a preparative digest of a single λ Charon 35 clone, subcloned into M13mp19, and sequenced (Figure 1).

A total of 2630 bp of the *C. capsici* cutinase gene were sequenced. The sequence of the 1.3-kbp *Pst*1 fragment spanned 1374 bp extending from the middle of the coding region to 800 bp upstream from the ATG (Figure 4). Only 1256 bp of the 5.1-kbp *Pst*1 fragment, extending from the middle of the coding region toward the 3′ end of the gene, were sequenced. The nucleotide sequence exactly matched the sequence of the cDNA except for a 57-bp insert in the genomic DNA. This insert had the usual intron border sequences found in other filamentous fungi and occurred at the same location in the coding region as the intron in the *F. solani* cutinase gene.

S1 Nuclease Mapping of the C. capsici Cutinase Gene. The transcriptional start and polyadenylation sites of the *C. capsici* cutinase gene were determined by S1 nuclease mapping. Four major start sites were found at −143, −140, −138, and −135 nt as measured from the translational start site (ATG). The

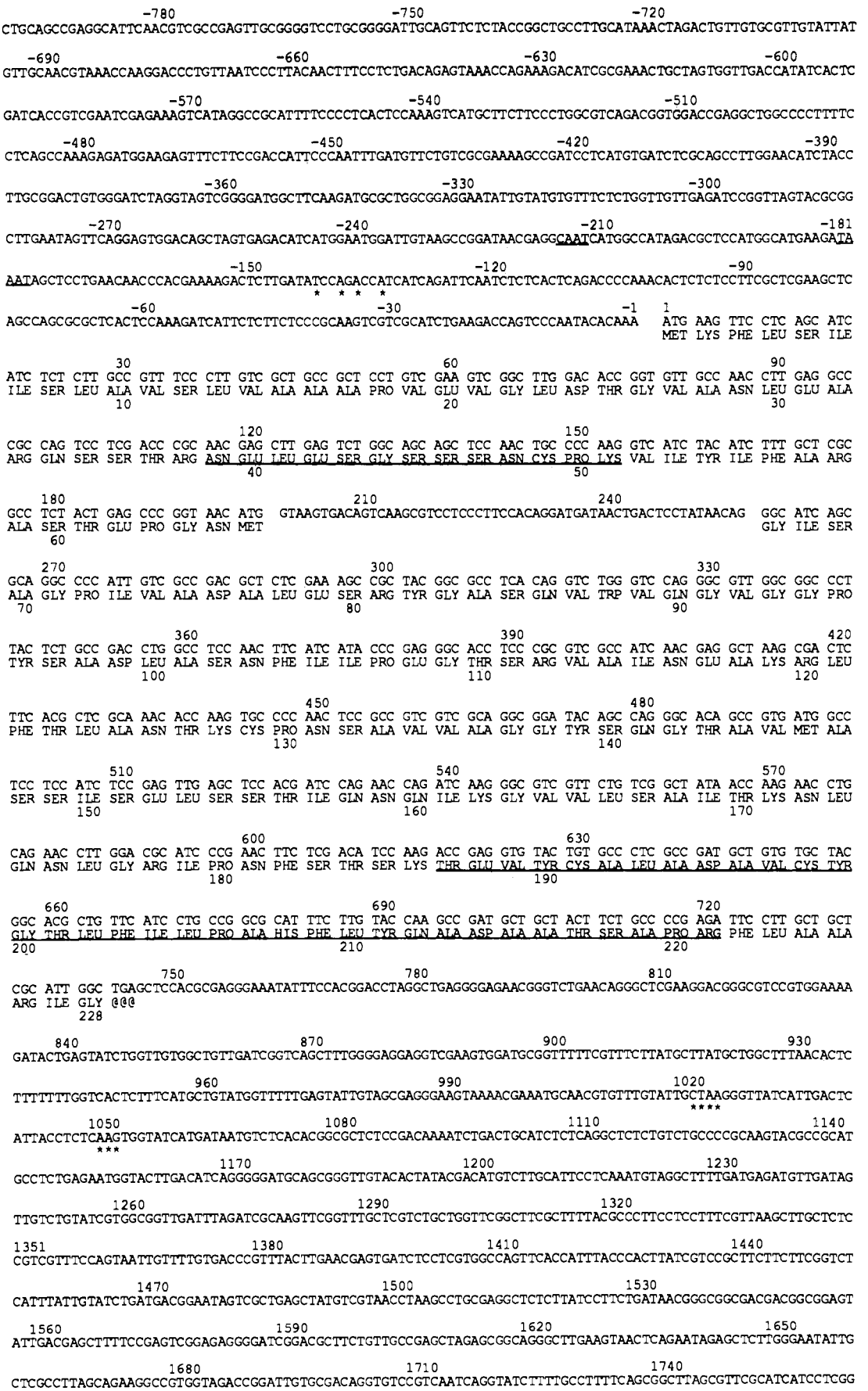

FIGURE 4: Nucleotide sequence of the cutinase gene and flanking regions cloned from *C. capsici*. Base pairs are numbered above the DNA sequence in relation to the "A" in the initiation codon ATG. The deduced amino acids of precutinase are numbered under the amino acids. Amino acids corresponding to sequenced peptides are underlined. The transcriptional start sites and polyadenylation sites are indicated with stars. The CAAT and TAAATA boxes are underlined. The position of the single intron is indicated by the gap in the amino acid sequence.

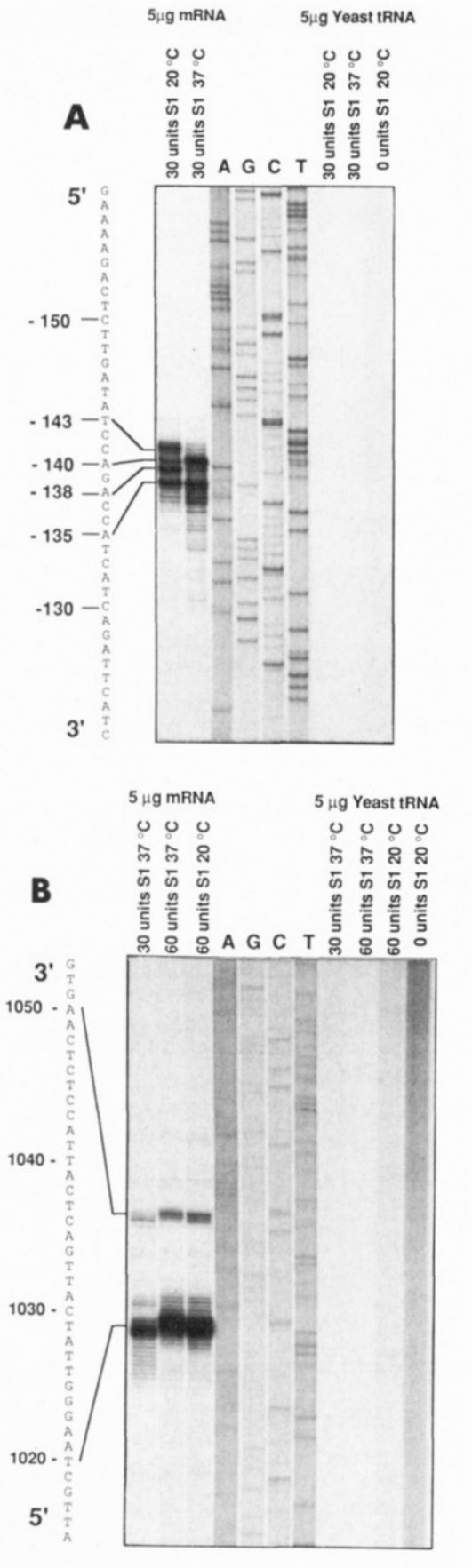

FIGURE 5: S1 nuclease mapping of the transcriptional start sites (A) and polyadenylation site (B) of *C. capsici* cutinase mRNA. As in Figure 4, the base pairs are numbered from the "A" in the initiation codon ATG. A, G, C, and T refer to lanes of dideoxy sequencing reaction used as a size standard.

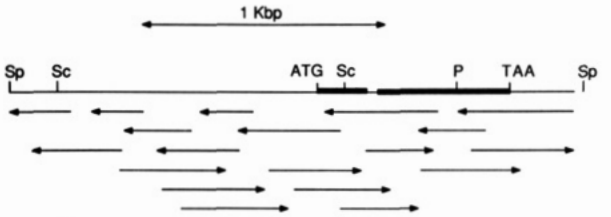

FIGURE 6: Sequencing strategy used for cloned cutinase gene from *C. gloeosporioides*. The gap in the open reading frame of the map indicates the position of the single intron. The arrows indicate the direction and length of the sequences. Restriction sites are *Sph*1 (SP), *Pst*1 (P), and *Sac*1 (Sc). Translation start and stop sites are indicated as ATG and TGA.

sequence in the transcript initiation area is *TCCAGACCA*, with the initiation nucleotides italicized (Figure 5). Since the protected fragment representing initiation at T is less obvious at 37 °C compared to that at 20 °C, this site may not be a real initiation site. There are two sites of polyadenylation of the primary transcript of the *C. capsici* cutinase gene. The major polyadenylation site occurs at 275–278 nt 3′ to the termination codon (1020 nt after the initiation codon). The minor polyadenylation site occurs 30 nt 3′ to the major site (304–306 nt 3′ to the termination codon or 1050 nt 3′ to the initiation codon). At no point between the termination codon and the polyadenylation site is there a consensus polyadenylation signal (AATAAA) found in most other eukaryotic genes (Nevins, 1983).

Sequence of the C. gloeosporioides Cutinase Gene. The *C. gloeosporioides* genomic library in EMBL 3 consisted of 6.8 × 10^4 recombinant phage. To screen the library, 16 000 pfu were plated onto four 85-mm Petri plates. The plaques were transferred to nitrocellulose and hybridized with the 838-bp *C. capsici* cutinase cDNA labeled with [^{32}P]dATP by nick translation. The primary screening of the library yielded 11 recombinant phage containing the cutinase gene. The positive phage were analyzed by Southern blotting. One of the recombinant clones produced a 2.2-kbp *Sph*1 fragment that contained the entire cutinase gene as shown by restriction mapping. The 2.2-kbp *Sph*1 fragment was isolated from a preparative digest of the EMBL 3 clone and subcloned into M13mp19 and sequenced (Figure 6).

The nucleotide sequence of 1749 bp of the *C. gloeosporioides* cutinase gene spans 823 bp of the DNA 5′ to the codon representing translation initiation, 727 bp of the cutinase coding region, and 199 bp of the DNA 3′ to the codon for translation termination (Figure 7). The open reading frame is interrupted by a 52-bp intron. The splice junction sequences of the putative intron match those of introns from other filamentous fungi and are at the same locations as the introns in the cutinase genes from *F. solani* and *C. capsici*. Although the transcription initiation site from the *C. gloeosporioides* gene has not been determined, there is an ATAAAT sequence at −168 nt (5′ to the ATG) and a CAAT sequence at −188 nt. These presumptive CAAT and TATA boxes are in a homologous location in the cutinase genes in both species of *Colletotrichum*. As with the *C. capsici* cutinase genes, the 3′ nontranslated region contains no identifiable polyadenylation signal (AATAAA). However, only a small portion of the 3′ region of this gene was sequenced.

Discussion

Detailed studies of the biochemistry of cutinase from *F. solani* f. sp. *pisi* strain T-8 have been conducted. These studies have clearly demonstrated that cutinase is essential for penetration of the cuticular layer of plants by pathogenic fungi

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          -810                                -780                                -750                                -720
AAACCACAAGAGCTATCCTCACCCAACAGGAATTACCCGCCGGAAACAGCCATAAGCCGCAGCAGGCCCCTCATTCCAGACCTCGAGCTATCTTTCCTGATGCTAGGTCTTGTCC

                -690                                -660                                -630                                -600
CGAGGCTCGCCCCCTTTTTCTGTCTGGCTTGCTATTGCTACAACTAGAGGGATTGCGGAGCGTCTTCCGACCATACCCAATTTGATGGAAACTTGCGAAATGGCGACCCTCACGT

                    -570                                -540                                -510                                -480
GGCATCGTGGGCCTTGGAACGCCCAGCTCACCGACTCTGGAATTTTGGTGATGGGGATTGCTTCAAGATGCGACTGGCGGAGGAATAACGGAGTCCATGACTCTGATTGAGGGAA

                        -450                                -420                                -390
CCTTGTTGACGAGCGATTTTGAGGATCAGGGTCAGCTAGGTTAGTCGCTGCTTTCAGGCATAGATCGAAGCTCGTGAAGGATCAAAGTCGTCAGGGTTCTCGATGGGATGAACTG

-360                                -330                                -300                                -270
GCATGTCACTTTGACATCTGTTCCTCATCGTGAACTCATTGAGCTTGCATGAGTTCTCTTGCGAGATATCGTACTTCTCATCGTTTGATCAACTGCAAGAGATCTTGTACTTGGT

    -240                                -210                                -180                                -150
ACATCTCACGGATCGACCTCCGCCTGTTATGAAGTGTCAACTGTAAGGTCGAAACGATGACAATCGTGGAAACCAAACAACATAAATAGGGCCTCACGATCCTCTCAAACACGGC

        -120                                -90                                 -60                                 -30
TTTCTTTTGGATCATCAGACTCAAGCCCTCGCTCAGACTGACAGCTCCTCCTTCTCCTTCGCTCCAAGCCGACGCAGCACATTCTCTCAGAGACACCTCTCTTCATACGACGCCC

                -1  1                                   30                                                  60
CGTCCCCAAACATCCAAA  ATG AAG TTC CTC AGC GTC TTG TCC CTG GCC ATC ACC CTC GCC GCC GCG GCT CCT GTC GAA GTT GAG ACC GGC
                    MET LYS PHE LEU SER VAL LEU SER LEU ALA ILE THR LEU ALA ALA ALA ALA PRO VAL GLU VAL GLU THR GLY
                      1                                     10                                                  20

                     90                                  120                                                 150
GTC GCC CTC GAG ACC CGT CAG TCG TCC ACC CGC AAC GAG CTC GAG ACC GGC AGC AGC TCC GCC TGC CCC AAG GTC ATC TAC ATC TTT
VAL ALA LEU GLU THR ARG GLN SER SER THR ARG ASN GLU LEU GLU THR GLY SER SER SER ALA CYS PRO LYS VAL ILE TYR ILE PHE
                     30                                  40                                                  50

                        180                                 210                                 240
GCC CGT GCC TCG ACT GAG CCC GGT AAC ATG   GTAAGGCTTCCTTCATCCTATCTTCAAAGGCCTATACTCACATTTCGAAAAG  GGA ATC AGC GCA GGC
ALA ARG ALA SER THR GLU PRO GLY ASN MET                                                          GLY ILE SER ALA GLY
                         60

                270                                 300                                 330
CCC ATC GTC GCC GAC GCC CTG GAG AGA ATC TAC GGC GCC AAC AAC GTC TGG GTC CAG GGC GTG GGC GGC CCC TAC CTC GCC GAC CTG
PRO ILE VAL ALA ASP ALA LEU GLU ARG ILU TYR GLY ALA ASN ASN VAL TRP VAL GLN GLY VAL GLY GLY PRO TYR LEU ALA ASP LEU
 70                                  80                                  90

                    360                                 390                                 420
GCC TCC AAC TTC CTG CCC GAC GGC ACC TCG TCG GCG GCC ATC AAC GAG GCC AGA CGC CTC TTC ACC CTC GCC AAC ACA AAG TGC CCC
ALA SER ASN PHE LEU PRO ASP GLY THR SER SER ALA ALA ILE ASN GLU ALA ARG ARG LEU PHE THR LEU ALA ASN THR LYS CYS PRO
       100                                 110                                 120

                        450                                 480                                 510
AAC GCG GCC ATC GTC TCG GGC GGC TAC AGC CAG GGC ACC GCC GTG ATG GCG GGC TCC ATC TCA GGC CTG AGC ACC ACG ATC AAG AAC
ASN ALA ALA ILE VAL SER GLY GLY TYR SER GLN GLY THR ALA VAL MET ALA GLY SER ILE SER GLY LEU SER THR THR ILE LYS ASN
           130                                 140                                 150

                            540                                 570                                 600
CAG ATC AAG GGC GTC GTG CTC TTC GGG TAC ACC AAG AAC CTG CAG AAC CTG GGC CGC ATC CCC AAC TTC GAG ACG TCC AAG ACC GAG
GLN ILE LYS GLY VAL VAL LEU PHE GLY TYR THR LYS ASN LEU GLN ASN LEU GLY ARG ILE PRO ASN PHE GLU THR SER LYS THR GLU
                160                                 170                                 180

                                630                                 660                                 690
GTC TAC TGC GAC ATT GCG GAT GCG GTC TGC TAC GGC ACC CTG TTC ATC CTG CCG GCG CAC TTT TTG TAC CAG ACT GAT GCG GCT GTT
VAL TYR CYS ASP ILE ALA ASP ALA VAL CYS TYR GLY THR LEU PHE ILE LEU PRO ALA HIS PHE LEU TYR GLN THR ASP ALA ALA VAL
                    190                                 200                                 210

                                    720                                 750                                 780
GCG GCC CCG CGC TTC CTC CAG GCC CGC ATT GGT TAAGAGGGTTGAAGGAGAACCGAAATTGGGGTTAGAGTCGAATAAGCATGGCGGGATCGAGGGGAAGGTGT
ALA ALA PRO ARG PHE LEU GLN ALA ARG ILE GLY @@@
                        220

            810                                 840                                 870                                 900
TTTTGGTTGCGGCTGTTGATCGGTCAGGGGGAGGAGTTGGCTGAGCTTGGTGCTCTACATTCAATTCTGTCACTCACTTCGTCATTCGCTTTTTATCACTCGCTAAGTACATGAT

            926
GCTTCTGGATAGCTGC
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FIGURE 7: Nucleotide sequence of the cutinase gene and flanking regions cloned from *C. gloeosporioides*. Base pairs are numbered above the DNA sequence in relation to the "A" in the initiation codon ATG. The amino acids of the protein are numbered under the amino acids. The CAAT and TAAATA boxes are underlined. The position of the single intron is indicated by the gap in the amino acid sequence.

(Kolattukudy, 1985). The primary structure of the *F. solani* cutinase has been deduced from the sequence of a cloned cDNA (Soliday et al., 1984). The hydrolysis products of cutin are very potent inducers of the cutinase gene (Woloshuk & Kolattukudy, 1986). A cutinase gene from *F. solani* f. sp. *pisi* strain T-8 has recently been sequenced (Kolattukudy, 1987). However, studies on the regulation of expression of the gene in *F. solani* f. sp. *pisi* strain T-8 are complicated by the fact that this organism produces two cutinase isozymes (Purdy & Kolattukudy, 1975) and contains more than one copy of the gene (Kolattukudy & Soliday, 1985). Enzymological studies of cutinase from *C. capsici* (W. F. Ettinger and P. E. Kolattukudy, unpublished results) and *C. gloeosporioides* (Dickman et al., 1982) indicate that these organisms produce a single cutinase isozyme and might therefore contain a single copy of the gene. *Fusarium* and *Colletotrichum* are taxonomically distinct organisms. *Fusarium* is the imperfect form of *Nectria*, and many of the *Colletotrichum* species from which perfect forms have been identified have been grouped in the genus *Glomerella*. In the class *Ascomycetes*, *Nectria* is in the order Hypocreales, and *Glomerella* is in the order Xylariales (Alexopoulos & Mims, 1979).

In order to study the *Colletrotrichum* cutinase genes, a specific cDNA probe for *C. capsici* cutinase was isolated from a λ gt11 cDNA library constructed from cutin hydrolysate induced mRNA. The *C. capsici* cutinase cDNA was 838 bp in length and coded for 80% of precutinase. Two tryptic peptides isolated from *C. capsici* cutinase, one tridecapeptide and another containing 35 amino acid residues, exactly matched the amino acid sequence deduced from the nucleotide sequence of the cDNA, confirming that the cloned cDNA was complementary to cutinase mRNA. This cDNA was used as a probe to detect cutinase coding sequences in digests of genomic DNA from *C. capsici*, *C. gloeosporioides*, *C. graminicola*, and *C. lindemuthianum*. All *Colletotrichum* species that contained the cutinase gene appeared to have only a single copy of the gene. However, the possibility that multiple genes, upon digestion with restriction enzymes, yield fragments of the same size cannot be completely ruled out. In similar studies of *Fusarium*, *F. solani* f. sp. *pisi* strain T-8 was one

```
C. gloeosporioides   CAATC..17..TAAATA..41...TCAGACT...11...TCAGACT...94..ATG
C. capsici           CAATC..29..TAAATA..34..TCCAGACCA..21...TCAGACC..106..ATG
F. solani f.sp. pisi CAAGC...1..TAAATA...7..TCCAGACCA..11...CCAGACCA..72..ATG
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FIGURE 8: Sequences of the 5′ noncoding regions that are homologous among the *C. gloeosporioides*, *C. capsici*, and *F. solani* cutinase genes. The translational start codon (ATG) of each gene is at the right border. The distance, in nucleotides, between the homologous sequences is indicated. The transcriptional start sites of the *C. capsici* gene are underlined. Nucleotides surrounding the core (CAGAC) of the repeated sequence which are homologous with other repeats are also included. The TAAATA and CAA(T/G) boxes are also displayed.

of several species that contained more than a single copy of the cutinase gene (Kolattukudy et al., 1985).

Initial attempts were made to screen the *C. capsici* genomic library with the cDNA from *F. solani* f. sp. *pisi*. Although there was sufficient homology between *F. solani* and *C. capsici* to detect some light hybridization, the degree of hybridization was not sufficient to allow for the reproducible screening of the recombinant λ plaques. Therefore, the cutinase cDNA from *C. capsici* was used to screen genomic clones from *C. capsici* and *C. gloeosporioides*. Sequences from the 5′ flanking regions found to be homologous among three cutinase genes are indicated (Figure 8). Other homologous sequences are found when comparing *C. capsici* with *F. solani*, *C. capsici* with *C. gloeosporioides*, or *F. solani* with *C. gloeosporioides* but not among all three genes. The transcription start sites in the *C. capsici* cutinase gene are not associated with a classical TATAA box found in most eukaryotic genes (Nevins, 1983). The *F. solani* cutinase gene lacks both consensus TATAA and CAAT boxes (Kolattukudy, 1987), and the *C. gloeosporioides* cutinase gene also lacks a consensus TATAA box. Although all three genes lack the consensus TATAA box, they have a TAAATA sequence in a common location. In the *C. capsici* gene, this TAAATA sequence is 34 nt 5′ to the transcription start sites, or 177 nt from the translational start site. In the *C. gloeosporioides* and *F. solani pisi* genes, the TAAATA sequence is 160 and 111 nt 5′ of the translational start sites, respectively. A distinct "TATA" box is usually absent in the genes of filamentous fungi (Clements & Roberts, 1986), particularly in *Neurospora* (Newbury et al., 1986). Furthermore, none of the TATA boxes that have been found in the genes of filamentous fungi have been functionally dissected. A CAAT sequence, preceding the TAAATA sequence in *C. capsici* by 30 nt and in *C. gloeosporioides* by 18 nt, is in a homologous location to CAAT boxes of genes of higher eukaryotes. A similar CAAG sequence is found in the *F. solani* gene but is only 2 nt from the TAAATA sequence. The CAGAC core of the TCCAGACCA sequence associated with the transcriptional start site in *C. capsici* is found duplicated 21 nt 3′ to the start sites. This CAGAC core is also found duplicated 11 nt apart within 66 nt 3′ of the TAAATA sequence in the *F. solani* and *C. gloeosporioides* genes. It is unknown whether the CAGAC core is located at the transcription initiation sites of these genes.

Cutinase is a serine esterase, containing the classical catalytic triad found in the serine hydrolases (Köller & Kolattukudy, 1982). The residues identified as being involved in the catalytic triad are located far apart in the primary structure. The active serine was identified by treating the *Fusarium* enzyme with [^{3}H]diisopropyl fluorophosphate, followed by isolation and sequencing of the tryptic peptide containing the modified residue (Soliday & Kolattukudy, 1983). The histidine of the catalytic triad was readily identified as being the sole histidine residue in the sequence. An essential carboxyl group in the *F. solani* enzyme was located by coupling a carbodiimide-activated carboxyl group with [^{14}C]glycine ethyl ester followed by isolation and sequence analysis of a tryptic peptide (W. F. Ettinger and P. E. Kolattukudy, unpublished results). The four cysteines in the structure are highly conserved. The two disulfide bridges in cutinase are critical for catalytic activity; reduction of the disulfide bridges results in the complete inactivation of the enzyme (W. Köller and P. E. Kolattukudy, unpublished results). The disulfide bridges must play a critical role in holding the catalytic residues in juxtaposition. In the aligned sequences of the enzyme (Figure 8) the serine and histidine of the catalytic triad and the cysteines are all in very strongly conserved positions.

The sequences of *C. capsici*, *C. gloeosporioides*, and *F. solani* cutinases contain highly homologous segments although they have undergone considerable evolutionary diversification. Aligning the primary sequences to achieve the highest degree of homology necessitates allowing for six gaps caused by the insertion or deletion of one or more amino acids (Figure 9). Taking these gaps into account, about 43% of the amino acids are directly conserved among all three enzymes. The longest stretches of conserved amino acids are three segments, each containing six amino acid residues. One of these surrounds the active serine. Conversely, the longest stretch without homology is an 11-residue segment in the putative signal sequence, in which there are three gaps. The N-terminal structure and the signal sequences of *Colletotrichum* cutinases have not been elucidated by direct chemical studies on the proteins. As expected, the enzymes from *Colletotrichum* share much more homology with each other (82%) than either of them do with *F. solani*. *C. capsici* and *F. solani* are 47% homologous, while *C. gloeosporioides* and *F. solani* are 48% homologous. It is not unusual for active serine enzymes to diverge considerably. Bovine trypsin and crayfish trypsin are about 40% homologous (Neurath, 1984). It is likely that cutinase has evolved independently from other peptidases and esterases which have the active serine mechanism, as seen from comparison of amino acid sequences around the active serine (Soliday & Kolattukudy, 1983).

Cloning of full-length cutinase cDNA in the expression vector might be difficult because of the potential harm to cell components by the catalytic activity of the translation product. The coding region in the 838-bp cDNA is 37 residues short of the start codon, although it is approximately the size of the mature protein. The added synthetic *Eco*RI linker on the cDNA puts the cutinase protein in the same open reading frame as β-galactosidase in pUC19. Of the 27 pUC19 recombinants screened with the 838-bp cDNA cloned into the *Eco*RI site, all had the insert in the 3′ to 5′ direction. No recombinants were isolated with the cDNA insert with the proper orientation to produce a β-galactosidase–cutinase fusion protein. Constructs could not be obtained that produced the fusion proteins possibly because the esterase activity of the fusion protein with β-galactosidase is deleterious. Such a possibility may also account for the fact that only the short 365-bp λ gt11 recombinant was obtained by antibody screening. Upon induction with high levels of IPTG, any full-length cutinase cDNAs in λ gt11 may have been selected against. Since cutinase is known to hydrolyze triglycerides (Kolattukudy, 1984), the toxicity might arise from hydrolysis of cellular lipids.

Bias in codon usage is evident in the primary structures of the *C. capsici* and *C. gloeosporioides* cutinases. Of the 61 nonterminating codons, 13 codons are not used in *C. capsici*, and 18 codons are not used in *C. gloeosporioides*. This level of bias is fairly moderate for the filamentous fungi. A much greater degree of bias is found in the H3 and H4 histone genes in which 32 codons are not used (Woudt, 1983). As described

C. gloeosporioides met lys phe leu ser val leu ser
C. capsici met lys phe leu ser ile ile ser
F. solani f. sp. *pisi* met lys phe phe ala leu thr thr

leu ala ile thr leu ala ala ala ala pro val glu val ... ... glu thr gly val ala
leu ala val ser leu val ala ala ala pro val glu val gly leu asp thr gly val ala
leu leu ala ala thr ala ser ala leu pro thr ser asn pro ala gln ... ...

... leu glu thr arg gln ser ... ... ser thr arg asn glu leu glu thr gly ser ser
asn leu glu ala arg gln ser ... ... ser thr arg asn glu leu glu ser gly ser ser
glu leu glu ala arg gln leu [gly] arg thr thr arg asp asp leu ile asn gly asn ser

ser ala [cys] pro lys val ile tyr ile phe ala arg ala ser thr glu pro gly asn met
ser asn [cys] pro lys val ile tyr ile phe ala arg ala ser thr glu pro gly asn met
ala ser [cys] arg asp val ile phe ile tyr ala arg gly ser thr glu thr gly asn leu

gly ile ser ala gly pro ile val ala asp ala leu glu arg ile tyr gly ala asn asn
gly ile ser ala gly pro ile val ala asp ala leu glu ser arg tyr gly ala ser gln
gly ... thr leu gly pro ser ile ala ser asn leu glu ser ala phe gly lys asp gly

val trp val gln gly val gly gly pro tyr leu ala [asp] leu ala ser asn phe leu ...
val trp val gln gly val gly gly pro tyr ser ala [asp] leu ala ser asn phe ile ile
val trp ile gln gly val gly gly ala tyr arg ala thr leu gly [asp] asn ala leu ...

pro asp gly thr ser ser ala ala ile asn glu ala arg arg leu phe thr leu ala asn
pro glu gly thr ser arg val ala ile asn glu ala lys arg leu phe thr leu ala asn
pro arg gly thr ser ser ala ala ile arg glu met leu gly leu phe gln gln ala asn

thr lys [cys] pro asn ala ala ile val ser gly gly tyr [ser] gln gly thr ala val met
thr lys [cys] pro asn ser ala val val ala gly gly tyr [ser] gln gly thr ala val met
thr lys [cys] pro asp ala thr leu ile ala gly gly tyr [ser] gln gly ala ala leu ala

ala gly ser ile ser gly leu ser thr thr ile lys asn gln ile lys gly val val leu
ala ser ser ile ser glu leu ser ser thr ile gln asn gln ile lys gly val val leu
ala ala ser ile glu asp leu asp ser ala ile arg asp lys ile ala gly thr val leu

phe gly tyr thr lys asn leu gln asn leu gly arg ile pro asn phe glu thr ser lys
ser ala ile thr lys asn leu gln asn leu gly arg ile pro asn phe ser thr ser lys
phe gly tyr thr lys asn leu gln asn arg gly arg ile pro asn tyr pro ala asp arg

thr glu val tyr [cys] asp ile ala asp ala val [cys] tyr gly thr leu phe ile leu pro
thr glu val tyr [cys] ala leu ala asp ala val [cys] tyr gly thr leu phe ile leu pro
thr lys val phe [cys] asn thr gly asp leu val [cys] thr gly ser leu ile val ala ala

ala [his] phe leu tyr gln thr asp ala ala val ala ala pro arg phe leu gln ala arg
ala [his] phe leu tyr gln ala asp ala ala thr ser ala pro arg phe leu ala ala arg
pro [his] leu ala tyr gly pro asp ala arg gly pro ala pro glu phe leu ile glu lys

ile gly
ile gly
val arg ala val arg gly ser ala

FIGURE 9: Primary structure of the *C. gloeosporioides*, *C. capsici*, and *F. solani* cutinases. Gaps have been inserted into the sequences where necessary to align the sequences to display the greatest amount of homology. Amino acids homologous between *C. gloeosporioides* and *C. capsici* are underlined under the *C. gloeosporioides* amino acids. Amino acids homologous between *C. capsici* and *F. solani* cutinases are underlined under the *C. capsici* amino acids. Amino acids homologous between the *C. gloeosporioides* and *F. solani* cutinases are underlined under the *F. solani* amino acids. The location of the single intron is indicated by the vertical bar. The cysteines, active serine, histidine, essential carboxyl, and N-terminal glycine residue of the *F. solani* cutinase are boxed along with the corresponding homologous residues of the other cutinases.

for *Neurospora* (Fincham, 1985), there is a strong bias against A in the third position of the cutinase codons.

The structural information presented in this paper also will aid in elucidating the molecular mechanism involved in the regulation of expression of cutinase gene. Since cutinase in *Colletotrichum* also appears to be induced by cutin hydrolysate, it will be possible to study how cutin monomers trigger the expression of cutinase gene in this organism. With the availability of cloned cutinase genes from organisms that attack different host plants, it will be possible to test whether the different cutin monomers found on these hosts will show specificity in triggering the expression of the cutinase gene from the different pathogens.

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