The Plant Cell, Vol. 4, 621-629, June 1992 © 1992 American Society of Plant Physiologists

RESEARCH ARTICLE

Cutinase Is Not Required for Fungal Pathogenicity on Pea

Dietmar J. Stahl and Wilhelm Schäfer[1]

Institut für Genbiologische Forschung Berlin GmbH, Ihnestrasse 63, D-1000 Berlin 33, Germany

Cutinase, a fungal extracellular esterase, has been proposed to be crucial in the early events of plant infection by many pathogenic fungi. To test the long-standing hypothesis that cutinase of *Nectria haematococca (Fusarium solani* f sp *pisi)* is essential to pathogenicity, we constructed cutinase-deficient mutants by transformation-mediated gene disruption of the single cutinase gene of a highly virulent *N. haematococca* strain. Four independent mutants were obtained lacking a functional cutinase gene, as confirmed by gel blot analyses and enzyme assays. Bioassays of the cutinase-deficient strains showed no difference in pathogenicity and virulence on pea compared to the wild type and a control transformant. We conclude that the cutinase of *N. haematococca* is not essential for the infection of pea.

INTRODUCTION

In contrast to human or animal disease development, the molecular mechanisms involved in establishing plant diseases are poorly understood. With fungal pathogens, only the genes for cutinase production (Dickman et al., 1989; Soliday et al., 1989) and pisatin demethylating ability (Weltring et al., 1988; Schäfer et al., 1989) of *Nectria haematococca* MP VI and the *b* locus of *Ustilago maydis* (Kronstad and Leong, 1989; Schulz et al., 1990) have been isolated and characterized as putative pathogenesis determinants. The direct involvement of the *b* locus in the pathogenic development of *U. maydis* was unequivocally demonstrated by gene replacement (Kronstad and Leong, 1990).

The crucial role of cutinase in infection of plants was suggested after investigation of the filamentous ascomycete *N. haematococca* mating population VI (anamorph: *Fusarium solani* f sp *pisi*), the causal agent of foot rot of pea. *N. haematococca* is a soil inhabiting fungus with a world-wide distribution. In pea seedlings, the initial center of attack by this pathogen is the cotyledonary attachment area, below-ground epicotyl, and upper taproot. Above-ground symptoms are not readily defined but consist primarily of yellowing of the basal foliage and stunted growth (Kraft et al., 1981). Indirect evidence has been interpreted to imply a pivotal role of cutinase in infection, especially during penetration of the underground hypocotyl. Cutinase, a serine esterase, could be detected immunocytologically at the penetration site where germinating spores were breaching the cuticle of etiolated, sectioned pea stems (Shayhk et al., 1977). Inhibitors of serine hydrolases and antiserum raised against cutinase prevented infection by *N. haematococca* (Maiti and Kolattukudy, 1979). Expression and release of cutinase in spores of *N. haematococca* are induced by monomers of cutin (Woloshuk and Kolattukudy, 1986; Podila et al., 1988), and repressed by glucose (Lin and Kolattukudy, 1978). An agricultural use of cutinase inhibitors as fungicides was proposed, but never reached the point of practical application. Insertion of the cutinase gene of this pea pathogen into *Mycosphaerella* spp, a wound pathogen of papaya fruits, enabled the transformants to infect unwounded papaya fruits, demonstrating the involvement of cutinase in the interaction of this recombinant pathogen with its host (Dickman et al., 1989). To evaluate directly the role of cutinase during the infection process of *N. haematococca* on its natural host, we constructed a cutinase-deficient mutant by gene replacement. The results are inconsistent with a crucial role for cutinase in disease development under normal conditions.

[1] To whom correspondence should be addressed.

RESULTS

Selection of an *N. haematococca* Strain for the Gene Replacement

An *N. haematococca* strain was desired that is highly virulent and carries only one cutinase gene for the construction of the null mutant. Isolates were tested for virulence on soil-grown pea plants. A 500-bp fragment corresponding to nucleotide position 161-659 of the cutinase gene of *N. haematococca* isolate T8 (Soliday et al. 1989) was amplified by polymerase chain reaction (PCR) of genomic DNA. The PCR product was cloned to create plasmid pCut22. The number of cutinase genes of each isolate was estimated by DNA gel blot analysis. All strains contained a common cutinase gene, whereas some strains

contained a second gene. Strain 77-2-3 was chosen for further experiments because it was highly virulent and the cutinase gene was unique in the genome of this fungal isolate.

Transformation-Mediated Gene Disruption

The cutinase gene of strain 77-2-3 was isolated for transformation-mediated gene disruption by screening a partial genomic library. One clone containing a 5.5-kb SacI-SmaI fragment (p8C77-1) was analyzed further. A 754-bp open reading frame interrupted by a 52-bp intron was identified after determining the nucleotide sequence of a 1700-bp segment of p8C77-1 (data not shown). The coding region and regulatory elements of the gene showed more than 99% identity with the cutinase gene of *N. haematococca* isolate T8 (Soliday et al., 1989).

The transformation vector pDC6 shown in Figure 1A was constructed for gene replacement. A 360-bp Eco47III-PpuMI fragment internal to the cutinase gene open reading frame was replaced by a selection marker. The selection marker consisted of the hygromycin B phosphotransferase (*hph*) gene of *Escherichia coli*, conferring resistance to hygromycin B, and regulatory elements of the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) and tryptophan synthase (*trpC*) genes of *Aspergillus nidulans* (Punt et al., 1987). Insertion of the marker gene into the coding region allowed the application of the "one-step gene replacement" technique (Rothstein, 1983), as depicted in Figure 1B. Deletion of a 360-bp fragment of the cutinase coding region during vector construction permitted the unequivocal proof of gene replacement by DNA gel blot analysis. Strain 77-2-3 was transformed with plasmid pDC6. Hygromycin B–resistant transformants were screened for gene replacement by DNA gel blot analysis. Three different types of transformants could be obtained, as shown in Figure 2. DNA from transformant 77-102 produced hybridization patterns consistent with replacement of the wild-type cutinase gene by the disrupted cutinase gene of plasmid pDC6 (Figures 2A and 2D). A 3.6-kb length increase of the cutinase signal of 77-102, as compared with the length of the wild-type gene (Figures 2A and 2B), when using pDC6 as a probe, reflected the addition of the hygromycin B selection marker. No signal was detectable for 77-102 after hybridizing with the 360-bp Eco47III-PpuMI fragment. In the case of transformant 77-100 (Figures 2A and 2E), the transformation vector had integrated by a single homologous recombination event without disturbing cutinase gene function. The ectopic transformant 77-109 (Figures 2A and 2C) represents the most common integration event.

A plate assay for extracellular esterase activity was designed to facilitate the identification of further null mutants. As cutinase is inducible by the cutin monomer 16-hydroxyhexadecanoic acid (Lin and Kolattukudy, 1978) and acetate is not nearly as repressive as glucose to the production of cutinase by cutin (Dantzig et al., 1986), transformants were grown on 16-hydroxyhexadecanoic acid–acetate agar. After 3 days of growth, colonies were overlaid with the esterase substrate

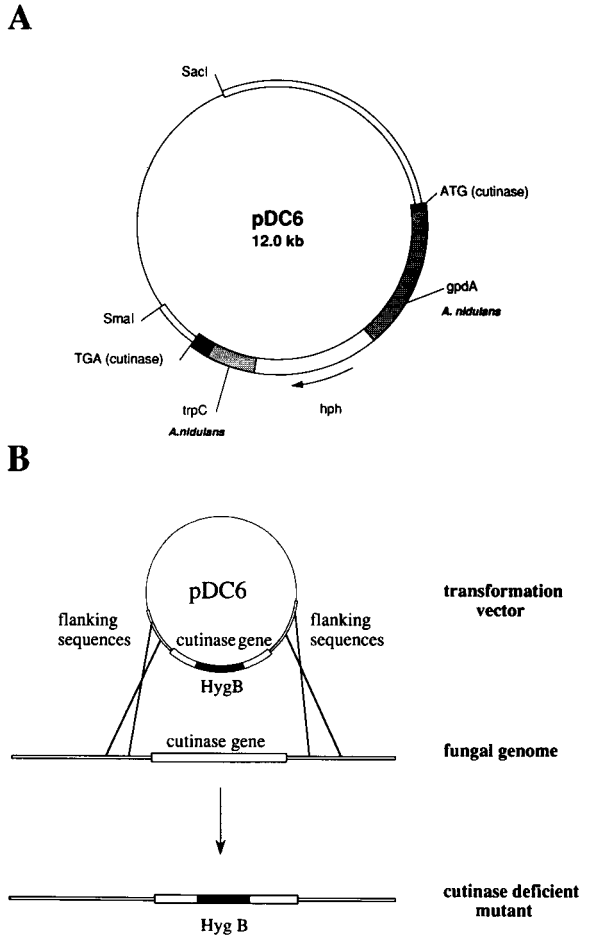

Figure 1. Transformation-Mediated Gene Disruption of the Cutinase Gene.

(A) For construction of the transformation vector pDC6, the cutinase gene of strain 77-2-3 located on a 5.5-kb SacI-SmaI fragment (thin white box) was cloned. The hygromycin B phosphotransferase (*hph*) gene of *E. coli* (thick white box) was inserted into the coding region of cutinase (black boxes). Expression of this selectable marker is controlled by regulatory elements of the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) and tryptophan synthase (*trpC*) genes of *A. nidulans* (shaded boxes). A 360-bp Eco47III-PpuMI fragment of the 753-bp coding region of the cutinase gene was replaced by the 4.0-kb selection marker. **(B)** Schematic presentation of the "one-step gene replacement" technique. The fungal cutinase gene is replaced by the disrupted cutinase gene from the transformation vector by twofold homologous recombination in the 5′ and 3′ regions flanking the cutinase gene.

para-nitrophenyl butyrate. Whereas wild-type 77-2-3 and most of the 100 transformants analyzed produced a large halo on these plates, as shown in Figure 3, 77-102 and three transformants, 77-5, 77-75, and 77-84, produced no halos, indicating the absence of extracellular esterase activity. DNA gel blot analysis of these transformants (data not shown) revealed the same replacement of the cutinase gene as that in 77-102. Overall,

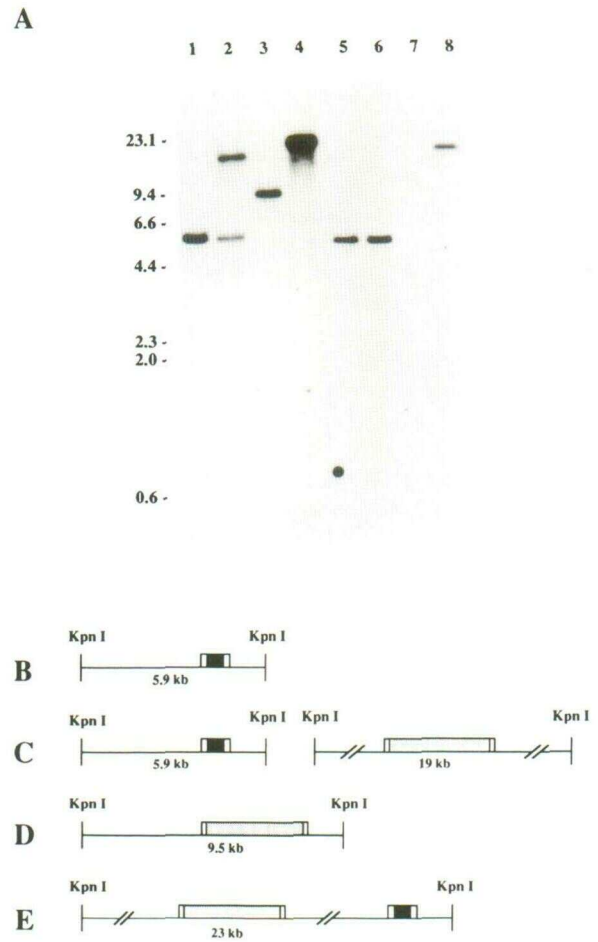

Figure 2. DNA Gel Blot Analysis of *N. haematococca* Wild-Type Strain and Transformants.

(A) DNA gel blot analysis of a KpnI digest of genomic DNA from wild-type isolate 77-2-3 and transformants. Lanes 1 and 5, wild-type 77-2-3; lanes 2 and 6, ectopic transformant 77-109; lanes 3 and 7, cutinase-deficient transformant 77-102; lanes 4 and 8, transformant 77-100. Lengths and migration of λ HindIII are shown at left. Markers are given in kilobases. Lanes 1 to 4 were probed with the gene replacement vector pDC6, and the lanes 5 to 8 with a 360-bp Eco47III-PpuMI cutinase fragment, which was deleted from the disrupted cutinase gene in the transformation vector pDC6.

(B) Restriction map of the cutinase locus from wild-type strain 77-2-3.

(C) Restriction map of the cutinase locus and the integrated copy of the transformation vector from ectopic transformant 77-109.

(D) Restriction map of the mutated cutinase locus from null mutant 77-102.

(E) Restriction map of the cutinase locus from the single homologous transformant 77-100.

The positions of the intact and the interrupted cutinase genes on the KpnI fragments are shown by boxes. The 360-bp Eco47III-PpuMI fragment of the cutinase gene used as a probe in lanes 5 to 8 in **(A)** is indicated by the black boxes in **(B)**, **(C)**, and **(E)**. The selection marker disturbing cutinase gene function is indicated by a stippled box in **(C)**, **(D)**, and **(E)**.

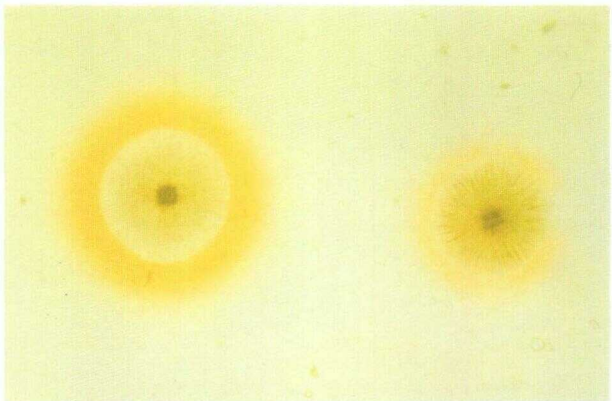

Figure 3. Plate Assay for Extracellular Esterase Activity.

Wild-type strain 77-2-3 at left and cutinase-deficient transformant 77-102 at right were grown on cutinase induction medium to yield colonies 2 cm in diameter. After being overlaid with *para*-nitrophenyl butyrate-containing agar, a yellow halo appeared around the colony of the wild-type strain indicating the secretion of esterase activity into the agar. The absence of a halo around 77-102 revealed the lack of extracellular esterase activity of this transformant.

4% of the transformants were mutated to the cutinase-deficient phenotype by the gene disruption procedure.

Transcriptional and Enzymatic Analysis of the Transformants

The influence of gene replacement at the transcriptional level was determined by RNA gel blot analysis. The wild type, null mutant 77-102, and transformant 77-100 were grown in liquid culture with apple skin as a crude source of cutin for cutinase induction and in medium containing glucose for repression of cutinase. RNA was isolated, and gel blots were hybridized with the radiolabeled 360-bp Eco47III-PpuMI fragment. A single 1050-nucleotide transcript was detected for the wild type and transformant 77-100 under conditions of cutinase induction, as shown in Figure 4. No transcript was detected for transformant 77-102. Neither the wild type nor transformants gave rise to cutinase transcript when grown in medium containing glucose, confirming the catabolite repression of cutinase (Lin and Kolattukudy, 1978).

Wild type, 77-100, and the four null mutants 77-5, 77-75, 77-84, and 77-102 were cultivated on purified apple cutin as carbon source. Whereas the wild type and transformant 77-100 grew equally well, growth of the null mutants was dramatically reduced. This result is due to replacement of the cutinase gene, because germination efficiency, growth rate, and colony morphology were identical when the strains were grown on complete medium.

To demonstrate the absence of the cutinase protein in transformant 77-102, the culture fluids from cutin-grown strains were treated with 1,3-^{14}C-diisopropyl fluorophosphate, an inhibitor

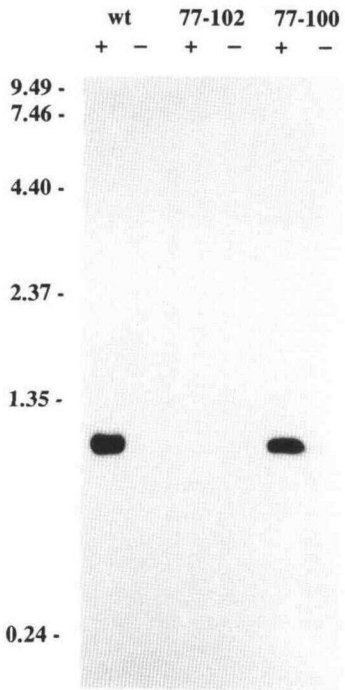

Figure 4. RNA Gel Blot Analysis of Cutinase Expression.

RNA of wild-type (wt) strain 77-2-3 and the transformants 77-100 and 77-102 grown under conditions of cutinase induction (+) or repression (−) was analyzed. Length (kb) and migration of the RNA ladder are shown at left. Total cellular RNA (24 μg) was loaded per lane, and the filter was hybridized with the internal 360-bp Eco47III-PpuMI fragment of the cutinase gene.

of serine hydrolases, including cutinase (Cohen and Oosterbaan, 1967; Köller et al., 1982a). A highly abundant protein of 24 kD, corresponding to the molecular mass of cutinase after 1,3-^{14}C-diisopropyl fluorophosphate treatment (Köller et al., 1982a), was detected with the wild type and transformant 77-100, but not for 77-102, as shown in Figure 5. Besides the 24-kD cutinase, four less abundant proteins (with molecular masses of 26.5, 29, and approximately 48 and 55 kD) were detected for transformant 77-100 and the wild type. After prolonged exposure, all proteins, with the exception of cutinase, were visible for transformant 77-102 (data not shown). This result suggests that at least five serine hydrolases are secreted by *N. haematococca* isolate 77-2-3 during saprophytic growth on cutin, and four of these proteins are secreted by the cutinase null mutant.

Culture fluids of the wild type, 77-100, and 77-102 cultivated on cutin were assayed daily for general esterase activity with *para*-nitrophenyl butyrate as substrate. The time course of extracellular esterase induction was similar for the wild type and 77-100. After 20 days, the enzyme activity of both cultures reached a plateau at a similar value. By contrast, 77-102 did not show an induction of esterase activity. After 20 days, an esterase activity accumulated corresponding to 0.3% of the wild-type activity. Table 1 shows that the four null mutants (77-5, 77-75, 77-84, and 77-102) produced only 0.13 to 0.95% of the activity measured for the wild-type strain 77-2-3. Transformant 77-100, carrying the transformation vector in the vicinity of the cutinase locus, displayed 82.2% of the wild-type activity. To prove that there was no residual cutinase activity in the culture fluids of the null mutants, we performed a specific cutinase assay that relies on release of soluble material from ^{3}H-cutin. The wild type and transformant 77-100 showed a significant level of cutinolytic activity, but no cutinase activity was detected for the four null mutants (Table 1).

Pathogenicity Tests

Different bioassays were performed to evaluate the importance of cutinase activity for pathogenicity. Pea seeds were planted in sterile soil and infected with conidia from the wild type and transformants. Plants were exposed to conidia concentrations corresponding to the level of inoculum found in the soil of infected pea fields (Kraft and Roberts, 1969). The infected plants were examined daily for 20 days, and no difference in the virulence of the wild type, the four null mutants, or transformant

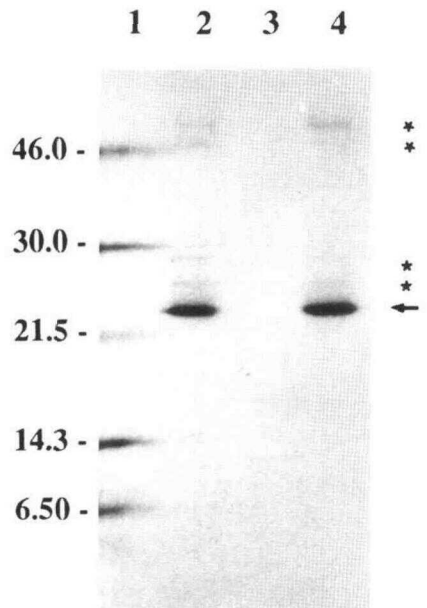

Figure 5. Detection of Proteins with Active Serine in the Culture Fluid of Cutin Grown Fungi.

Fluorography of proteins with active serine after 1,3-^{14}C-diisopropyl fluorophosphate treatment and separation on 15% SDS–polyacrylamide gels. Equal volumes of culture fluid of cutin grown fungi were analyzed. Lane 1, molecular mass standards (^{14}C-labeled proteins) with markers given in kilodaltons; lane 2, transformant 77-100; lane 3, null mutant 77-102; lane 4, wild-type strain 77-2-3. Besides the 24-kD cutinase, marked by an arrow, four minor proteins, marked by asterisks, are visible for the wild type and transformant 77-100.

Table 1. Comparison of Esterase and Cutinase Activities in Culture Fluid from the Wild Type and Transformants Cultivated for 3 Weeks on Cutin

	Esterase[a]		Cutinase[b]	
Strain	(nkat/mL)	(%)[c]	(cpm/100 µL/hr)	(%)[c]
Wild type	554.0	100.0	33115 ± 2318	100.0
77-100	455.4	82.2	26523 ± 2121	78.0
77-102	0.74	0.13	3018 ± 156	0.0
77-5	1.94	0.35	3386 ± 148	0.0
77-75	5.3	0.95	3166 ± 332	0.0
77-84	0.73	0.13	3155 ± 417	0.0
Control[d]	0.0	0.0	3223 ± 525	0.0

[a] Culture fluid was assayed for esterase activity using *para*-nitrophenyl butyrate as a substrate.
[b] Cutinase activity was measured by determining the release of radioactivity from ^{3}H-cutin. Results are the average of two replicative determinations.
[c] Relative activities (%) are given as the difference of culture fluid value and control value compared with the wild-type activity (100%).
[d] Control activities show total esterase activity or cutinase activity after incubation with culture medium alone.

77-100 was observed. All infected plants displayed the typical symptoms described for *N. haematococca* (Kraft et al., 1981). These included yellowing of the basal foliage and stunted growth, as can be seen for the wild type and cutinase-deficient mutant 77-102 in Figure 6A. A brown discoloration of the hypocotyl and the upper tap root localized the point of fungal attack (Figure 6B). Pea seedlings were examined 6 and 8 days after planting in soil infested with conidia of the wild type or the null mutant 77-102 for the analysis of the early stage of infection. The appearance of small brown lesions on the tap root and on the hypocotyl showed that both organs were independently colonized equally well by the wild type and the null mutant. Therefore, the colonization of the hypocotyl need not be caused by a spread of the pathogen from the root into the stem base.

Results from other experiments performed by Maiti and Kolattukudy (1979) and Köller et al. (1982b) suggested that cutinase is essential for plant infection, but these experiments were performed with sections of etiolated pea stems. We therefore evaluated our strains by this assay system. The hypocotyls of etiolated peas were cut in pieces of 2 to 3 cm in length, and the unwounded surface was inoculated with a droplet of conidial suspension. Symptoms were checked 3 to 4 days after incubation in the dark. Infection caused brown lesions due to invading hyphae, as shown for wild type and null mutant 77-102 in Figure 6C. The presence of hyphae in infected plant tissue was confirmed by microscopic analysis (D. J. Stahl, C. Hofmann, and W. Schäfer, manuscript in preparation). Many of the conidia of the droplet were converted to a mass of wet mycelium (Figure 6C). This phenomena, resembling saprophytic fungal growth on agar plates, is typical for this assay and was never observed in assays with intact, living plants. Virulence of each strain with this assay was determined, and the results are summarized in Table 2. Wild-type strain 77-2-3, transformant 77-100, and null mutant 77-102 displayed a mean virulence of 77, 80, and 81%, respectively, in four independent experiments. Further experiments with the three null mutants 77-5, 77-75, and 77-84 revealed no decrease in infectivity of these transformants in comparison to the wild type (Table 2). We concluded that the cutinase-deficient mutants display the same pathogenicity as the wild type or control transformant in two different bioassays.

DISCUSSION

N. haematococca, a fungal pea pathogen, can grow on cutin as a sole carbon source and makes a substrate-induced, catabolite-repressed cutinase (Lin and Kolattukudy, 1978). We produced fungal lines that contain null mutations of the cutinase gene and thus cannot produce cutinase. All disruptants lacked cutinase, whereas all other transformants possessed the enzyme. Without cutinase, fungal growth in culture with cutin as a sole carbon source was strongly inhibited. By contrast, its pathogenicity was unaltered.

Evidence for the direct involvement of cutinase of *N. haematococca* in the disease development of this fungus on pea has relied on the analysis of infection studies with polyclonal antibodies (Maiti and Kolattukudy, 1979) or chemical inhibitors (Maiti and Kolattukudy, 1979; Köller et al., 1982b) in the inoculum. These studies depended on the specificity of the inhibitor. To our knowledge, the specificity of the polyclonal antibody in the presence of germinating conidia on sectioned, etiolated pea stems has not been shown. The chemical inhibitors used are the organophosphorous compounds diisopropyl fluorophosphate and diethyl *para*-nitrophenyl phosphate. These are general inhibitors of serine hydrolases, including esterases and proteases (Cohen et al., 1967). Therefore, the inhibition of other enzymes besides cutinase could have been responsible for the observed reduction in pathogenicity. Following 3 weeks of growth on cutin as carbon source, at which time cutinase activity contributes up to 99.7% of the total esterase activity, four other proteins were labeled with diisopropyl fluorophosphate in addition to cutinase. That this inhibitor is not specific for cutinolytic enzymes is in agreement with that reported for *Colletotrichum lagenarium* in which diethyl *para*-nitrophenyl phosphate was tested. Although the growth of *C. lagenarium* was not affected, there was a reduction of appressoria formation in the presence of the inhibitor. This could only be attributed to a disturbance of fungal metabolic functions (Bonnen and Hammerschmidt, 1989).

Genetic approaches were used to elucidate the role of cutinase in virulence of phytopathogenic fungi. Mutants reduced or deficient in cutinolytic activity were induced by chemical treatment of *C. gloeosporioides* (Dickman et al., 1987) and *C. lagenarium* (Bonnen and Hammerschmidt, 1989) or by UV irradiation of *N. haematococca* (*F. s. pisi*)

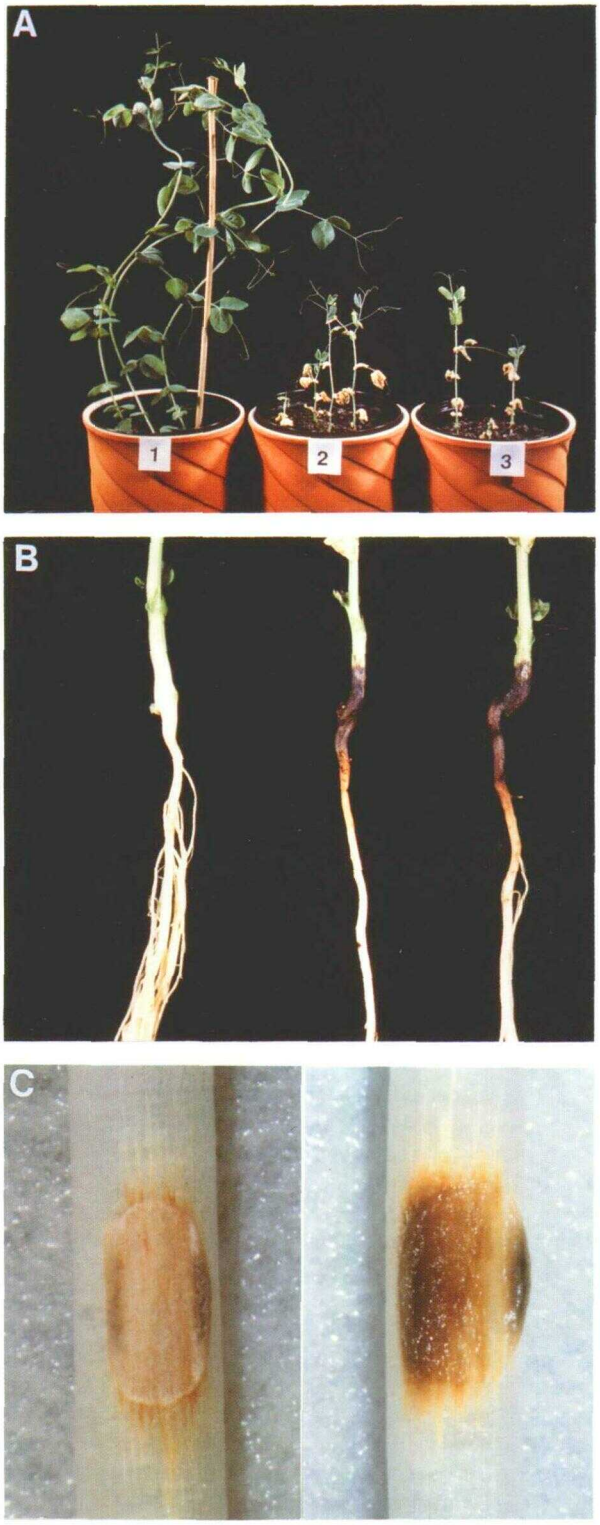

Figure 6. Pathogenicity Tests of the Wild-Type of *N. haematococca* and a Cutinase-Deficient Mutant.

Table 2. Virulence of *N. haematococca* Transformants in Comparison to the Wild-Type Strain 77-2-3 on Segments of Etiolated Pea Stems

	Degree of infection (%)[a]	
Strain	Noninfected	Infected
Wild type[b]	23	77
77-100[b]	20	80
77-102[b]	19	81
77-5[c]	20	80
77-75[c]	0	100
77-84[c]	0	100
Water[b]	100	0

[a] Infectivity and virulence were expressed as the percentage of infected segments.
[b] Values are the average of four independent experiments using 10 to 20 pea segments, respectively.
[c] Results from one experiment using 20 pea segments.

and *C. gloeosporioides* (Dantzig et al., 1986; Dickman et al., 1987). Whereas cutinase-deficient mutants of *C. gloeosporioides* lost pathogenicity on papaya fruits, the extensive investigations of 13 different *C. lagenarium* mutants (Bonnen and Hammerschmidt, 1989) revealed no correlation between cutinase activity and pathogenicity. The single *N. haematococca* mutant described possessed an 80 to 90% reduction in cutinase activity and reduced virulence in the sectioned pea stem assay. In general, mutants obtained after chemical or UV mutagenesis require a series of backcrosses to eliminate additional, undetected mutations. This can only be circumvented by analyzing several independent mutants.

The availability of a transformation system and the cloned cutinase gene allowed the construction of a recombinant strain where the endogenous cutinase gene was replaced by a mutated functionless copy. The ascomycete *N. haematococca* is a haploid fungus, and the isolate used in this study contains

(A) Symptoms on peas grown 20 days in soil infested with wild-type strain 77-2-3 and cutinase-deficient mutant 77-102 are shown in pot 2 and pot 3, respectively. Each pot was infested with 5×10^6 conidia. Yellowing of basal foliage and stunted growth of the above-ground plant parts were caused to the same extent by both fungi. Uninoculated control plants are shown in pot 1. Ten to 15 replicate plants were used per treatment. The experiment was repeated three times with results similar to those shown here.

(B) Detailed picture of the root and the lower stem of plants shown in **(A)**. The root and lower stem of an uninoculated control plant are shown at left. The dark brown of upper tap root and the below-ground epicotyl at center is caused by infection with the wild-type strain. The same symptoms are caused by infection with the cutinase-deficient mutant 77-102, as shown at right.

(C) Lesions on etiolated pea segments, caused by germinating conidia of wild-type strain 77-2-3 (left) and null mutant 77-102 (right).

only one copy of the cutinase gene. Every change in the genotype can directly affect the phenotype of the transgenic strain. The construction of a precise gene disruption mutant was recently shown for *Cochliobolus carbonum*, a maize pathogen. The disruption of an endopolygalacturonase gene did not lead to a different pathogenic behavior of the mutant (Scott-Craig et al., 1990). In *Magnaporthe grisea*, a rice pathogen, the only cutinase gene that is homologous to the cutinase genes of *Colletotrichum capsici* (Ettinger et al., 1987) and *N. haematococca* was destroyed by a one-step gene replacement experiment. The resultant mutants were indistinguishable from wild-type strains in pathogenicity tests on three different hosts. However, the mutants retained cutinase activity in the standard cutinase enzyme assay (Valent and Chumley, 1991). Our results do not exclude the possibility of other yet undetected cutinases, although we could not measure residual cutinolytic activity in the mutants in vitro. If present, these undetected cutinases must fulfill the following criteria: (1) they do not cross-hybridize to DNA or RNA of the cloned gene and to the 360-bp middle fragment of the gene used as a probe; (2) they are not expressed during saprophytic growth and are not induced when grown with cutin as a sole carbon source; and (3) they are only induced by yet unknown substances in the plant.

Cutinolytic enzyme activity was first described for *Penicillium spinulosum* (Heinen, 1960), a ubiquitous saprophyte common in soil, decaying vegetation, and foods (Pitt, 1979). It is known now that cutinase is produced by a variety of fungi (Kolattukudy, 1981) and bacteria (Heinen and de Vries, 1966). Until now, cutinase has been mainly discussed as a pathogenicity factor; the ecological aspect of its activity has been neglected. Because cutin degradation is a feature of many saprophytic microorganisms, the cutinolytic activities of these saprophytes are probably responsible for the degradation of cutin in the litter and the soil after leaf fall (Köller, 1991). Therefore, we propose that the cutinase of *N. haematococca* is primarily used for cuticle decomposition in plant debris. This function is in agreement with the ecology of this soil-borne facultative parasite and the plant inducibility of the cutinase gene (Woloshuk and Kolattukudy, 1986; Podila et al., 1988).

METHODS

Fungal Culture and Cutinase Gene Isolation

Nectria haematococca MP VI isolates were obtained from H. D. VanEtten (University of Arizona, Tucson) and maintained on V8 juice agar plates (200 mL of V8 juice, 3 g of $CaCO_3$, 25 g of agar per liter) at 23°C. Saprophytic growth on cutin was performed as described by Kolattukudy et al. (1981) with slight modifications. Mineral medium (10 mL) supplemented with 50 mg of apple cutin was inoculated with 1×10^6 spores of each fungal isolate, grown on V8 juice agar, and incubated at 23°C.

Preparation of fungal genomic DNA was done as described by Garber and Yoder (1983). Two oligonucleotides, a 25-mer (TTATGCCCGAGGTTCAACAGAGACG) and a 24-mer (TGCAGCAACGATCAAGCTACCAGT), were synthesized and corresponded to nucleotide positions 1122 to 1146 and 1598 to 1621, respectively, of the cutinase gene of *N. haematococca* isolate T8 (Soliday et al., 1989). The oligonucleotides (40 nM) were used as primers in the PCR with 1 μg of genomic DNA isolate T8. The reaction was carried out in a DNA Thermal Cycler using reagents from GeneAmp PCR Reagent Kit (Perkin-Elmer Cetus, Norwalk, CT). Amplification was performed for 40 cycles of 1 min at 92°C, 1 min at 37°C, and 6 min at 70°C. The amplified 500-bp PCR product was ligated into the SmaI-digested phagemid vector pT7T318U using standard procedures (Sambrook et al., 1989).

Transformation-Mediated Gene Disruption

For the gene replacement, the vector pDC6 was constructed. An internal 360-bp Eco47III-PpuMI fragment of the cutinase gene was deleted from plasmid p8C77-1, and a 4.0-kb BglII-HindIII fragment of vector pAN7-1 (Punt et al., 1987) was blunt-end ligated into linearized p8C77 to create pDC6.

Transformation of *N. haematococca* was done according to Yelton et al. (1984) with some modifications. Strain 77-2-3 was cultivated for 2 to 3 weeks on V8 juice agar plates to produce conidia. Conidia were suspended in water, separated from mycelium by passage through two layers of cheesecloth, and transferred into a 2000-mL flask containing 100 mL of a glucose-asparagine medium (VanEtten and Stein, 1978). The flask was shaken vigorously (200 rpm) at 28°C, and the mycelium was harvested after 12 to 15 hr. Mycelium was resuspended in 25 mL of STC high osmolarity medium (1.2 M sorbitol, 50 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.0) containing 1% Novozym 234 (Novo Industries, Copenhagen, Denmark) and gently agitated at 28°C for 2 hr. Protoplasts were filtered through two layers of cheesecloth and one layer of 20-μm Nybolt membrane, mixed with one volume of ST buffer (0.6 M sorbitol, 100 mM Tris-HCl, pH 7.0), and pelleted by centrifugation. Protoplasts were washed two times in STC and resuspended in STC containing 2 mM aurintricarboxylic acid. Circular plasmid DNA (20 to 30 μg) was mixed with 10^7 protoplasts (100 μL), and the suspension was treated with two volumes of polyethylene glycol (60% PEG 4000, 10 mM Tris-HCl, pH 7.0, 50 mM $CaCl_2$). Aliquots of transformed protoplasts were plated in 10 mL of regeneration agar (20% sucrose, 0.1% yeast extract, 0.1% casein enzymatic hydrolysate, and 1.6% agar). Regeneration plates were incubated at 28°C and overlaid after 18 hr with 10 mL of 1% agar containing hygromycin B to yield a final concentration of 50 μg/mL. Routinely, 20 transformants were obtained per 10^7 protoplasts. Transformants were purified by propagating colonies from single isolated conidia.

RNA Gel Blot Analysis

N. haematococca strains were cultivated in 100 mL of mineral medium (VanEtten and Stein, 1978) in a 1000-mL flask containing either 1% apple skin or 1% glucose as carbon source. Cultures were shaken at 28°C, and mycelium was harvested after 10 days, lyophilized, and ground to powder. Total cellular RNA was isolated from ground mycelium by the method of Logemann et al. (1987). RNA was glyoxylated, fractionated by gel electrophoresis, and transferred to a nylon membrane (GeneScreen Plus, Du Pont) as described by Williams and Mason (1985). The filter was hybridized with a radiolabeled internal fragment of the cutinase gene. An RNA ladder (Gibco–Bethesda Research Laboratories) was used as the size standard.

Enzyme Assays

To identify cutinase-deficient mutants, a plate assay was designed to determine extracellular esterase activity. As cutinase is secreted extracellularly, cutinase-lacking transformants can be detected when overlaid with the esterase substrate *para*-nitrophenyl butyrate. Plugs of mycelium were placed onto plates containing induction medium (glucose-free asparagine medium, 1% sodium acetate, 0.1% 16-hydroxyhexadecanoic acid, 1.6% agar) and incubated at 28°C. After 3 days, 2-cm-diameter colonies had formed. The plates were overlaid with staining agar (50 mM potassium phosphate, pH 8.0, 0.5 mM *para*-nitrophenyl butyrate, 1% agar). Only transformants with an intact cutinase gene showed a large, yellow halo around the fungal colony. Colonies without this halo had lost the ability to secrete the *para*-nitrophenyl butyrate hydrolyzing activity and were analyzed further.

General esterase activity of fungal isolates grown on cutin was measured spectrophotometrically at 405 nm using *para*-nitrophenyl butyrate as described by Kolattukudy et al. (1981). Cutinase activity was assayed with tritiated apple cutin (^{3}H-cutin) according to Köller et al. (1982a). Cutin was radiolabeled by the tritium labeling service of Amersham International (London).

Treatment of culture fluid with ^{14}C-diisopropyl fluorophosphate was a modification of a published procedure of Köller et al. (1982a). In brief, 80 μL of culture filtrate of cutin-grown fungi was incubated with 10 μL of 1,3-^{14}C-diisopropyl fluorophosphate (2.0 μCi) and 10 μL of 0.2 M sodium phosphate, pH 7.5, for 3 hr at 23°C. The proteins were separated on 15% SDS–polyacrylamide gels, using ^{14}C-labeled Rainbow proteins (Amersham International) as molecular mass standards and identified by fluorography.

Pathogenicity Tests

Virulence of the strains was assessed using a pot assay described by VanEtten (1978) with slight modifications. Pea seeds (cultivar Alaska 2B, Asgrow Seed Co., Kalamazoo, MI) were surfaced sterilized with 3% sodium hypochlorite and grown in steamed soil contained in plastic pots (12 × 12 cm). Soil was infested with 40 mL of conidial suspension (1.25×10^5 per mL), prepared from 10- to 14-day-old cultures grown on V8 juice agar, immediately after the pea seeds had been planted. Plants were incubated in an environmental growth chamber with a 16-hr photoperiod and 28°C day/23°C night temperatures.

Pea stem bioassays were done as described by Köller et al. (1982a). Pea seedlings (cultivar Alaska 2B) were grown for 6 to 9 days in the dark in a growth chamber at 23 to 25°C. Sections of the hypocotyl (2 to 3 cm) were washed with distilled water, placed on moist filter paper in Petri dishes, and inoculated with 5-μL droplets of conidial suspension (10^8 per mL) in water. Droplets were carefully placed on the surface of the segments, and inoculated segments were incubated at 23 to 25°C in the dark. Virulence was evaluated 3 to 4 days after inoculation.

Received March 5, 1992; accepted April 1, 1992.

REFERENCES

Bonnen, A.M., and Hammerschmidt, R. (1989). Role of cutinolytic enzymes in infection of cucumber by *Colletotrichum lagenarium*. Physiol. Mol. Plant Pathol. **35,** 475–481.

Cohen, J.A., Oosterbaan, R.A., and Berends, F. (1967). Organophosphorus compounds. Methods Enzymol. **11,** 686–702.

Dantzig, A.H., Zuckerman, S.H., and Andonov-Roland, M.M. (1986). Isolation of a *Fusarium solani* mutant reduced in cutinase activity and virulence. J. Bacteriol. **168,** 911–916.

Dickman, M.B., and Patil, S.S. (1986). Cutinase deficient mutants of *Colletotrichum gloeosporioides* are nonpathogenic to papaya fruit. Physiol. Mol. Plant Pathol. **28,** 235–242.

Dickman, M.B., Podila, G.K., and Kolattukudy, P.E. (1989). Insertion of cutinase gene into a wound pathogen enables it to infect intact host. Nature **342,** 446–448.

Ettinger, W.F., Thukral, S.K., and Kolattukudy, P.E. (1987). Structure of cutinase gene, cDNA and the derived amino acid sequence from phytopathogenic fungi. Biochemistry **26,** 7883–7892.

Garber, R.C., and Yoder, O.C. (1983). Isolation of DNA from filamentous fungi and separation into nuclear, mitochondrial, ribosomal, and plasmid components. Anal. Biochem. **135,** 416–422.

Heinen, W. (1960). Über den enzymatischen Cutin Abbau. I. Mitteilung: Nachweis eines "cutinase systems." Acta Bot. Neerl. **9,** 167–190.

Heinen, W., and de Vries, H. (1966). Stages during the breakdown of plant cutin by soil microorganisms. Arch. Microbiol. **54,** 331–338.

Kolattukudy, P.E., Purdy, R.E., and Maiti, I.B. (1981). Cutinases from fungi and pollen. Methods Enzymol. **71,** 652–664.

Köller, W. (1991). Plant cuticles: The first barriers to be overcome by plant pathogens. In The Fungal Spore and Disease Initiation in Plants and Animals, G.T. Cole and H.C. Hoch, eds (New York: Plenum), pp. 219–246.

Köller, W., Allan, C.R., and Kolattukudy, P.E. (1982a). Role of cutinase and cell wall degrading enzymes in infection of *Pisum sativum* by *Fusarium solani* f. sp. *pisi*. Physiol. Plant Pathol. **20,** 47–60.

Köller, W., Allan, C.R., and Kolattukudy, P.E. (1982b). Protection of *Pisum sativum* from *Fusarium solani* f. sp. *pisi* by inhibition of cutinase with organophosphorus pesticides. Phytopathology **72,** 1425–1430.

Kraft, J.M., and Roberts, D.D. (1969). Influence of soil water and temperature on the pea root rot complex caused by *Pythium ultimum* and *Fusarium solani* f. sp. *pisi*. Phytopathology **59,** 149–152.

Kraft, J.M., Burke, D.M., and Haglund, W.A. (1981). *Fusarium* diseases of beans, peas, and lentils. In *Fusarium*: Diseases, Biology, and Taxonomy, P.E. Nelson, T.A. Toussoun, and R.J. Cook, eds (University Park, PA: The Pennsylvania State University Press), pp. 142–156.

Kronstad, J.W., and Leong, S.A. (1989). Isolation of two alleles of the *b* locus of *Ustilago maydis*. Proc. Natl. Acad. Sci. USA **86,** 978–982.

Kronstad, J.W., and Leong, S.A. (1990). The *b* mating-type locus of *Ustilago maydis* contains variable and constant regions. Genes Dev. **4,** 1384–1395.

Lin, T.S., and Kolattukudy, P.E. (1978). Induction of a biopolyester hydrolase (cutinase) by low levels of cutin monomers in *Fusarium solani* f. sp. *pisi*. J. Bacteriol. **133,** 942–951.

Logemann, J., Schell, J., and Willmitzer, L. (1987). Improved method for the isolation of RNA from plant tissues. Anal. Biochem. **163,** 16–20.

Maiti, I.B., and Kolattukudy, P.E. (1979). Prevention of fungal infection of plants by specific inhibition of cutinase. Science **205,** 507–508.

Pitt, J.I. (1979). The Genus Penicillium and its Telemorphic States Eupenicillium and Talaromyces, J.I. Pitt, ed (New York: Academic Press), pp. 175–180.

Podila, G.K., Dickman, M.B., and Kolattukudy, P.E. (1988). Transcriptional activation of a cutinase gene in isolated fungal nuclei by plant cutin monomers. Science **242,** 922–925.

Punt, P.J., Oliver, R.P., Dingemanse, M.A., Pouwels, P.H., and van den Hondel, C.A.M.J.J. (1987). Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. Gene **56,** 117–124.

Rothstein, R.J. (1983). One-step gene disruption in yeast. Methods Enzymol. **101,** 202–211.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Schäfer, W., Straney, D., Ciuffetti, L., VanEtten, H.D., and Yoder, O.C. (1989). One enzyme makes a fungal pathogen, but not a saprophyte, virulent on a new host plant. Science **246,** 247–249.

Schulz, B., Banuett, F., Dahl, M., Schlesinger, R., Schäfer, W., Martin, T., Herskowitz, I., and Kahmann, R. (1990). The *b* alleles of *U. maydis*, whose combinations program pathogenic development, code for polypeptides containing a homeodomain-related motif. Cell **60,** 295–306.

Scott-Craig, J.S., Panaccione, D.G., Cervone, F., and Walton, J.D. (1990). Endopolygalacturonase is not required for pathogenicity of *Cochliobolus carbonum* on maize. Plant Cell **2,** 1191–1200.

Shaykh, M., Soliday, C., and Kolattukudy, P.E. (1977). Proof for the production of cutinase by *Fusarium solani* f. *pisi* during penetration into its host, *Pisum sativum*. Plant Physiol. **60,** 170–172.

Soliday, C.L., Dickman, M.B., and Kolattukudy, P.E. (1989). Structure of the cutinase gene and detection of promoter activity in the 5′-flanking region by fungal transformation. J. Bacteriol. **171,** 1942–1951.

Valent, B., and Chumley, F.G. (1991). Molecular genetic analysis of the rice blast fungus, *Magnaporthe grisea*. Annu. Rev. Phytopathol. **29,** 443–467.

VanEtten, H.D. (1978). Identification of additional habitats of *Nectria haematococca* mating population VI. Phytopathology **68,** 1552–1556.

VanEtten, H.D., and Stein, J.I. (1978). Differential response of *Fusarium solani* isolates to pisatin and phaseollin. Phytopathology **68,** 1276–1283.

Weltring, K.M., Turgeon, B.G., Yoder, O.C., and VanEtten, H.D. (1988). Isolation of a phytoalexin-detoxification gene from the plant pathogenic fungus *Nectria haematococca* by detecting its expression in *Aspergillus nidulans*. Gene **68,** 335–344.

Williams, J.G., and Mason, P.J. (1985). Hybridisation in the analysis of RNA. In Nucleic Acid Hybridisation, B.D. Hames and S.J. Higgins, eds (Oxford: IRL), pp. 139–160.

Woloshuk, C.P., and Kollattukudy, P.E. (1986). Mechanism by which contact with plant cuticle triggers cutinase gene expression in the spores of *Fusarium solani* f. sp. *pisi*. Proc. Natl. Acad. Sci. USA **83,** 1704–1708.

Yelton, M.M., Hamer, J.E., and Timberlake, W.E. (1984). Transformation of *Aspergillus nidulans* by using a *trp*C plasmid. Proc. Natl. Acad. Sci. USA **81,** 1470–1474.