Structure of the Cutinase Gene and Detection of Promoter Activity in the 5'-Flanking Region by Fungal Transformation

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The cutinase gene from Fusarium solani f. sp. pisi (Nectria hematococa) was cloned and sequenced. Sau3A fragments of genomic DNA from the fungus were cloned in a λ Charon 35 vector. When restriction fragments generated from the insert were screened with 5' and 3' probes from cutinase cDNA, a 5.5-kilobase Srl fragment hybridized with both probes, suggesting the presence of the entire cutinase gene. A 2,818-base pair segment was sequenced, revealing a 690-nucleotide open reading frame that was identical to that found in the cutinase cDNA with a single 51-base pair intron. Transformation vectors were constructed containing a promoterless gene for hygromycin resistance, which was translationally fused to flanking sequences of the cutinase gene. When protoplasts and mycelia were transformed with these vectors, hygromycin-resistant transformants were obtained. Successful transformation was assessed by Southern blot analysis by using radiolabeled probes for the hygromycin resistance gene and the putative promoter. The results of Southern blot analysis indicated that the plasmid had integrated into the Fusarium genome and that the antibiotic resistance was a manifestation of the promoter activity of the cutinase flanking sequences. Transformation of Colletotrichum capsici with the same construct confirmed the promoter activity of the flanking region and the integration of the foreign DNA. Transformation and deletion analysis showed that promoter activity resided within the 360 nucleotides immediately 5' to the cutinase initiation codon.

Several lines of evidence strongly suggested that the ability to produce cutinase might determine whether a fungal pathogen can penetrate intact plant organs and infect them (22). In vitro translation of mRNA from cultures induced to produce cutinase (25) revealed that the primary translation product of cutinase was 2,100 daltons larger than the mature enzyme (13). By using the induced mRNA, a cDNA library was constructed, cutinase cDNA was isolated, and the nucleotide sequence was determined, revealing an open reading frame for a 23,951-dalton protein (44). The availability of the cloned cDNA provided a probe for the cutinase gene.

Recently, strong evidence was presented that fungal spores sense contact with a plant surface by using the small amount of cutinase carried by the spore. Upon contact with the cuticle, the enzyme generates small amounts of cutin monomers and the most unique monomers trigger cutinase synthesis (47). Dot blot analysis with cloned cutinase cDNA as the probe demonstrated that cutinase gene transcripts became measurable within 15 min after the spores contacted cutin. To determine how the cutin monomers trigger cutinase gene expression, a knowledge of the cutinase gene structure is essential. In this paper we report the cloning of the cutinase gene from Fusarium solani f. sp. pisi and its nucleotide sequence, including the sequences of the flanking regions. We also report the transformation of F. solani f. sp. pisi and Colletotrichum capsici using the hygromycin resistance gene as a selectable marker and the cutinase 5'-flanking region as the promoter. The results demonstrate that the 360-base pair (bp) segment immediately 5' to the cutinase coding region contains promoter activity.

MATERIALS AND METHODS

Fungal and bacterial strains. F. solani f. sp. pisi field isolates T-8 and T-30 were provided by Hans van Etten, Cornell University. C. capsici ATCC 48574, an isolate from pepper, was from Ralph Nicholson, Purdue University. Single-spore isolates were obtained and routinely maintained on 10% vegetable juice agar (10% V-8 juice, 0.2% CaCO3, 2% agar). A mineral medium (37) containing 1% glucose as the carbon source was inoculated with conidia, and after 10 days of growth in Roux culture bottles at 30°C, mycelia were collected for isolation of fungal genomic DNA. Escherichia coli KH802 was used to host lambda phage infection and its propagation. Plasmids as well as bacteriophage M13 derivatives were propagated in E. coli JM101 or DH5 (17).

Enzymes and chemicals. Hygromycin B, lithium acetate, β-glucuronidase, diselase, d-sorbitol, cetyltrimethylammonium bromide, and polyethylene glycol 4000 and 8000 were obtained from Sigma Chemical Co., St. Louis, Mo. [α-32P]deoxyxynucleotide triphosphates were purchased from New England Nuclear Corp., Boston, Mass. Restriction enzymes, T4 DNA ligase, T4 DNA polymerase, and E. coli DNA polymerase I were obtained from Pharmacia Diagnostics, Piscataway, N.J. or New England BioLabs Inc., Beverly, Mass. Novozym 234 was from Novo Industries, and Miracloth was from Calbiochem-Behring, La Jolla, Calif.

Isolation of nucleic acids. Lyophilized mycelia were ground in liquid nitrogen with a mortar and pestle, and the extracted nucleic acids were precipitated with cetyltrimethylammonium bromide as described elsewhere (33). Bacteriophage and plasmid DNAs were isolated by using an alkaline lysis procedure (8). Minipreparations of plasmid DNA were done as described by Maniatis et al. (26). Final purification of the genomic and plasmid DNAs was done by centrifugation in a CsCl step gradient (14). To prepare large quantities of bacteriophage lambda DNA, E. coli cultures were infected by the appropriate bacteriophage and grown until complete
ysis of the culture occurred as described by Maniatis et al. (26). The bacteriophage was isolated from the lysed cultures and purified (49), and lambda DNA was obtained as described (26).

Genomic library construction and screening for the cutinase gene. Analysis of \textit{F. solani} f. sp. \textit{pisi} DNA digested with varying amounts of \textit{Sau}3A restriction enzyme for 1 h at 37°C indicated that 0.06 U of \textit{Sau}3A per µg of DNA provided the greatest number of DNA fragments in the 15 to 20-kilobase (kb) range. A 3-µg portion of DNA, enriched in 15 to 20-kb fragments by 5 to 20% NaCl density gradient centrifugation, and 9 µg of arms from a lambda Charon 35 vector were ligated by using a standard ligation reaction with T4 DNA ligase. The ligated DNA (1 µg) was mixed with a lambda DNA packaging system (Promega Biotech, Madison, Wis.) and incubated at 20°C for 3 h. About 12,000 PFU were plated in each of two 157-mm petri dishes by established procedures (5). Plaques were transferred to nitrocellulose and hybridized with a cutinase cDNA probe which had been labeled with [α-32P]deoxyribonucleotide triphosphates by nick translation (40). The genomic clones containing cutinase gene inserts were subjected to restriction enzyme mapping. The localization of particular sequences of DNA within restriction fragments was accomplished by the Southern transfer technique (45).

DNA sequence analysis. Selected DNA fragments from restriction endonuclease digests of lambda DNA containing the cutinase gene were subcloned into M13 mp18 and M13 mp19. The procedures of Messing and Vieira (30) were followed for restriction endonuclease digestion, DNA fragment isolation, ligation, transformation, M13 bacteriophage propagation, isolation of single-stranded DNA, isolation of replicative forms, and subclone evaluation.

The nucleotide sequence of the resulting single-stranded DNA was determined by the dideoxy-chain termination method (29, 41), with modifications made for the use of reverse transcriptase (19) in place of the large fragment of DNA polymerase I. The single-stranded copy generated by reverse transcriptase was primed with either a pentadecamer complementary to an M13 region just 3' to the polylinker region or a synthetic 20-mer complementary to a region of cloned DNA whose nucleotide sequence had just been determined. Oligonucleotides were synthesized by phosphoramidite chemistry in a 380A DNA synthesizer (Applied Biosystems Inc., Foster City, Calif.) and purified by polyacrylamide gel electrophoresis on 20% polyacrylamide containing 7 M urea in 0.5× TBE (89 mM Tris, 89 mM boric acid, 1 mM EDTA) (41) before use as primers.

Construction of transformation vectors. The hygromycin resistance gene from pTiT23 (20) (Eli Lilly & Co., Indianapolis, Ind.) was restricted with \textit{Bam}HI and \textit{Hind}III, and the resulting 1.7-kb fragment encoding hygromycin phosphotransferase was gel purified and subcloned into pUC19 which had been previously digested with the same enzymes and gel purified. A pUC19 derivative containing the entire cutinase structural gene including nearly 4 kb of DNA upstream from the cutinase translation initiation codon was linearized with \textit{Bgl}I, blunt ended with T4 DNA polymerase, and then digested with \textit{Ssp}I. The 3.7-kb segment containing 5' non-coding sequences and the cutinase ATG codon was ligated into pUC19 containing the hygromycin B gene. This plasmid was prepared for ligation with digestion with \textit{Kpn}I, treated with T4 DNA polymerase, and cut with \textit{Ssp}I. Transformants were selected on carbenicillin (50 µg/ml). Large-scale plasmid preparations were performed by alkaline lysis followed by CsCl-ethidium bromide density gradient centrifugation (26). The resulting vector, pCT57, was the parent transformation vector.

Restriction of pCT57 with \textit{Acc}I and religation after purification of the fragment by agarose gel electrophoresis gave pCT45 containing a 1.5-kb upstream sequence. The plasmid pCT72 containing 360 bp of upstream sequence was constructed by \textit{Pvu}I digestion of pCT45, blunt-ending with T4 DNA polymerase, \textit{Bam}HI digestion, gel purification of the fragment containing the 360-bp 5'-flanking region, and ligation into pUC19 containing the hygromycin resistance gene opened at \textit{Bam}HI and \textit{Sma}I. \textit{Bsu}I digestion of pCT45 generated pCT57 containing 750 bp from the 3' end of the upstream segment.

Transformation of protoplasts. \textit{Fusarium} protoplasts were prepared by a modification of the procedures of Yelton et al. (50). Approximately 0.5 × 10⁶ to 1.0 × 10⁶ conidia were inoculated into 100 ml of mineral medium (37) containing 2% glucose. After the cultures were shaken for 30 to 36 h at 75 rpm at room temperature, mycelia were recovered by filtration with sterile Miracloth in a Buechner funnel and washed with 0.6 M MgSO₄. Mycelia were suspended in 1.2 M MgSO₄, 10 mM morpholinepropanesulfonic acid (MOPS, pH 7.0), and β-glucuronidase-H2 (10% [vol/vol]), and driselase (10 mg/ml) and Novozymes 594 (20 μg/ml) were added. The suspension was incubated at 30°C for 2 h with shaking at 75 rpm. The suspension was passed through two layers of cheesecloth, and then through 100-μm nylon mesh followed by passage through 30-μm nylon mesh. Protoplasts were pelleted by centrifugation at 5,000 × g for 5 min at 4°C and washed three times by centrifugation in STC buffer (1.2 M sorbitol, 10 mM Tris hydrochloride [pH 7.5], 10 mM CaCl₂) and resuspended in STC. A 100-ml culture yielded approximately 10⁷ protoplasts. For transformation, 10⁶ protoplasts in 100 μl of STC were gently mixed with 25 μl of STC containing 1 to 20 μg of vector DNA and incubated at room temperature for 30 min. Polyethylene glycol 4000 (60% [w/v] in 10 mM Tris hydrochloride [pH 7.5] containing 10 mM CaCl₂) was added in steps of 200, 200, and 800 μl, with mixing after each addition. This solution was left at room temperature for an additional 30 min, centrifuged as above, and washed with STC. The pellet was resuspended in STC and spread at various dilutions on regeneration medium containing 1.2 M sorbitol, mineral salts (37), and 2% agar. Overlays of 1% agar containing hygromycin were added 24 h later. Colonies appeared in about 1 week.

Transformation with alkali cations. The transformation procedure was similar to that described by Dhande et al. (12). About 10⁶ spores (from cultures 7 to 10 days old) were transferred to mineral medium (37) containing 2% glucose. After 36 h of shaking, the mycelia collected by vacuum filtration were washed twice with 10 mM Tris hydrochloride (pH 7.8) containing 1 mM EDTA, suspended in 0.1 M lithium acetate (5 ml/g of tissue), and incubated with shaking at 30°C for 30 min. The mycelia collected by centrifugation were resuspended in as small a volume of 0.1 M lithium acetate as possible. To this thick slurry, 5 to 20 µg of vector DNA in 20 µl of the Tris-EDTA buffer was added and incubated at 30°C for 30 min with shaking. Ten volumes of 40% polyethylene glycol 4000 in 0.1 M lithium acetate was added, and the incubation continued for 90 min under the same conditions. The tissue was then heat shocked for 5 min at 37°C, centrifuged, washed twice with sterile water, resuspended in sterile water, and plated on vegetable juice agar. After keeping the plates for 20 h at room temperature (25°C), 0.75% agar overlays containing hygromycin (final concentration, 100 μg/ml) were then added.
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FIG. 1. Southern blot hybridization of the 5.5-kb fragment in the Ssrl digest of the 21-kb insert of the genomic DNA from F. solani f. sp. pisi with the 32P-labeled 125-bp probe from the 5' end of cutinase cDNA. A 150-bp probe from the 3' end of the cutinase cDNA also gave an identical result. Numbers are molecular size markers (in kilobases).

Analysis of DNA from transformants. DNA from transformants was isolated by the miniprep procedure of Biel and Parrish (7). For Southern blot hybridization of DNA from the transformants, DNA probes were labeled by nick translation to a specific activity of $5 \times 10^7$ to $5 \times 10^8$ cpm/μg (40).

RESULTS

To construct a genomic library of F. solani f. sp. pisi isolate T-8, randomly cleaved DNA fragments averaging 17 kb were inserted into the BamHI site of the lambda Charon 35 vector. Eleven clones of the cutinase gene were located among 24,000 genomic clones by hybridization of labeled cutinase cDNA. DNA was isolated from three randomly selected cutinase genomic clones, and restriction enzyme mapping showed them to contain 8.2-, 11-, and 21-kb genomic inserts. The first 125 bp of the 5' end and 150 bp of 3' end of the cutinase cDNA were used as probes for Southern blots of restriction digests of the genomic inserts. The 21-kb insert was found to contain a 5.5-kb Ssrl fragment which hybridized with both the 5' and 3' end probes from the cDNA (Fig. 1). A restriction enzyme map of the 5.5-kb Ssrl fragment was constructed to develop a strategy for the determination of the nucleotide sequence of this genomic DNA. This 5.5-kb Ssrl fragment contains two BglII sites, three HindIII sites, and five AccI sites. BamHI, EcoRI, PstI, and XbaI did not cleave this fragment.

A map of the cleavage sites for these restriction enzymes was constructed (Fig. 2) from comparisons of single and multiple restriction enzyme digests of the 5.5-kb Ssrl fragment and a 3-kb AccI fragment generated from within the 5.5-kb segment and by analysis of Southern blots of these digests hybridized with cDNA probes. Later sequencing results confirmed the presence of the predicted restriction enzyme cleavage sites.

A 510-bp HindIII fragment and a 1.530-bp HindIII-AccI fragment from the 5.5-kb Ssrl genomic segment, as well as the complete 5.5-kb Ssrl fragment, were subcloned into M13 bacteriophage for sequence determination by the dideoxy-nucleotide method. With five subclones from three fragments, the use of eight primers made it possible to read more than 60% of the DNA sequence from both strands and to independently sequence more than 80% of the DNA two or more times. The sequencing strategy is summarized in Fig. 2.

A continuous sequence of 2,818 nucleotides was determined in which 690 nucleotides code for the amino acids of...
procutinase (Fig. 3). The open reading frame is interrupted by a short 51-nucleotide intron which begins 193 nucleotides from the ATG initiation codon. The intron begins and ends with typical intron junctions, and there is some internal sequence homology common with introns of some of the other known fungal gene sequences (21, 23, 34, 48). A Sau3A site at +1829 is followed by 50 nucleotides of the lambda Charon 35 linker sequence preceding the SsrI site, indicating this to be the site of insertion between a Sau3A-cleaved genomic DNA fragment and the BamHI-cleaved vector. Therefore, this 3.5-kb SsrI fragment, which contains the cutinase structural gene, is located at the 3' end of the 21-kb genomic DNA insert.

Southern blot analysis of the genomic DNA from the T-8 isolate of F. solani f. sp. pisi showed two bands when three different restriction enzymes were used (Fig. 4). Since the gene does not have internal cut sites for these enzymes, it appears that the genome of this isolate contains two copies of the cutinase gene. On the other hand, T-30, another isolate of F. solani f. sp. pisi, showed only one band, indicating the presence of only one copy of the gene.

To test for the functional significance of the various regions of the cutinase gene and its flanking regions, a transformation system was developed for F. solani f. sp. pisi. Resistance to hygromycin B, a broad spectrum aminoglycoside antibiotic (39), was chosen as a selectable marker.
because it has been shown to inhibit growth of yeasts, certain other fungi, and plant and mammalian cells (20).

F. solani f. sp. pisi was transformed by the two methods previously described. While both procedures proved to be successful, as assessed by the generation of stable colonies with an Hm' phenotype and verification by blot hybridization, the lithium acetate protocol was more rapid and simple. It was also reliable and reproducible and has become the primary method for our DNA transformation experiments. The results presented here are based on this protocol.

The hygromycin coding region without the initiation codon was translationally fused to a 3.7-kb segment of the cutinase upstream region including the initiation codon as illustrated in Fig. 5. This vector, pCT43, was used to transform F. solani f. sp. pisi, and the transformants were selected by their hygromycin resistance. Spermidine and heparin had negligible effects on transformation frequency. The 3.7-kb segment of Fusarium DNA of pCT43 was shortened by elimination of an AccI fragment, resulting in a 1.5-kb promoter, and this construct, pCT45 (Fig. 6), yielded hygromycin-resistant transformants.

Hygromycin-resistant colonies appeared 5 to 14 days following treatment with lithium acetate and pCT43. Transformation with the hygromycin B resistance gene containing plasmids without cutinase 5'-flanking sequences never resulted in colonies exhibiting the transformed phenotype. In addition, spontaneous revertants to hygromycin resistance in wild-type fungi were never observed. Mitotic stability was evaluated by growing Hm' colonies on rich nonselective

![Image](EcoRI_HindIII_PstI_T8_T30_T8_T30_T8_T30.png)

FIG. 4. A Southern blot of electrophoretically separated restriction endonuclease digests of genomic DNA isolated from cultures of T-8 and T-30 strains of F. solani f. sp. pisi. The blot was hybridized with 32P-labeled cutinase cDNA.

![Diagram](pCT43.png)

FIG. 5. Construction of pCT43, the parent transformation vector used to test promoter activity of the 5'-flanking region of the Fusarium cutinase gene. The nucleotide sequence of the region showing translational fusion of cutinase to the hygromycin resistance gene is given at the bottom.
media (vegetable juice agar) followed by transfer back to media containing 100 μg of hygromycin per ml. About 50% of the transformants lost their resistance to the antibiotic and were presumed to be abortive. The remaining 50% had growth rates and colony morphologies similar to those of the wild-type F. solani f. sp. pisi on vegetable juice agar. Resistant transformants were purified by single-spore isolation to eliminate heterokaryons. We obtained an average of 10 mitotically stable transformants per μg of vector DNA per g of tissue. Circular plasmids were used in each case. Stable transformants were routinely maintained on media containing 100 μg of hygromycin B per ml, which was determined to be the optimum concentration.

To verify that the integrative transfer of foreign DNA occurred, resulting in transcription and expression of the hygromycin resistance gene, Southern analysis was performed. When gel blots of undigested genomic DNA from the wild-type and four pCT45 transformants were probed with the 32P-labeled nick-translated hygromycin resistance gene, high-molecular-weight bands were observed, indicating that hygromycin phosphotransferase DNA was contained in these transformants and that integration of this DNA into the Fusarium genome had occurred (Fig. 7, top panel, A). The absence of any low-molecular-weight DNA bands suggests that pCT45 does not exist in F. solani f. sp. pisi as a free plasmid, although this possibility cannot be ruled out. However, we did not observe hybridization to lower-molecular-weight DNA fragments even in autoradiograms generated with extended exposure periods. The absence of a detectable signal in the wild-type lane indicated that hygromycin bears no homology to DNA sequences found in the Fusarium genome.

Further characterization of the integration events of the transforming DNA was done by Southern analysis of restriction-digested genomic DNA from the transformants. Probing of Ssr1-digested DNA was done with each component of the vector (Fig. 7, middle panel). Ssr1 digestion of the genome of wild-type F. solani f. sp. pisi resulted in a 5.5-kb fragment containing the entire cutinase structural gene as well as the flanking sequences. There was one Ssr1 site in the vector. Probing with a fragment containing cutinase 5'-flanking sequences showed hybridization in the 5.5-kb region of each transformant as well as in the wild type. Radiolabeled probes for hygromycin resistance and pUC19 also gave signals at 5.5 kb in each transformant but not in the wild type. Therefore, it appears that the entire vector had integrated, and the hybridization pattern suggested that either tandem copies of the transforming DNA had integrated or a Ssr1 site happens to be present very near the insertion site in the fungal genome. The pattern of integration for filamentous fungi, whether a homologous or nonhomologous recombination, has not been consistently established and can be viewed only on a case-by-case basis.

To test further whether the 5' segment of the cutinase gene from F. solani f. sp. pisi exhibits promoter activity, C. capsici, which has no detectable homology with the Fusarium cutinase gene as determined by Southern blot hybridization (data not shown), was used as the host for transformation. The sensitivity of C. capsici to hygromycin was found to be less than that of F. solani f. sp. pisi, and therefore transformants of this fungus were selected on 300 μg of hygromycin per ml. Under the same transformation conditions as those used for F. solani f. sp. pisi, C. capsici was successfully transformed with pCT45 and numerous hygromycin-resistant colonies were obtained. The frequency of transformation for C. capsici was up to twofold higher than that obtained with F. solani f. sp. pisi. Southern blot analysis of genomic DNA showed the presence of the hygromycin resistance gene in the transformants but not in the wild type, indicating successful DNA transfer and chromosomal integration (Fig. 7, top panel, B). When the PstI digest of the genomic DNA was subjected to Southern blot analysis with the cutinase 5'-flanking region, the hygromycin resistance gene, and pUC19 as probes, all three probes revealed hybridization bands at about 4 kb (Fig. 7, bottom panel). PstI should cut pCT43 in half, to yield fragments at 4 kb. The single hybridizing band found at about 4 kb in the Southern blots of the transformants is consistent with that expected from integration of the plasmid.

To further define cis-acting sequences, the length of the 5'-flanking sequence of cutinase inserted into the transforming vector was shortened. The Fusarium cutinase promoter activity could be detected when smaller segments of 5'-flanking region of the cutinase gene were tested as promoters. BAL 31 digestion of pCT45 yielded promoter regions containing 750, 550, 350 bp of the 5'-flanking region. PvuI cleavage of pCT45 gave pCT72 containing 360 bp of the 5'-flanking region of the cutinase gene (Fig. 6). All of these constructs yielded hygromycin-resistant transformants of F. solani f. sp. pisi. Genomic DNA from these transformants revealed hybridization with dot blot analysis (not shown). Since the precise region of the 5'-flanking segment required for promoter activity has not been determined, we can conclude only that cutinase promoter activity is within the 360 bp upstream from the coding region.

**DISCUSSION**

We have determined the nucleotide sequence of a DNA fragment containing the cutinase gene of F. solani f. sp. pisi.
FIG. 7. Southern hybridization of genomic DNA from hygromycin-resistant transformants generated by pCT43. (Top panel) Undigested genomic DNA from four transformants of *F. solani* f. sp. *pisi* (A) and three transformants of *C. capsici* (B). Lane 1 in both cases represents wild-type DNA. Electrophoresis was on 0.7% agarose, and the $^{32}$P-labeled hygromycin resistance gene (*BamHI*-HindIII, Fig. 5) was used as the probe. (Middle panel) Southern hybridization of *SstI* digests of genomic DNA from pCT43 transformants of *F. solani* f. sp. *pisi* shown in the top panel (A). (Bottom panel) Southern hybridization of *PstI* digests of genomic DNA from pCT43 transformants of *C. capsici* shown in the top panel (B). In both bottom and middle panels, the $^{32}$P-labeled *PvuI*-BglII fragment from the 5'-flanking region of the cutinase gene from *F. solani* f. sp. *pisi* was used as the probe. Since the figure is a composite of experiments, direct comparisons of relative intensities are not valid. All electrophoresis was conducted on 0.7% agarose, and experimental details are in the text.

The sequence of the DNA fragment contains a region that is completely homologous to the previously sequenced cutinase cDNA (44), except for a 51-nucleotide interruption of the open reading frame which most probably represents an intron. The predicted polypeptide has a molecular weight consistent with that estimated for the in vitro translated procutinase (13) and contains the amino acid sequences of several cutinase peptides which have been isolated and
sequenced, representing about 40% of the primary structure of mature cutinase (44). Thus, the particular open reading frame in this sequenced region of the fungal genome surely represents the authentic coding region for cutinase that is induced by cutin.

The 940 nucleotides of sequence in the 5'-flanking region preceding the ATG start codon that should include the transcription initiation site of the cutinase gene contain neither typical TATAAA or CAAT homologies. These sequence elements have been implicated as important functional components of gene promoters in eucaryotes, including genes from several filamentous ascomycetes such as the NADP-glutamate dehydrogenase gene (21) and ADP-ATP carrier protein gene (2) from Neurospora crassa, the glucoamylase gene from Aspergillus niger (10), the spoCI-C gene from Aspergillus nidulans (15), and the glucoamylase gene from Aspergillus awamori (34). In the cutinase gene at positions -117 to -111 a TAAATAT sequence exists, and this has a resemblance to the TATA sequence. The CAAG sequence found in close proximity is also a common feature of eucaryotic genes. However, whether this sequence or this region has any functional significance remains to be determined. The CT-rich region found in the vicinity of the initiation codon is also found in the cutinase gene. As with the cutinase gene, there are other examples of fungal genes that lack the classical promoter elements in their 5'-flanking regions, which include genes for the N. crassa qa cluster (1), N. crassa trp-1 (42), A. nidulans trpC (32), and N. crassa his-3 (24). In fact, a recent functional analysis of the upstream region of the trpC gene from A. nidulans showed that the CCAAT and TATAA sequences are not essential for the expression of this gene (16).

In the 3'-flanking region of most higher eucaryotic genes, the AATAAA sequence precedes the site of polyadenylation (36). However, there are examples of polyadenylation in the absence of the AATAAA sequence, including mRNAs of filamentous ascomycetes (10, 34). The 3'-flanking region of the cutinase structural gene does not contain this polyadenylation signal, but it does have a very similar AAAATTTA sequence at positions +635 to +642 just 120 nucleotides from the TGA stop codon. At positions +648 to +651, there is a TAGT sequence in the 3'-flanking region of the cutinase gene as found in several genes of S. cerevisiae where such sequences were postulated to be involved in polyadenylation or transcription termination (51). In a proposed model for polyadenylation of pre-mRNA, a U4 small nuclear RNA hybridizes to the AAUAAA sequences and the CAYUG sequence to allow polymerization of poly(A) (6). In the 3'-flanking region of the cutinase gene, a CAATG sequence occurs at positions +609 to +613 and +619 to +651. This latter CAATG sequence is just 3' to the AATAAA sequence. The functional significance of each of these specific sequences identified in the 3'-flanking region of this gene remains unclear, since no functional analysis has yet been conducted.

The one intervening sequence localized in the cutinase gene is very short (51 nucleotides), which is typical of introns found in fungal nuclear genes. The 5' splice junction fits the GTNNGT pattern also noticed in yeast introns (35), and the 3' end has a pronounced CT-rich region before the AG (31). As observed with other genes of filamentous fungi, internal sequences in the intron show only partial homology with yeast introns (24, 36). Other areas of homology among introns, such as the GAGCTGACTG sequence in both introns of exo-cellobiohydrolase I from Trichoderma reesei (43), which show partial homologies with A. niger glucoamylase introns (10), are not found in the cutinase gene intron.

A survey of the codons found in the cutinase gene shows a moderate degree of bias toward the utilization of certain codons over the choices possible for each amino acid. Out of the 61 possible triplets, 50 codons are used, and there are clear preferences of codon usage for each amino acid. This is in contrast to the extreme codon bias observed in several highly expressed genes of Saccharomyces cerevisiae (4) and the histone genes of N. crassa (48), in which only 33 or 29 codons are utilized, respectively. Normally there is little bias in codon utilization observed in the genes of filamentous ascomycetes, but there are patterns of codon preference. A similar pattern of codon usage is seen in the Neurospora genes for the proteolipid component of mitochondrial ATP synthase (46), histones (48), and NADP-glutamate dehydrogenase (21). For example, the third position of the codon is almost always a pyrimidine except for the use of guanine when a purine has to be used. Also, the AGN codons for arginine and serine and the UUPu leucine codons are rarely used. Several highly regulated genes among the filamentous ascomycetes (10, 11, 24, 34, 42, 43) show a strong preference for G and C in the third position with varying usage of U, and they use the AGN and UUPu codons. Codon utilization for the cutinase gene tends to fit with this group.

Since the genomic DNA appeared to contain two segments of DNA that hybridize with the cDNA, it is likely that these represent two copies of the cutinase gene in this organism. Since the organism is known to produce two cutinases (38), it is possible that the two copies code for the two proteins. The amino acid sequence of two peptides from the two proteins showed that they are highly homologous but distinctly different in the primary structure. For example, Ala and Val in cutinase I were replaced by Thr and Ile, respectively, in cutinase II (22). The nucleotide sequence of the cDNA representing the induced cutinase and the cloned gene described in the present paper completely agree with the amino acid sequence of the peptides from cutinase I, the major cutinase induced by cutin and its monomers (44). Therefore, it is likely that the present clone of the genomic DNA represents the cutinase gene that is inducible. The other copy of the gene has a sequence that is highly homologous to the gene for cutinase I but must have nucleotide substitutions in the amino acid substitutions. For example, each of the two substitutions observed in the peptide presented in this paper would involve at least a single-base substitution. Isolate T-30, which is known to be much less virulent in intact (unwounded) organs than T-8, has a low constitutive level of cutinase, and the induced level remains less than 20% of that generated by T-8 (22). This isolate showed only one copy of the cutinase gene. Since the cloned copy of the gene from T-8 appears to be the highly inducible gene, it is highly likely that this gene is involved in pathogenesis. Therefore, we decided to concentrate our efforts on this copy of the gene.

To study the functional aspects of the cutinase promoter, we had to develop a transformation system for *F. solani* f. sp. *pisi*. As indicated in this report, a promoterless hygromycin resistance gene turned out to be a suitable system for detecting promoter activity of the 5'-flanking regions of the cutinase gene. In our experience, the lithium acetate method is a simple one to introduce foreign DNA into *F. solani* f. sp. *pisi* and *C. capsici*. The use of protoplasts did yield two to four times higher transformation frequencies in absolute terms. The transformation frequency was adequate for ready isolation of transformants on the basis of hygromycin resis-
tance, probably because we were using constructs containing the putative cutinase promoter rather than randomly fragmented DNA.

The results presented here show the nucleotide sequence of the cutinase gene, which matches completely with that of the cDNA induced by cutin. The 5'-flanking region of this cloned cutinase gene manifests promoter activity. From the limited number of deletion fragments tested for promoter activity, it appears clear that the 360-bp region immediately 5' to the translation initiation codon effectively directed transcription. Since the present studies did not use reporter genes for quantitative evaluation of the promoter strength, precise definition of the essential regions of the promoter is not possible.

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