Variation in Cutinase from Two Populations of *Colletotrichum gloeosporioides* from Citrus

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ABSTRACT

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Strains belonging to two distinct genetic populations of *Colletotrichum gloeosporioides* from citrus all secreted esterases with cutinolytic activity when induced with cutin monomers. The molecular weights of the two major serine esterases detected after active-site labeling with ^{3}H-di-isofluorophosphate were correlated with the genetic population of the strain. One population of strains, SGO (slow growing, orange-colored), secreted 26- and 19-kDa esterases, whereas the second population of strains, FGG (fast growing, gray-colored), secreted 24- and 21-kDa esterases. A DNA probe containing the cutinase gene from an isolate of *C. gloeosporioides* from papaya hybridized strongly to DNA from FGG strains but hybridized poorly or not at all to SGO strains. Thus, distinct cutinase genes may be present in the two types of *C. gloeosporioides* from citrus.

Cutinolytic enzymes have been purified and characterized from various plant pathogens (5,6,9), including *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz. The single cutinase isozyme produced by a strain of *C. gloeosporioides* isolated from papaya fruit had a molecular weight of 24,000 (3). Comparison of the DNA sequences for this cutinase gene and a gene isolated from a strain of *Nectria haematococca* (*Fusarium solani* f. sp. *pisi*), however, revealed considerable dissimilarity. Although both cutinase genes shared homologous regions critical for activity and structural integrity, only 43% of the amino acids were directly conserved (4). Differences in cutinase DNA sequences seem to exist even among *Colletotrichum* species (4,7). A cDNA clone of the cutinase gene from a strain of *Colletotrichum capsici* hybridized with genomic DNA from representative strains of *C. graminicola, C. lindemutheanum,* and *C. gloeosporioides* but not with DNA from strains of *C. orbiculare* (syn. *C. lagenarium*) or *C. coccodes.*

Although not extensively investigated, cutinase diversity also may be reflected in enzymatic parameters. For example, cutinolytic activity of esterases purified from *N. haematococca* (14) and *Fusarium roseum culmorum* (18) was highest under alkaline con-

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ditions (pH 10), whereas an optimum of pH 6.5 was determined for the enzyme derived from *Venturia inaequalis* (10). These differences in enzymatic properties may be involved in the tissue specificity of pathogens (23). Substrate differences also have been reported for the cutinases produced by strains of *N. haematococca* and *C. gloeosporioides*: Only the enzyme from the latter accepted palmitate as a model substrate, and the specific esterase activity was substantially lower with both *p*-nitrophenyl butyrate and polymeric cutin (3).

Thus, although cutinase diversity has been examined for various strains within the genus *Colletotrichum* and among representatives of other fungal species, the degree of variation within a single fungal species has not been well documented. The objective of this investigation was to determine the degree of variation in cutinase within and between populations of the species *C. gloeosporioides*. Two distinct populations of *C. gloeosporioides* isolated from citrus have been described recently (13) based on a variety of morphological, physiological, and genetic criteria. We have determined that population differences are reflected in cutinase isozymes and characteristics of the cutinase genes.

MATERIALS AND METHODS

Strains. Strains of *C. gloeosporioides* included in this study represent RFLP-defined (restriction fragment length polymorphism) SGO (slow growing, orange-colored) and FGG (fast growing, gray-colored) populations (13). The strain numbers, geographic origins, and hosts are listed in Table 1. All strains used in this study were derived from single spores. Fungal DNA was extracted according to a method previously described by Liyanage et al (13). Restriction enzymes were purchased from either New England BioLabs (Beverly, MA) or Bethesda Research Laboratories (Gaithersburg, MD); digestions were conducted at 37 C for 6–12 h, according to the manufacturers' recommendations.

Southern hybridization. A clone of the cutinase gene from a *C. gloeosporioides* isolate from papaya was obtained from Martin Dickman (Department of Plant Pathology, University of Nebraska). Clone pU5-11 contains the entire cutinase coding sequence and flanking DNA on a 2.2-kb *Sph*I fragment (4). Agarose gel electrophoresis, DNA transfer to nylon membranes, and Southern hybridization (19) using ^{32}P-labeled probes were carried out according to methods described by Sambrook et al (17). High stringency hybridization and washing of blots were conducted at 68 C. First and second washes were in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1× SSC, respectively, with 0.1% SDS (sodium dodecyl sulfate). Low-stringency hybridization was performed at 65 C; first and second washes with 2× SSC and 0.1% SDS were performed at 55 C. Conditions for pulsed-field electrophoresis were described previously (13).

Induction and identification of cutinolytic esterases. Induction of cutinase expression with cutin hydrolysate, enzyme assays, protein determination, active-site labeling of serine esterases with tritium-labeled di-isopropyl fluorophosphate (^{3}H-DFP), and electrophoretic separation of labeled esterases was carried out according to procedures described elsewhere (10,11). In brief, mycelium from nine colonies (each 15 mm in diameter) of each strain grown on potato-dextrose agar was homogenized in a Waring blender with 20 ml of water. The mycelial suspension (1 ml) was transferred to roux bottles containing 100 ml of minimal medium. Under inductive conditions, cutin hydrolysate (0.25 mg per milliliter) was added to the medium. After incubation at 24 C for 2 wk, fungal mycelium was removed by filtration. Esterase activity was assayed with *p*-nitrophenyl butyrate (PNB) and *p*-nitrophenyl palmitate (PNP) as substrates (15); cutinase activity was tested with tritiated grapefruit cutin (10).

TABLE 1. Isolates of *Colletotrichum gloeosporioides* isolated from citrus[a]

Strain	Host	Location and year of isolation
SGO (slowly growing, orange) isolates		
H-1	Tahiti lime	Immokalee, FL 1989
H-3	Tahiti lime	Immokalee, FL 1989
H-9	Tahiti lime	Homestead, FL 1988
H-25B	Tahiti lime	Homestead, FL 1988
H-36[b]	Sweet orange	Ft. Pierce, FL 1989
IMB-3[c]	Sweet orange	Immokalee, FL 1990
LP-1[c]	Sweet orange	Lake Placid, FL 1990
Maran[c]	Sweet orange	Indiantown, FL 1990
OCO[c]	Sweet orange	Arcadia, FL 1990
FGG (fast growing, gray) isolates		
H-4	Tahiti lime	Immokalee, FL 1989
H-12	Tahiti lime	Homestead, FL 1988
H-46[b]	Sweet orange	Vera Cruz, Mexico 1989
H-48[b]	Sweet orange	Vera Cruz, Mexico 1989
180269[d]	Sweet orange	Belize
226802[d]	Sweet orange	Belize

[a] All strains were isolated from diseased tissue and tested for their ability to blight citrus petals, thus reproducing the symptoms of citrus postbloom fruit drop disease.

[b] Strains were provided by R. Sonoda, Agricultural Research and Education Center, University of Florida, Fort Pierce.

[c] Strains were provided by L. W. Timmer, Citrus Research and Educational Center, University of Florida, Lake Alfred.

[d] Strains were obtained from Commonwealth Institute of Mycology, Kew, London, U.K. (provided by R. Sonoda).

All other strains were obtained from the culture collection at Tropical Research and Education Center, University of Florida, Homestead.

RESULTS

Cutin monomers act as specific inducers of cutinase gene expression in *N. haematococca* (1,8,12), *V. inaequalis* (11), and *C. capsici* (4). A cAMP-responsive element within the 5′ flanking region of the cutinase gene of *N. haematococca*, involved in cutin monomer-induced expression and catabolite repression, was also identified in the 5′ region of the cutinase genes of *C. capsici* and a papaya isolate of *C. gloeosporioides* (1,8). In agreement with these regulatory similarities, esterase and cutinase activity were cutin-monomer induced in all citrus strains of *C. gloeosporioides* tested (Table 2). The extracellular esterase activity in the absence of cutin hydrolysate never exceeded 5% of the levels found under inductive conditions. Differences among strains were apparent with regard to substrate specificities of secreted esterases. Both model substrates were hydrolyzed, but the ratio between PNBase and PNPase activities ranged from 18.6 to 4.4 (Table 2). A similar variation was observed for the pH dependency of cutinolytic activities. Although cutinases were consistently more active at pH 6.0 than at pH 9.5, the ratios of activities ranged from 30.8 to 1.6 (Table 2).

Neither the absolute amounts of esterase and cutinolytic activities nor the relative rates of model ester and cutin hydrolysis were correlated with the population of the strains. A clear correlation with population became apparent, however, when extracellular proteins were labeled with ^{3}H-DFP, used as an active-site probe for serine esterases (Fig. 1). The molecular weight of these proteins differed among strains but was correlated to *C. gloeosprodioides* RFLP-defined populations (13). All SGO strains contained labeled proteins of 26 and 19 kDa, whereas all FGG strains contained labeled proteins of 24 and 21 kDa. These molecular weights are within the range of known fungal cutinases (5,6,9,22). An additional esterase with a molecular weight of approximately 70,000, which was not reported for the papaya strains of *C. gloeosporioides* (3), was present in all strains. The molecular weight of this esterase was slightly larger for SGO strains. The relative contribution of the high molecular-weight esterase to the total esterase and cutinase activities remains unknown. However, the enzyme might be similar to the 60-kDa alkaline, cutinolytic esterase isolated for *C. lagenarium* (2) or the 54-kDa nonspecific esterase of *N. haemotococca* (15).

The genomic clone pU5-11, containing the entire cutinase gene, was ^{32}P-labeled and used to probe *Sph*I-digested DNA from *C. gloeosporioides* strains from citrus. The probe hybridized to a 2.2-kb *Sph*I fragment only in FGG strains (Fig. 2). DNA from

TABLE 2. Enzymatic activities of extracellular esterases produced by isolates of *Colletotrichum gloeosporioides* in the presence of cutin hydrolysate

Isolate	Esterase[a]			Cutinase[b]		
	PNB (μkat mg^{-1})	PNP (μkat mg^{-1})	Ratio PNB/PNP	(kBq h^{-1} mg^{-1}) pH 6.0	pH 9.5	Ratio pH 6.0/pH 9.5
SGO (slow growing, orange) isolates						
H-1	4.73	0.26	18.6	18.7	2.0	9.4
H-3	3.68	0.30	12.2	23.9	4.5	5.3
H-9	2.19	0.33	6.7	18.8	4.2	4.4
H-25B	3.63	0.49	7.4	27.5	5.5	5.0
H-36	1.85	0.37	5.0	23.7	5.8	4.1
IMB-3	6.48	0.35	18.5	25.4	2.8	9.1
LP-1	5.84	0.50	11.7	28.6	8.3	3.4
Maran	3.58	0.48	7.4	23.1	10.3	2.2
OCO	1.46	0.33	4.4	12.8	7.9	1.6
FGG (fast growing, gray) isolates						
H-4	8.78	0.75	11.7	57.5	8.0	7.2
H-12	4.69	0.55	8.5	40.0	1.3	30.8
H-46	0.48	0.05	9.6	5.9	1.5	3.9
H-48	4.10	0.66	6.2	27.0	6.1	4.4
180269	4.67	0.77	6.1	28.1	4.9	5.7
226802	4.70	0.78	6.0	49.3	10.7	4.6

[a] Esterase activities were assayed with *p*-nitrophenyl butyrate (PNB) or *p*-nitrophenyl palmitate (PNP) as model substrates. Values are the mean of three measurements. Standard errors were <5%.
[b] Cutinase activity was tested with tritiated grapefruit cutin. Values are the means of three measurements. Standard errors were <15%.

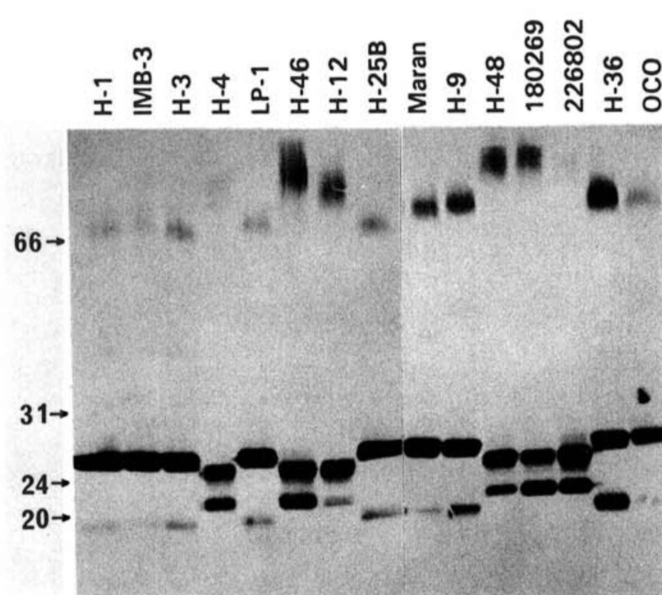

Fig. 1. Fluorography of ^{3}H-DFP labeled proteins after SDS- (sodium dodecyl sulfate) polyacrylamide gel electrophoresis of extracellular fluid from cultures of *C. gloeosporioides* grown on cutin as the sole, carbon source. Numbers at the left indicate the molecular weight of size standards in kilodaltons.

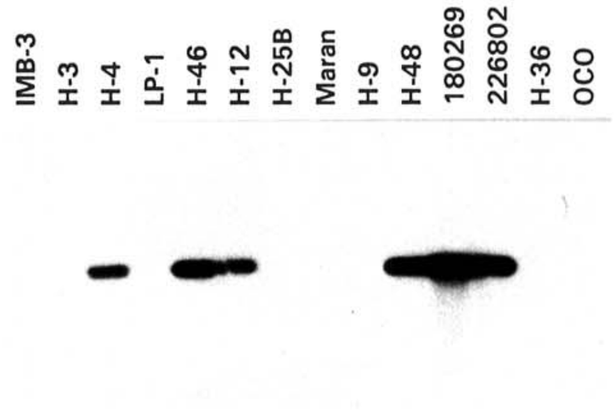

Fig. 2. Hybridization of a cutinase gene to DNA from strains of *C. gloeosporioides*. Total DNA was digested with *Sph*I and hybridized to ^{32}P-labeled clone pU5-11. Hybridization to a 2.2-kb fragment is shown.

SGO strains showed no detectable level of hybridization under high-stringency conditions. However, at less-stringent conditions, extremely weak hybridization occurred to a 7.4-kb *Hin*dIII fragment and 3–4 other *Hin*dIII fragments, ranging in size from 4.8 to 9 kb—hybridization was detected only by long exposure (>1 mo) of blots to X-ray film. The same probe was hybridized to a Southern blot of chromosome sized DNA separated by pulsed-field gel electrophoresis. The cutinase-gene probe hybridized to a 4.7 million base-pair chromosome only in FGG strains (data not shown).

DISCUSSION

All strains of *C. gloeosporioides* isolated from citrus were induced to produce cutinases in response to cutin monomers. The slightly acidic preference of cutinolytic activities corresponded to the properties of cutinases produced by pathogens that infect aerial parts of plants (10,16,23). Although all strains secreted two active-serine esterases with molecular weights in the range of fungal cutinases, the banding pattern of the two esterases was population specific. Southern hybridization of a cloned cutinase gene from *C. gloeosporioides* to genomic DNA also was population specific. Only DNA from FGG strains, which secreted esterases of 24 and 21 kDa, contained a 2.2-kb *Sph*I fragment that hybridized to the cutinase gene isolated from a papaya strain of *C. gloeosporioides*. The papaya strain was reported to produce a single 24-kDa cutinase (3) encoded on a 2.2-kb *Sph*I fragment (4).

The poor hybridization between DNA from SGO strains and the cutinase gene probe, despite the fact that the strains have abundant cutinase activity, suggests considerable evolutionary divergence among cutinase-gene sequences. Cutinase divergence within the genus *Colletotrichum* has been observed previously. A cDNA clone of cutinase from *C. capsici* hybridizes readily with the genomic DNA from *C. graminicola* and the *C. gloeosporioides* strain from papaya but not to DNA from other *Colletotrichum* species (7).

DNA from FGG strains has only one restriction fragment that hybridizes to the cutinase gene probe. Howeveer, the strains may produce two cutinase isozymes under inductive conditions. This suggests that two cutinase isozymes could arise from a single cutinase-gene product. But, considering the lack of hybridization of SGO strains and an example from *Magnaporthe grisea* (20,21), an alternative hypothesis can be tested: Are two (or more) cutinase

isozymes encoded by separate genes with little similarity in DNA sequence? Disruption of the cutinase gene, CUT1, in a strain of *M. grisea* had no effect on pathogenicity and did not abolish cutinase activity secreted in the presence of cutin. No second gene for cutinase could be found in *M. grisea* by Southern hybridization, yet a high-degree of similarity was noted between CUT1 and the cutinase gene from the papaya strain of *C. gloeosporioides*. The results indicate that distinct differences among cutinases are not only observed for different species or strains, but that distinctly different cutinase genes may exist even within a pathogen species or individual strain. Although the presence of cutinase isozymes has been reported for other pathogens (14, 15,23), their genetic relationships and any functional relevance to biological and pathogenic diversity remains unknown.

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