Effect of Disruption of a Cutinase Gene (*cutA*) on Virulence and Tissue Specificity of *Fusarium solani* f. sp. *cucurbitae* race 2 Toward *Cucurbita maxima* and *C. moschata*

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Received 25 September 1996. Accepted 17 December 1996.

A 3.9-kb genomic DNA fragment from the cucurbit pathogen *Fusarium solani* f. sp. *cucurbitae* race 2 was cloned. Sequence analysis revealed an open reading frame of 690 nucleotides interrupted by a single 51-bp intron. The nucleotide and predicted amino acid sequences showed 92 and 98% identity, respectively, to those of the *cutA* gene of the pea pathogen *F. solani* f. sp. *pisi*. A gene replacement vector was constructed and used to generate *cutA*⁻ mutants that were detected with a polymerase chain reaction (PCR) assay. Seventy-one *cutA*⁻ mutants were identified among the 416 transformants screened. Vector integration was assessed by Southern analysis in 23 of these mutants. PCR and Southern analysis data showed the level of homologous integration was 14%. Disruption of the *cutA* locus in mutants was confirmed by RNA gel blot hybridization. Neither virulence on *Cucurbita maxima* cv. Delica at any of six different inoculum concentrations, nor pathogenicity on intact fruit of four different species or cultivars of cucurbit or hypocotyl tissue of *C. maxima* cv. Crown, was found to be affected by disruption of the *cutA* gene.

Additional keyword: *Nectria haematococca*.

The plant cuticle is the first surface barrier that must be breached before phytopathogenic fungi such as *Fusarium solani* (Mart.) Sacc. (teleomorph *Nectria haematococca* Berk. & Broome) can invade and colonize their hosts. Cutin is a major component of the plant cuticle and can be hydrolyzed by the serine esterase cutinase. Genes encoding cutinase have been cloned from several fungi including *Alternaria brassicicola* (Yao and Köller 1994), *Aspergillus oryzae* (Ohnishi et al. 1995), *Colletotrichum capsici* and *C. gloeosporioides* (Ettlinger et al. 1987), *Magnaporthe grisea* (Sweigard et al. 1992a),

and *Fusarium solani* f. sp. *pisi* (Soliday et al. 1984, 1989). The gene from *F. solani* f. sp. *pisi* has been the subject of extensive study that has included determination of the three-dimensional structure of recombinant cutinase (Martinez et al. 1992), analysis of regulation (Podila et al. 1988; Bajar et al. 1991; Kamper et al. 1994), targeting of secretion (Podila et al. 1995), transformation-mediated gene disruption (Rogers et al. 1994; Stahl and Schäfer 1992; Stahl et al. 1994), and transformation-mediated genome augmentation (Dickman et al. 1989; Oeser and Yoder 1994).

Experimentation over the last two decades has suggested a crucial role for cutinase in determining pathogenicity and tissue specificity of several phytopathogenic fungi. For example, cutinase has been found at the site of penetration of pea by *F. solani* f. sp. *pisi* (Shaykh et al. 1977). Cutin monomers were found to activate transcription of cutinase in *F. solani* f. sp. *pisi* (Podila et al. 1988). Inhibition of conidial cutinase activity was found to reduce penetration and disease development for *F. solani* f. sp. *pisi* (Köller et al. 1982a; Maiti and Kolattukudy 1979) and *C. graminicola* (Pascholati et al. 1993). Cutinase-deficient mutants of *Alternaria alternata* lost the ability to cause black spot of Japanese pear (Tanabe et al. 1988) whereas those of *F. solani* f. sp. *pisi* showed reduced virulence. However, this could be restored by addition of exogenous purified cutinase (Dantzig et al. 1986; Dickman and Patil 1986; Köller et al. 1982b). The involvement of cutinase in penetration of host tissue has also been demonstrated by heterologous transformation of *Mycosphaerella* spp., a cutinase-deficient wound pathogen of papaya fruit (Dickman et al. 1989). Transformants expressing *cutA* from *F. solani* f. sp. *pisi* acquired the capacity to infect unwounded papaya fruit (Dickman et al. 1989). Further, virulence of *F. solani* f. sp. *pisi* isolates on pea was found to be positively correlated with their level of expression of cutinase, and gene disruption of cutinase in *F. solani* f. sp. *pisi* isolate 77-2-3 resulted in a decrease in virulence on pea (Rogers et al. 1994).

By contrast, evidence suggesting cutinase plays little role in pathogenesis has also been reported. For example, a poor correlation was observed between cutinase activity and pathogenicity in mutants of *C. lagenarium* (Bonnen and Hammer-schmidt 1989). Over-expression of the *F. solani* f. sp. *pisi*

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Nucleotide and/or amino acid sequence data are to be found at GenBank as accession number U63335.

cutinase gene in *Cochliobolus heterostrophus* did not result in a change in pathogenicity on corn, its natural host, or on pea (Oeser and Yoder 1994). Disruption of the *cut1* gene in *M. grisea* had no effect on pathogenicity (Sweigard et al. 1992b) nor did disruption of the *CUTAB1* gene in *A. brassicicola* (Yao and Köller 1995). However, both the *cut1*⁻ and *CUTAB1*⁻ transformants continued to secrete enzymes with “cutinase-like” activity. Disruption of cutinase did not alter infection of gerbera flowers or tomato fruit by *Botrytis cinerea* (van Kan et al. 1997). Disruption of cutinase in *F. solani* f. sp. *pisi* strain 77-2-3 resulted in no difference in pathogenicity and virulence on pea, compared with the wild-type strain (Stahl and Schäfer 1992; Stahl et al. 1994). This finding led to the suggestion that cutinase is primarily responsible for cuticle decomposition in plant debris and functions solely in a saprophytic life-cycle phase (Stahl and Schäfer 1992).

The conflicting results reported for *cutA* gene-disrupted mutants in *F. solani* f. sp. *pisi* (Rogers et al. 1994; Stahl and Schäfer 1992; Stahl et al. 1994) have meant there is no clear resolution of the question of the importance of cutinase in the pathogenicity of *N. haematococca*. However, the species is heterogeneous, being grouped into at least seven genetically distinct mating populations (MPI to MPVII; Matuo and Snyder 1973). The pea pathogen (*F. solani* f. sp. *pisi*) belongs to MPVI. It is possible that analysis of the role of cutinase in pathogenicity of members of other mating populations of *N. haematococca* may lead to a clarification of the role of this enzyme in pathogenicity in this species.

We are interested in *N. haematococca* MPI and MPV, which correspond to the race 1 and 2 phenotypes of *F. solani* f. sp. *cucurbitae* previously identified by Toussoun and Snyder (1961). These two races cause storage rots of cucurbit fruit (Hawthorne 1988), in particular fruit of buttercup squash (*Cucurbita maxima* Duchesne). Race 1 and race 2 isolates show differences in pathogenic phenotype. Race 1 isolates can induce aggressive rots on intact roots and stems. However, considerable variability in virulence on fruit has been observed between race 1 isolates on fruit (Hawthorne et al. 1994). In contrast, race 2 isolates are aggressive pathogens of intact fruit but are incapable of initiating disease on intact root or stem tissue. Penetration of cucurbit fruit by both races is direct and formation of an appressorium has not been observed. Isolates of both races cause disease on all plant parts once the cuticle has been breached (Hawthorne et al. 1994) and rots induced by both races are similar. Since cucurbit fruit, including buttercup squash, possess a thick waxy cuticle, the ability to secrete cutinase would appear advantageous in the early stages of pathogenesis of these fruit by *F. solani*. However, preliminary analyses suggest that cutinase activity in race 1 is negligible when these isolates are grown in liquid medium supplemented with cutin purified from a number of different cucurbit species or cultivars as the sole source of carbon (B. T. Hawthorne and J. Rees-George, unpublished). In contrast, secretion of cutinase by race 2 isolate PGB153 into culture medium was observed to be influenced by the origin of cutin used during the assay (B. T. Hawthorne and J. Rees-George, unpublished).

We wished to determine the contribution of cutinase to the differences in pathogenic phenotype observed between race 1 and race 2 on their cucurbit hosts. This paper describes the cloning of the cutinase gene (*cutA*) from the race 2 isolate

PGB153, the generation of transformation-mediated gene replacement *cutA*⁻ mutants, and evaluation of the pathogenicity and tissue specificity of these *cutA*⁻ mutants.

RESULTS

Characterization of the cutinase gene (*cutA*) from *F. solani* f. sp. *cucurbitae* race 2 isolate PGB153.

At low stringency, the *F. solani* f. sp. *pisi* cutinase cDNA (Soliday et al. 1984) probe hybridized to single high molecular weight fragments in *Clal*, *SstI*, *Sall*, and *XbaI* digests of PGB153 genomic DNA. This probe hybridized to a single low molecular weight fragment of approximately 4 kb in *BglII*, 1.8 kb in *EcoRV*, and 2.1 kb in *XhoI* digests of PGB153 DNA, and to two fragments in *EcoRI*, *HindIII*, and *PstI* digests of PGB153 DNA. This pattern of hybridization to genomic DNA of PGB153 indicated the presence of a single copy of a sequence with homology to the *F. solani* f. sp. *pisi* cutinase cDNA (data not shown). At low stringency, the probe hybridized nonspecifically to digested DNA of race 1 isolate BTH869 resulting in a smear. Increasing stringency resulted in loss of hybridization signal (data not shown). This suggested that race 1 isolates may lack a homologue closely related to the *cutA* gene of *F. solani* f. sp. *pisi*.

Plasmid clones exhibiting homology to the *F. solani* f. sp. *pisi* cutinase cDNA probe were obtained from the PGB153 genomic DNA *BglII* and *XhoI* genomic libraries. pXX1 contained a 2.1-kb *XhoI* genomic DNA fragment (Fig. 1A) while pBB1 contained a 3.94-kb *BglII* genomic DNA fragment (Fig. 1B). Sequence data from the 5′ and 3′ terminal regions of pXX1 and pBB1, and from subclones containing internal *HindIII* fragments, showed that pXX1 extended past the 3′ terminus of pBB1 by 33 bases but included only 235 bases upstream of the translation initiation codon. pBB1 was therefore used during further analysis.

A continuous sequence of 3,938 nucleotides was determined for both strands of the PGB153 genomic DNA insert in pBB1. A single open reading frame of 690 nucleotides was present and was predicted to code for a 230 amino acid protein (Fig. 2). The genomic DNA insert in pBB1 included 2,127 and 1,056 bp of the 5′ and 3′ flanking regions, respectively, of which positions −747 to 1472 (relative to the translation initiation codon) are presented in Figure 2. The open reading frame was interrupted by a 51-bp intron. The intron is identical in position to that in the *F. solani* f. sp. *pisi* *cutA* sequence (Soliday et al. 1989) relative to the translation initiation codon. The sequences around the 5′ (GTTCG) and 3′ (TAG) splice sites are consistent with the consensus sequences found in other filamentous fungi (Rambosek and Leach 1987; Edelmann and Staben 1994). The sequence immediately preceding the ATG translation initiation codon (CACCATG) closely resembles the consensus sequence (CA(c/a)(c/a)ATG) found in *Neurospora crassa* (Edelmann and Staben 1994). The genomic DNA sequence was 92% identical to that from *F. solani* f. sp. *pisi* (Soliday et al. 1989). The 5′ noncoding region of the cucurbit pathogen sequence differs from the pea pathogen sequence in a number of positions. The most striking difference occurs at positions −82 to −72, where an 11-bp insertion (5′-ACATACATTCA-3′) is present in the PGB153 sequence with respect to the *F. solani* f. sp. *pisi* *cutA* gene. These 11 bp form the first part of an imperfect 11-bp direct

repeat (5'-ACATACATTCAACATACCTTCA-3'). Each 11-bp unit of the repeat has an internal, imperfect, inverted, subrepeat structure. The function of this sequence has not been ascertained.

The concentration of purified cutin in the mineral medium used for induction of cutinase was found to significantly affect both nonspecific esterase and cutinase activity in culture filtrates of PGB153 (Fig. 3). Activity increased with increasing cutin concentration up to approximately 0.5 g of cutin per liter in the culture medium (Fig. 3). Consequently, a 10-fold higher cutin concentration (5 g/liter) was used in mineral medium during cloning of the *cutA* cDNA to ensure a high level of ex-

pression of *cutA*. The sequence of the *cutA* cDNA was identical to that of exons 1 and 2 of the genomic clone, confirming the position of the intron in the genomic clone and showing that no errors were introduced during polymerase chain reaction (PCR)-based cloning of the molecule. The predicted amino acid sequence of the *F. solani* f. sp. *cucurbitae* gene showed 98% identity to that from *F. solani* f. sp. *pisi* (Fig. 4). The predicted amino acid sequence of the cucurbit pathogen *cutA* gene suggests one amino acid replacement in the signal peptide and three amino acid replacements in the mature protein relative to the pea pathogen sequence. Superposition onto the three-dimensional structure of cutinase (Martinez et al. 1992) reveals that all three polymorphisms in the mature protein occur on external loops of the protein and are thus unlikely to affect substrate specificity or catalytic activity.

Selection of *cutA*⁻ gene-disruption transformants.

Transformation of PGB153 with either the *Sma*I or the *Eco*RV replacement vectors (Fig. 1C) resulted in transformants that grew to visible size after 2 to 4 days. This is consistent with previous observations regarding transformation of this fungus (Crowhurst et al. 1992). Four hundred transformants resulting from integration of the *Sma*I replacement vector and 20 transformants resulting from integration of the *Eco*RV replacement vector were selected at random and transferred to selection medium plates. Two colony types could be distinguished among the transformants. These types included transformants with colony morphology and coloration identical to wild-type PGB153 and transformants exhibiting an increase in aerial growth resulting in a "fluffy white" appearance to the colony. Tufts of a similar growth-type are commonly seen on potato dextrose agar (PDA) cultures of PGB153 older than 14 to 21 days.

Disruption of *cutA* was expected to result from a double recombination event between the cutinase DNA sequences flanking the hygromycin resistance cassette on the integrating linear vector DNA and the corresponding homologous regions of the PGB153 genome (Fig. 1C). The PCR-based assay to detect homologous integration of vector DNA at the *cutA* locus was performed on conidial suspensions from 416 of the 420 hygromycin-resistant PGB153 transformants, irrespective of colony morphology type. The remaining four transformants failed to grow following subculture. A 3.1-kb PCR product was expected for the wild-type locus when amplified with the primers employed. A 5.3-kb PCR product was expected for the disrupted *cutA* locus with the gene replacement strategy employed herein. The PCR assay was not designed to provide information on ectopic integration events. Typically, amplification profiles contained one to 11 fragments (Fig. 5). The majority of these PCR products were common to all transformants and most probably resulted from primer binding at non-homologous sites due to use of a suboptimal annealing temperature.

The PCR product profiles could be subdivided into three groups. A 3.1-kb but not a 5.3-kb PCR product was present in profiles of 111 transformants resulting from integration of the *Sma*I replacement vector and 11 transformants resulting from integration of the *Eco*RV replacement vector. These transformants therefore contained a wild-type *cutA* locus as well as at least one ectopic integration of the appropriate gene replacement vector. These transformants were discarded. A 5.3-kb

Fig. 1. (A) Restriction map of the 2.1-kb genomic DNA fragment in plasmid clone pXX1. (B) Restriction map and nucleotide sequencing strategy of the 3.94-kb *Bgl*II genomic DNA fragment in clone pBB1 and the PGB153 *cutA* cDNA clone. Stippled box indicates position of the *cutA* gene in the clone; solid box indicates position of the single intron. (C) Single-step gene replacement strategy used to disrupt the *cutA* locus. Positions of primers RC31 and RC40 used during the polymerase chain reaction screening procedure are indicated. Double-crossing-over events that must occur in order to disrupt the *cutA* locus are indicated. Primer RC40 resides 656 bp upstream of the 5' *Sma*I site; primer RC31 resides 86 bp upstream of the 3' *Sma*I site. Graphic representations of *cutA* genomic DNA locus and gene replacement vector are not drawn to same scale. Restriction endonuclease abbreviations: A = *Aat*II; Bm = *Bam*HI; B = *Bgl*II; E = *Eco*RV; H = *Hind*III; P = *Pst*I; S = *Sma*I; and X = *Xho*I.

-747	TGTTTCTCTAGGTGAGGTAGGTAGGTTGACCCACCCATGATGAGCGGCAGGGTCAGGTAG	-688
-687	GGCGGTAGATATCCGAGTCGCTGATTTGCGGATGAACGAGTCCAAGCTTGGCATAATCTG	-628
-627	TTGATCTCAAAACCTCCAATCACCATAAAGAGGCACCCGTAAAAGCTCCTGATGCCCTCT	-568
-567	CCACCATCAGGTAGGTGGTGTCTATGCGCGCTCTGACAATCTTCGCAAGGTATGACAGAA	-508
-507	TAGGCATGGTGGTTTTTCAGGGTTTCATCTCTAAAACCAAACCCACGCTTGTCAAAGTG	-448
-447	ACAGCTGAACAGCCGATTTTCGCTGATTGGGCTCTTTCATGTTTGCGGGAACGTTCCAT	-388
-387	CCTACCGGTTTAGCGATCGGCGCGGGTTGCAACTAGAAGCGGAAGACCTTGCGGGGAGGG	-328
-327	GCACGGAGTGGTTTCTGAAGCGACCGGGTTGCCTGAACTATCACGACTCATAGCCTCG	-268
-267	AGCGCATGGTCTAGTATCAAGAGTTTTCACGCTCCTTTGATCAAAACCGCCCTCTCTGA	-208
-207	CGCTAGAAACCGAGGAAATGGATCGCGAGCCGAGGCTCGATTTTCAGAGCTTGACGATGA	-148
-147	TAGTTTCATCTGTTCAAGCTTAAATATCGTCGAGCCAGACCACTGGGACGAAACCAGAC	-88
-87	AACAAC <u>ACATACATTCAACATACCTTC</u> ACTTCATCAACATTCACTTCTATTCTTCACCGC	-28
-27	GTCCTTTTCGCTCTTTACCATCTTCACCATTGAAGTTCTTCGCTCTCACCACATTTCTCGC	32
	MetLysPhePheAlaLeuThrThrPheLeuAl	
33	CGCCACGGCTTCGGCTCTGCCTACCTCTAACCTGCTCAGGAGCTTGAGGCGCGTCAGCT	92
	aAlaThrAlaSerAlaLeuProThrSerAsnProAlaGlnGluLeuGluAlaArgGlnLe	
93	TGGTAGAACAACTCGCGACGATCTGATCAACGGCAACAGCGCTTCCTGCGCCGATGTCAT	152
	uGlyArgThrThrArgAspAspLeuIleAsnGlyAsnSerAlaSerCysAlaAspValIl	
153	CTTCATTTATGCCCAGGTTCAACAGAGACGGGCAACTTGGTTCGTAGAATTTGTTCTGA	212
	ePheIleTyrAlaArgGlySerThrGluThrGlyAsnLeu	
213	TTACAACACCACCTTCTTACACATACATTAGGGAACACTCGGTCCGAGCATCGCCTCCAA	272
	GlyThrLeuGlyProSerIleAlaSerAs	
273	CCTTGAGTCCGCCTTCGGAACGGACGGTGTCTGGATCCAGGGCGTTGGCGGTGCCTACCG	332
	nLeuGluSerAlaPheGlyThrAspGlyValTrpIleGlnGlyValGlyGlyAlaTyrAr	
333	AGCCACTCTTGGGGACAACGCTCTGCCCCGCGGAACCTCTAGCGCCGCCATCCGGGAGAT	392
	gAlaThrLeuGlyAspAsnAlaLeuProArgGlyThrSerSerAlaAlaIleArgGluMe	
393	GCTCGGTCTCTTCCAGCAGGCCAACACCAAGTGCCCTGACGCGACTCTGATCGCCGGTGG	452
	tLeuGlyLeuPheGlnGlnAlaAsnThrLysCysProAspAlaThrLeuIleAlaGlyGl	
453	CTACAGCCAGGTTGCTGCACCTTGACGCGCCTCCATCGAGGACCTTGACTCGGCTATTTCG	512
	yTyrSerGlnGlyAlaAlaLeuAlaAlaAlaSerIleGluAspLeuAspSerAlaIleAr	
513	CGACAAGATCGCCGGAACCTGTTCTGTTTGGCTACACCAAGAACCTGCAGAACCGTGGCCG	572
	gAspLysIleAlaGlyThrValLeuPheGlyTyrThrLysAsnLeuGlnAsnArgGlyAr	
573	AATTCCTCAACTACCTGCTGACAGGACCAAGGTCTTCTGCAACGTGGGCGATCTCGTTTG	632
	gIleProAsnTyrProAlaAspArgThrLysValPheCysAsnValGlyAspLeuValCy	
633	CACCGGTAGCTTGATCGTTGCTGCACCTCACTTGGCCTATGGTCTGATGCTCGTGGCCC	692
	sThrGlySerLeuIleValAlaAlaProHisLeuAlaTyrGlyProAspAlaArgGlyPr	
693	TGCCCCTGAGTTCCTCATCGAGAAGGTTCCGGCTGTCCGTGGTTCTGCTTGAGGAGGATG	752
	oAlaProGluPheLeuIleGluLysValArgAlaValArgGlySerAla***	
753	AGAATTTTAACAGGCGGGCCTGTAAATCATAACGATGTTTCAAGTTTTTCTTTTGTGGAA	812
813	TAGCCATGATGGATTGGTTCAACACTCAATCTACTACAATGCCCATGTTTCAACTTAAAG	872
873	AAGTTATGAATGGTGATCTACACATCGCTTTGTCCAAGAAATCTCAACCAAGCTTCCATA	932
933	CCCTCAGCCAGTCGAGTACAAATTCGTTGCCCTCGTTTGAATCTTGCCAGGAAAGGCTG	992
993	ATAGACAAATCATGGCCTTTGACAGACGGGCACTTACCTGCACGAATGGACCCGCGAG	1052
1053	TGGGCGCTCAAGGCAAAGTTCGCCTGATTTATAGACCACCTCTCATCCCCATCATGCACA	1112
1113	TCCGTCCTGACTCGATTTCTCCATCAATAACACCGAGATTGGTTACAATCCAGGGATAT	1172
1173	CTCGCGATACCTTTTGTCTGATCTCCGTGATACTCCTGCCAATCATGCACTAGCTTCAT	1232
1233	CAAGCCAACAATGTTGTTTTTCAGCCGGCATTCAGCCTTCCTCGATATCCCCACGGGC	1292
1293	GATCCTTGATGCTGACCATATCTCCTTCTCAAGATCATGGACAGGTTGGCTTTCCACGTT	1352
1353	GTTGGCCCGGGCTGTGGCCAAATATCCGCAACCAGGTCGGAGTCAAACGTGTGGTGGATG	1412
1413	GTCGACACGCAGTTCTGCACCTTCTCTGGGCATCAGCTGCATTGTCTTTGTTAGGTAC	1472

Fig. 2. Nucleotide sequence and predicted amino-acid sequence (3 letter code) of the genomic DNA insert in clone pBB1 containing a cutinase-encoding gene from *Fusarium solani* f. sp. *cucurbitae* isolate PGB153. The 11-bp direct repeat units in the promoter region are italicized and underlined. The 11-bp repeat unit not present in the *F. solani* f. sp. *pisi* gene sequence is in bold type.

but not a 3.1-kb PCR product was present in profiles of 70 *Sma*I and one *Eco*RV replacement vector transformants, indicating that homologous integration had occurred in these transformants. The remainder of the transformants possessed product profiles including both 3.1- and 5.3-kb products. PCR analysis of five single-spore progeny from each of 17 transformants of this type consistently resulted in both 3.1- and 5.3-kb products (e.g., see Figure 5D). Consequently, this type of transformant was also discarded.

Single-spore progeny were produced for 23 transformants possessing the desired PCR product pattern. Re-screening of three to five single-spore progeny for each of these transformants showed that all progeny produced PCR profiles containing a 5.3-kb fragment (e.g., see Figure 5C). One single-spore progeny of each of these transformants was selected for further analysis. This included a single-spore progeny of mutant E3, the sole gene-disruptant resulting from integration of the *Eco*RV replacement vector. The growth of the majority of these transformants was not significantly different from wild-type PGB153 (50.2 ± 0.96 mm) over the duration tested (13 days) on PDA. However, three transformants did show statistically significant reductions in growth, compared with wild-type PGB153. These were S201 (46.7 ± 1.07 mm; $P = 0.0046$), S360 (46.3 ± 1.07 mm; $P = 0.0014$) and S402 (46.7 ± 0.66 mm; $P = 0.0015$). Transformants with normal PGB153 colony morphological phenotype were E3, S84, S99, S201, S203, S307, and S321. The remaining transformants possessed the "fluffy" growth phenotype described above.

Southern analysis and RNA gel blot hybridization.

Hybridization of the 2.6-kb *Sma*I fragment (Fig. 1C) probe to *Bgl*II-, *Cla*I-, and *Sma*I-digested genomic DNA of wild-type PGB153 resulted in the detection of restriction fragments of the expected size (described above). The 1-kb *Aat*II-*Bam*HI fragment probe from the *hph* gene (Fig. 1C) did not hybridize to wild-type PGB153 DNA. Southern analysis revealed that

Fig. 3. Comparison of esterase and cutinase activity in culture filtrate from wild-type PGB153. Esterase activity (●) was assayed with *p*-nitrophenyl butyrate as a substrate. Cutinase activity (□) was measured by determining release of radioactive fragments from ³H-cutin (cv. Crown). Results are average of eight replicate determinations.

these probes hybridized to multiple fragments of different signal intensities in *Sma*I-digested DNA of transformants E3, S84, S99, S201, and S203 but to single fragments in the remaining transformants. A 4.8-kb fragment was seen in *Sma*I-digested DNA from all transformants except S203. Hybridization signal intensities detected in *Bgl*II-digested DNA of transformants E3, S84, S99, S201, and S203 suggested that the probes hybridized to at least one high molecular weight fragment whereas a single 6.1-kb fragment was detected in the remaining transformants (Fig. 6). The 4.8- and 6.1-kb fragments conform to the restriction fragment sizes expected for the *cutA* locus when disrupted by the strategy employed herein and when digested with *Sma*I or *Bgl*II, respectively.

RNA gel blots of total RNA from transformants, excluding S201, S203, and S303, showed no hybridization to the PGB153 *cutA* cDNA probe, confirming that the *cutA* locus had been disrupted in these transformants (Fig. 7). Hybridization to the *cutA* cDNA probe was detected in total RNA (Fig. 7) from S201, S203, and S303, showing that these transformants retained a functional *cutA* locus. This finding is of interest because all of the transformants were originally selected on the basis of possessing a 5.3-kb recombinant but not a 3.1-

Fig. 4. Comparison of predicted amino acid sequences (single letter codes) derived from the *cutA* genes of *Fusarium solani* f. sp. *cucurbitae* PGB153 (upper sequence) and *F. solani* f. sp. *pisi* (lower sequence). Sequences were aligned with the Wisconsin Sequence Analysis Package program Bestfit. Position of residues in the active site of the *F. solani* f. sp. *pisi* cutinase and corresponding residues in the *F. solani* f. sp. *cucurbitae* sequence are in bold underlined type. Amino acids forming the catalytic triad are indicated by bold type and an "*" below the residue.

kb wild-type product when assayed by PCR. However, Southern analysis showed that neither S201 nor S203 (S303 was not analyzed) possessed fragments of the size expected for a func-

Fig. 5. Polymerase chain reaction (PCR)-based screening of transformants. **(A and B)** Selected gels showing “typical” amplification product profiles observed for transformants. **(C)** Examples of amplification product profiles of three single-spore progeny (ss1 to ss3) resulting from two of the transformants in **B**. **(D)** Example of amplification product profiles of five single-spore progeny resulting from a transformant (S79) possessing both 3.1-kb “wild-type” and 5.3-kb “recombinant” PCR products and from a transformant (S99) possessing a 5.3-kb “recombinant” PCR product. Cycling regime for gels in **A** and **B** was 35 cycles of 30 s at 94°C, 30 s at 50°C, 6 min at 68°C. Cycling regime for gels in **C** and **D** was identical to that used for amplification reactions in **A** and **B** except that the 30 s at 50°C step was not performed. Selected fragments of the size marker (SM) (1-kb ladder; Life Technologies) are indicated in kilobases (kb) to the side of each gel image.

tional wild-type locus for either the *Bgl*II or *Sma*I digests (Fig. 6A,B). Additional Southern analysis of *Xho*I- and *Pst*I-digested DNA of E3, S99, S201, and S203, using the *Xho*I-*Pst*I wild-type locus fragment (Fig. 1A) as a probe, did reveal the presence of a hybridizing 831-bp fragment in DNA of S201 and S203 but not in DNA of E3 or S99. Disruption of the *cutA* locus by the strategy employed herein should result in the replacement of this 831-bp fragment in the genome.

Fig. 6. Southern analysis of wild-type PGB153 and selected transformants. **(A)** *Bgl*II-digested genomic DNA. **(B and C)** *Sma*I-digested genomic DNA. Each DNA blot was hybridized with the 2.6-kb *Sma*I fragment probe from the wild-type *cutA* locus. Selected size marker fragments (1-kb ladder; Life Technologies) are indicated to left of each blot image.

These hybridization patterns suggest that integration must have occurred at the homologous locus in S201 and S203 but in such a manner as to leave the locus functional.

By combining the data from the PCR screening assay and from Southern analysis of transformants, the level of homologous integration in *F. solani* f. sp. *cucurbitae* race 2 isolate PGB153 was determined to be 14%.

Nonspecific esterase activity of *cutA*⁻ transformants.

Activity of both nonspecific esterase and cutinase in culture filtrates of PGB153 increased with increasing cutin concentration up to 0.5 g of cutin per liter in the culture medium (Fig. 3). For all subsequent assays, cutin was used at 5.0 g/liter and only nonspecific esterase activity was measured.

Nonspecific esterase activity was measured for PGB153 and two randomly selected *cutA*⁻ mutants (S321 and S351). Esterase activity was detected in culture filtrates of PGB153 grown in the presence of either buttercup squash cutin or butternut cutin in the growth medium (Table 1). Addition of 0.1% glucose to culture medium had no significant effect on esterase activity. Esterase production was greater when PGB153 was grown in mineral medium containing buttercup squash cutin than in medium containing butternut cutin. The activity detected in the culture filtrates of the two mutants when grown on either cutin type was not significantly different (*P* > 0.01) from that when grown on mineral medium alone.

Whole fruit and detached hypocotyl pathogenicity assays.

Frequency of infection of buttercup squash fruit was observed to increase with increasing inoculum dosage, reaching a plateau at a dosage of 2.5×10^3 conidia applied per site (5×10^5 conidia per ml) (Fig. 8A) for all isolates tested. Lesion size was also observed to increase with increase in inoculum dosage, peaking at a dosage of 2.5×10^3 conidia applied per inoculation site for mutants S347, S351, S360, and S400 (Fig. 8B). For the wild type and mutant S321, lesion size was observed to plateau at a dosage of 2.5×10^4 conidia applied per inoculation site (Fig. 8B). These results suggest that differences in virulence were not masked by inoculum concentration. Both wild-type PGB153 and the five *cutA*⁻ transformants tested responded similarly at each of the six inoculum dosages used, suggesting that disruption of *cutA* in these mutants did not affect virulence.

All transformants were found to be capable of colonization of host tissue and of pathogenic ability similar to that of wild-type PGB153 on each of the four fruit types inoculated (Fig. 8C,D) at the single inoculum concentration used for these ex-

periments. For each fruit type, there was no significant difference in mean lesion sizes induced by the mutants or wild-type PGB153. The frequency of occurrence of lesions with diameters within arbitrary size ranges was similar for wild type and the mutants tested (e.g., see Figure 9).

Buttercup squash proved to be the most susceptible of the fruit types tested while butternut was the least susceptible. Inoculation of detached hypocotyls of cv. Crown with PGB153, the *cutA*⁺ mutant S203, and seven *cutA*⁻ mutants (E3, S99, S302, S345, S360, S364, and S400) resulted in no difference in pathogenic phenotype between mutants and wild type. Lesions were produced on all wounded detached hypocotyl segments but not on unwounded hypocotyl segments.

DISCUSSION

It has been suggested that cutinase is involved in the penetration phase of fungal pathogenesis (Kolattukudy 1985). This involvement has been investigated for a number of filamentous fungal phytopathogens including *F. solani*, in which the focus has been directed toward the pea pathogen *F. solani* f. sp. *pisi*. The involvement of cutinase in pathogenesis of *F. solani* f. sp. *pisi* has been a matter of considerable debate (Rogers et al. 1994; Stahl and Schäfer 1992; Stahl et al. 1994). *F. solani* is a genetically heterogeneous entity and little is known about the involvement of cutinase in the pathogenicity of the other formae speciales of *F. solani*. In this manuscript we addressed this lack of information, cloning the *cutA* homologue from *F. solani* f. sp. *cucurbitae* race 2 and analyzing its function in pathogenesis/virulence by creating gene-disrupted mutants at the *cutA* locus.

The *F. solani* f. sp. *cucurbitae* race 2 *cutA* gene was found to be almost identical to the *cutA* gene from the closely related

Fig. 7. RNA gel blot hybridization analysis of the wild-type strain and selected transformants. Total RNA (30 µg per lane) isolated from cultures grown in the presence of purified cv. Crown cutin was hybridized with the *cutA* cDNA.

Table 1. Esterase activity in culture filtrates of wild-type PGB153 and two *cutA*⁻ mutants

Medium ^a	Isolate	Esterase activity (nkat/ml) ^b	Relative activity (%) ^c
MM + Bc	Wild-type	116.26 ± 16.25	100.00
MM + Bc	S321	18.41 ± 0.29	0.10
MM + Bc	S351	19.19 ± 0.34	0.89
MM + Bc + G	Wild-type	137.40 ± 9.06	100.00
MM + Bc + G	S321	19.48 ± 0.51	0.98
MM + Bc + G	S351	19.72 ± 0.46	1.18
MM + Bn	Wild-type	93.39 ± 11.73	100.00
MM + Bn	S321	18.35 ± 0.17	0.04
MM + Bn	S351	17.83 ± 0.17	0.00
MM + Bn + G	Wild-type	77.56 ± 6.93	100
MM + Bn + G	S321	19.35 ± 0.40	1.74
MM + Bn + G	S351	15.96 ± 2.32	0.00
MM	Wild-type	16.97 ± 0.15	...
MM	S321	16.55 ± 0.11	...
MM	S351	16.85 ± 0.16	...
MM + G	Wild-type	18.52 ± 0.18	...
MM + G	S321	17.86 ± 0.16	...
MM + G	S351	17.78 ± 0.18	...
MM	Medium only	18.31 ± 0.12	0.00

^a Medium composition: MM = mineral medium; Bc = 5 g of buttercup squash cutin per liter; Bn = 5 g of butternut cutin per liter; G = 0.1% glucose.

^b Mean of six replicate determinations.

^c Relative esterase activity in culture filtrates, in which activity in control (medium only) filtrate is taken to be 0% and that in wild-type culture filtrate is 100%.

Fig 8. (A) Percentage of infection of buttercup squash fruit following inoculation with wild-type PGB153 and five *cutA*⁻ mutants at each of six different inoculum concentrations (expressed as conidia per ml). (B) Mean lesion size induced on buttercup squash fruit by wild-type PGB153 and five *cutA*⁻ mutants at each of six different inoculum concentrations. (C) Percentage of infection of intact fruit of butternut and the *Cucurbita maxima* cvs. Crown, Delica, and Triamble after inoculation with wild-type PGB153, 16 *cutA*⁻ mutants, and three *cutA*⁺ mutants. (D) Mean lesion size induced on intact fruit of butternut and the *C. maxima* cvs. Crown, Delica, and Triamble after inoculation with wild-type PGB153, 16 *cutA*⁻ mutants, and three *cutA*⁺ mutants.

F. solani f. sp. *pisi*. Recently, Kamper et al. (1994) identified four important elements in the 5' noncoding region of the *F. solani* f. sp. *pisi* gene that are involved in regulation of cutinase expression. Of these, the G-rich region and palindrome 2 are identical in the promoter region of the *cutA* gene cloned from PGB153. However, the position of these sequences relative to the ATG translation initiation codon is different in the PGB153 *cutA* promoter due to the presence of an additional 11 bp. The significance of this 11-bp sequence, which forms the first of two 11-bp tandem direct repeat units, has yet to be de-

termined. The remaining promoter regions identified by Kamper et al. (1994) are highly conserved between the PGB153 and the pea pathogen sequence.

Detection of putative gene-disrupted transformants typically requires confirmation of integration type by Southern analysis. Where the level of homologous recombinant is high, it is a relatively efficient process to identify desired mutants among transformants directly by Southern analysis. For example, Rikkerink et al. (1994) and Bowen et al. (1995) detected levels of homologous integration of between 55 and 95% during

Fig. 9. Frequency of occurrence of the size of lesions induced by either wild-type PGB153 or one of 16 *cutA*⁻ mutants on intact buttercup squash fruit.

one-step gene disruption in *Glomerella cingulata*, using either a truncation (Shortle et al. 1982) or replacement (Rothstein 1983) strategy. However, detection of desired mutants by Southern analysis can become an inefficient process when the level of homologous integration is low. During this study we were able to determine the level of homologous integration in *F. solani* f. sp. *cucurbitae* race 2 isolate PGB153. Coupling the results of our PCR-based screen and those from Southern analysis showed that the level of homologous integration in PGB153 was 14%. We have yet to determine whether this will be a general level for homologous integration in *F. solani* f. sp. *cucurbitae* race 2 or whether it represents a locus-specific targeting efficiency.

PCR-based screening of transformants proved to be an efficient tool for identifying transformants for the subsequent Southern and RNA gel blot hybridization analyses necessary for confirmation of integration type and *cutA* gene transcription, respectively. By performing the assay directly on conidia, the necessity for isolating genomic DNA was obviated and the time taken for the selection of suitable transformants was decreased. Such an approach proved successful in identifying gene-disruption events in *Aspergillus fumigatus* transformants (Aufauvre-Brown et al. 1993). An unexpected result of our PCR screening was the high proportion of transformants possessing amplification products indicative of both wild-type and recombinant (gene-disrupted) *cutA* loci. This type of transformant could have arisen in a number of ways. If uncut pBB4 DNA contaminated the linear replacement vector fragment used for gene disruption, then the uncut pBB4 would likely integrate at ectopic sites. Transformation of PGB153 by an intact vector is extremely efficient and multiple integration events are common (Crowhurst et al. 1992). Because binding sites for the primers, used during the PCR assay of integration type, are present in both the wild-type locus and intact pBB4, the primers would amplify both the 3.1-kb wild-type and 5.3-kb recombinant products in those transformants in which uncut pBB4 had integrated. Ectopic integration of contaminating uncut pBB4 as well as the replacement vector might also explain the complex restriction fragment patterns detected by Southern analysis in some transformants. However, steps were taken to minimize carryover of uncut pBB4 vector. These included digestion of the integrating linearized vector fragment with *DraI* and gel purification of the appropriate fragment. An alternative explanation for the occurrence of transformants carrying both wild-type and recombinant loci may be that these transformants are the result of (i) protoplast fusion events occurring during the transformation process, or (ii) transformation of multinucleate protoplasts where not all nuclei carry disruptions of the *cutA* locus. In either case, the PCR screen procedure efficiently identified this type of transformant and enabled us to focus solely on those transformants having the desired transformation events.

Analysis of the pathogenic abilities of wild-type PGB153 and of 16 *cutA*⁻ mutants on fruits of four cucurbit hosts and on stem tissue of one of these hosts showed that it was not possible to differentiate between the pathogenic ability of the wild-type and these *cutA*⁻ mutants in the plant challenge assays employed. Our data suggest, therefore, that the *cutA* gene is not required for lesion development on intact cucurbit fruit or in determining tissue specificity of race 2 isolate PGB153. This finding is significant in that these fruit have thick, waxy

cuticles. These findings for *cutA* in *F. solani* f. sp. *cucurbitae* race 2 are similar to those reported for the involvement of *cutA* in pathogenicity and virulence of *F. solani* f. sp. *pisi* (Stahl and Schäfer 1992; Stahl et al. 1994), a pathogen causing foot rot of pea. However, this finding is in contrast to that of Rogers et al. (1994) for *F. solani* f. sp. *pisi*. Rogers et al. (1994) raised questions regarding the methodologies applied (Stahl and Schäfer 1992) during assessment of virulence of *cutA*⁻ mutants of *F. solani* f. sp. *pisi* and identified the use of several different inoculum levels during assessment of pathogenicity/virulence as being crucial for accurate detection of a reduction in virulence. They reported that use of spore concentrations of 10⁶ per ml or higher resulted in rapid infection that masked differences in virulence between isolates. In recognition of this finding, we assessed virulence on buttercup squash at six different inoculum dosages but found that the inoculum dosage did not mask virulence of *cutA*⁻ mutants or the wild type.

We have previously shown considerable genetic variation to exist between isolates of *F. solani* f. sp. *cucurbitae* race 1 and race 2 (Crowhurst et al. 1991). Yet, these races are presently recognized as MPI and MPV, respectively, of *Nectria haematococca*. It is more likely that mating populations of *N. haematococca* represent different species, as suggested by Van Etten and Kistler (1988). Differences between *N. haematococca* MPI, MPV, and MPVI, in the importance of various genes in pathogenicity, may therefore not be unexpected. The present state of the taxonomy of *N. haematococca* will lead to confusion in any discussion of the roles of enzymes such as *cutA* in which the anamorphic state of a given pathogen is referred to as *F. solani* without further reference to its forma specialis status. However, the virulence of isolates of both *F. solani* f. sp. *cucurbitae* race 1 and race 2 on stem tissue with intact cuticle does not coincide with the observed correlation between cutinase production and virulence noted for isolates of *F. solani* f. sp. *pisi*. Rogers et al. (1994) have noted that the higher the cutinase production by an isolate, the higher its virulence on tissue with an intact cuticle. Further, those isolates exhibiting high cutinase expression also possessed multiple cutinase genes. Our data suggest that PGB153 possesses a single gene for cutinase while the evidence to date for the isolates examined suggests that race 1 isolates do not possess a gene homologous to *cutA*. Yet, while race 2 isolates are capable of high expression of cutinase, they are not capable of attacking intact stem tissues. On the other hand, race 1 isolates are highly virulent on intact stem tissue and capable of attacking intact fruit but as yet cutinase production has not been detected in isolates of this race either by molecular techniques or by analysis of culture filtrates with cutin purified from several of the pathogen's hosts (buttercup squash, butternut, or cv. Crown) as the sole carbon source.

It is possible that the difference in phenotype between race 1 and race 2 on intact stem tissue is related to the differential triggering of a host defense response or to differences in expression of toxins by these two pathogens. We have observed that inoculation of unwounded hypocotyls of cv. Crown with isolates of race 2 results in either no visible symptom or very small, dark-colored, superficial specks (Hawthorne et al. 1994) reminiscent of a hypersensitive response reaction. However, if such a reaction is involved in governing the difference in pathogenicity of races 1 and 2 on intact stem tissue,

then evidence from the *cutA*⁻ mutants investigated herein suggests that expression of *cutA* is not a factor in triggering such a reaction.

Interestingly, Yao and Köller (1995) have pointed out that all cutinase genes cloned to date encode cutinases that are predominantly expressed during saprophytic growth of the respective pathogens. They suggest that other cutinases are induced following interaction with the host. The PGB153 *cutA* gene was cloned by homology to the pea pathogen gene (Soliday et al 1984). Although we have ruled out the involvement of *cutA* in pathogenicity and virulence of PGB153, there remains the possibility that other as yet undetected enzymes with "cutinase-like" activity are induced in PGB153 or race 1 isolates following contact with their cucurbit hosts and play some role in pathogenesis.

MATERIALS AND METHODS

Fungal isolates, bacterial strains, bacterial transformation, and plasmids.

Single-spore cultures of wild-type *F. solani* f. sp. *cucurbitae* race 2 isolate PGB153, cutinase disrupted transformants of PGB153, and *F. solani* f. sp. *cucurbitae* race 1 isolates BTH869 and ATCC18099 were stored as conidial suspensions dried on filter paper disks at -20°C. The origin, mating population, random amplified polymorphic DNA profile, and pathogenicity of PGB153, BTH869, and ATCC18099 have been described previously (Crowhurst et al. 1991; Hawthorne et al. 1992).

Escherichia coli strains DH5 α or DH5 α F' IQ (Life Technologies Inc., Gaithersburg, MD) were used for propagation of plasmids. Preparation of electroporation competent cells and subsequent transformation were performed according to Smith et al. (1990). Plasmid DNA was isolated with either Magic or Wizard Minipreps or Maxipreps DNA Purification Systems (Promega Corporation, Madison, WI). Plasmid pGEM-7Zf(-) (Promega) was used for subcloning fragments for sequence analysis. pAN7-1 (Punt et al. 1987) was obtained from R. Oliver (University of East Anglia, Norwich, UK). The *F. solani* f. sp. *pisi* *cutA* cDNA was obtained from P. E. Kolattukudy (Ohio State Biotechnology Center, Ohio State University, Columbus).

Southern analysis of cutinase genes in race 2 isolate PGB153 and race 1 isolate BTH869.

Genomic DNA was isolated from BTH869 and PGB153 as described previously (Crowhurst et al. 1992). Restriction endonuclease digested DNA was size fractionated by electrophoresis in 0.8% TAE (40 mM Tris, pH 8.0, 40 mM sodium acetate, and 1 mM EDTA) agarose gels (5 μ g per lane). Restriction endonucleases used were *Bgl*II, *Cl*aI, *Eco*RI, *Eco*RV, *Hind*III, *Pst*I, *Sal*I, *Sst*I, *Xba*I, and *Xho*I. Following depurination in 0.2 M HCl for 30 min, DNA was directly transferred from the gel onto Hybond N⁺ (Amersham, Buckinghamshire, England) membrane with 0.4 M NaOH. A cDNA clone of the *F. solani* f. sp. *pisi* cutinase mRNA (Soliday et al. 1984) was labeled with α -³²P-dCTP (Amersham), using a RadPrime Labeling System (Life Technologies). Labeled probe was hybridized in 1 M NaCl, 1% sodium dodecyl sulfate (SDS), 1% skim milk powder (Anchor, Auckland, New Zealand) at 60°C overnight. Filters were washed three times in 500 ml of 2 $\times$ SSC (1 $\times$ SSC is 0.15 M

NaCl plus 0.015 M sodium citrate) 1% SDS at 60°C, 20 min per wash. The washed filters were exposed to Curix X-ray film (Agfa, Mortsel, Belgium) for 24 h at -70°C.

Cloning *cutA* from PGB153.

PGB153 genomic DNA digested with *Bgl*II or *Xho*I was size fractionated by electrophoresis on 1% agarose gels in TAE buffer (Sambrook et al. 1989). Fragments between 2 to 6 kb (*Bgl*II) or 0.5 to 4 kb (*Xho*I) were recovered with the GlassMAX DNA Isolation Spin Cartridge System (Life Technologies). Fragments were ligated into *Bam*HI (for *Bgl*II fragments) or *Xho*I cleaved pGEM-7Zf(-) and used to transform DH5 α F' IQ cells. Cells were grown for 5 h at 37°C. Aliquots containing approximately 4,000 colonies were plated onto replicate Hybond N⁺ filters. Filters were screened for homology to the *F. solani* f. sp. *pisi* cutinase cDNA clone.

Isolation of the *cutA* cDNA from PGB153.

C. maxima cv. Crown cutin was purified by the method of Walton and Kolattukudy (1972). Roux culture flasks containing 100 ml of liquid mineral medium (Lin and Kolattukudy 1978), supplemented with 5 g of purified Crown cutin per liter and 1 g of glucose per liter, were inoculated with 6 $\times$ 10⁷ conidia obtained from 13-day-old PDA cultures of PGB153. Flasks were incubated, without shaking, at 26°C for 11 days. Mycelium was harvested by filtration through Miracloth (Calbiochem-Novabiochem Corp., La Jolla, CA) and lyophilized. RNA was extracted by homogenization of lyophilized mycelium in 10 ml of TRIZOL (Life Technologies) with a mortar and pestle. Polyadenylated RNA was purified from total RNA by a mRNA Purification Kit (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) and cDNA synthesized from mRNA by a Superscript Choice System (Life Technologies) with the oligo(dT) primer RC16 (5'-GGGATCCGCGG CCGCTCTAGAdT₁₅-3'). Primers RC16 and RC30 (5'-CGC TGAGCTCAATATTCAAAATGAAGTTCTTCGTC-3') were used to amplify a product from cDNA in an amplification reaction containing 50 mM Tris-HCl, pH 8.3, 2.5 mM MgCl₂, 100 μ M each of dATP, dCTP, dGTP, and dTTP, 200 nM each primer, and 1 unit of *Taq* DNA polymerase (Boehringer Mannheim, Mannheim, Germany). Amplification reactions were performed in a TwinBlock System Easy Cycler (Ericomp Inc., San Diego, CA) programmed for 4 min at 94°C, followed by 35 cycles of 60 s at 94°C, 60 s at 42°C, and 90 s at 72°C. Following completion of the last cycle, reactions were incubated at 72°C for 7 min. To facilitate cloning of amplified products, primer RC16 included an *Xba*I restriction endonuclease cleavage site and primer RC30 included an *Sst*I restriction endonuclease cleavage site. RC30 was also designed to modify the context of the start codon from CACCAATG in PGB153 to CAAAATG in order to facilitate later expression in yeast. Expression of the *G. cingulata* *pnlA* gene in yeast was increased six- to 10-fold when the context of the start codon was so mutated (Templeton et al. 1994). The amplification product was digested with *Sst*I and *Xba*I, ligated to *Sst*I- and *Xba*I-digested pGEM-7Zf(-), and used to transform DH5 α F' IQ cells.

Determination of DNA sequence.

Systematic deletion subclones were generated by the method of Lin et al. (1985). Unique *Xba*I and *Kpn*I restriction

sites in the vector polylinker 5' to the original *Bam*HI cloning site in pBB1 were used in the generation of "XK-" series deletion subclones. No suitable pair of unique restriction sites existed 3' of the *Bam*HI cloning site. Therefore the unique vector polylinker restriction endonuclease site *Nsi*I was coupled with a unique *Pst*I site internal to clone pBB1 (Fig. 1) and used in the generation of "NP-" series deletion subclones. Purified DNaseI-digested plasmid was digested with *Xba*I or *Nsi*I overnight at 37°C, purified by the GlassMAX DNA Isolation Spin Cartridge System, adjusted to 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂ containing 2 units Klenow fragment (Boehringer Mannheim) for 60 min at room temperature, the DNA purified by a Magic DNA Clean-Up System, and self-ligated overnight at 16°C. Following DNA purification, samples were subdivided into two equal aliquots and subjected to restriction endonuclease cleavage with the second of the paired restriction endonucleases, then used to transform electroporation-competent DH5 α F' IQ cells.

Where necessary, additional subclones were generated by restriction endonuclease digestion and ligation to pGEM-7Zf(-) cleaved with the appropriate restriction endonuclease. Synthetic oligonucleotide primers were employed during sequencing where no subclones were available for a given region. The nucleotide sequence of both strands was determined by automated sequencing with an Applied Biosystems Model 373A automated sequencer (Biochemistry Department, University of Otago, Dunedin, New Zealand). Sequence analysis was performed with programs from the Wisconsin Sequence Analysis Package (Genetics Computer Group, Madison, WI).

Construction of gene replacement vectors.

Plasmid pBB2 was constructed by removal of the *Xho*I, *Eco*RI, *Kpn*I, *Sma*I, *Csp*I, and *Cl*aI sites from the multiple cloning site region of pBB1. Plasmid pBB4 was constructed by replacing the 831-bp *Xho*I to *Pst*I fragment in pBB2 (comprising 271 bp of 5' noncoding and 559 bp of the *cutA* coding region) with the 3,046-bp *Xho*I to *Hind*III fragment (*hph* cassette) from pAN7-1. The *hph* cassette contains the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase promoter fused to the *E. coli* hygromycin phosphotransferase gene (*hph*) and coupled to terminator sequences from the *A. nidulans* *trpC* gene. Both the *Pst*I terminus of pBB2 and the *Hind*III terminus of the hygromycin resistance cassette fragment were converted to blunt termini prior to ligation. Linear gene replacement vector DNA was prepared by digesting pBB4 with either *Sma*I or *Eco*RV. These digests were subsequently digested with *Dra*I (which cleaves the pGEM-7 derived sequences). The 4.8-kb *Sma*I and 4-kb *Eco*RV fragments were gel purified and thereafter termed the *Sma*I or *Eco*RV replacement vector, respectively (Fig. 1C). The *Sma*I replacement vector shares 1,048 and 799 bp of homology at its 5' and 3' ends, respectively, with the PGB153 *cutA* locus. The 5' and 3' ends of the *Eco*RV replacement vector share 511 and 610 bp of homology, respectively, with the PGB153 *cutA* locus.

Generation of *cutA* gene-disruption transformants.

Disruption of cutinase in PGB153 employed a one-step gene-disruption strategy (Rothstein 1983) (see Figure 1C). Fungal protoplasts were isolated and transformed with the appropriate replacement vector by previously described methods (Crowhurst et al. 1992). Hygromycin-resistant transformants

were transferred to selection medium (PDA supplemented with 80 μ g of hygromycin B per ml [Boehringer Mannheim]). Putative *cutA* gene disruptants were detected with conidial suspensions (5×10^6 conidia per ml) from individual transformants in a PCR-based screening assay. Amplification reactions were performed in 25- μ l volumes containing 350 μ M each of dATP, dCTP, dGTP, and dTTP, 300 nM upstream primer (RC40), 300 nM downstream primer (RC31), 50 mM Tris-HCl, pH 9.2, 14 mM (NH₄)₂SO₄, 1.75 mM MgCl₂, 2.6 units of Expand Long Template Enzyme Mix (Boehringer Mannheim), and approximately 2×10^4 conidia. Amplification reactions were performed in a TwinBlock System Easy Cycler (Ericomp) programmed for 1 min at 94°C, followed by 35 cycles of 20 s at 94°C, 30 s at 50°C, and 6 min at 68°C. Reaction products were size fractionated by electrophoresis in 1% TAE agarose gels. Transformants identified by this initial PCR assay were subject to single-spore isolation and three to five resulting single-spore progeny per original transformant were tested with the above PCR-based assay, except that the 30 s at 50°C annealing step was not performed. Growth and colony morphology of putative *cutA* gene-disruptants selected by this procedure were compared with wild-type PGB153 on PDA plates after 13 days at 24°C.

DNA isolation and Southern analysis of transformants.

Conidia from wild-type PGB153 and selected transformants were inoculated into 100-ml volumes of GM (Crowhurst et al. 1992) and incubated at 24°C, 100 rpm, on an orbital shaker (Labline Orbit, Lab-Line Instruments, Melrose Park, IL) for 5 days. Mycelium was harvested by filtration through Miracloth, snap frozen in liquid nitrogen, and lyophilized. Genomic DNA was extracted from the lyophilized mycelium by homogenization in DNazol reagent (Life Technologies) according to the manufacturer's recommendations. DNA preparations were further purified by sequential extraction with phenol, phenol-chloroform, and chloroform. Aliquots (5 μ g) of genomic DNA were digested with *Bgl*II, *Sma*I, or *Cl*aI and size fractionated by electrophoresis in 1% TAE agarose (Sambrook et al. 1989). Digested DNA was transferred to Hybond-N⁺ membrane. The 2.6-kb *Sma*I fragment from the wild-type *cutA* gene locus was gel purified and labeled with α -³²P-dCTP, using a RadPrime Labeling System, and hybridization performed in 0.5 M sodium phosphate (pH 7.2), 7% SDS at 60°C overnight. Filters were washed three times in 100-ml volumes of 1 $\times$ SSC, 1% SDS at 60°C, 20 min per wash. The washed filters were exposed to Curix X-ray film (Agfa) for 3 to 24 h at -70°C. Following autoradiography, this probe was removed and filters re-hybridized with the 1-kb *Aat*II-*Bam*HI fragment of the *hph* gene from the hygromycin resistance cassette of pAN7-1.

RNA gel blot hybridization analysis of transformants.

Total RNA was extracted from cultures as described above, size fractionated (30 μ g per lane) on 1% denaturing formaldehyde gels (Sambrook et al. 1989), and transferred to Hybond-N⁺ membrane by capillary transfer in 0.05 M NaOH. Filters were hybridized with the *cutA* cDNA clone labeled with α -³²P-dCTP, using a RadPrime Labeling System. Labeled probe was hybridized in 0.5 M sodium phosphate, 7% SDS at 60°C overnight. Filters were washed four times in 100-ml volumes of 1 $\times$ SSC, 1% SDS at 60°C, 20 min per wash. The washed

filters were exposed to Curix X-ray film (Agfa) for 1 to 24 h at -70°C .

Nonspecific esterase and cutinase assays.

Cutin was purified from *C. maxima* cvs. Delica (buttercup squash) and Crown and from *C. moschata* Duchesne (butternut) by previously described methods (Walton and Kolattukudy 1972). The effect of cutin concentration in the growth medium on both nonspecific esterase activity and cutinase activity of PGB153 was determined with Crown cutin. Roux culture flasks containing 100 ml of liquid mineral medium (Lin and Kolattukudy 1978) supplemented with either 0.1, 0.5, 2.5 or 5.0 g of Crown cutin per liter were inoculated with 6×10^7 conidia obtained from a 13-day-old culture of PGB153 grown on PDA at 24°C . Flasks were incubated, without shaking, at 26°C for 13 days. Esterase activity in fungal culture filtrates was measured with *p*-nitrophenylbutrate as substrate (Purdy and Kolattukudy 1973). Cutinase activity was measured by previously described methods (Baker and Bateman 1978). Tritium labeling of purified cutin from *C. maxima* cv. Crown (200 mg) was performed by Amersham International (Buckinghamshire, UK). Twenty milligrams of the labeled product (1.33 Ci/mg) was diluted 200-fold with nonlabeled Crown cutin and washed as described by Baker and Bateman (1978). The final, diluted, labeled cutin (^3H -cutin) used in cutinase assays contained approximately 7×10^5 dpm/mg.

Nonspecific esterase activity of wild-type and *cutA*⁻ mutants was determined as described above except that 5 g of either purified buttercup squash or butternut cutin per liter was used. Where appropriate, glucose (0.1% wt/vol) was added to the growth medium.

Whole fruit and detached hypocotyl pathogenicity assays.

To assess whether inoculum concentration affected virulence, 10-fold serial dilutions (5×10^7 to 5×10^2 conidia per ml) were prepared for wild-type PGB153 and five *cutA*⁻ transformants (S321, S347, S351, S360, and S400). Each of 20 buttercup squash fruit was inoculated with all dilutions of all isolates. A 5- μl droplet of the appropriate dilution of the appropriate isolate was applied per site. The effective inoculum applied per site therefore ranged from 2.5 to 2.5×10^5 conidia. A 5- μl droplet of sterile water was applied as a control for each set of dilutions of each isolate. Droplets were left to air dry, then individual fruit were placed in plastic bags containing a water-moistened paper towel. Bags were incubated at 24 to 28°C for 7 days, then fruit were removed from the bags. Incubation was continued for a further 5 days, after which time the number of sites exhibiting lesion development was counted and lesion diameters measured following removal of the outermost layers of the fruit surface with a scalpel.

Pathogenicity of transformants was assessed on intact fruit of butternut and the *C. maxima* cvs. Crown, Delica, and Triamble. Export-quality fruit, harvested at commercial maturity, were stored at ambient temperature until inoculated. For each of two sites per inoculum (excluding PGB153) on each of 20 fruit of each fruit type, a 5- μl droplet of inoculum was placed on the fruit surface without wounding. Inoculum consisted of either conidial suspension (5×10^7 conidia per ml in sterile water) or sterile water as a control. Each pair of sites for each inoculum was separated by a single inoculation site

for PGB153 (5- μl droplet of a 5×10^7 conidia per ml suspension). Fruit were bagged, incubated, and assayed as described above.

Pathogenicity of transformants was also assessed on detached hypocotyls of Crown. Seeds were surface sterilized by soaking in 5% hypochlorite solution for 10 min followed by rinsing in running water for 60 min. The surface-sterilized seeds were placed on sterile Whatman No. 1 filter disks in sterile petri dishes and incubated in a Hearson Germinator (Charles Hearson, London) at 25°C for 3 to 4 days. Germinated seeds were transplanted to sterile sand and raised for 7 days. The assay was thereafter performed as previously described (Hawthorne et al. 1994).

ACKNOWLEDGMENTS

This project was funded by the New Zealand Foundation for Research, Science and Technology (contract no. CO6402).

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