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Transformation-Mediated Chromosome Loss and Disruption of a Gene for Pisatin Demethylase Decrease the Virulence of *Nectria haematococca* on Pea

Catherine C. Wasmann and Hans D. VanEtten

Department of Plant Pathology, Room 204 Forbes Building, University of Arizona, Tucson, AZ 85721 U.S.A.
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Isolates of the fungus *Nectria haematococca* pathogenic on pea are able to detoxify the phytoalexin pisatin via cytochrome P450-mediated demethylation. To examine the role of pisatin demethylating ability (Pda) in pathogenicity, mutants of *N. haematococca* deficient in Pda were created by transformation-mediated gene disruption (gdr). Eleven Pda$^-$ transformants were obtained as determined biochemically by their inability to demethylate pisatin. The transformants were of two types. Seven of the Pda$^-$ transformants were gdr mutants, while the other four were Pda$^-$ because they had lost the 1.6-Mb chromosome which carries the gene (*PDA1*) for Pda in the recipient isolates. All of the Pda$^-$ mutants were more sensitive to pisatin and less virulent on pea than the Pda$^+$ recipient isolates. However, only the four chromosome-deletion transformants were reduced to virulence equivalent to naturally occurring Pda$^-$ isolates of *N. haematococca*. There was no evidence that the Pda$^-$ gdr mutants contained a cryptic *PDA* gene induced in planta. Lesion tissue produced by these mutants did not demethylate pisatin while that produced by the wild-type isolates did. In addition, the four chromosome-deletion transformants were as virulent on ripe tomato fruit as the wild-type isolate. These results support the hypothesis that (i) lack of Pda reduces but does not eliminate the virulence of *N. haematococca* on pea and (ii) an additional gene(s) required for high virulence on pea but not tomato is located on the dispensable chromosome that contains *PDA1*.

Additional keywords: Fusarium solani, phytoalexin detoxification, B chromosomes, supernumerary chromosomes, *Pisum sativum.*

All isolates of *Nectria haematococca* Berk. & Br. mating population (MP) VI (anamorph: *Fusarium solani*) pathogenic on pea can detoxify the pea phytoalexin pisatin by a cytochrome P450-mediated demethylation (Matthews and VanEtten 1983). Natural isolates of this fungus vary quantitatively in pisatin demethylating ability (Pda) but have one of three whole cell phenotypes: inability to detoxify pisatin (Pda$^-$); production of low levels of Pda after long exposure to pisatin (Pda^L); and rapid, pisatin-induced production of moderate to high levels of Pda (Pda^H) (In previous publications the Pda^L phenotype has been referred to as Pda^n or Pda^{LL} and the Pda^H phenotype as Pda^i, Pda^{SH}, or Pda^{SM}) (Mackintosh et al. 1989). Conventional genetic analyses have identified seven *PDA* genes, three Pda^H genes (*PDA1*, *4*, and *5*) and four Pda^L genes (*PDA2*, *3*, *6-1* and *6-2*). Only Pda^H isolates are highly virulent on pea and, despite numerous attempts, the genetic linkage between virulence and the presence of Pda^H has not been broken in genetic crosses (Mackintosh et al. 1989; Kistler and VanEtten 1984b, Tegtmeier and VanEtten 1982; D. Funnell and H. D. VanEtten, unpublished). Additional evidence in support of a role for Pda in virulence on pea comes from experiments involving the transformation of a *PDA*^{H} gene into Pda$^-$ isolates of MPVI or other fungi. When *PDA*^{H} gene was transformed into the maize pathogen *Cochliobolus heterostrophus,* the chickpea pathogen *Ascochyta rabiei,* or a Pda$^-$ isolate of *N. haematococca* MPVI, the transformants generally made larger lesions on pea than the recipient isolate without the *PDA* gene (Oeser and Yoder 1994; Barz and Welle 1992; Schäfer et al. 1989; Ciuffetti and VanEtten 1996).

During the course of genetic studies to characterize the *PDA* genes, unexpected loss of Pda occurred in some progeny (Miao and VanEtten 1992; Mackintosh et al. 1989). Miao et al. (1991a) demonstrated that in crosses involving *PDA6-1* the unexpected loss of Pda was due to the partial or complete loss of the 1.6-megabase (Mb) chromosome carrying *PDA6-1*. Subsequent studies have indicated that the meiotic instability common to all of the genetically characterized *PDA* genes is due to the loss of dispensable (DS) chromosomes or dispensable regions of the *PDA*-containing chromosomes (Miao et al. 1991b, D. Funnell and H. D. VanEtten, unpublished). While the presence of *PDA* genes on DS chromosomes has important implications for the interpretation of past and present genetic studies, the linkage between the Pda^H phenotype and virulence on pea in these studies had remained unbroken (VanEtten et al. 1994; D. Funnell and H. D. VanEtten, unpublished). In the present study we have broken the linkage between Pda^H and pathogenicity on pea by using transformation-mediated gene disruption to construct Pda$^-$ mutants in three virulent isolates of *N. haematococca.*

RESULTS

Transformation-mediated gene disruption and chromosome loss.

To construct the gene disruption vector, an internal region of the *PDA1* homolog, *PDAT9* (Fig. 1; Maloney and VanEtten

Corresponding author: H. D. Van Etten, email: vanetten@ag.arizona.edu

1994), was replaced with a hygromycin resistance gene (*hph*) fused to the glucoamylase promoter (*glaA*) of *Aspergillus niger* and the *A. nidulans trpC* transcription terminator to form pKO1. In pKO1, the *glaA-hph-trpC* chimera (hyg^R) is flanked by 692-bp 5′ and 888-bp 3′ of the *PDAT9* gene, thus recombination between the *PDAT9* fragments and *PDA1* in the recipient fungus should result in replacement of a 657-bp fragment of *PDA1* with hyg^R. Ascospore isolates of *N. haematococca* (77-13-4, 77-13-5, and 77-13-7) containing a single *PDA* gene and virulent on pea were transformed with the circular form of pKO1. Transformants were selected on hygromycin and screened for their ability to demethylate pisatin. Of the 431 (171 of 77-13-4, 69 of 77-13-5 and 191 of 77-13-7) transformants tested, 11 (five of 77-13-4, three of 77-13-5, three of 77-13-7) were Pda^-.

Genomic DNA of recipients and transformants was analyzed by Southern hybridization after digestion with *Xho*I, which does not digest within *PDA1* or hyg^R, and hybridized with the 1,474-bp *Eco*RV fragment of *PDAT9* (Fig. 1B). The *Eco*RV probe hybridized to the 7.1-kb fragment containing *PDA1* (Maloney and VanEtten 1994) in the untransformed parent isolates 77-13-7 (Fig. 2A), 77-13-5, and 77-13-4 (not shown) and to this same-sized fragment as well as one of greater than 20 kb in Tr1.1 (Fig. 2A), a Pda^+ transformant. The hybridization pattern of Tr1.1 is consistent with ectopic integration of pKO1. A precise gene replacement in which a single copy of the hygromycin resistance gene is substituted for the internal fragment of *PDA1* should yield an *Xho*I fragment of 8.7 kb in the Pda^- transformants. Only DNA from Tr7.8 contained a fragment of approximately 8.7 kb that hybridized with the *PDAT9* probe. Additional analyses (described below) indicated that the Pda^- transformants can be divided into two groups: One group consists of Pda^- transformants which result from the integration of one to several cop-

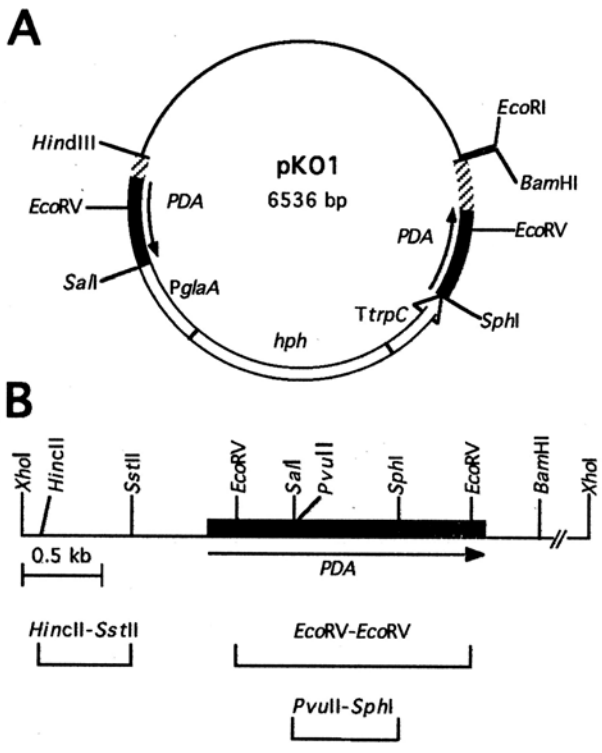

Fig. 1. Restriction map of pKO1 and the *PDAT9* gene. **A,** Restriction map of pKO1. Solid bars represent the coding sequence of *PDAT9*; cross hatched bars represent regions flanking the coding sequence of *PDAT9*. The open bar represents the hygromycin resistance gene (*hph*), *glaA* promoter (P*glaA*) and *trpC* terminator (T*trpC*) which have replaced a portion of the *PDAT9* coding sequence. The *Hin*dIII and *Eco*RI restriction sites derive from the polylinkers of plasmids used to construct pKO1. **B,** Partial restriction map of the 7.1-kb *Xho*I fragment of *N. Haematococca* genomic DNA containing the *PDAT9* gene and its flanking regions. The solid bar represents the genomic copy of the coding sequence of *PDAT9*. Positions of the DNA fragments of *PDAT9* used as probes in Southern hybridizations are shown below the diagram. Not all restriction sites are shown.

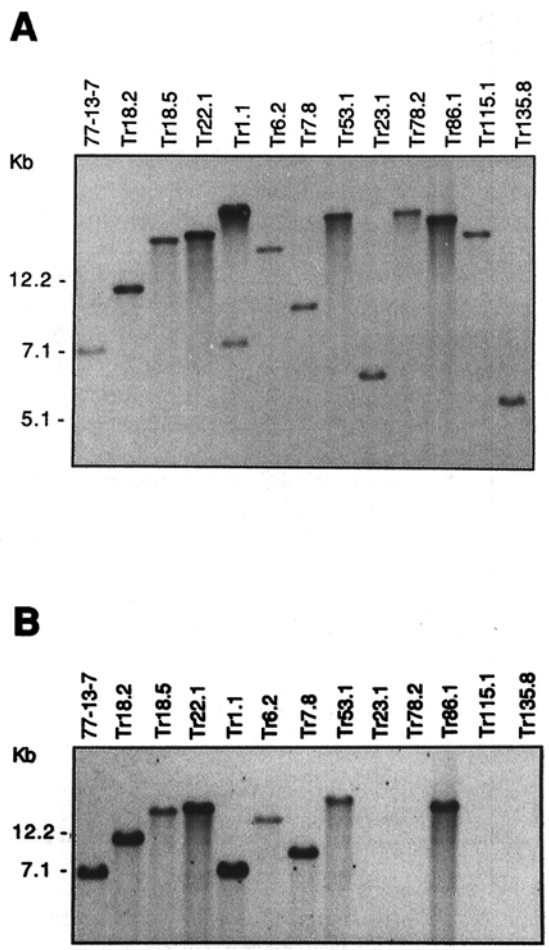

Fig. 2. DNA gel blot analysis of the transformants and a recipient isolate of *N. haematococca*. **A,** DNA gel blot analysis of *Xho*I-digested genomic DNA from a parent isolate, 77-13-7, and transformants probed with the internal *Eco*RV fragment of *PDAT9*. The positions of DNA molecular weight (Kb) markers are indicated to the left. **B,** DNA gel blot analysis of a *Xho*I-digested genomic DNA from a recipient isolate, 77-13-7, and transformants probed with a *Hinc*II-*Sst*II fragment from the 5' untranslated region of *PDAT9*. The positions of DNA molecular weight (Kb) markers are indicated to the left.

ies of pKO1 at the *PDA1* locus; the second group consists of transformants which are Pda⁻ because they have lost the 1.6-Mb chromosome containing *PDA1*.

A single hybridizing *Xho*I fragment larger than 8.7 kb would be expected if there had been integration of more than one copy of pKO1 at the *PDA1* locus but two of the Pda⁻ transformants (Tr23.1 and Tr135.8) had hybridizing *Xho*I fragments smaller than 7.1 kb (Fig. 2A). None of the Pda⁻ transformants hybridized with the 627-bp *Pvu*II-*Sph*I fragment of *PDAT9* (Fig. 1B) which is predicted to be absent if a gene replacement had occurred, indicating that this internal portion of *PDA1* is absent in all the Pda⁻ transformants (data not shown).

To determine whether the DNA flanking *PDA1* was altered in the Pda⁻ transformants, the *Xho*I digested DNA was hybridized with a 579-bp *Hinc*II-*Sst*II fragment (Fig. 1B) from the 5′ region flanking *PDA1*. In *Xho*I-digested DNA from 77-13-7 and seven of the Pda⁻ transformants (Tr18.2, Tr18.5, Tr22.1, Tr6.2, Tr7.8, Tr53.1, and Tr86.1) the *Hinc*II-*Sst*II fragment hybridized to the same sized *Xho*I fragments as hybridized to the *Eco*RV fragment (Fig. 2B), indicating that the 5′ region flanking *PDA1* is intact. Hybridization of *Xho*I-digested DNA of these transformants with a fragment of the hygromycin resistance gene produced the same pattern (data not shown). The hybridization pattern of these seven transformants is thus consistent with their being Pda⁻ because of a gene disruption (gdr) at the *PDA1* locus resulting from the integration of multiple copies (except in Tr7.8) of the transforming DNA at that locus. The *Hinc*II-*Sst*II fragment hybridized to the 7.1-kb *PDA1*-containing *Xho*I fragment in the Pda⁺ transformant, Tr1.1, but did not hybridize to the approximately 20-kb *Xho*I fragment that hybridized with the *Eco*RV fragment. This pattern of hybridization is again consistent with an ectopic integration of pKO1 in Tr1.1.

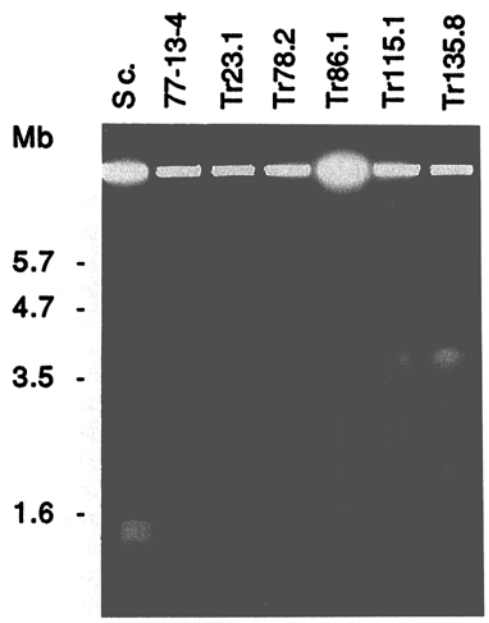

Fig. 3. PFGE analysis of 77-13-4 and the Pda⁻ transformants of 77-13-4. Chromosome-sized DNAs from 77-13-4 and Tr23.1, Tr78.2, Tr86.1, Tr115.1 and Tr135.8 were resolved by PFGE. Chromosomes of *Saccharomyces cerevisiae* (Sc) are shown to the left. The approximate sizes of some of the chromosomal DNAs are indicated to the left of the figure.

The DNA of the other four Pda⁻ transformants (Tr23.1, Tr78.2, Tr115.1, and Tr135.8) did not hybridize to the *Hinc*II-*Sst*II fragment (Fig. 2B), a result consistent with deletion of part of the region surrounding the *PDA* gene. All four of these are transformants of 77-13-4. To gain additional information on the extent of the loss which occurred in the four Pda⁻ transformants that had lost DNA flanking the *PDA1* gene, the chromosome-sized DNAs (ChDNAs) of the transformants and recipient isolates were separated by pulsed-field gel electrophoresis (PFGE). The recipient isolates 77-13-4, 77-13-5, and 77-13-7, Tr1.1, all of the Pda⁻ transformants of 77-13-5 and 77-13-7 and the single gdr Pda⁻ transformant of 77-1-34 contained the same ChDNAs as their recipient isolates (Fig. 3, data not shown). In contrast, the four Pda⁻ transformants (Tr23.1, Tr78.2, Tr 115.1, and Tr135.8) of isolate 77-13-4 that lacked the DNA flanking *PDA1* were found to lack a 1.6-Mb ChDNA that is present in 77-13-4 (Fig. 3) and is known from previous studies (Miao et al. 1991b) to carry *PDA1*. No other alterations were observed in the karyotypes of these transformants. Thus, these transformants are Pda⁻ because they have lost the *PDA1*-bearing chromosome rather than because of a *PDA1*-specific gene disruption. Transformation-induced loss of chromosomes by *N. haematococca* has been reported previously (Kistler and Benny 1992).

Table 1. Growth in the absence and presence of pisatin and numbers of microconidia and macroconidia produced by the parent isolates, the transformants and ascospore isolates

Isolate	Pda	Growth[a] (mm)	Inhibition[b] (%) 160 µg pisatin/ml	Microconidia[c] (×10⁴)	Macroconidia[c] (×10⁴)
77-13-7	+	11.4 (0.5)	27	73 (13)	88 (17)
Tr18.2	–	11.8 (0.4)	64	91 (25)	91 (18)
Tr18.5	–	12.0 (0.0)	65	92 (30)	91 (11)
Tr22.1	–	11.0 (0.0)	60	61 (13)	72 (6)
Tr1.1	+	12.0 (0.0)	31	66 (15)	82 (12)
77-13-5	+	11.0 (0.0)	36	67 (15)	88 (7)
Tr6.2	–	11.5 (0.7)	59	114 (22)*	66 (5)
Tr7.8	–	11.5 (0.7)	59	117 (6)*	78 (13)
Tr53.1	–	11.3 (1.1)	58	121 (20)*	83 (15)
77-13-4	+	11.5 (0.7)	30	88 (31)	75 (21)
Tr23.1	–	12.3 (0.4)	59	130 (23)*	18 (12)
Tr78.2	–	12.0 (0.0)	63	126 (62)*	25 (19)
Tr86.1	–	10.8 (0.4)	65	113 (18)*	12 (7)
Tr115.1	–	12.5 (0.7)	58	95 (17)*	26 (9)
Tr135.8	–	12.5 (0.7)	56	112 (47)*	29 (5)
44-100	–	13.5 (0.7)	58	59 (6)	92 (15)
156-2-1	–	Not tested	Not tested	44 (6)	252 (41)

[a] The radii of the mycelial colonies after 3 days of growth on medium without pisatin. Entries are the average of two experiments. Numbers in parentheses are the standard deviations of the means (n = 2).

[b] Entries are the % inhibition of growth of each test isolate on pisatin compared to the growth in the absence of pisatin.

[c] Conidia were collected from 5-day-old cultures grown on solid medium lacking pisatin as described in the Materials and Methods. The numbers in parentheses are the standard deviations of the means (n = 2). Values which differ significantly ($P \leq 0.05$) from that for the corresponding recipient are denoted by an asterisk.

Growth characteristics and pisatin sensitivity of the Pda⁻ transformants.

All the Pda⁻ transformants had a similar growth rate and were inhibited similarly and to a greater degree by pisatin than were the Pda⁺ transformant and recipient isolates (Table 1). The degree of inhibition by pisatin was similar to that of a Pda⁻ ascospore isolate (44-100) from a laboratory cross. The colony morphologies of the Pda⁺ and Pda⁻ transformants of 77-13-7 and 77-13-5 were indistinguishable from their untransformed counterparts on the media used in this study. All of the transformants of 77-13-4 produced more aerial mycelia than 77-13-4.

Since conidiation in this fungus is sensitive to subtle differences in physiological conditions (Dietert et al. 1983), it was used as an indicator of changes, beyond the loss of Pda, which might have occurred in the genomes of the transformants. The number and type of conidia produced after growth for 5 days on potato dextrose agar at 27°C in the dark were determined. All isolates produced both microconidia and macroconidia and the numbers produced by all the transformants of 77-13-7 were similar to the recipient isolate (Table 1; $P \leq 0.05$ by Student's t test; Sokal and Rohlf 1981). Transformants of 77-13-5 made the same number of macroconidia ($P \leq 0.05$) but more microconidia than 77-13-5 (Table 1; $P \leq 0.05$). While all the transformants of 77-13-4 produced different numbers of both microconidia and macroconidia than 77-13-4, the total numbers of conidia produced were similar to 77-13-4 (Table 1; $P \leq 0.05$).

Table 2. Virulence of the transformants, parent isolates and Pda⁻ ascospore isolates on pea and ripe tomato fruit

		Pea		Tomato
Isolate	Pda[a]	Mean length[b] (mm)	Reduction[c] (%)	Mean diameter[d] (mm)
77-13-7	+	11.8 (2.4)		23.8 (6.4)
Tr18.2	–	9.0 (2.1)*	23.7	24.0 (5.3)
Tr18.5	–	9.4 (2.1)*	20.3	23.5 (3.3)
Tr22.1	–	9.4 (2.3)*	20.3	NT
Tr1.1	+	13.3 (3.3)	–11.3	25.5 (4.7)
156-2-1	–	4.0 (1.1)		NT
44-100	–	3.9 (0.3)		NT
T488		NT[f]		0.37 (0.64)
Blind 1[e]				
77-13-7	+	13.8 (4.1)		
Tr18.5	–	8.7 (2.7)	37.0	
Blind 2[e]	–			
77-13-7	+	16.6 (7.4)		
Tr18.5	–	9.0 (1.9)	46.0	
77-13-5	+	13.9 (3.2)		NT
Tr6.2	–	8.2 (1.8)*	41.0	NT
Tr7.8	–	8.6 (2.3)*	38.1	NT
Tr53.1	–	8.4 (1.3)*	40.0	NT
156-2-1	–	4.0 (1.1)		NT
44-100	–	3.4 (1.3)		NT
77-13-4	+	9.7 (2.3)		18.4 (5.7)
Tr23.1	–	4.2 (0.6)*+	56.7	15.3 (5.3)
Tr78.2	–	3.7 (1.1)*+	61.9	18.1 (4.2)
Tr86.1	–	6.5 (1.3)*	33.0	16.5 (3.7)
Tr115.1	–	3.3 (0.7)*+	66.0	17.8 (3.4)
Tr135.8	–	3.7 (0.8)*+	61.9	14.1 (4.0)
156-2-1	–	3.3		NT
44-100	–	4.3 (1.1)		NT
T488		NT		3.4 (4.7)

[a] Pda phenotype.

[b] Virulence assays on pea stems were performed as described in Materials and Methods with the exception that the blind assay performed by experimenter 1 utilized the dwarf pea variety Little Marvel. Entries are the averages of the mean lengths of the lesions produced in nine experiments (77-13-7, Tr18.5, Tr18.2, Tr1.1), six experiments (Tr22.1, 77-13-5, Tr6.2, Tr7.8, Tr53.1) or, seven experiments (77-13-4, Tr23.1, Tr78.2, Tr86.1,Tr115.1, Tr135.8). Values differing significantly ($P \leq 0.05$) from that of the corresponding recipient are denoted by an asterisk Values differing significantly ($P \leq 0.01$) from that of the gdr Pda⁻ transformant Tr86.1 are denoted by +.

[c] Entries are the % reduction in the mean lesion size of the transformant relative to the corresponding recipient isolate.

[d] Virulence assays on ripe tomato fruit were performed as described in Materials and Methods. Entries are the averages of the diameters of the lesions produced in three experiments. Numbers in parentheses are the standard deviations of the means.

[e] Mean sizes of the lesions produced in a "blind" pea stem bioassay performed by two experimenters. Entries are the results obtained in one assay (blind 1) or two assays (blind 2). The numbers in parentheses are the standard deviations of the means.

[f] Not tested.

Virulence of the Pda⁻ transformants.

Virulence was assessed by the "test tube" virulence assay as described previously (VanEtten et al. 1980). The Pda⁻ gdr mutants produced lesions that were on average 20 to 47% shorter than those produced by the corresponding recipient isolate (Table 2). These differences were significant by Student's t test at $P \leq 0.05$ (Sokal and Rohlf 1981). Lesions produced by Tr1.1 were not significantly different in size than those produced by 77-13-7. The differences in the mean lengths of the lesions produced by the gdr transformants and their respective recipient isolates also were significant ($P < 0.0156$) when compared using the nonparametric Wilcoxan signed ranks test (Sokal and Rohlf 1981). In addition to being smaller than the lesions produced by the recipient isolates, the lesions produced by these gdr Pda⁻ transformants were qualitatively different from those produced by the recipients. While the recipients generally made lesions that were dark brown and often water-soaked, the transformants made lesions that were light brown and tended to be dry with a cork-like appearance (Fig. 4A, data not shown). Transformant Tr18.5, the first characterized Pda⁻ gdr transformant, has been tested most extensively and the reduction in its virulence has been verified in "blind" pea stem bioassays (Table 2).

In contrast to the moderate reduction in virulence of the gdr Pda⁻ transformants, the four Pda⁻ transformants that had lost the 1.6-Mb chromosome were greatly reduced in virulence and produced only small (3.3 to 4.2 mm) light tan lesions resembling those produced by 44-100, a Pda⁻ ascospore isolate (Table 2; Fig. 4A). Since there was a gdr Pda⁻ transformant (Tr86.1) of 77-13-4, the virulence of the four Pda⁻ transformants that had lost the chromosome were compared with both the recipient isolate and Tr86.1. The mean lengths of the lesions produced by the chromosome-deficient transformants were 57 to 66% shorter than those produced by 77-13-4 and 35 to 49% shorter than those produced by Tr86.1 (Table 2) and the differences in the mean lengths of the lesions produced were significant at $P \leq 0.01$ by Student's t test. When the differences were compared using the Wilcoxan signed ranks test, they were different with a level of significance similar to that obtained using Student's t test ($P = 0.0078$).

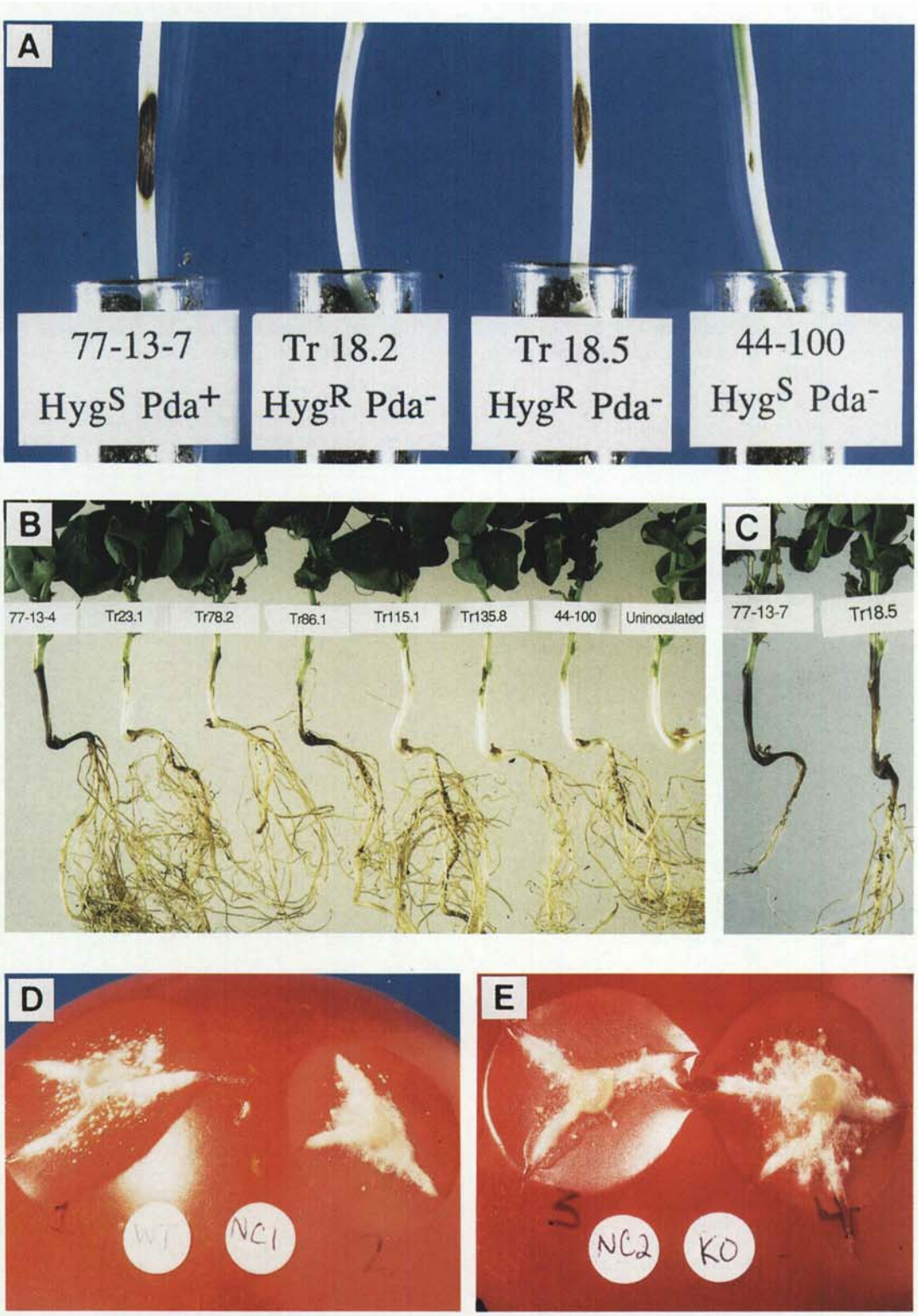

Fig. 4. Virulence of selected recipient isolates and transformants on pea and tomato fruit. **A,** Virulence on pea of a Pda$^+$ ascospore isolate (77-13-7), a Pda$^-$ ascospore isolate (44-100), and gdr Pda$^-$ mutants of 77-13-7 (Tr18.2 and Tr18.5). Lesions were produced using the protocol for the test tube assay of virulence and the plants were photographed 6 days after inoculation. **B, C** Virulence on pea of Pda$^+$ recipients (77-13-4, 77-13-7), a Pda$^-$ ascospore isolate (44-100), Pda$^-$ gdr mutants (Tr86.1, Tr18.5) and Pda$^-$ chromosome deletion mutants (Tr23.1, Tr78.2, Tr115.1, Tr135.8). Plants were photographed 23 days after inoculation in a pot asssay of virulence. **D, E** Virulence on ripe tomato fruit of a natural Pda$^+$ isolate (WT, 77-13-4), a gdr Pda$^-$ mutant of 77-13-4 (KO, Tr86.1) and two Pda$^-$ chromosome deletion mutants of 77-13-4 (NC1, Tr23.1; NC2, Tr78.2). The fruit were photographed six days after being inoculated as described in the Materials and Methods.

Some of the transformants were also tested for virulence in a "pot assay" in which pea seedlings are inoculated with a spore suspension shortly after emergence, and a qualitative assessment of disease is made 4 weeks later (VanEtten 1978). Both the Pda⁻ gdr mutants and the Pda⁺ transformant caused symptoms with a disease rating similar to those produced by the recipient isolate 77-13-7 (Table 3; Fig. 4B, C) but the disease-producing abilities of the transformants which had lost the 1.6-Mb chromosome were less than those of the gdr Pda⁻ mutant (Tr86.1) and markedly less than either their recipient isolate 77-13-4 or Tr18.5 and its recipient isolate 77-13-7 (Fig. 4B, C). Uninoculated control plants and plants inoculated with 44-100, a Pda⁻ ascospore isolate, were free of lesions. Decreasing the amount of inoculum or incubating the plants for a shorter period of time after inoculation did not markedly change the relative disease rating from that obtained under standard conditions (Table 3).

We sought to determine whether Pda might provide a competitive advantage in planta by inoculating the stems of pea plants in a test tube virulence assay with mycelial plugs grown from spore suspensions containing different ratios of a gdr Pda⁻ transformant (Tr18.5) and its corresponding recipient isolate (77-13-7) or the Pda⁺ transformant Tr1.1. Six days after inoculation the lesions were excised and conidia allowed to develop on the lesion tissue. The ratios of Pda⁻ and Pda⁺ isolates recovered from conidia were similar to the inocula ratios (Table 4).

To determine whether detectable changes in the molecular structure of the *PDA* locus occurred in planta, Southern analysis was carried out on one reisolate each of Tr18.2 and Tr1.1 and on two reisolates of Tr18.5, each from a different lesion. The pattern of hybridization of *Eco*RV-digested DNA probed with the *Eco*RV fragment of *PDAT9* was identical to that before passage through the plant (data not shown).

Table 3. Virulence of selected transformants, parent isolates and Pda⁻ ascospore isolates on pea plants as evaluated by a pot virulence assay

	Disease rating[a]							
					Exp. 4			
Isolate	Exp. 1	Exp. 2	Exp. 3	Exp. 5	10^4	10^5	10^6	10^7
77-13-7	9.3	7.5	8.5	NT	5.5	4.0	9.0	10.0
Tr18.2	8.8	7.3	NT	NT	NT	NT	NT	NT
Tr18.5	8.5	7.8	6.0	NT	3.0	6.5	8.5	9.5
Tr1.1	9.3	8.3	NT	NT	NT	NT	NT	NT
77-13-4			8.0	10.3				
Tr23.1			1.0	0.5				
Tr78.2			3.5	1.0				
Tr86.1			4.5	5.3				
Tr115.1			1.0	0.5				
Tr135.8			1.0	1.0				

[a] Six- to eight-day-old pea seedlings grown five to a pot were inoculated with 25 ml of a spore suspension at 10^7 spores/ml (experiments 1, 2, 3, and 5) or with a range of spore concentrations (experiment 4). Disease symptoms were evaluated at 4 weeks (experiments 1, 2, 4, and 5) or at 3 weeks (experiment 3). Entries are a numerical ranking of the severity of the symptoms and are the averages of the scores for four pots (experiments 1, 2, and 5) or two pots (experiments 3 and 4). Scores represent the range of symptom severity with a maximum of 11 (all plants damped off), and a minimum of 0 (no lesions or lesions < 1 mm) (VanEtten 1978). Each experiment also included uninoculated plants and plants inoculated with 25 ml of a spore suspension at 10^7 spores/ml of either of two Pda⁻ ascospore isolates, 44-100 or 156-2-1, all of which produced a disease rating of 0.

Pisatin content in lesion tissue and expression of Pda in planta and in vitro.

Pisatin concentration was measured in lesions caused by gdr Pda⁻ transformants Tr18.2 and Tr18.5, and compared to that found in lesions made by 77-13-7, Tr1.1, and a Pda⁻ ascospore isolate, 156-2-1. Substantially more pisatin accumulated in the lesions produced by the Pda⁻ transformants and the Pda⁻ ascospore isolate than in lesion tissue of the Pda⁺ recipient or the Pda⁺ control (Table 5). The tissue immediately adjacent to lesions produced by all isolates contained similar amounts of pisatin.

To determine whether cryptic pda activity is induced in the Pda⁻ transformants in planta, excised tissue from 2-day-old lesions was incubated in the presence of radiolabeled pisatin. Tissue from lesions produced by the wild type Pda⁺ isolate 77-13-7 or the Pda⁺ transformant Tr1.1 was capable of degrading

Table 4. Pda phenotypes of isolates recovered from lesions on plants that had been inoculated with mixtures of a Pda⁻ transformant and either a Pda⁺ transformant or the Pda⁺ recipient isolate

		Pda Phenotype[b]	
Inoculum	Mean lesion length[a] (mm)	Pda⁻	Pda⁺
Experiment 1			
77-13-7	9.9 (3.0)		
Tr1.1	8.1 (4.0)		
Tr18.5	7.7 (2.3)		
Tr18.5:77-13-7 (1:1)	9.6 (3.4)	22	19
Tr18.5:Tr1.1 (1:1)	6.0 (1.9)	23	19
Experiment 2			
77-13-7	Not tested		
Tr18.5	10.8 (1.6)		
Tr1.1	15.8 (4.5)		
Tr18.5:Tr1.1 (1:1)	12.3 (1.5)	9	12
(1:10)	11.3 (2.2)	0	44
(10:1)	12.0 (1.7)	35	4

[a] Mean lengths of the lesions produced on pea plants six days after inoculation with spores of individual isolates or with a mixture of two isolates in the ratios indicated. Numbers in parentheses are standard deviations of the means.

[b] Numbers of each phenotype isolated from the lesions incited by the indicated mixutres of Pda⁻ and Pda⁺ isolates.

Table 5. Pisatin recovered from lesions and adjacent tissue after inoculation with Pda⁺ and Pda⁻ transformants, the Pda⁺ recipient isolate or a Pda⁻ ascospore isolate[a]

	Mean lesion	Pisatin (μg/g fresh weight)	
Isolate	length (mm)[b]	Lesion tissue	Adjacent tissue[c]
Experiment 1			
77-13-7	9.6 (4.1)	76	174
Tr18.2	8.9 (4.1)	7,644	676
Tr18.5	6.9 (3.0)	4,192	120
Tr1.1	9.5 (4.9)	48	331
156-2-1	3.2 (1.8)	6,138	286
Experiment 2			
77-13-7	18.6 (5.4)	282	103
Tr18.2	11.4 (1.2)	2,371	76
Tr18.5	11.4 (2.2)	3,761	178
Tr1.1	20.6 (5.1)	323	33
156-2-1	5.4 (1.2)	674	23

[a] Tissue for extraction of pisatin was produced using the test tube assay of virulence and was collected 6 days after inoculation.

[b] Mean sizes of the lesions produced in each experiment. The numbers in parentheses are standard deviations.

[c] Tissue adjacent to a lesion was collected from the border of the lesion to about four mm from the lesion border.

the added pisatin (Table 6). Uninoculated tissue and tissue from lesions produced by the Pda⁻ transformant Tr18.5 did not degrade pisatin as the decline in [^{14}C] pisatin was similar to that observed in autoclaved tissue.

The assay routinely employed to measure Pda detects the loss of [^{14}C] from pisatin labeled with [^{14}C] at the 3-*O* methyl group of pisatin (VanEtten et al. 1980). To exclude the possibility that the Pda⁻ transformants were able to degrade pisatin by another means not detected by this assay, Tr18.5 and 77-13-7 were grown in glucose asparagine medium amended with 12 μg of pisatin per ml and, after 24 h, the pisatin content of the medium was quantified by reverse-phase HPLC. All of the pisatin added to the culture inoculated with Tr18.5 was recovered and no pisatin was detected in the culture inoculated with 77-13-7 (data not shown).

Virulence on tomato fruit.

To determine whether the loss of Pda and of the *PDA*-bearing 1.6-Mb chromosome affected the virulence of these transformants on plants other than pea, some of the transformants were tested for virulence on ripe tomato fruit (Table 2). The differences in the mean diameters of the lesions produced by the transformants and recipient isolates were not significant at $P = 0.05$ by Student's t test. In contrast to pea where in seven assays consisting of eight plants inoculated per isolate, Tr23.1, Tr78.2, Tr115.1, and Tr135.8 never produced a large lesion (largest lesion produced, 5.2 mm), these transformants were able to make lesions on tomato fruit as large (>18.4 mm) as the average size lesion made by Tr86.1 and 77-13-4 (Table 2, Fig. 4, data not shown). T488, a *N. haematococca* MPI isolate and a pathogen of cucurbits, was unable to make large lesions on tomato (Table 2, data not shown).

DISCUSSION

In this study seven Pda⁻ transformants from three isolates were obtained in which *PDA1* had been mutated by the integration of transforming DNA. For three of these gdr Pda⁻ mutants, there was no indication that additional changes had occurred elsewhere in the genome. However, the ratio of macroconidia to microconidia produced (Table 1) by the other four gdr transformants and the colony morphology of one (Tr86.1) are of alterations in addition to the mutation of the *PDA1* locus. Nevertheless, all of the gdr Pda⁻ mutants showed a similar reduction in the size of lesion they produced on pea stems (Table 2), indicating that the one feature common to all the gdr transformants, a nonfunctional *PDA1* gene, was the reason for their reduced virulence.

Table 6. Determination of Pda in planta

Tissue	cpm Recovered[a]		
	0 h	24 h	36 h
Experiment 1			
Uninoculated		3,272 (71)	3,168 (21)
77-13-7 lesion		1,498 (153)	1,162 (392)
Tr18.5 lesion		3,421 (56)	3,290 (60)
Tr1.1 lesion		3,103 (30)	1,023 (155)
Autoclaved	3,578 (31)	3,394 (128)	3,131 (238)
No tissue	3,602[b]	3,591[b]	
Experiment 2			
Uninoculated		2,956 (11)	2,927 (167)
7-13-7 lesion		2,994 (15)	1,708 (408)
Tr18.5 lesion		3,301 (44)	3,260 (23)
Tr1.1 lesion		2,809 (228)	1,565 (251)
Autoclaved	3,440 (9)	3,093 (82)	2,593 (148)
No tissue	3,458 (51)	3,449 (24)	

[a] Fifty milligrams of lesion tissue was incubated in the presence of 31 μg/ml (.1mM) [^{14}C] labeled pisatin for the specified amount of time and the radioactivity measured. Entries are the averages of two samples. Numbers in parentheses are standard deviations.
[b] Single sample.

Transcripts of *PDA1* can be detected in infected pea tissue as early as 12 h after inoculation with isolate 77-2-3 (Hirschi and VanEtten 1996), indicating that the fungus has the ability to degrade pisatin shortly after invasion and colonization. The lower concentrations of pisatin found in the lesions produced by the Pda⁺ isolates compared to the Pda⁻ isolates (Table 5) and the observation that lesion tissue from Pda⁺ isolates contains demethylating activity (Table 6) are also consistent with pisatin detoxification occurring in planta in lesion tissue produced by the Pda⁺ isolates. However, despite the apparent functioning of this detoxification during infection and the observation that the loss of Pda reduced the virulence of *N. haematococca* on pea, it is clear that the gdr Pda⁻ mutants remain pathogenic on pea and are more virulent than natural Pda⁻ isolates or Pda⁻ isolates produced in laboratory crosses (Tables 2 and 3; Mackintosh et al. 1989; Kistler and VanEtten 1984b; Tegtmeier and VanEtten 1982; D. Funnell and H. D. VanEtten, data not shown).

The retention of the ability of the gdr mutants to cause disease on pea appears to conflict with the results of earlier genetic studies, which had unfailingly linked high virulence and the Pda^{H} phenotype. In light of the work of Miao et al. (1991a), demonstrating that some *PDA* genes are located on dispensable (DS) chromosomes in *N. haematococca,* we proposed that the results of the earlier genetic studies can be explained by the presence on the *PDA*^{H}-containing DS chromosomes of additional pea pathogenicity loci that are required, but not sufficient, for full virulence (VanEtten et al. 1994). We proposed that these additional genes (*PEP*^{D} = pea pathogenicity, dispensable chromosome) are missing from all naturally occurring Pda⁻ isolates or Pda⁻ isolates resulting from crosses of *N. haematococca* because they lack this DS chromosome or the portion of the chromosome containing *PDA*^{H} and the *PEP*^{D} gene(s). Thus, the genetic linkage between *PDA*^{H} and high virulence on pea occurred not only because the Pda^{H} and Pda⁻ isolates differ with respect to the presence of the *PDA*^{H} gene, but because the Pda⁻ isolates also lacked *PEP*^{D} (VanEtten et al. 1994).

The virulence of the Pda⁻ transformants of 77-13-4 is consistent with our model. *PDA1* is located on a 1.6-Mb chromosome (Miao et al. 1991b) and the gdr Pda⁻ transformant Tr86.1 retains the 1.6-Mb chromosome (Fig. 3). While Tr86.1 is less virulent on pea than 77-13-4, it is more virulent than the four Pda⁻ transformants of 77-13-4 which have lost the 1.6-Mb chromosome (Tables 2 and 3) and are similar in virulence to Pda⁻ field and ascospore isolates (VanEtten et al. 1980; Tegtmeier and VanEtten 1982; Table 2, Fig. 3).

Additional observations that support the hypothesis that *PDA*^{H} genes are located on DS chromosomes along with *PEP*^{D} gene(s) are the following: (i) Examination of low virulence Pda⁻ progeny from a previously reported cross (cross 44) and the parents of 77-13-4 (Kistler and VanEtten 1984b)

showed that they lack the *PDA1*-containing DS chromosome present in the Pda$^+$ parent and progeny (D. Funnell and H. D. VanEtten, data not shown). (ii) Loss of the DS chromosome can be induced in vegetative cells of Tr18.5 by growth in the presence of benomyl (S. Jorgenson and H. D. VanEtten, unpublished). All 12 of the DS chromosome-minus strains produced by the benomyl treatment are low in virulence (lesions <3 mm). These isolates are also benomyl-tolerant and grow more slowly than untreated Tr18.5, suggesting that changes in addition to loss of the DS chromosome have occurred. However, while benomyl-tolerant isolates that retained the 1.6-Mb chromosome grew slowly, they retained their virulence on pea (S. Jorgenson and H. D. VanEtten, data not shown). (iii) Kistler et al. (1996) have truncated about 100 kb of the DS chromosome containing *PDA1* in Tr18.5 by using a transformation vector containing telomeres and a portion of *PDA* to cause chromosome breakage at the *PDA* locus. Transformants, which are Pda$^-$, with truncated chromosomes are as low in virulence (<3 mm lesions) as natural Pda$^-$ isolates, implying that the *PEP*^{D} gene(s) are located in the deleted 100-kb region along with *PDA1*. All of these isolates grow normally in culture.

The four transformants of 77-13-4 that have lost the 1.6-Mb chromosome retain their pathogenicity on ripe tomato fruit (Table 2, Fig. 4) implying that genes needed for pathogenicity on this tissue are not on this chromosome and that loss of the *PDA1*-chromosome does not affect the general vitality of this organism. The ability of the chromosome-minus transformants to make lesions on tomato as large as 77-13-4 contrasts sharply with their apparent inability to do so on pea where, in seven assays involving a total of 56 plants per isolate, none has been observed to make a lesion approaching the average size of the lesions produced by 77-13-4. Taken together, the above data are consistent with the presence of an additional gene(s) on the *PDA1*-chromosome that is essential for high virulence on pea.

The moderate effect that the lack of Pda had on the virulence of the gdr transformants of *N. haematococca* is also consistent with the relatively moderate effect observed when Pda is transformed into *PDA*-chromosome-deficient (thus, Pda$^-$) isolates of *N. haematococca* MPVI (Ciuffetti and VanEtten 1996). Some of these Pda$^+$ transformants can make lesions on pea epicotyls that are more than twice as large as the lesions made without Pda, but none of these transformants produced large lesions.

The observation that *PDA1* and *PEP*^{D} may be linked to other genes conditioning pathogenicity on pea and are contained on DS chromosomes suggests a parallel between the DS chromosomes of this fungus and dispensable genetic elements in other plant-associated microorganisms. The Ti plasmid of *Agrobacterium tumefaciens* carries genes which condition the ability to colonize and infect specific host plants; without the Ti plasmid this bacterium exists as a soil saprophyte (Zambryski 1992). A similar phenomenon may exist for *N. haematococca* MPVI (VanEtten et al. 1994). Although some isolates of this MP are pathogens of pea (*Pisum sativum* L.) and such isolates commonly have been called *Fusarium solani* "f. sp. *pisi*," members of this MP, as identified by genetic means, can occupy diverse biological habitats and have been found to exist as soil-inhabiting saprobes and as pathogens of plants other than pea and of animals (VanEtten and Kistler 1988). Although the species has a broad host range, individual isolates vary greatly in their pathogenicity on specific plants (Matuo and Snyder 1972; VanEtten 1978). Most Pda$^-$ isolates lack a chromosome in the 1.6-Mb size range and it has been confirmed recently that a gene for detoxifying the phytoalexins medicarpin and maackiain from another host, chickpea (*Cicer arientinum* L.), is also located on a DS chromosome (Covert et al. 1996). Thus, the DS chromosomes may carry genes that determine the host range of this pathogen (VanEtten et al. 1994).

The observation that Pda$^-$ gdr mutants can produce significant lesions on pea even in the presence of large amounts of pisatin (Table 5) raises questions regarding the relative importance of Pda for tolerance to this phytoalexin. Clearly, Pda is a tolerance mechanism to pisatin as its activity results in a less toxic metabolite (VanEtten 1976) and when *PDA* genes are transformed into Pda$^-$ fungi their tolerance to pisatin increases (Weltring et al. 1995; Ciuffetti and VanEtten 1996; Table 1). However, Pda$^-$ isolates of *N. haematococca* MPVI, whether naturally occurring or gdr mutants, are substantially more tolerant of pisatin than related fungi such as *N. haematococca* MPI (Denny et al. 1987). This "non-degradative tolerance" (NDT) of *N. haematococca* MPVI to pisatin is an induced property which may involve a change in membrane structure or function that decreases the net intracellular concentration of pisatin (Denny et al. 1987). The gene(s) responsible for NDT is not located on the DS chromosome since DS chromosome-deficient isolates express NDT. Thus, *N. haematococca* MPVI apparently utilizes at least two types of pisatin tolerance mechanisms to circumvent the toxic environment created by the synthesis of pisatin in infected pea tissue. This redundancy of tolerance mechanisms may explain why gdr Pda$^-$ mutants, though reduced in virulence, remain pathogenic on pea.

The results of the current study are consistent with our model, which proposes that *PDA1* is a virulence factor (i.e., it affects the amount of disease) for *N. haematococca* MPVI on pea and that at least one other gene that also affects the virulence of *N. haematococca* is located on the same DS chromosome as *PDA1*. Additional tests including the reintroduction of *PDA1* into Pda$^-$ gdr mutants and isolation of a *PEP*^{D} gene(s) are needed to confirm this model.

MATERIALS AND METHODS

Fungal strains and growth conditions.

Three virulent isolates (77-13-4, 77-13-5, and 77-13-7; Kistler 1983) of *N. haematococca*, each containing a single *PDA1* gene, were used for transformation. Ascospore isolates 44-100 (Kistler and VanEtten 1984a) and 156-2-1 (Miao and Van Etten 1992) were used as Pda$^-$ controls. All isolates were maintained on V8 juice agar (medium 29: Stephans 1974) at 25 to 27°C. Mycelia for the preparation of protoplasts or the extraction of DNA were obtained from cultures inoculated with conidia collected from cultures grown on V8 juice agar plates and grown in 100 ml of glucose asparagine medium (VanEtten et al. 1980) in a 250-ml Erlenmeyer flask. Cultures to be used for the preparation of protoplasts were grown at room temperature on a gyratory shaker (175 to 200 rpm) for 14 to 18 h. Those to be used for DNA extraction were grown for 48 to 72 h.

Construction of the vector for gene-disruption.

The transformation vector used to disrupt the *PDA1* gene, pKO1,was made by subcloning a 1,332-bp *Sca*I-*Sst*II fragment containing the glucoamylase (*glaA*) promoter and 777 bp of the *hph* gene from pDH33 (Smith et al. 1990) into the *Eco*RV and *Sst*II sites of pBluescript (Stratagene, La Jolla, CA); the 1,803-bp *Eco*RI-*Sph*I fragment containing the *trpC* terminator from *Aspergillus nidulans* and part of a bacterial hygromycin phosophotransferase (*hph*) coding sequence from pDH25 (Cullen et al. 1987) was subcloned into the *Sph*I and *Eco*RI sites of pUC18 (Vieira and Messing 1982); and the 2,236-bp *Hin*dIII-*Bam*HI fragment from pdH (Hirschi 1994) containing the coding sequence of *PDAT9* (Maloney and VanEtten 1994), 155 bp of 5′ untranslated and 319 bp of 3′ untranslated sequence was subcloned into the *Bam*HI and *Hin*dIII sites of pUC8 (Vieira and Messing 1982). These fragments were then combined to form pKO1 (Fig. 1). *PDAT9* has >99% sequence identity with *PDA1* (Straney and VanEtten 1994; K. McCluskey, Fungal Genetics Stock Center, Kansas City, KS, personal communication).

Fungal transformation and selection.

Protoplasts for transformation were prepared as described by Miao et al. (1991b) with the exception that the protoplasts were washed two times in SE (1 M sorbitol, 50 mM EDTA, pH 8), once in STC (1.2 M sorbitol, 25 mM Tris-HCl, pH 7.0, 25 mM $CaCl_2$) and suspended at a concentration of approximately 5×10^7 per milliliter. Transformation and plating procedures were adapted from Farman and Oliver (1992). Each transformation reaction contained 5×10^6 protoplasts (100 µl), 2 mM aurintricarboxylic acid, 10 mM spermidine, and 5 to 10 µg of CsCl-purified circular plasmid DNA. The transformation mixture was incubated 30 min on ice, a total of 1 ml of PTC (20% polyethylene glycol 4000, 25 mM $CaCl_2$, 25 mM Tris-HCl, pH 7.5) was added in a stepwise fashion (200, 200, and 600 µl) and stirred with the pipettor tip. The transformation mixture was added to 4 ml of molten (48°C) regeneration top agar (0.1% yeast extract, 0.1% Casamino Acids, 1.2 M sucrose, 1% agar) and overlayed on petri dishes containing 15 ml of solidified regeneration basal agar (0.1% yeast extract, 0.1% Casamino Acids, 0.6 M sucrose, 1.5% agar). In some cases maltose was substituted for sucrose in both top and basal regeneration agar. The plated protoplasts were incubated in the dark for 22 h at 27°C and each plate was overlayed with 5 ml of 1% water agar containing 250 µg of hygromycin B (Sigma Chemical Co., St. Louis, MO) per ml. The plates were incubated for a further 7 to 10 days at 27°C. Transformed fungal colonies generally began to appear after 3 to 4 days. Transformants were purified by isolating single germinated macroconidia or hyphal tips. The efficiency of transformation was variable and ranged from 0 to 4 transformants per microgram of DNA.

Transformants were assessed for their ability to demethylate pisatin in a "vial assay" as described by Mackintosh et al. (1989). Individual transformants were grown 6 days in the dark at 27°C in 7-ml plastic scintillation vials containing 0.25 ml of PGA medium (VanEtten et al. 1980) supplemented with 3-*O*-methyl-[^{14}C] pisatin (31 µg pisatin/10,000 dpm/5 µl DMSO/ml medium). Scintillation fluid (4 ml 0.5% 2,5-diphenyloxazole in toluene) was added and the amount of [^{14}C] determined in a Beckman Instruments model LS 6000SC scintillation counter.

Characterization of the transformants.

Tolerance to pisatin was assessed in mycelial growth bioassay as described by VanEtten et al. (1980) with the exception that the final concentration of pisatin was 160 µg/ml. Colony radii were measured after 3 days of growth in the dark at 27°C.

The ability of isolates to produce asexual microconidia, and microconidia was tested by inoculating the center of a petri dish (60 × 150 mm) containing 5 ml of potato dextrose agar (Difco) with a 5-mm agar plug containing actively growing hyphae. Two dishes per isolate were inoculated and incubated in the dark at 27°C for 5 days. Conidia were collected in 2 ml of deionized H_2O and 150 µl of $CHCl_3$ added to prevent germination. Conidia were counted in a hemacytometer.

Virulence assays.

Virulence was assessed in the "test-tube assay" as previously described (VanEtten et al. 1980) with the exception that the inoculum plug was 1/2 of an agar plug 6 mm in diameter. Virulence was quantified by measuring the lengths of the lesions produced by each isolate. In addition to the test isolates each experiment included the recipient isolate and a Pda$^-$ ascospore isolate (44-100 or 156-2-1). Each test isolate was inoculated onto at least eight seedlings per experiment.

To prepare inoculum for the in planta competition assay 0.2 ml of conidial suspension (10^6 conidia/ml) containing conidia in the desired ratio were spread onto the surface of a petri dish containing 20 ml of M100 agar medium (Stephans 1975). The dishes were incubated in the dark for 2 days at 27°C and inoculum plugs prepared as above.

For the "pot assay" (VanEtten 1978) a dwarf variety of pea (cv. Little Marvel, Carolina Biological Supply Company, Burlington, NC) was used. Five seeds were planted in steam-sterilized potting mixture in 10-cm plastic pots and placed in a controlled environment chamber at 27 to 28°C with 12 h light. Six- to 8-day-old seedlings were inoculated by pouring 25 ml of 10^7 conidia/ml into each pot. At least two pots were used for each isolate. Disease development was evaluated 4 weeks after inoculation using the numerical coding system described by VanEtten (1978) to rank the virulence of the isolates.

To test the virulence of fungal isolates on tomato fruit, greenhouse-grown ripe tomatoes were surface sterilized with 95% ethanol. To inoculate the fruit, 5-mm-diameter agar plugs were placed mycelial side down onto a small wound on the tomato's surface made using a 25 gauge needle. Each fruit was inoculated with five to seven isolates and each isolate was inoculated onto a minimum of 10 fruits. Inoculated fruit were incubated under normal room illumination at 23 to 25°C for 6 days in covered plastic containers containing wet paper towels to maintain high humidity. The size of a lesion was determined by the mean of two orthogonal diameters.

Isolation of fungi from infected tissue.

Fungi were isolated from stem lesions produced in the test tube assay essentially as described by VanEtten (1978). Lesions were excised and placed on glass or stainless steel rods in petri dishes containing moistened sterile filter paper and incubated at room temperature for 2 days. Conidia were then collected and spread onto water agar and single germinated conidia or hyphal tips were isolated.

Measurement of pisatin and Pda in lesion tissue.

Six-day-old lesion tissue (0.02 to 0.17 g) was extracted three to five times with 0.5 to 0.75 ml of hexanes, and the solvant evaporated to dryness under vacuum. The residue was dissolved in 25 or 50 µl of ethanol and centrifuged to remove debris. The pisatin was determined by C-18 reverse-phase HPLC at 309 nm by comparing the area under the pisatin peak to a standard curve. To determine Pda in planta, lesion tissue (50 mg) was placed in a scintillation vial with 0.5 ml of 50 mM potassium phosphate buffer (pH 6.5) containing 3-*O*-methyl-^{14}C pisatin (31 µg of pisatin/10,000 dpm/5 µl of DMSO/ml of buffer). The vials were incubated at room temperature on a gyratory shaker and the reaction stopped by the addition of 4 ml of scintillation fluid (0.5% diphenyloxazole in toluene).

DNA manipulations and reagents.

Cloning, digestion with restriction endonucleases and preparative DNA gel electrophoresis were as described by Sambrook et al. (1989). DNA fragments excised from agarose gels were purified on QIAEX beads according to the manufacturer's directions (QIAGEN, Chatsworth, CA). *N. haematococca* genomic DNA was extracted from lyophilized mycelia as described by Miao et al. (1991b). For DNA hybridization, DNA was digested by restriction endonucleases and the DNA fragments separated by electrophoresis in 0.6% agarose gels in Tris-acetate buffer (Sambrook et al. 1989). The DNA fragments were transferred to Hybond N$^+$ (Amersham Life Science Inc., Arlington Heights, IL) using 10× SSC (Sambrook et al. 1989). DNA detection and labeling were by enzyme-linked immunoassay of digoxigenin-conjugated deoxyuridine triphosphate incorporated into the probe by random hexanucleotide priming of the large subunit of *E. coli* DNA polymerase I. The protocol for prehybridization, hybridization, posthybridization washing and development was essentially as described by Helentjaris (T. Helentjaris, Pioneer Hibred, personal communication). Prehybridization and hybridization were carried out at 65°C in 5× SSC, 0.1% N-lauroyl sarcosine, 0.02% SDS and 0.1% blocking agent (I-Block, TROPIX, Bedford, MA). DNA blots with hybridized probe were washed twice for 3 min at room temperature in 0.1× SSC, 0.1% SDS followed by 30 min wash at 65°C in the same solution. All subsequent steps were carried out at room temperature. The blots were then washed 3 times for 5 min in buffer I (0.15 M NaCl, 0.01 M Tris-HCl, pH 7.5) 30 min in blocking buffer (buffer I, 0.1% blocking agent), and 30 min in blocking buffer containing 2.5 µl/50 ml alkaline phosphatase-conjugated anti-digoxigenin FAB fragment (Boehringer Mannheim, Indianapolis, IN). The blots were washed 3 times for 5 min in buffer I and once in DEA buffer (0.1 M diethanolamine, 1 mM $MgCl_2$, 0.02% Na azide, pH 10.0). Lastly, the blots were washed with a minimal volume of DEA Buffer containing 10 µl/ml AMPPD or CSPB (TROPIX, Bedford, MA) and exposed to Kodak XAR film at room temperature or 37°C.

Pisatin and 3-*O*-methyl-[^{14}C] pisatin were isolated and prepared as described previously (Mackintosh et al. 1989).

Analysis of transformants by pulsed field gel electrophoresis.

Protoplasts for PFGE were prepared essentially as descibed by Miao et al. (1991b) except that the protoplasts were embedded at 1.5 × 10^8 protoplasts/ml in 0.6% low gelling-temperature agarose and the lysing was carried out in NDS containing 1 mg/ml pronase E (Sigma Chemical Co., St. Louis, MO) at 50°C.

Chromosome-sized DNAs (chDNAs) were fractionated by contour-clamped homogeneous field electrophoresis in 0.6% agarose gels (SeaKem Gold Agarose, FMC BioProducts, Rockland, ME) in 0.28× Tris-borate EDTA (TBE) buffer (Sambrook et al. 1989) using a CHEF DRIII System (Bio-Rad, Richmond CA). The gels were run at 1.2 or 1.3 v/cm for 168 h at 12°C with a ramped 20- to 60-min switch time. Commercially prepared (FMC BioProducts, Rockland, ME) chromosomes of *Saccharomyces cerevisiae* and *Saccharomyces pombe* were used as size standards. The chDNAs were stained with 0.05 mg/ml ethidium bromide in 0.28× TBE and destained in 0.28× TBE.

Statistical analysis.

In each trial, the mean lesion length or diameter from a minimum of eight replicates was used as a measure of virulence. The Wilcoxan signed ranks test and Student's *t* test (Sokal and Rohlf 1981) were used in pairwise comparisons across trials.

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