

# Virulence of a Pisatin Demethylase-Deficient *Nectria haematococca* MPVI Isolate Is Increased by Transformation with a Pisatin Demethylase Gene

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Previous studies have shown that all isolates of the fungus *Nectria haematococca* mating population (MP) VI pathogenic on pea are able to detoxify the pea phytoalexin pisatin by demethylation. This demethylation is catalyzed by pisatin demethylase (pdm), a cytochrome P450, and genes (*PDA*) encoding this cytochrome P450 have been cloned. Naturally occurring isolates of *N. haematococca* that lack the ability to demethylate pisatin (*Pda*<sup>-</sup>) normally lack *PDA* genes and are not pathogenic on pea. In this study, the effect of *Pda* on virulence was evaluated by introducing a *pdm*-encoding gene, *PDAT9*, into a *Pda*<sup>-</sup> isolate. Of the 146 *Pda*<sup>+</sup> transformants obtained, 52 were significantly more virulent on pea than the *Pda*<sup>-</sup> recipient and control transformants but none was highly virulent on pea. An analysis of three *Pda*<sup>+</sup> transformants revealed that all three transformants, though differing in virulence, were equally tolerant of pisatin and produced similar *pdm* activity. The results suggest that ability to detoxify pisatin can increase the virulence of *N. haematococca* MPVI but that for high virulence on pea additional genes are needed.

**Additional keywords:** *Fusarium solani*, isoflavonoids, pterocarpin.

Phytoalexin production has long been proposed to function as a resistance mechanism in plants. It has been suggested that some fungi can circumvent this resistance mechanism by enzymatically detoxifying the phytoalexins produced by their host (Anderson 1991). The filamentous ascomycete *Nectria haematococca* Berk. & Br. mating population (MP) VI (anamorph: *Fusarium solani*) detoxifies the pea phytoalexin pisatin by a specific demethylation reaction (VanEtten et al. 1989). All naturally occurring field isolates of this fungus that are pathogenic on pea produce pisatin demethylase (*pdm*), a substrate-inducible enzyme. All isolates that lack pisatin demethylating activity (*Pda*), whether they are field isolates or

ascospore progeny from laboratory crosses, are non-pathogenic or of low virulence on pea.

Pisatin demethylase is a microsomal cytochrome P450 monooxygenase (Matthews and VanEtten 1983). Cytochrome P450s catalyze a diverse group of biosynthetic and degradative reactions but because of the high degree of conservation at the amino acid (aa) level the genes (*CYP* genes) encoding these enzymes are considered members of one "superfamily" (Nelson et al. 1993). *CYP* genes whose deduced aa sequences are ≥40% identical belong to the same family and the sequence of the first characterized *PDA* gene (*PDAT9*) was sufficiently different from other known cytochrome P450s to define a new family (*CYP57*) (Maloney and VanEtten 1994).

Microsomal eukaryotic P450s require NADPH-cytochrome P450 reductase for enzymatic activity. Functionally, the reductase is a highly conserved protein in fungi (Scala et al. 1988) as well as other organisms and thus can complement the activity of any cytochrome P450. Consequently, introduction of a foreign cytochrome P450 into an organism will normally confer enzymatic activity characteristic of that P450. When the *PDAT9* gene of *N. haematococca* was transformed into *Aspergillus nidulans*, *Cochliobolus heterostrophus*, and *Ascochyta rabiei*, none of which are pathogens of pea, they acquired the ability to rapidly demethylate and thus detoxify pisatin (Schäfer et al. 1989; Weltring et al. 1995; Weltring et al. 1988). When the *Pda*<sup>+</sup> transformants of *C. heterostrophus* and *A. rabiei* were placed on wound sites of pea stems they produced more necrosis than nontransformed isolates (Schäfer et al. 1989; Oeser and Yoder 1994; Barz and Welle 1992). This supports the hypothesis that phytoalexin detoxifying ability helps neutralize a resistance mechanism based on phytoalexin production and indicates that detoxification is an important property for pathogenicity on pea.

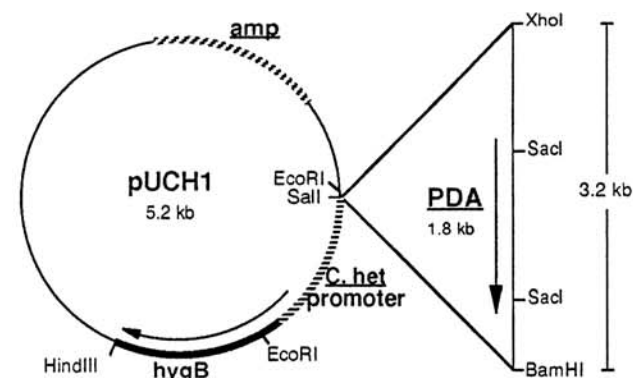
The increase in virulence of the *C. heterostrophus* and *A. rabiei* *Pda*<sup>+</sup> transformants was small, which is not surprising because, although these fungi are plant pathogens, neither is a pathogen on pea and may therefore lack a number of traits required to be an aggressive pathogen of this plant. In one of the genetic analyses relating *Pda* in *N. haematococca* to its virulence on pea, a three-generation backcross was completed and by the third generation (cross 94) most of the *Pda*<sup>+</sup> progeny were fairly uniform in virulence, while all *Pda*<sup>-</sup> isolates were low in virulence (Kistler and VanEtten 1984b). This means that all the genes needed for virulence were present in most

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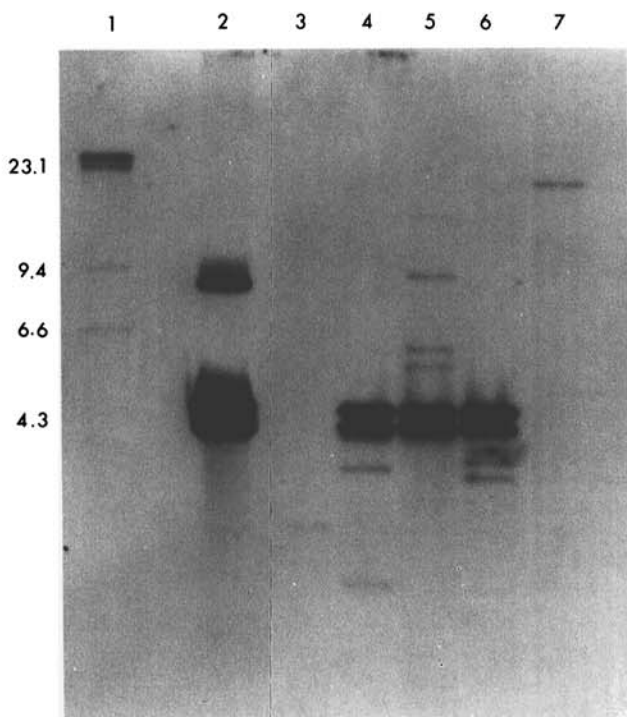
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Pda<sup>+</sup> isolates and suggests that all the same genes, except *PDA*, could be present in the Pda<sup>-</sup> progeny of this cross. Thus, if the low level of virulence observed in the Pda<sup>-</sup> progeny is due to the absence of the pisatin detoxifying ability, addition of a *PDA* gene to these isolates should significantly increase their virulence. The purpose of this study was to test this hypothesis as a means to further evaluate the importance of Pda in the virulence of fungi on pea.



**Fig. 1.** Restriction map and features of pUCH1-PDA. The 3.2-kb *XhoI/BamHI* insert is *Nectria haematococca* DNA containing the 1.8-kb *PDA* gene with an arrow indicating direction of transcription. *hygB* is the *Escherichia coli* hygromycin  $\beta$ -phosphotransferase gene (*hph*) under the control of a promoter element from *Cochliobolus heterostrophus*.



**Fig. 2.** Southern hybridization of transformants and isolates of *Nectria haematococca*. Genomic DNA from *N. haematococca* was digested with *EcoRI* and hybridized with pUCH1-PDA. Lane 1 = lambda *HindIII*, 2 = pUCH1-PDA, 3 = 94-6-1, 4 = Tx1, 5 = Tx2, 6 = Tx3, and 7 = T9. The band at about 8 kb in lane 2 represents undigested pUCH1-PDA.

## RESULTS

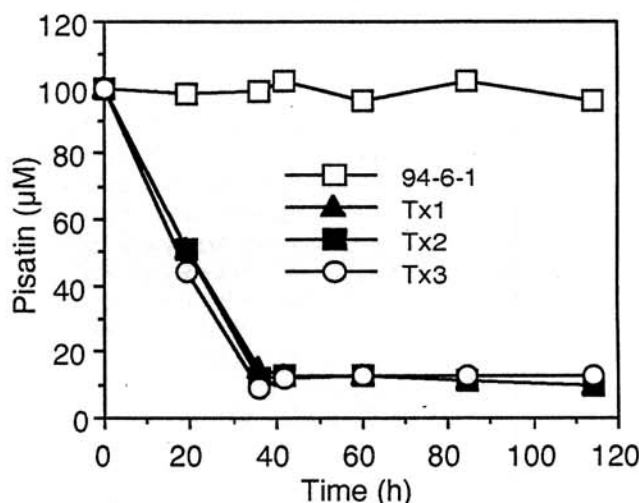
### Transformation of a Pda<sup>-</sup> isolate of *N. haematococca* to Pda<sup>+</sup>.

Resistance to hygromycin B was used as a selectable marker to transform a *PDA* gene into 94-6-1, a Pda<sup>-</sup> isolate from the above-mentioned backcross series. The transformation vector, pUCH1, contains *hph*, a hygromycin resistance gene fused to a *C. heterostrophus* promoter (Turgeon et al. 1987). pUCH1 and pUCH1 with a 3.2-kb *XhoI-BamHI* fragment containing the *PDA* gene of *N. haematococca* (pUCH1-PDA, Fig. 1) were used to transform *N. haematococca* to hygromycin resistance.

Initially, three transformants (Tx1, Tx2, Tx3) were obtained with pUCH1-PDA. Southern hybridization analysis indicated that all three transformants had integrated pUCH1-PDA (Fig. 2). The presence in the transformants of strong hybridizing bands of the same size as the *EcoRI* fragments in the vector along with weakly hybridizing bands of other sizes is consistent with multiple and possibly tandem copies of the transforming plasmid being present in the transformants. Southern analysis of undigested transformant DNA confirmed that transformation was integrative (data not shown). The ca. 20-kb band hybridizing in the Pda<sup>+</sup> strain T9 (Fig. 2, lane 7) is due to T-9's wild-type *PDA* genes.

All three transformants were Pda<sup>+</sup> (Fig. 3). When first tested for virulence on pea, two of the transformants produced lesions larger than those produced by 94-6-1 but none approached the size produced by a virulent Pda<sup>+</sup> isolate, 94-1-6 from the backcross series (Fig. 4). Repeat assays with these transformants indicated that two of the three transformants produced significantly larger lesions than 94-6-1 (Table 1). When tested for their sensitivity to pisatin, all three transformants showed a similar increase in tolerance to pisatin compared to 94-6-1 (Fig. 5).

Although the transformants showed similar rates of removal of pisatin from the media (Fig. 3), this assay does not measure the amount of pdm produced after induction with pisatin. The



**Fig. 3.** Demethylation of pisatin by pUCH1-PDA transformants of the Pda<sup>-</sup> isolate 94-6-1. A mycelial plug was transferred to vials containing 0.2 mM 3-*O*-methyl-<sup>14</sup>C pisatin in a peptone-glucose medium and the amount of pisatin remaining in the medium assayed at the indicated times.

level of inducible pdm activity has been linked to virulence in past studies (Kistler and VanEtten 1984b; Mackintosh et al. 1989). To determine if the transformants differed in this regard, the pdm activity was measured 6 h after induction with pisatin. All three isolates had similar rates [120 (Tx1), 119 (Tx2), and 114 (Tx3) pmoles of pisatin demethylated/min/mg (fresh wt.) of mycelium]. These rates are similar to those induced in naturally occurring virulent Pda<sup>+</sup> isolates of *N. haematococca* (Kistler and VanEtten 1984b).

#### Virulence of additional Pda<sup>+</sup> transformants of 94-6-1.

An additional 143 Pda<sup>+</sup> transformants of 94-6-1 were evaluated for virulence on pea to determine if a high level of virulence could be attained by Pda<sup>+</sup> transformants of this isolate. A number of these transformants were more virulent than the recipient isolate and the most virulent of these transformants produced lesions averaging 7.1 mm in size (Fig. 6 and Table 1). As a control for the possible effect of transformation per se on the virulence of 94-6-1, 10 pUCH1 vector only transformants were tested for their virulence on pea. None of these transformants produced lesions larger than 94-6-1 (Fig. 6, insert and Table 1).

As a further test of the role of the *PDA* genes in virulence, Pda<sup>-</sup> isolate 241-19-6 and a moderately virulent Pda<sup>+</sup> isolate (T474) were transformed with pUCH1-PDA and tested for virulence on pea. The one Pda<sup>+</sup> transformant of 241-19-6 obtained produced slightly larger lesions on pea that were significantly larger than produced by 241-19-6, while pUCH1-PDA transformants of T474 were not more virulent than T474 (Table 1).

#### Attempts to disrupt the *PDA* gene in virulent Pda<sup>+</sup> isolates.

Another approach to evaluate the importance of Pda in the virulence of *N. haematococca* besides the addition of a *PDA* gene to a Pda<sup>-</sup> isolate is to create a Pda<sup>-</sup> mutant of a virulent Pda<sup>+</sup> isolate. A transformation vector (pUCH1-SAC) was constructed in which a 1.3-kb internal *SacI* fragment from *PDAT9* was cloned into the *SacI* site of pUCH1. This vector should

give disruptions of the wild-type *PDA* gene if a single cross-over were to occur at the *PDA* locus. pUCH1-SAC was used to transform three virulent isolates that contain a single copy of *PDA*. Although 3, 25, and 11 hygromycin resistant isolates of T474, 94-1-6, and 77-2-3, respectively, were obtained, all were Pda<sup>+</sup>.

## DISCUSSION

Addition of the *PDAT9* gene to 94-6-1 increased virulence in many but not all transformants (Fig. 6). In those cases where there was a significant increase in virulence, the ability to demethylate pisatin would appear to be the cause rather than some event associated with transformation itself, as vector-only transformants never produced lesions larger than the recipient isolate (Fig. 6, insert).

Why Pda<sup>+</sup> transformants of 94-6-1 did not all show a similar increase in virulence is unclear. Transformants Tx1, Tx2, and Tx3 represent transformants that varied in their increase in virulence on pea (Table 1). However, all three transformants showed similar tolerance to pisatin in vitro, had similar times for induction for pisatin demethylation (Figs. 3 and 5), and produced similar levels of pdm after induction. The tolerance level, lag period before expressing pdm, and level of pdm induced by pisatin are the same as produced by naturally occurring highly virulent Pda<sup>+</sup> isolates of *N. haematococca* (Kistler and VanEtten 1984b; Mackintosh et al. 1989). The occurrence of transformants with nonuniform phenotypes is common in plants and is often attributed to positional effects of the integrating DNA influencing the level of expression of the introduced gene (Fujiwara and Beachy 1993). However, this has not been as commonly reported in fungi (Judelson et al. 1993) and how a positional effect would have unequal effects on virulence of the above transformants is not as obvious as the transformants expressed equivalent pdm activity in vitro. Nevertheless, a reasonable interpretation of our data is that the increase in virulence of a number of Pda<sup>+</sup> transformants of 94-6-1 is due to the presence of *PDAT9*.

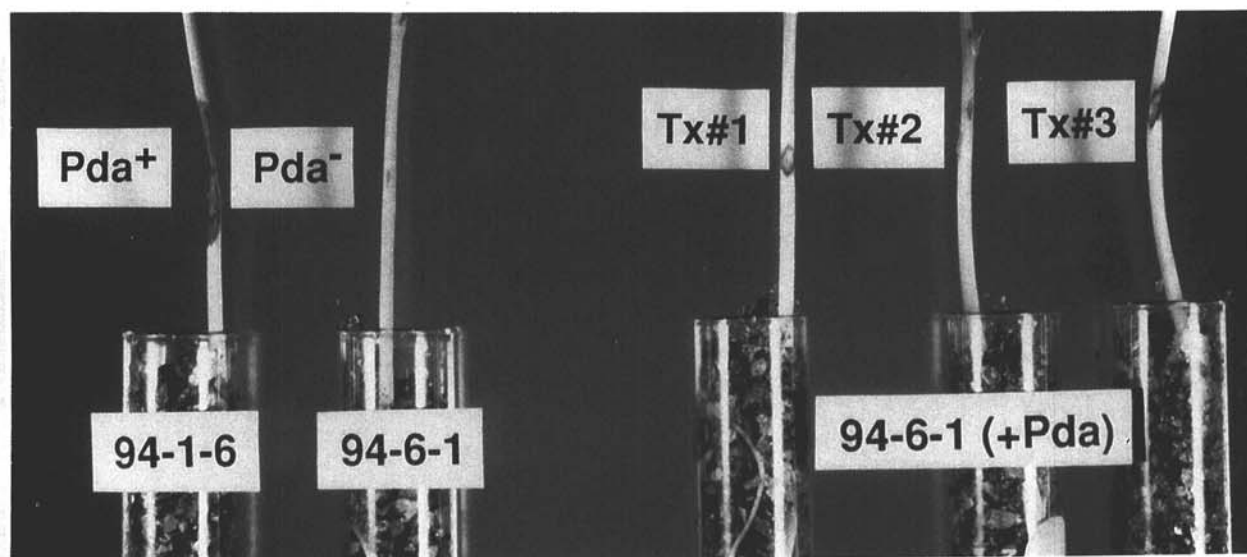


Fig. 4. Virulence on pea of Pda<sup>+</sup> isolate 94-1-6, Pda<sup>-</sup> isolate 94-6-1, and 94-6-1 pUCH1-PDA transformants Tx1, Tx2, and Tx3. Plants were photographed 6 days after inoculation.



Even though there was an increase in the virulence of most of the 94-6-1 Pda<sup>+</sup> transformants, the most virulent transformant produced lesions that averaged 7.1 mm (Fig. 6; and Table 1). In contrast, nearly all Pda<sup>+</sup> progeny in cross 94 produced lesions larger than 7.1 mm (Table 1; Kistler and VanEtten 1984b). It is clear from previous genetic studies that genes in addition to *PDA* are needed for the pathogenicity of this fungus on pea (Tegtmeier and VanEtten 1982). Perhaps 94-6-1 lacks some of these other pathogenicity genes and therefore a high level of virulence is not obtained despite the presence of pdm activity. An attempt to address this possibility by transforming another Pda<sup>-</sup> isolate (241-19-6) from a different genetic background to Pda<sup>+</sup> was inconclusive because only one Pda<sup>+</sup> transformant was obtained. Also, the addition of extra

copies of *PDA* to a moderately virulent Pda<sup>+</sup> isolate (T474) did not increase its virulence, presumably because it already had sufficient pdm activity.

As an alternative approach to evaluate the role of pdm in the virulence of *N. haematococca* on pea, we attempted to create a specific mutation in the *PDA* gene of a virulent Pda<sup>+</sup> isolate that contained a single *PDA* gene. However, no Pda<sup>-</sup> transformants were produced. Three different Pda<sup>+</sup> isolates were transformed with a vector that was designed to cause a disruption of the resident *PDA* gene if there was a single crossover event at the *PDA* locus. Although gene disruption transformants have been made in *N. haematococca*, the vectors employed cause gene replacement events which require a double crossover (Stahl and Schäfer 1992; Wasmann and VanEtten 1996). It may be that gene disruption events in this fungus are more easily accomplished with vectors that require a double rather than a single crossover event.

This study shows that addition of *PDAT9* and its concurrent expression in a Pda<sup>-</sup> isolate can increase virulence on pea. However, since these Pda<sup>+</sup> transformants did not produce large lesions, it is evident that other virulence-associated characteristics are required and are missing from the Pda<sup>-</sup> isolate 94-6-1. The existence of genes encoding other pea virulence traits is further supported in the accompanying article (Wasmann and VanEtten 1996) which describes the specific mutagenesis of a *PDA* gene by gene disruption and its effect on virulence.

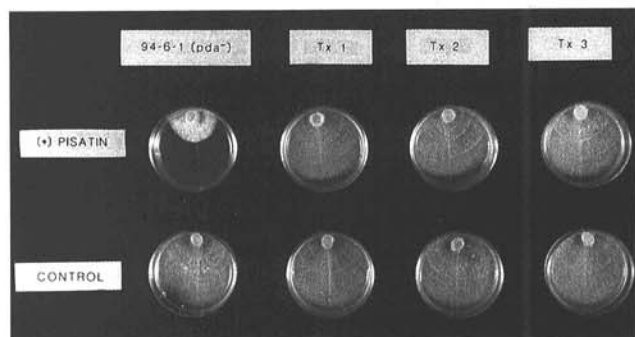


Fig. 5. Growth of 94-6-1 and its pUCH1-PDA transformants (Tx1, Tx2, and Tx3) on a peptone-glucose medium containing 0.5 mM pisatin. Plates were photographed 5 days after inoculation.

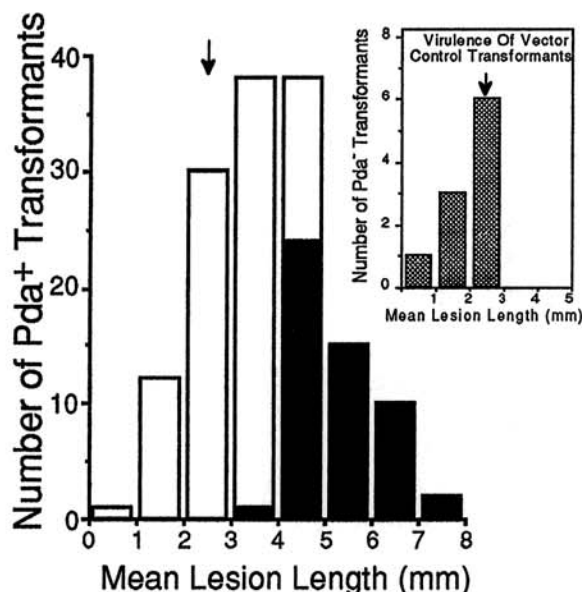


Fig. 6. Virulence on pea of pUCH1 (insert) and pUCH1-PDA transformants of 94-6-1. Lesion length was recorded 6 days after inoculation. Arrow indicates the mean lesion size of 94-6-1 (2.4 mm). Heights of the bars are the number of transformants that produced lesions of the mean size in the size range indicated. Solid data bars are number of transformants that were significantly different than the recipient isolate at a confidence level of >0.99 using the Dunnett's T test.

Table 1. The effect of the addition of a pisatin demethylase gene on the virulence of *Nectria haematococca* isolates

Isolate	Phenotype	Plants (no.) <sup>a</sup>	Virulence <sup>a</sup>
Recipient 94-6-1	Pda <sup>-</sup>	77	2.4 ± 1.1
Transformed with pUCH1-PDA			
Tx1	Pda <sup>+</sup>	35	3.5 ± 1.7
Tx2	Pda <sup>+</sup>	38	5.3 ± 2.2*
Tx3	Pda <sup>+</sup>	32	5.0 ± 1.6*
13A3-3	Pda <sup>+</sup>	12	7.1 ± 1.1*
8A-7	Pda <sup>+</sup>	20	7.1 ± 1.6*
Transformed with pUCH1			
9A3-1	Pda <sup>-</sup>	44	2.7 ± 1.2
Recipient: 241-19-6	Pda <sup>-</sup>	16	3.1 ± 1.3
Transformed with pUCH1-PDA			
9B1-5	Pda <sup>+</sup>	24	4.4 ± 1.6*
Recipient: T474	Pda <sup>+</sup>	16	10.4 ± 2.2
Transformed with pUCH1-PDA			
13C1-2	Pda <sup>+</sup>	24	8.7 ± 3.2
13C1-3	Pda <sup>+</sup>	24	10.0 ± 2.4
13C2-1	Pda <sup>+</sup>	8	8.0 ± 0.9
13C2-2	Pda <sup>+</sup>	8	8.5 ± 3.7
94-1-6	Pda <sup>+</sup>	62	16.5 ± 4.9

<sup>a</sup> Epicotyl lesions were measured 6 days after inoculation and each assay used ≤ 8 plants per isolate. Values reported are means of all assays. \*Indicates the value was significantly different than the recipient isolate at a confidence of >0.99 using the Dunnett's T test and number in parenthesis is the standard deviation.

## METHODS AND MATERIALS

### Isolates.

Stock isolates of *N. haematococca* MPVI were grown as described previously (VanEtten et al. 1980). Isolates 94-6-1 and 241-19-6 are Pda<sup>-</sup> ascospore isolates from previous studies (Kistler and VanEtten 1984b; Miao and VanEtten 1992). Isolate T9 is a field isolate from which *PDAT9* was cloned (Weltring et al. 1988). Ascospore isolates 94-1-6 and 77-2-3 contain *PDA1*, a homolog of *PDAT9* (Kistler and VanEtten 1984b; Maloney and VanEtten 1994) and T474 is a field isolate with a single *PDA* gene that has a high degree of sequence similarity to *PDAT9* (Hirschi 1994).

### Transformation of *N. haematococca*.

Fungal transformation was based on modifications of the procedures of Yelton et al. 1984 and Turgeon et al. 1987. Conidia from 7- to 10-day-old cultures grown on V8 juice agar slants were incubated at 25°C with shaking (200 rpm) for about 15 h in 80 ml of GA medium (glucose-asparagine medium, VanEtten and Stein 1978). Mycelia were harvested by filtration and washed with 0.6 M MgSO<sub>4</sub>. One gram of mycelium was added to 10 ml of 1.2 M MgSO<sub>4</sub>, 10 mM sodium phosphate containing 80 to 100 mg of Novozyme 234 (Novo Industries), and 80 to 100 µl of sterile β-glucuronidase (Sigma) and the suspension incubated with gentle agitation at 25°C for 2 to 3 h. The suspension was filtered through 45 µm Nytex, layered on a 0.6 M sorbitol, 100 mM Tris-HCl, pH 7.0, solution and centrifuged at 5,000 rpm for 15 min in a Beckman Swinging Bucket JS-13.1 rotor. The band of protoplasts was removed and washed twice in STC [1.2 M sorbitol, 10 mM Tris-HCl (pH 7.5), 10 mM CaCl<sub>2</sub>] with centrifugation at 600 rpm for 3 min in a Sorvall SS34 rotor. The protoplast number was adjusted to 2 × 10<sup>7</sup> per 200 µl of STC and kept on ice until used.

ATA (0.5 µl of 20 mM aurintricarboxylic acid), plasmid DNA, and PEG 4000 (60% in 50 mM CaCl<sub>2</sub>, 10 mM Tris) to a final concentration of 40% were added to protoplasts and gently mixed. After the addition of 1 ml of STC, the suspension was embedded in molten regeneration medium (1.2 M sorbitol, 0.1% yeast extract, 1.5% agar, Turgeon et al. 1987) at a concentration of 2 × 10<sup>6</sup> protoplasts per plate. The plates were incubated in the dark for 14 to 16 h at 25°C and hygromycin in 2% water agar was added as a 10-ml overlay to give a final concentration of 35 to 40 µg/ml. After an additional 3 to 5 days of incubation in the dark at 25°C, transformants appeared on the surface of the medium. Spores were collected from these colonies after several more days of growth and stored temporarily in water at 4°C.

### Plasmid DNA used for transformation.

The transformation vector pUCH1 was described by Turgeon et al. 1987 and the pUCH1-PDA vector that contained *PDAT9* from *N. haematococca* on a 3.2-kb *Xho*I/*Bam*HI fragment was that in Schäfer et al. (1989) called pUP1.

### Measurement of Pda.

Hygromycin B-resistant transformants obtained from transformation with pUCH1-PDA were initially screened for Pda by the "vial" assay (Mackintosh et al. 1989). A plug of mycelium from each transformant, grown on hygromycin-

supplemented regeneration medium, was transferred to a 7-ml scintillation vial containing 250 µl of M-2 agar medium (Mackintosh et al. 1989) supplemented with 0.2 mM 3-*O*-methyl-<sup>14</sup>C pisatin, ("<sup>14</sup>C pisatin", specific activity 1.1 × 10<sup>5</sup> dpm/µmole) and whether a transformant was Pda<sup>+</sup> or Pda<sup>-</sup> was deduced by measuring the amount of toluene-extractable radioactivity remaining in the medium after growth for 7 days. The <sup>14</sup>C extracted into the toluene phase was determined with a Beckman LS355 scintillation spectrometer. Replicate vials were extracted at intervals to measure the demethylation of pisatin over time (Fig. 3).

A previously described procedure (Kistler and VanEtten 1984a) was used to determine the amount of pisatin demethylase induced after treatment with pisatin. Flasks containing mycelial suspensions (30 mg fresh wt/ml) were incubated on a reciprocal shaker (90 strokes min<sup>-1</sup>) at 25°C and treated with 0.1 mM pisatin. Six hours later <sup>14</sup>C pisatin was added to the mycelial suspension to give a <sup>14</sup>C pisatin concentration of 0.1 mM (specific activity 1.1 × 10<sup>5</sup> dpm/µ mole) and aliquots (0.5 ml) were withdrawn at 5-min intervals over the following 20 min and the content of <sup>14</sup>C pisatin in each sample measured as above. Pisatin demethylation rates were determined by calculating the linear regression of pisatin concentration v. time, and were expressed as pmoles of pisatin demethylated min<sup>-1</sup> mg<sup>-1</sup> (fresh wt) of mycelium.

### Pisatin tolerance assay.

Tolerance to pisatin was determined by measuring the degree of inhibition of radial growth of a mycelial colony as described previously (VanEtten et al. 1980).

### Preparation of genomic DNA.

Transformants were grown on regeneration medium slants in the absence or presence of 35 µg of hygromycin B/ml. Spores were harvested from 5- to 7-day-old cultures and placed in 250-ml flasks that contained 100 ml of GA medium with 10 µg of hygromycin B/ml. Flasks were shaken at 250 rpm at 25°C for 2 to 3 days. Mycelia were harvested by filtration, washed with sterile water, lyophilized, and ground in liquid nitrogen. The procedure for isolation of fungal DNA was as described by VanWert and Yoder 1992.

### Southern hybridization analysis.

Southern hybridization analysis was carried out by established procedures (Sambrook et al. 1989). *Eco*RI digests of pUCH1-PDA and genomic DNA from transformed and untransformed isolates of *N. haematococca* were probed with <sup>32</sup>P-labeled pUCH1-PDA.

### Virulence of the Pda<sup>+</sup> transformants on pea.

Virulence was measured using the "test tube" assay (VanEtten et al. 1980). Spores from each transformant and control isolates were placed on *Ustilago* genetics minimal medium (medium M-100, Stevens 1974) and grown for 2 days. A small wound in the pea epicotyl (about 0.5 mm) was made with a 25 gauge needle. The inoculum plug was obtained from the edge of an actively growing mycelial colony by removing a 4-mm-diameter disk with a cork borer. The mycelial surface of the plug was placed on the wound in the epicotyl. Four to eight epicotyls were inoculated per isolate and inoculated plants were placed in a high-humidity cham-

ber. Lesion length was measured (mm) after 6 days. Most transformants were assayed at least twice using eight plants per assay. At the time the plants were inoculated, a sample of mycelia used for plant inoculation was tested to verify its Pda phenotype and hygromycin sensitivity.

### Statistical analysis.

The general linear model procedure of the Statistical Analysis System (The SAS Institute, Inc., Carey, NC) was used to determine the statistical significance of the differences in the virulence, as determined by lesion length, of the transformants versus the recipient isolates. The Dunnett's T-Test was used to compare the lesion length of each transformant to that of its corresponding recipient isolate. A confidence level of 99% for a one-tailed test was used to determine which transformants were significantly different from their corresponding recipient.

### Chemicals.

Pisatin and 3-*O*-methyl-<sup>14</sup>C pisatin were from previous studies (Mackintosh et al. 1989). All other chemicals were obtained from commercial sources.

## ACKNOWLEDGMENTS

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