Pathogenesis by *Cochliobolus heterostrophus* Transformants Expressing a Cutinase-Encoding Gene from *Nectria haematococca*

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***Nectria haematococca*, a pathogen of pea stems and roots, produces cutinase (pH optimum 9.5), an enzyme that degrades cutin found in the plant epidermis. A cutinase encoding gene (*CUT-T8*) from *N. haematococca* was overexpressed in transformants of *Cochliobolus heterostrophus* (a pathogen of corn foliage) with or without simultaneous over-expression of *PDA-T9*, a gene from *N. haematococca* encoding pisatin demethylase (PDA), which detoxifies the pea phytoalexin pisatin. Transformants were compared with each other and with wild type for ability to attack roots, stems, or leaves of pea, and roots or leaves of corn. Overexpression of PDA alone caused increased virulence to pea of several independently generated *C. heterostrophus* transformants, confirming a previous report, which was based on analysis of one transformant. *N. haematococca* cutinase alone had no detectable effect on virulence of *C. heterostrophus* to pea, but in combination with PDA caused distinctively dark-colored lesions on nonwounded pea stems, suggesting cutinase (in addition to PDA) as a possible factor in this heterologous plant/fungus interaction. All transformants caused normal symptoms (indistinguishable from those of wild-type *C. heterostrophus*) on corn leaves, but no detectable damage to roots of corn or pea. Thus, over-expression of heterologous cutinase did not change the organ specificity of *C. heterostrophus* on either its own host (corn) or a nonhost (pea).**

Additional keyword: penetration.

Cochliobolus heterostrophus (Drechs.) Drehs. is a natural pathogen of corn. Its host range can be extended to pea (Schäfer *et al.* 1989) if it is made to overexpress the *Nectria haematococca* Berk. & Br. *PDA-T9* gene, which encodes pisatin demethylase (PDA) and thus allows the fungus to detoxify the pea phytoalexin pisatin (VanEtten *et al.* 1989). The virulence of PDA-producing *C. heterostrophus* toward pea, as measured by lesion length on stems, is low compared with that of *N. haematococca*. This suggests that *N. haematococca* produces additional virulence factors that *C. heterostrophus* lacks.

One attractive candidate for such a factor is cutinase, since *C. heterostrophus* is known to be a low producer of this enzyme (Baker and Bateman 1978). Cutinase has been suggested as a requirement for fungal penetration through the plant cuticle (Kolattukudy 1985). Several lines of evidence have been advanced in support of cutinase as a pathogenicity factor for *N. haematococca*. The enzyme is found at the site of penetration (Shaykh *et al.* 1977), inhibitors of cutinase activity reduce fungal penetration efficiency (Köller *et al.* 1982a; Maiti and Kolattukudy 1979), induced mutants and natural variants with low cutinase activity have reduced virulence or are nonpathogenic (Dantzig *et al.* 1986; Köller *et al.* 1982b), and transformation of a weakly virulent, low cutinase-producing strain with a cloned cutinase encoding gene caused the recipient strain to become more virulent (Kolattukudy *et al.* 1989). The most persuasive experiment pointing to a role for cutinase in pathogenesis involved heterologous gene expression (Dickman *et al.* 1989). The *N. haematococca* cutinase gene was transformed into a *Mycosphaerella* spp. which is a natural wound pathogen of papaya fruit; it does not normally attack intact tissues or produce cutinase. Transformants expressing the gene were able to invade nonwounded papaya fruits, thus indicating that this single enzyme confers penetration ability to this particular fungus.

Another genetic test of the same *N. haematococca* cutinase produced no evidence that this enzyme is involved in pathogenicity of *N. haematococca* toward pea (Stahl and Schäfer 1992). The chromosomal copy of the cutinase gene was replaced (via homologous recombination) by a disrupted copy of the gene. The resulting transformants produced no detectable cutinase transcript or enzyme activity, yet they were as virulent in two different types of plant test as wild-type control strains. Thus it appears that cutinase may be involved in some plant-fungal interactions but not in others and that its role in pathogenesis must be examined on a case-by-case basis.

In the case of *C. heterostrophus*, penetration occurs directly through the cuticle (Wheeler 1977), suggesting a possible role for cutinase even though this fungus secretes only small amounts (Baker and Bateman 1978). Moreover, *C. heterostrophus* is specific for foliar plant parts and is rarely if ever found colonizing roots. This observation is especially interesting since previous work (Köller and Parker 1989; Trail and Köller 1990) has associated fungal organ specificity with cutinase pH optimum. Fungi that attack foliage produce primarily cutinase

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Table 1. *Cochliobolus heterostrophus* transformants chosen for analysis

Transformant[a]	Plasmid	Approximate copy number	Integration event[b]	Expression[c] of: *PDA-T9*	Expression[c] of: *CUT-T8*
C2-pUCH1-1	pUCH1	4	Homologous		
C2-pUCH1-2*	pUCH1	4	Ectopic		
C2-pUCH1-3	pUCH1	4	Homologous		
C2-PDA-1[d]	pUP1	13	Homologous	+	
C2-PDA-2*	pUP1	8	Homologous	+	
C2-PDA-3	pUP1	12	Homologous	+	
C2-PDA-4	pUP1	14	Ectopic	+	
C2-CUT-1	pCHN1	7	Homologous		+
C2-CUT-2*	pCHN1	7	Homologous		+
C2-CUT-3[e]	pCHN1	2	Homologous		+
C2-PDA/CUT-1	pCPNH86	12	Ectopic	+	+
C2-PDA/CUT-2*	pCPNH86	11	Ectopic	+	+
C2-PDA/CUT-3	pCPNH86	11	Homologous	+	+
C2-PDA/CUT-4	pCPNH86	4	Ectopic	+	+

[a] Asterisks indicate transformants chosen to represent each of the four groups of transformants in assays described in Tables 4–7 (except for C2-CUT-1 in Table 6). All transformants shown here were assayed in experiments described in Tables 4–7 and each gave the result of its representative.
[b] The homologous integration site is promoter 1 (Turgeon *et al.* 1987).
[c] + = Over-expression of the indicated heterologus gene. Blank = no gene over-expression.
[d] This transformant, described previously as C2-P (Schäfer *et al.* 1989), originally had an estimated 18 copies of *PDA-T9*. Upon recovery from storage for experiments reported here its copy number was estimated to be 13. There was no apparent change in its phenotype.
[e] This transformant conidiated poorly and was pale orange rather than white (the color typical of progenitor strain C2). Genetic analysis indicated that the "orange/reduced conidia" phenotype segregated as a single gene (designated *Orc1*) unlinked to *hygB*.

Table 2. Cutinase (esterase) activity of *Cochliobolus heterostrophus* and *Nectria haematococca* grown on medium containing apple cutin as sole carbon source

Strain	ΔOD_{400}/min/μg protein	Strain	ΔOD_{400}/min/μg protein
C2	0.08	T8[a]	3.8
C2[b]	0.00	T8[a,b]	0.14
C2-pUCH1-1	0.14	C2-CUT-1	10.5
C2-pUCH1-2	0.04	C2-CUT-2	18.6
C2-pUCH1-3	0.16	C2-CUT-3	4.1
C2-PDA-1	0.06	C2-PDA/CUT-1	13.4
C2-PDA-2	0.07	C2-PDA/CUT-2	12.5
C2-PDA-3	0.17	C2-PDA/CUT-3	13.9
		C2-PDA/CUT-4	5.0

[a] *N. haematococca.*
[b] Glucose replaced cutin in the growth medium.

Table 3. Growth of *Cochliobolus heterostrophus* on medium containing 1 mM pisatin

Strain	Colony radius[a] (mm)	Strain	Colony radius[a] (mm)
C2	8	C2-pUCH1-1	8
C2[b]	17	C2-pUCH1-1[b]	21
C2-PDA-1	23	C2-PDA/CUT-1	21
C2-PDA-2	18	C2-PDA/CUT-2	18
C2-PDA-3	22	C2-PDA/CUT-3	19
C2-PDA-4	22	C2-PDA/CUT-4	13

[a] Average of two plates.
[b] Growth on medium containing DMSO (the solvent for pisatin) but not pisatin.

with a slightly acidic pH optimum (about 6), whereas those that attack roots produce primarily cutinase with an alkaline pH optimum (about 9); *C. heterostrophus* cutinase has a pH optimum of 6.5 (Trail and Köller 1990). Significantly, PDA overproduction by *C. heterostrophus* allows attack of only pea foliage (not roots), thus maintaining the natural organ specificity of the fungus (Schäfer and Yoder 1994).

Our investigation centered on two questions, based on the foregoing observations: 1) Is the penetration efficiency or virulence of *C. heterostrophus* (a low cutinase producer) affected by over-expression of heterologous cutinase? and 2) Can organ specificity be altered by changing the pH optimum of the predominant cutinase? Both questions were addressed using *C. heterostrophus* transformants over-expressing, either alone or in combination, the *N. haematococca CUT-T8* gene (Soliday *et al.* 1984), which encodes cutinase with a pH optimum of 9.5, and/or the *N. haematococca PDA-T9* gene (Weltring *et al.* 1988), which encodes pisatin demethlyase.

RESULTS

Construction of *C. heterostrophus* transformants.

C. heterostrophus strain C2 was transformed with pUCH1 (control), pUP1 (*PDA-T9*), pCNH1 (*CUT-T8*), or pCPNH86 (*PDA-T9* and *CUT-T8*). Transformants were chosen (Table 1) that were morphologically stable, conidiated well (except for C2-CUT-3), and grew on medium containing 1 mg of hygromycin B per milliliter. Southern blot analysis revealed that in some transformants integration was ectopic, whereas in others the vector integrated by homologous recombination at the promoter 1 site (Table 1). Copy numbers ranged from four to 14 (except for the unusual transformant C2-CUT-3). Growth rate comparisons showed that four or more copies of *hygB* enabled *C. heterostrophus* to grow normally on 1 mg of hygromycin B per milliliter; transformant C2-CUT-3, with two copies of *hygB*, grew significantly slower.

When grown on medium containing apple cutin, the cutinase activity of *C. heterostrophus* transformants carrying *N. haematococca CUT-T8* was approximately 100 times higher than that of untransformed strain C2 and as high as, or higher than, that of *N. haematococca* wild-type strain T8 (Table 2). When grown on medium containing glucose, cutinase activity of transformants was reduced only slightly, indicating that *CUT-T8* is constitutively expressed in *C. heterostrophus* (data

not shown). In *N. haematococca*, it is induced by cutin and repressed by glucose (Lin and Kolattukudy 1978). *C. heterostrophus* transformants carrying *PDA-T9* grew as fast in the presence of pisatin as in its absence, indicating expression of the heterologous gene and *in vivo* activity of its product; growth of strains lacking *PDA-T9* was inhibited by pisatin (Table 3).

Pea stem assays.

Long lesions were caused by *N. haematococca* on wounded pea stems, whereas *C. heterostrophus* strain C2 with or without pUCH1 or *CUT-T8* caused small superficial lesions (Table 4). *C. heterostrophus* transformants expressing *PDA-T9* caused significantly larger lesions (1.5–2 times longer) of the type described earlier for transformant C2-PDA-1 (Schäfer *et al.* 1989). Expression of both *PDA-T9* and *CUT-T8* together did not alter the effect on wounded pea stems, compared with expression of *PDA-T9* alone.

The pea stem assay was repeated using unwounded stems, which were incubated for 10 days after inoculation rather than 6 days as reported elsewhere (Schäfer and Yoder 1994). The longer incubation period allowed development of symptoms caused by *C. heterostrophus* control strains (C2, C2-pUCH1-2) that are not seen after shorter incubation. No significant differences in lesion length were observed among any of the *C. heterostrophus* transformants or control strains (Table 5). However, lesions caused by transformants expressing both *PDA-T9* and *CUT-T8* were substantially darker than those caused by control strains or transformants expressing either *PDA-T9* or *CUT-T8* alone (Fig. 1), although there was an occasional exception (Table 5). Microscopic observation of stem cross sections stained in cotton blue suggested that the darker lesion color was not caused by more extensive fungal invasion of the stem tissue. Although the significance of the dark-colored lesions is not known, it is clear that any change effected by expression of *CUT-T8* is on fungal virulence and not on organ specificity, since the PDA-producing transformants can attack pea stems with or without

Table 4. Lesion lengths on wounded pea stems caused by *Nectria haematococca* and *Cochliobolus heterostrophus*

Strain	Lesion/Length (mm)[a]
77-2-3[b]	17.8 c (mean of five assays)
C2	1.3 d (mean of six assays)
C2-pUCH1-2	1.6 d (mean of four assays)
C2-CUT-2	1.7 d (mean of three assays)
C2-PDA-2	2.7 e (mean of three assays)
C2-PDA/CUT-2	2.8 e (mean of three assays)

[a] Data are compiled from six different assays; strain C2 was included in each. There were eight pea stems inoculated/strain/assay. For each assay, data were analyzed by the pairwise Student's *t* test; the statistically significant (5% level) differences shown here (numbers followed by the same letter are not different) were consistently observed in each assay.
[b] *N. haematococca.*

Table 5. Lesions on unwounded pea stems caused by *Nectria haematococca* and *Cochliobolus heterostrophus*[a]

Strain	Number of plants with lesions			
	None	Small light[b]	Small dark[c]	Large dark[d]
T8[e]	0	0	0	8
C2	1	7	0	0
C2-pUCH1-2	3	5	0	0
C2-PDA-2	1	6	1	0
C2-CUT-2	2	5	1	0
C2-PDA/CUT-2	0	0	8	0

[a] Data are from one of two assays, which together included all transformants shown in Table 1. All assays included eight stems/strain and gave results similar to those shown here.
[b] Light-colored lesions 1–2 mm long (Fig. 1).
[c] Dark-colored lesions 1–2 mm long (Fig. 1).
[d] Dark-colored lesions 20–30 mm long.
[e] *N. haematococca.*

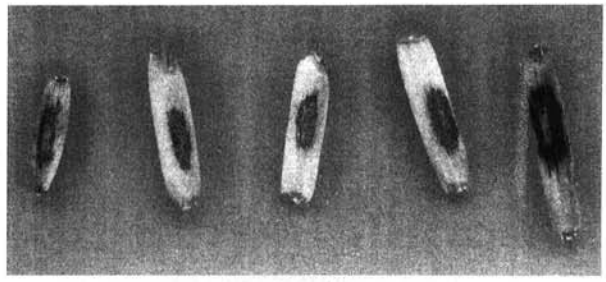

Fig. 1. Lesions on unwounded pea stems caused by *Cochliobolus heterostrophus*. Strains used for inoculum, from left: wild-type strain C2 (control), transformant C2-pUCH1-2 (vector control), transformant C2-PDA-2 (over-producing PDA), transformant C2-CUT-2 (over-producing cutinase), and transformant C2-CUT/PDA-2 (over-producing both PDA and cutinase; note the distinctively darker color of the lesion). Roots and shoots were excised before the picture was taken.

Table 6. Growth of corn or pea plants in soil infested with conidia from *Cochliobolus heterostrophus* or *Nectria haematococca*[a]

Strain	Shoot fresh weight (g/plant)		Root fresh weight (g/plant)	
	Pea	Corn	Pea	Corn
None	4.26 ± 1.27	3.64 ± 0.27	2.03 ± 0.06	2.89 ± 0.35
T8[b]	0.88[c] ± 1.02	5.11 ± 1.47	0.50[d] ± 0.53	3.93 ± 1.10
C2	3.22 ± 0.14	5.59 ± 3.08	2.01 ± 0.62	3.70 ± 0.62
C2-pUCH1-2	3.43 ± 0.12	6.63 ± 1.01	1.59 ± 0.46	2.82 ± 0.44
C2-PDA-2	4.48 ± 0.56	4.56 ± 1.06	2.01 ± 0.17	2.95 ± 0.91
C2-CUT-1[e]	3.72 ± 0.38	3.90 ± 0.70	2.55 ± 0.40	2.77 ± 0.44
C2-PDA/CUT-2	3.25 ± 0.56	5.08 ± 0.56	2.27 ± 0.22	1.80 ± 1.10

[a] Data (± standard deviation) were compiled from four different assays, each of which included uninoculated plants and the control strians. There were six to eight plants weighed/strain/assay.
[b] *N. haematococca.*
[c] Each stem had a black base.
[d] Roots were severely discolored.
[e] Strain C2-CUT-2 was used for the corn root assays.

production of the heterologous cutinase. The large lesions produced on unwounded pea stems by *N. haematococca* strain T8 (Table 5) were also observed when strain 77-2-3 was used, but rarely (Schäfer and Yoder 1994).

Root assays.

Pea roots were infected and severely damaged only when plants were grown in soil infested with *N. haematococca* (Table 6). The stem base was black, roots were discolored and their development was poor. Inoculation of corn roots with either *N. haematococca* or *C. heterostrophus* (including the transformants) did not inhibit plant development compared to control plants grown in uninfested soil (Table 6).

Leaf assays.

To quantitatively evaluate fungal virulence to corn leaves, the number of conidia that gave rise to lesions was determined (Table 7). Virulence of all transformants (carrying either pUCH1, *PDA-T9*, *CUT-T8*, or both *PDA-T9* and *CUT-T8*) was indistinguishable from that of strain C2, which caused normal *C. heterostrophus* lesions. The pea pathogen *N. haematococca* did not infect corn leaves.

In contrast, all *C. heterostrophus* transformants carrying the *PDA-T9* gene killed more (60–75%) pea leaves (Fig. 2; Table 7) than did transformants or controls without *PDA-T9* (5–20%). Expression of *CUT-T8*, with or without *PDA-T9*, did not significantly change the effect of *C. heterostrophus* on pea leaves. Under these experimental conditions, *PDA-T9* expression in *C. heterostrophus* caused total collapse and dehydration of inoculated leaves (Fig. 2).

DISCUSSION

Cutinase has been proposed to mediate two different processes in fungal pathogenesis: penetration of the epidermis (Kolattukudy 1985; Köller 1991) and organ specificity (Trail and Köller 1990). Our results suggest that cutinase is not a limiting factor in penetration and that its pH optimum does not play a major role in organ specificity of *C. heterostrophus*. This fungus (a leaf pathogen) is normally a low producer of cutinase with a pH optimum of 6.5 (Trail and Köller 1990). Causing it to produce large amounts of *N. haematococca* cutinase, which has a pH optimum of 9.5, did not affect the efficiency with which it penetrated the epidermis of either its own host (corn) or a nonhost (pea), even if the fungus was tolerant of the pea phytoalexin pisatin. These negative results, however, must be considered, along with our observation that over-expression of *CUT-T8* (along with *PDA-T9*) in *C. heterostrophus* caused unusually dark-colored lesions on non-wounded pea stems (Fig. 1). To further evaluate the possible role of cutinase in pathogenesis by *C. heterostrophus*, experiments based on disruption and over-expression of *C. heterostrophus* cutinase gene(s) will be performed.

On the issue of organ specificity, it has been noted that fungi which are leaf pathogens produce cutinase with an acidic pH optimum, whereas fungi which are root and stem pathogens produce cutinase with an alkaline pH optimum; fungi which attack both roots and shoots produce both types of cutinase (Köller and Parker 1989; Trail and Köller 1990; Trail and Köller 1993). Moreover, when a stem-specific isolate of the bean pathogen *Rhizoctonia solani* (which produces cutinase with an alkaline pH optimum) was inoculated onto bean leaves in the presence of supplemental cutinase with an acidic pH optimum (from the leaf-specific pathogen *Venturia inaequalis*), disease symptoms were produced; there was no disease if the inoculum was not supplemented, or supplemented with cutinase having an

Table 7. Infection of corn or pea leaves with *Nectria haematococca* or *Cochliobolus heterostrophus*

Strain	Infections/cm² corn leaf[a]	Collapsed pea leaves[b]
T8[c]	0 ± 0	...
77-2-3[c]	...	0/0
C2	7 ± 2	1/0
C2-pUCH1-2	8 ± 1	2/1
C2-CUT-2	8 ± 2	4/2
C2-PDA-2	6 ± 2	15/16
C2-PDA/CUT-2	6 ± 1	12/14

[a] Data are compiled from five assays, which together included all transformants shown in Table 1. Numbers (± standard deviations) indicate averages from all assays.
[b] The two numbers for each strain reflect the results of two independent assays. Each assay included 20 inoculated leaves/strain.
[c] *Nectria haematococca*.

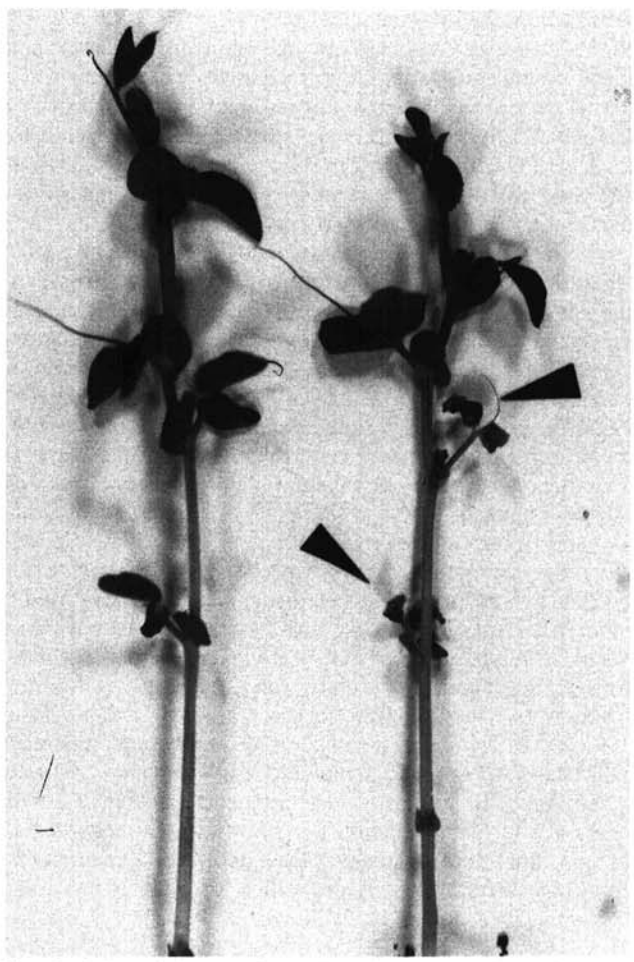

Fig. 2. Collapsed leaves of pea plants inoculated with *Cochliobolus heterostrophus*. Left, control transformant C2-pUCH1 (inoculated leaves, corresponding to positions indicated by arrowheads at right, are unaffected). Right: transformant C2-PDA-2, which over-produces PDA (inoculated leaves, at arrowheads, are completely withered; healthy leaves above arrowheads emerged subsequent to inoculation).

alkaline pH optimum (from *N. haematococca*, a root and stem-specific pathogen) (Trail and Köller 1990).

These results support the hypothesis that the organ specificity of a fungus can be changed if the pH optimum of its predominant cutinase is changed. To test this prediction in our system, we over-expressed cutinase with an alkaline pH optimum (from *N. haematococca*) in *C. heterostrophus*, a leaf pathogen that normally produces cutinase with an acidic pH optimum. Transformants produced at least 100 times more heterologous than native cutinase activity. The hypothesis predicts that the transgenic *C. heterostrophus* strains would now be pathogenic on corn roots, which are not attacked by wild-type *C. heterostrophus*. However, this did not occur. We reasoned that perhaps *N. haematococca* cutinase is not effective on corn, a nonhost of *N. haematococca*. Therefore, we over-expressed *N. haematococca* cutinase in *C. heterostrophus* in combination with PDA, an enzyme that enables *C. heterostrophus* to attack pea, but only leaves and stems. The assumption was that transformants producing both enzymes might be able to attack pea roots. This also did not occur. Thus, our results suggest that cutinase is not a primary determinant of organ specificity in *C. heterostrophus*.

A previous study (Schäfer *et al.* 1989) showed that *C. heterostrophus* could attack pea, but only if it over-produced PDA. This report was based on just one PDA over-producing transformant, prompting concern that the phenomenon may not be generally valid with a larger number of transformants. Here we have shown that seven independently generated PDA (or PDA plus cutinase) over-producing transformants all had increased ability to attack pea (both leaves and stems), thus confirming the previous observation.

Several lines of evidence support the hypothesis that PDA is required by *N. haematococca* for pathogenicity to pea: 1) there is a genetic correlation between the ability of *N. haematococca* to produce PDA, to tolerate pisatin in culture, and to cause disease of pea (VanEtten *et al.* 1989); 2) when a pda-, nonpathogenic strain of *N. haematococca* is transformed with *PDA-T9*, at least 20% of transformants have enhanced ability to attack pea (Ciuffetti *et al.* 1988; L. Ciuffetti and H. D. VanEtten, personal communication); and 3) when *C. heterostrophus* is transformed with *PDA-T9*, all transformants tested are able to attack pea (Schäfer *et al.* 1989; this report). Recently, technology for site-specific gene disruption has become available for *N. haematococca* and has been used to more rigorously assess the role of PDA in pathogenesis. When PDA production by *N. haematococca* was eliminated by specific mutation of a PDA gene, the resulting transformants were still pathogenic to pea (Wassman and VanEtten 1992; H. D. VanEtten, personal communication). This result suggests that PDA is not required by *N. haematococca* for pathogenesis to pea. However, although PDA may not be necessary for pathogenicity, our results with a heterologous fungus imply that PDA can, at least under certain conditions, contribute significantly to the ability of a fungus to cause symptoms on pea.

MATERIALS AND METHODS

Fungal strains.

The *C. heterostrophus* laboratory strain C2 (ATCC 48329; Leach *et al.* 1982), an albino mutant (*alb1*) that has normal virulence to corn but cannot survive in nature (Fry *et al.* 1984), was used as recipient in transformation experiments. Three *N. haematococca* isolates, T8, T30, and 77-2-3 (Miao *et al.* 1991), were provided by H. D. VanEtten. Experiments involving inoculation of pea with *C. heterostrophus* included a *N. haematococca* strain, either T8 or 77-2-3, as a control; the two strains gave similar results under the experimental conditions used for this report. All strains were stored in 25% glycerol at –70° C and were recovered fresh from storage for each experiment.

Molecular techniques and vector construction.

Previously described protocols were followed for fungal (VanWert and Yoder 1992) and bacterial DNA preparation, restriction enzyme digestions, ligation, transformation of *E. coli* strain DH5α, and Southern blots (Ausubel *et al.* 1987). Vectors pUCH1 (pUC18 carrying the *hygB* gene fused to *C. heterostrophus* promoter 1) and pUP1 (pUCH1 carrying the *N. haematococca PDA-T9* gene) have been described (Turgeon *et al.* 1993; Schäfer *et al.* 1989). *CUT-T8*, which was cloned previously (Soliday *et al.* 1984; Soliday *et al.* 1989), was isolated independently by us from a plasmid library of strain T8 using an internal fragment of *CUT-T8* (Stahl and Schäfer 1992) as probe. To distinguish *CUT-T8* from a gene in strain T8 known to encode a minor cutinase (Soliday *et al.* 1989), RFLPs of *CUT* genes in genomic DNAs of strains T8 and T30 were compared with those of the cloned gene. pCN17 carries *CUT-T8* on a 1.8-kb *Acc*I-*Pvu*II fragment inserted in the pUC19 *Sma*I-*Acc*I site. To construct pCNH1, pUCH1 was digested with *Sma*I and *Sac*I and ligated to a 1.8-kb *Sma*I/*Sac*I fragment from pCN17 bearing *CUT-T8*. The *PDA-T9* gene of *N. haematococca*, on a 3-kb *Bam*HI fragment from pUP1, was inserted into pCNH1 to produce pCPNH86, which carries both *CUT-T8* and *PDA-T9*.

Transformation of *C. heterostrophus*.

The transformation protocol described earlier (Yoder 1988) was used, except that the concentrations of the enzymes Novozym, Driselase, and chitinase were reduced 25%; for transformation 70 μg of CsCl purified DNA was added to 5×10^6 to 10^7 protoplasts. Transformants carrying multiple plasmid copies were identified by their high resistance to hygromycin B. When the transformant colonies reached a diameter of 1 cm, each was cut into 25 pieces, which were transferred individually to plates containing complete medium with 1 mg of hygromycin B per milliliter. Those that grew at approximately normal rates were purified and tested for resistance to 1 mg of hygromycin B per milliliter, for good conidiation, and for mitotic stability of the transformed phenotype after five serial transfers to nonselective medium.

Characterization of transformants.

Transformants chosen for analysis grew at the wild-type rate in culture and conidiated normally except where noted. No differences were observed between the behavior of strain C2 vs. strain C2-pUCH1 (control transformant) either in culture or on plants. All transformants expressing the same heterologous gene(s) gave the same result in plant tests, thus allowing one member of a group with the same genotype to represent the entire group; except where noted, the second transformant in each group (Table 1) was used to illustrate the

performance of that group in plant tests (Tables 4–7). No position effects were observed when transformants carrying the same gene at homologous and ectopic sites were compared.

Cutinase production was quantified by growing still cultures (15 ml of Czapek-Dox medium in 300-ml Erlenmeyer flasks) with apple cutin (75 mg) as the sole carbon source. Esterase activity in culture filtrate was determined using *p*-nitrophenylbutyrate as the substrate (Kolattukudy *et al.* 1981). The assay was performed at pH 8.0 (the substrate is unstable at acidic pH), which favors activity of *N. haematococca* cutinase but not native *C. heterostrophus* cutinase (pH optimum 6.5). A specific cutinase assay was not necessary since the goal was to quantify the increase in activity caused by introduction of *CUT-T8* (a single gene known to encode only cutinase) into *C. heterostrophus*. Relative pisatin demethylase activity was determined by measuring colony radii after 2 wk of growth on 1 mM pisatin at 30° C according to methods described previously (Weltring *et al.* 1988).

To estimate integrated vector copy number, genomic DNA of each transformant was digested with *Hin*dIII or *Eco*RI, separated in 1% agarose, transferred to a nylon membrane and probed with ^{32}P-labeled vector. Band intensities were quantified with a densitometer and copy number was calculated by dividing the intensity of signal(s) of the vector by the intensity of both border fragments and adding one.

Plant assays.

The inoculation procedure for roots of corn (W64AN) and pea (Alaska 2B) was modified from that of VanEtten (1978). Clay pots (10 cm diameter) were filled to 75% of capacity with a 1:1:1 mixture of soil, peat, and sand. The soil surface was covered with 35 ml agarose (0.3%) containing 10^7 conidia. Seeds of corn (18 per pot) and pea (9 per pot) were surface sterilized, placed on the agarose, and covered with the soil mixture. After 4 wk of incubation at 30° C in a growth chamber (14 hr light/day), symptoms were noted and fresh weights of roots and shoots were measured. In all cases where symptoms were produced, the fungus was reisolated from the infected tissue and confirmed to have the phenotype of the original inoculum.

The corn leaf assay was modified from that described previously (Yoder and Gracen 1975). Conidia concentration was adjusted to 2×10^4/ml. The number of viable conidia (> 98%) was determined by plating an aliquot of each suspension on agar medium. Ten milliliters of conidial suspension was sprayed on six corn plants growing in a 10-cm pot. Two days after inoculation leaves were stained in 0.1% Calcofluor for 10 min, washed in water for 10 min, and observed under fluorescence optics. The number of infecting conidia per lesion and the number of lesions per square centimeter of leaf surface were determined.

For assays with pea stems, the procedures of VanEtten *et al.* (1980) were modified. Wounded and unwounded pea stems (unexcised) were inoculated with agar cylinders bearing mycelium; symptoms were recorded after 10 days of incubation in a moist chamber.

For assays with pea leaves, plants were grown under the same conditions as for the stem assay except that a light regime (7–8 hr/day) was imposed. Eleven days after planting, leaves were inoculated with 50 µl of conidial suspension (2×10^6 conidia per milliliter of water containing 0.1% Tween 20). The number of collapsed leaves was recorded 7 days after inoculation. This assay is efficient and easily quantified, and is modified from an alternative described elsewhere (Schäfer and Yoder 1994).

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