MPMI Vol. 9, No. 6, 1996, pp. 483-491. Publication no. M-1996-0621-01R. © 1996 The American Phytopathological Society

Expression of the Pisatin Detoxifying Genes (*PDA*) of *Nectria haematococca* in Vitro and in Planta

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Received 1 December 1995. Accepted 1 April 1996.

The phytopathogenic fungus *Nectria haematococca* detoxifies pisatin, a phytoalexin produced by pea. Pisatin demethylating ability (a phenotype called Pda) is due to pisatin demethylase (pdm) and the genes encoding this enzyme are called *PDA*. Some isolates rapidly acquire a high to moderate rate of pisatin demethylating activity in culture in response to pisatin (phenotypes Pda^{SH} and Pda^{SM}), while other isolates only slowly demethylate pisatin (phenotype Pda^{LL}). Here we report that *PDA*-specific RNA levels increased more quickly in response to pisatin in isolates with *PDA* genes confering a Pda^{SH} or Pda^{SM} phenotype than in isolates with genes confering a Pda^{LL} phenotype. In addition, the pdm activity of transformants of *N. haematococca* containing chimeric constructs of PDA^{SH} and PDA^{LL} genes in which the 5′ regulatory regions of these genes had been switched supports the conclusion that differential expression of *PDA* genes is responsible for the different Pda phenotypes detected *in vitro*. Northern analysis of pea tissue infected with isolates carrying PDA^{SH} or PDA^{LL} genes indicated that differential induction of these genes also occurred *in planta*. Only PDA^{SH}-specific RNA is readily detected in tissue infected with isolates containing PDA^{SH} and PDA^{LL} genes. Recently a pisatin biosynthetic gene, isoflavone reductase (*IFR*), has been identified. Using the polymerase chain reaction, qualitative detection of *IFR* and PDA^{SH} transcripts in infected tissue were made to assess the relative timing of these genes' expression. No transcripts were detected 6 h after inoculation, but transcripts of both genes were detected at 12 h, suggesting an interplay between the regulatory systems controlling the plants's defense response and the pathogen's counter response.

Additional keywords: *Fusarium solani,* cytochrome P450, pterocarpan.

A mode of pathogenesis for some necrotrophic fungi, including the pea pathogen *Nectria haematococca* mating population (MP) VI Berk. and Br. (anamorph: *Fusarium solani*), appears to be the neutralization of the plant's inducible defenses rather than preventing their expression (Lamb et al. 1989; Gabriel and Rolfe 1990; Keen 1992). Pisatin, the major phytoalexin of pea (*Pisum sativum* L.), is produced by the plant in response to infection by *N. haematococca* (Pueppke and VanEtten 1974); however, this pathogen detoxifies this xenobiotic (Fig. 1, VanEtten et al. 1989). A substrate-inducible cytochrome P450 monooxygenase, pisatin demethylase (pdm), catalyzes this detoxification (Matthews and VanEtten 1983; Maloney and VanEtten 1994).

Conventional genetic analysis has identified at least six loci in *N. haematococca* for genes encoding this cytochrome P450 (VanEtten et al. 1989). Among the characterized *PDA* genes, three in vitro whole cell phenotypes corresponding to the lag period for induction of the enzyme and to the final level of enzyme activity have been recognized: short lag, high activity (Pda^{SH}); short lag, moderate activity (Pda^{SM}); and long lag, low activity (Pda^{LL}). An association between pda phenotypes and pathogenicity on pea has been established; only isolates with *PDA* genes that encode a Pda^{SH} or Pda^{SM} phenotype have been shown to be pathogenic on pea (Kistler and VanEtten 1984; Mackintosh et al. 1989).

The sequences of the first two cloned *PDA* genes, *PDA*T9 and *PDA*6-1, which confer the Pda^{SH} and Pda^{LL} phenotypes, respectively, reveal that these genes are 90% identical at the deduced amino acid level and 88% identical at the nucleic acid level (Maloney and VanEtten 1994; Reimann and VanEtten 1994). The open reading frames contain conserved amino acid motifs found among all cytochrome P45's and thus these genes are members of the P450 superfamily (Nelson et al. 1993). The *PDA* genes are the first two members of the *CYP57A* subfamily (Maloney and VanEtten 1994; Reimann and VanEtten 1994).

The sequence and restriction site information of these *PDA* genes allowed for the selection of specific DNA probes and restriction enzymes that produce diagnostic sets of restriction fragments permitting a rapid restriction fragment length polymorphism (RFLP) comparison of the other known and unknown *PDA* genes (Miao et al 1991; Maloney and VanEtten 1994; K. Hirschi, unpublished). These analyses of reference strains and field isolates of *N. haematococca* indicate that most of the *PDA* genes in this species are similar to the two previously characterized genes. However, the RFLP analyses placed the *PDA* genes into two groups, one that contains the PDA^{SH} and PDA^{SM} genes and the other that contains the PDA^{LL} genes (VanEtten et al. 1994b). Recently a gene conferring the Pda^{SM} phenotype has been sequenced and it was 98% identical to *PDA*T9 and 89% identical to *PDA*6-1 at the nucleotide level (K. Hirschi, unpublished). Thus, this is in agreement with the RFLP analysis that placed PDA^{SH} genes in the same group with PDA^{SM} genes. The RFLP screen of field

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isolates also identified one *PDA* homolog, termed *Phda* (*PDA* Hybridizing DNA) that is apparently nonfunctional in that no pisatin demethylase activity is detected in isolates with only *Phda* (Miao and VanEtten 1991; Hirschi, University of Arizona, unpublished). Its sequence puts it in the PDA^{SH} and PDA^{SM} group (K. Hirschi, unpublished).

Biochemical analysis of *PDA* gene products associated with each whole cell phenotype suggests that these enzymes possess similar catalytic properties (George, unpublished; K. Hirschi, unpublished). These functional similarities suggest that the Pda phenotypes are due to levels of RNA accumulation in response to pisatin rather than due to the biochemical characteristics of the *PDA* gene products.

The purpose of this study is to determine if the difference in phenotypes encoded by the different *PDA* genes is due to differential expression of these genes. We were also interested in determining whether qualitative differences in expression of the various *PDA* genes occurs in planta. Finally, we wished to determine if pisatin is the possible regulatory signal for induction of *PDA* in planta by monitoring production of *PDA* transcripts in infected tissue along with production of transcripts of a gene specifically involved in pisatin biosynthesis. This was possible because of the recent identification of a gene, isoflavone reductase (*IFR*), which apparently is the first of several genes involved in the terminal steps of pisatin biosynthesis (Fig. 1; Paiva et al. 1994).

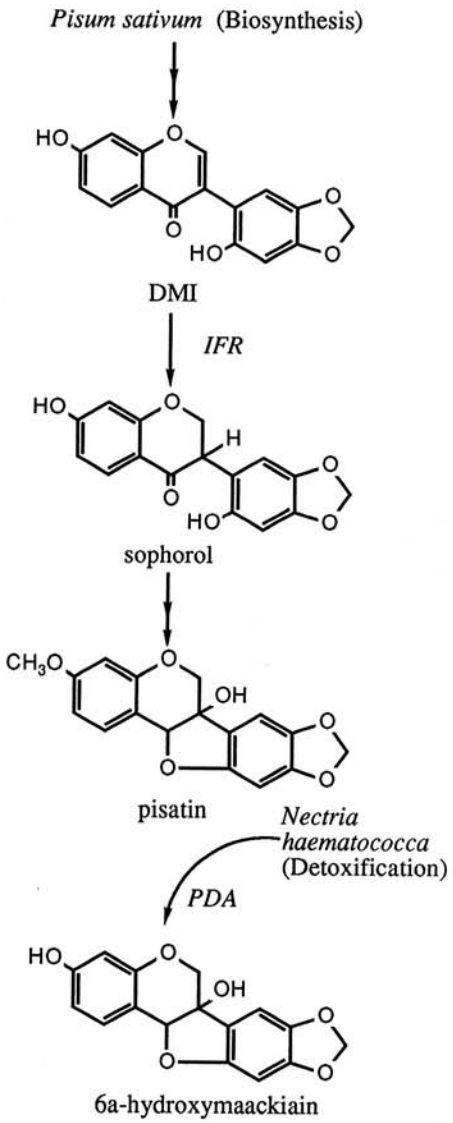

Fig. 1. Late steps in the proposed pathway for the biosynthesis of the phytoalexin pisatin in *Pisum sativum* and its detoxification by *Nectria haematococca*. *IFR* refers to the gene encoding isoflavone reductase in *P. sativum*, *PDA* refers to the gene in *N. haematococca* encoding pisatin demethylase and DMI refers to 7,2′-dihydroxy-4′,5′-methylenedioxyisoflavone.

RESULTS

In Vitro

Accumulation of *PDA*-specific RNA in isolates with different *PDA* genes.

Freshly growing mycelia of *N. haematococca* were suspended in phosphate buffer and exposed to pisatin. At given time intervals, total RNA was isolated and hybridized to a *PDA*-specific probe. Because each isolate analyzed had previously been shown to contain a single *PDA* gene that confers a specific phenotype we could determine whether accumulation of *PDA*-specific RNA correlated to in vitro phenotypes. It was readily apparent that *PDA*-specific RNA had undergone a significant increase by 8 h or sooner after pisatin treatment in isolates with either a PDA^{SH} or PDA^{SM} gene (Fig. 2A). The Pda^- isolate harboring *Phda* also showed induction of *PDA*-specific RNA after 8 h of pisatin treatment. Under similar conditions, *PDA*-specific RNA was not detected within 8 h in

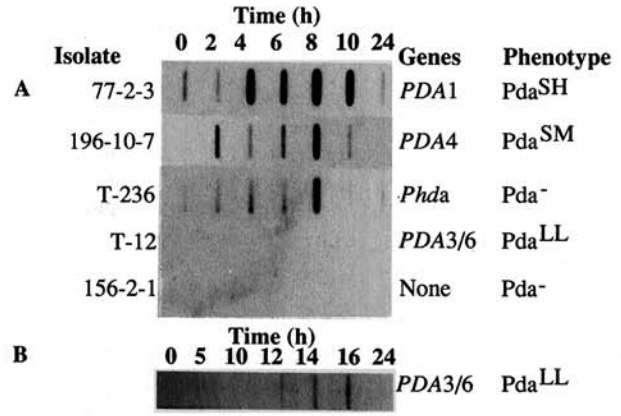

Fig. 2. Pisatin-induced accumulation of *PDA* RNA in mycelia of isolates of *Nectria haematococca* carrying different *PDA* genes. **A,** Total RNA was isolated from mycelia of *N. haematococca* isolates following treatment with pisatin and hybridized to a *PDA*-specific probe that is capable of detecting all known *PDA* genes (Maloney and VanEtten 1994). The isolate number (left) *PDA* genes and whole cell phenotypes are indicated (right). Hybridization contained between 4 and 10 µg of RNA per slot (10 µg per slot in the T-12 lanes), but all slots within a time series contained the same amount of RNA. Film exposure times were adjusted so that the relative amounts of *PDA*-specific RNA within a time series could be visualized accurately. With the Pda^{SH}, Pda^{SM}, and *Phda* isolates, *PDA*-specific RNA could be detected at each time point by exposing the blots for extended periods of time. Neither the Pda^{LL} nor the Pda^- isolate accumulated detectable levels of *PDA* specific transcripts. **B,** Hybridization of RNA (10 µg per slot) from the Pda^{LL} isolate T-12 to a 1.65-kb *Hin*dIII-*Bgl*II portion of *PDA*6-1, a PDA^{LL} gene (Reimann and VanEtten 1994).

the isolate that contains a PDA^{LL} gene. If a PDA^{LL}-specific probe and extended exposure times were used, a *PDA*-specific RNA could be detected in this isolate only after 12 h exposure to pisatin (Fig. 2B).

Although it was not possible to precisely compare the relative amount of *PDA*-specific RNA produced between isolates because of differences in the specificities of the probes (Fig. 2A vs 2B), it is clear that *PDA*-specific RNA increases sooner after exposure to pisatin in isolates with PDA^{SH} and PDA^{SM} and *Phd*a genes than in the isolate with a PDA^{LL} gene. In fact, the inability of a PDA^{LL}-specific probe to detect any *PDA*-specific RNA at time points earlier than 12 h after exposure to pisatin further suggests differential regulation of these genes. Given the restraints of the *PDA*-specific probe, it was possible to directly compare relative levels of *PDA*-specific RNA within the same isolate. Levels increased more than 10-fold 8 h after pisatin induction in isolate 77-2-3 (PDA^{SH}), fourfold in isolates 196-10-7 (PDA^{SM}), and ninefold in isolate T-236 (*Phd*a). These studies confirm earlier reports of pisatin-induced expression of genes associated with the Pda^{SH} phenotype (Weltring et al. 1988; Straney and VanEtten 1994). After detection at 12 h following pisatin addition *PDA* transcripts increased fourfold in isolate T-12 (PDA^{LL}). These results demonstrate that isolates with short lag period *PDA* genes (PDA^{SH} and PDA^{SM}) accumulate measurable levels of *PDA*-specific RNA after exposure to pisatin more quickly than an isolate with the long lag period *PDA* gene (PDA^{LL}).

Induction of pdm in isolates with chimeric *PDA* genes.

Chimeric *PDA* genes were constructed in which the 5′ regions were exchanged (Fig. 3A) in order to determine if the 5′ region determines the differences in the biochemical whole cell phenotypes. The chimera with the 5′ regulatory region from a PDA^{SH} gene and the structural gene from a PDA^{LL} (pSL), the reverse construct (pLH), and control constructs in which the 5′ regions were religated to the same *PDA* gene (pSH and pLL) were transformed into a Pda^{-} isolate and tested for pdm activity in *A. nidulans* and *N. haematococca*. *N. haematococca* transformants that harbored a chimeric *PDA* gene containing the 5′ regulatory region from a PDA^{SH} gene (i.e., pSL and pSH) had readily measurable rates of whole cell pdm (>5pmole/min/mg) activity that peaked 6 to 8 h after exposure to pisatin as do isolates that contain endogenous PDA^{SH} genes (Fig. 3B; Mackintosh et al. 1989; Straney and VanEtten 1994). The Pda^{+} transformants containing *PDA* genes with the 5′ regulatory region from a PDA^{LL} gene (i.e., pLH and pLL) had pdm activity through out the course of the experiment that was too low (<5pmole/min/mg) to be accurately quantified in the short reaction period (25 min) used to assay enzyme activity at each time point. This is the same pattern of response to pisatin that is observed in isolates with endogenous PDA^{LL} genes (Kistler and VanEtten 1984; Mackintosh et al. 1989).

To ascertain if *trans*-acting factors are necessary for high pdm induction in isolates of *N. haematococca* that only contain a PDA^{LL} gene, T-12 was transformed with pUCHPda (Schäfer et al. 1989) which contains an unaltered PDA^{SH} gene. T-12 transformants containing pUCHPda responded to pisatin similarly as the transformants with pSL and pSH (Fig. 3B) and are comparable to reference isolates harboring PDA^{SH} genes (Straney and VanEtten 1994).

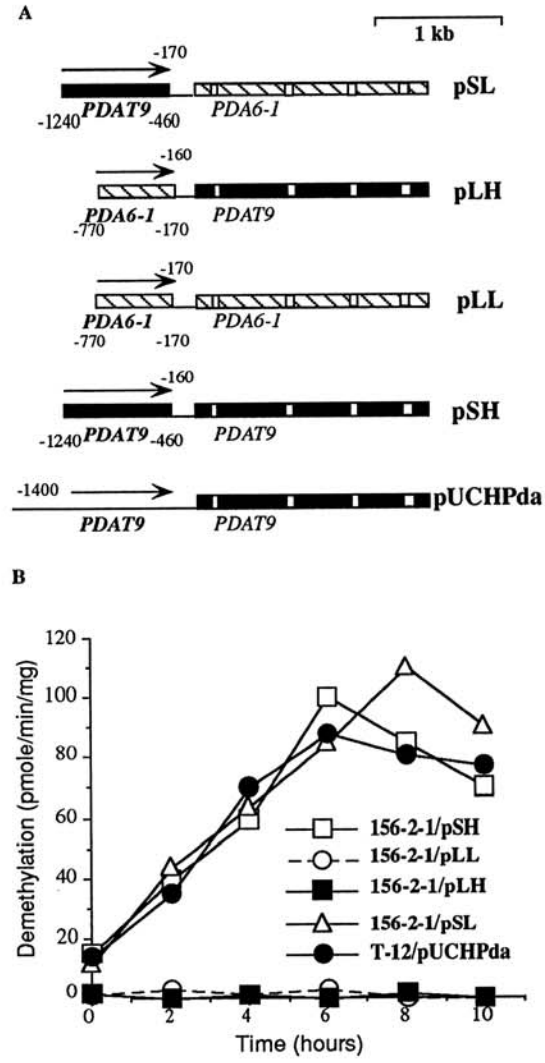

Fig. 3. Pisatin demethylase activity of chimeric *PDA* genes in *N. haematococca*. **A,** Schematic of the *PDA* chimeric gene constructs and pUCHPda (Schäfer et al. 1989). Solid boxes mark regions of *PDA*T9 (a PDA^{SH} gene) and hatched boxes represent regions of *PDA*6-1 (a PDA^{LL} gene). Open boxes represent introns. The bold lettering and arrows indicate the source of 5′ regulatory regions used for the chimeric genes and the thin lines represent the normal DNA from the noncoding regions preceding the structural gene. The numbers above the construct correspond to the last nucleotide associated with the start of the open reading frame of the structural gene (with 1 corresponding to the A in the initiation ATG codon). The numbers below a construct correspond to the nucleotide positions in the native gene of the 5′ regulatory region used in the chimeric constructs. *A. nidulans* and *N. haematococca* transformants harboring these chimeric constructs were screened for demethylation of radiolabelled pisatin in a "vial assay" (Weltring et al. 1988) to determine if they were Pda^{+}. **B,** Pisatin demethylase activity of isolate 156-2-1 (Pda^{-}) transformed with chimeric *PDA* genes and isolate T-12 (PDA^{LL}) transformed with pUCHPda. Thirty mg/ml of mycelia were resuspended in buffer and treated with 0.1 mM pisatin at time 0. The rate of pisatin demethylation was assayed at the indicated times. Activity is expressed as pmoles of pisatin demethylated^{-1} min^{-1} mg (dry wt.) of mycelium. Strains are designated by the parent isolate followed by the name of the *PDA* construct transformed into these strains. Three or more transformants obtained with each gene were assayed and since all transformants with the same gene gave similar results, only the results of one transformant are shown.

In Planta

Accumulation of different *PDA*-specific RNAs in pea tissue infected with *N. haematococca.*

Pea plants were inoculated with isolates carrying either PDA^{SH} or PDA^{LL} genes or both. After 3 days RNA was isolated from diseased tissues and hybridized to a general *PDA*-specific probe (SacB) or probes specific for PDA^{SH} (prSH) or PDA^{LL} (prLL) genes. *PDA*-specific RNA was detected only in pea tissues infected with isolates containing PDA^{SH} genes (Fig. 4). The size of the lesions produced by T-12 (PDA^{LL}) and the Pda⁻ isolate 156-2-1 were small (<3 mm). However, the detection of *N. haematococca* tubulin RNA in the lesion tissue indicates that some growth of these isolates had occurred in the pea tissue, thus the inability to detect *PDA* transcripts was not because of a lack of mycelium in the infected tissue. As an additional control, tissue infected with an isolate (T-2) that contains both a PDA^{SH} and a PDA^{LL} gene and produces large lesions (>6 mm) was assayed for PDA^{SH} and PDA^{LL} RNA. In this isolate only the PDA^{SH} RNA was detected (Fig. 4).

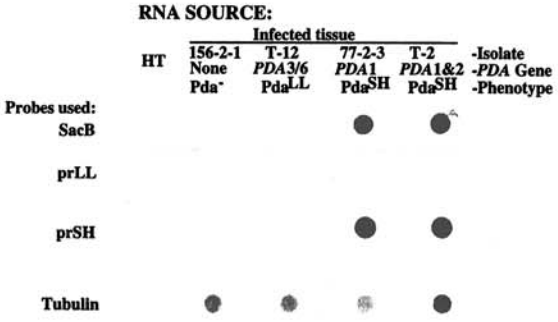

Fig. 4. Accumulation of PDA^{SH} RNA in *N. haematococca* infected pea tissue. Total RNA was isolated from pea epicotyls 3 days after inoculation with a *N. haematococca* isolate with a PDA^{SH} gene (77-2-3), a PDA^{LL} gene (T-12), a PDA^{SH} and PDA^{LL} gene (T-2) or no *PDA* gene (156-2-1). HT indicates RNA from healthy pea tissue that was not infected with a fungal isolate but was otherwise treated the same as inoculated tissue. RNA was blotted on to Hybond N^+ and then probed. *Sac*B is a general probe for all *PDA* RNA (Maloney and VanEtten 1994), prSH is a PDA^{SH} specific probe consisting of a 70-bp region in *PDA*T9 (a PDA^{SH} gene) that differs from the *PDA*6-1 gene (a PDA^{LL} gene) and prLL is a PDA^{LL} specific probe consisting of a 45-bp region in *PDA*6-1 that differs from that present in *PDA*T9. The probe used for hybridization to tubulin consisted of a 2.5-kb fragment of the *Neurospora* tubulin gene and was used to verify the presence of fungal transcripts.

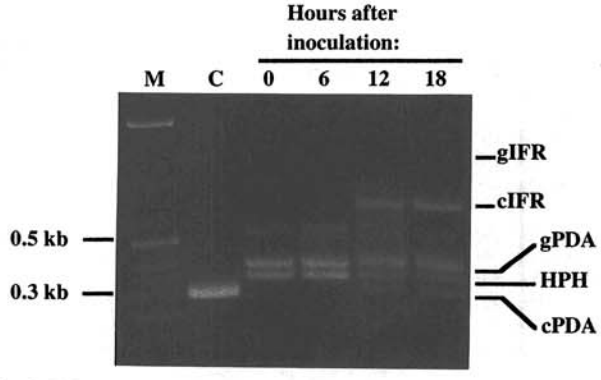

Fig. 5. Polymerase chain reaction (PCR) analysis of *PDA* and *IFR* transcription in infected pea tissue. Plant, fungal, and control transcripts from infected tissue were detected by subjecting extracted RNA to cDNA synthesis and amplification by PCR using primers for *PDA*, *IFR* and *hph*. Products were fractionated by size on a 2.0% agarose gel and stained with ethidium bromide. Lanes are identified by M, molecular weight markers, C, cDNA from isolate 77-2-3, and times postinoculation. Molecular weight markers were made by digesting pBR322 with *Hin*fI. Indicated on the right margin are expected sizes of the PCR products from genomic *IFR* (gIFR), cDNA of *IFR* (cIFR), genomic *PDA*1 (gPDA), *hph* gene (HPH) and cDNA of *PDA*1 (cPDA). The faint gIFR band and contaminating PCR fragment were reproducible. The gIFR band was characterized by hybridization to an *IFR* specific probe.

Temporal expression of a PDA^{SH} gene and a pisatin biosynthesis gene (*IFR*) during infection.

Once it was established that the PDA^{SH} genes were expressed in planta, we were interested in characterizing the timing of expression of a PDA^{SH} gene in relationship to the induction of pisatin biosynthesis during infection. To facilitate this experiment a new infection assay was developed that did not require wounding of the plant tissue. This was done to insure that the pathogen, and not the inoculation procedure, elicited the plant's defense response. The assay involved the direct application of a spore suspension to specific sites on intact roots. Visible symptoms of infection generally appeared 48 h postinoculation.

Pisatin biosynthesis was monitored by assaying isoflavone reductase (*IFR*) transcripts. The polymerase chain reaction (PCR) was employed to detect the appearance of both *IFR* and PDA^{SH} transcripts during infection. A virulent PDA^{SH} isolate (77-2-2 Trn 1.2) containing the hygromycin detoxifying gene (*hph*) was used as the fungal inoculum. If no genomic contaminating sequences were present in the cDNA synthesis, it was anticipated that the *hph* transcript could be used as a constitutive transcript to standardize the amount of fungal transcripts in the infected tissue. The *hph* gene was not induced by pisatin or water-soluble plant extracts from infected pea tissue (data not shown).

The oligonucleotide primers used to detect PDA^{SH} transcripts flanked an intron so that the size of PCR amplified products could be used to distinguish contaminating genomic DNA sequences from cDNA sequences. Though the number and exact location of the *IFR* gene introns have not been determined, this gene likely contains introns (Paiva et al. 1994) and preliminary tests indicated that the primers used in this study allow us to distinguish genomic *IFR* from cDNA sequences.

Throughout the course of this experiment two PCR products were amplified corresponding to the PCR product expected from amplification of the genomic *PDA* gene (410 bp) and the faint 700-bp product expected from amplification of the plant genomic *IFR* gene (Fig. 5). This indicates that both fungal and plant tissue was present at the inoculation site analyzed. A 300-bp product corresponding to the size expected for the *PDA* cDNA amplified product was not detected until 12 h after inoculation. Both the 410- and 300-bp PCR products hybridized to a *PDA* specific probe (SacB), verifying that these are PCR-amplified products of the genomic *PDA* and *PDA* cDNA, respectively. A product greater than 500 bp, the size expected for the PCR product from the *IFR* cDNA (526 bp) was detected 12 h after inoculation (Fig. 5). Both the faint 700-bp genomic product and the 520-bp cDNA product hybridized to an *IFR*-specific probe.

Treatment of the infected-tissue extract with RNAase prior to PCR resulted in no amplification of the 526 and 300 bp products, respectively. However, the 340-bp product characteristic of the hph gene, as well as the genomic fragments from the PDA and IFR genes were detected. This indicated that the samples were contaminated with genomic DNA and thus the hph-derived product could not be used to quantititate fungal transcripts.

When a similar analysis was done using an isolate (T-200) that contained only a PDA^{LL} gene, PCR products from both the *PDA* and *IFR* transcripts could be detected at the earliest time point tested (24 h after inoculation). The observation that PDA^{LL} transcripts can be seen through PCR analysis but not by Northern analysis (when using a strain with a similar *PDA* gene) suggests that this group of *PDA* genes are expressed but at low levels during infection.

DISCUSSION

In Vitro analysis.

At least two models can be proposed to explain the Pda phenotypes: 1) Genes which confer the Pda^{SH} or Pda^{SM} phenotypes encode enzymes that more efficiently catalyze the demethylation of pisatin than the genes associated with the Pda^{LL} phenotype; or 2) *PDA* gene products are enzymatically equivalent but the genes differ in their rates of expression. To further define the mechanisms underlying whole-cell Pda phenotypes, we initially examined the *PDA*-specific RNA accumulation in mycelium after induction by pisatin in culture. The *PDA* genes associated with a short lag phase all had rapid accumulation of *PDA*-specific RNA with a maximum at around 8 h after exposure to pisatin. Previous studies of two isolates with the Pda^{SH} phenotype have also demonstrated that induction of pdm activity occurs at the level of transcriptional activation (Weltring et al. 1988; Straney and VanEtten 1994). *PDA* genes associated with the Pda^{LL} whole cell phenotype were not rapidly induced in culture. Slower induction of transcription may result in the long lag phase in pisatin demethylase activity observed in isolates with these genes. Transformation of Pda^{-} isolates with chimeric constructs which fused the 5′ regulatory region of a PDA^{SH} gene to the PDA^{LL} structural gene and its complement, the PDA^{LL} 5′ regulatory region fused to a PDA^{SH} structural gene, resulted in transformants that expressed whole cell phenotypes as determined by the 5′ regulatory region and not the structural gene. Furthermore, a PDA^{LL} isolate was shown to contain the necessary *trans*-acting factors to allow rapid induction of an ectopic PDA^{SH} gene.

A preliminary analysis of transcriptional activation of a PDA^{SH} gene has shown that a 35-bp region positioned –504 to –469 relative to the first mRNA start site acts as a binding site for a 220,000 Da protein that is produced in response to pisatin (Straney and VanEtten 1994). When multiple copies of the 35-bp binding site were transformed into *N. haematococca* containing only a PDA^{SH} gene induction of pdm activity was significantly delayed, suggesting titration of a *trans*-acting factor involved in activation (Straney and VanEtten 1994). These results suggest this 35-bp region contains a pisatin responsive activator site.

The cloning and sequencing of *PDA* genes that encode different Pda whole cell activities allow a comparison of their putative pisatin-responsive activator binding (*PRAB*) sites in the different genes (Fig. 6). *PDA* genes which are rapidly induced in response to pisatin contain the conserved PRAB site. A *PDA* gene that confers the Pda^{LL} phenotype has nine mismatches compared to the consensus and is interrupted by an insertion of 5 bp. Although the chimeric constructs used in this study differ in the length of 5′ sequence, they both contain the gene's putative PRAB site. The results of this study are further evidence that this region is associated with the induction of pdm activity.

We have also shown that addition of pisatin caused the induction of high levels of *Phd*a-specific RNA in a Pda^{-} isolate, T-236, which contains *Phd*a. Thus, absence of pdm activity in this isolate is not because of lack of transcription of *Phd*a. In fact, the PRAB site is identical to the short lag phase gene consensus sequence (Fig. 6). *Phd*a lacks a stop codon at the position where it is found in the functional *PDA* genes (K. Hirschi, unpublished) and this may account for the lack of pdm activity in T-236.

In planta *PDA* expression.

To parasitize pea, *N. haematococca* must have the ability to grow in the potentially noxious environment of the infected pea tissue. Tissue infected with *N. haematococca* may be comprised of up to 5% pisatin (dry weight) (Pueppke and VanEtten 1974). The high expression in planta of PDA^{SH} genes in lesion tissue (Fig. 2) suggests that pisatin may be the natural inducer of PDA^{SH} gene expression in planta. This is in agreement with studies which showed that pisatin is a specific inducer of the expression of pdm in vitro (VanEtten and Barz 1981; Straney and VanEtten 1994). Our in planta Northern analyses also show that fungal PDA^{LL} transcripts are not expressed at significant levels in the infected tissue when compared to PDA^{SH} transcripts. This difference in expression was evident in a fungal isolate (T-2) which contains both a PDA^{SH} and PDA^{LL} gene and indicates that the differences are due to structural elements of the genes' 5′ regulatory region rather than to a deficiency in some *trans*-acting element. Thus the difference in growth in infected tissue between isolates that contain a PDA^{SH} gene and isolates that contain only PDA^{LL} genes is not the reason that PDA^{LL} transcipts are recovered in small amounts.

The synthesis of phytoalexins constitutes part of an active response in plants that is induced by many invading microbes (Anderson 1991). The transcription of genes specifically involved in the latter steps of phytoalexin biosynthetic pathways is apparently repressed in healthy cells, but is induced after inoculation with a pathogen. Thus, transcripts of pisatin syn-

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CONSENSUS   GGCCGATCTTATCTCCGATTAACA      CCTCGTGAGATGATGAA
PDA1(Pda^SH)  ------------------------      ----------------
PDAT9(Pda^SH) ------------------------      ----------------
PDA4(Pda^SM)  ------------------------      ----------------
PDA6(Pda^LL)  ----CT--A---G-T----CT-A-TACGG-----A----------
Phda (Pda^-)  ------------------------      ----------------
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Fig. 6. Comparison of the 35-bp pisatin-responsive activator binding (PRAB) site in the sequenced *PDA* genes and *PDA* homolog. The putative PRAB sites are compared in *PDA*1 (Straney and VanEtten 1994), *PDA*T9 (Maloney and VanEtten 1994), *PDA*6-1 (Reimann and VanEtten 1994), *PDA*4 and *Phd*a (Hirschi, unpublished). The phenotype each gene confers immediately follows the genes' name. The dashed lines "-" indicate that the bases are identical to the consensus sequence. The break in the sequences represents inserted bases present only in *PDA*6-1.

thetic genes should be detectable soon after inoculation of the pea tissue. The time postinfection at which *IFR* transcripts are detected (Fig. 5) is consistent with the timing of pisatin accumulation in elicited pea tissue (Teasdale et al. 1974). The response of the attacking fungus may be to express a specific enzyme (pdm) to detoxify pisatin. Presumably, transcription of *PDA* would be repressed until sufficient pisatin accumulates in the infection zone to induce expression. We have shown that PDA^{SH} transcripts reach a certain threshold level that can be detected 12 h after infection as are transcripts of a gene specifically involved in pisatin biosynthesis. Presumably, during this interval postinfection, the plant accumulates sufficient levels of pisatin that result in the induction of *PDA* transcripts in *N. haematococca*. Therefore, while triggering the initiation of pisatin biosynthesis, *N. haematococca* is also prepared to respond to the plant's defense with the synthesis of an enzyme capable of detoxifying pisatin.

Previous studies suggest that the efficiency with which *N. haematococca* detoxifies newly synthesized pisatin is a determining factor in the outcome of the interaction between pea and this fungus (VanEtten et al. 1989). Strong support for this supposition has been the observation that only Pda^{SH} and Pda^{SM} isolates are pathogenic on pea and Pda^{LL} and Pda^{-} isolates are not. However, recent results have shown that the virulence of PDA^{SH} gene-disruption mutants while reduced, is not reduced to the level of naturally occurring Pda^{-} or Pda^{LL} isolates (C. Wasmann and H. D. VanEtten, unpublished; VanEtten et al. 1994b). This has lead to the hypothesis that the previously unbroken association between high virulence on pea and the presence of the Pda^{SH} or Pda^{SM} phenotype was due to an unbroken genetic linkage between *PDA* genes encoding these phenotypes and other (unidentified) pea virulence genes called PEP^{D} (VanEtten et al. 1994b). Thus, according to this model, high virulence on pea required both a PDA^{SH} (or PDA^{SM}) gene and PEP^{D} gene.

One possibility that has been offered to explain why the gene-disruption Pda^{-} mutants are still pathogenic even though they lack the ability to detoxify pisatin is that *N. haematococca* has another means to resist the inhibitory effects of pisatin (VanEtten et al. 1994a). An inducible "non-degradative" mechanism that increases the tolerance of this fungus to pisatin has been described previously (Denny et al. 1987). Such a redundant tolerance mechanism may relieve the fungus from a dependency on pdm for pathogenicity and allow the pdm to acquire another function such as the first step in the catabolism of pisatin as a carbon source (VanEtten et al. 1994b). The observation that pdm is glucose repressed suggests that this enzyme could be part of a nutritional pathway (Straney and VanEtten 1994). The high concentrations of pisatin found in lesion tissue and the ability to use pisatin as a carbon source could give *N. haematococca* an advantage over other necrotrophic microorganisms at the infection site. Whatever eventual role(s) is (are) demonstrated for pdm, the results of the current study suggest that *PDA* genes are expressed during pathogenicity by *N. haematococca* on pea and that expression of these genes starts early in the infection process.

MATERIALS AND METHODS

Fungal strains, plasmids, and DNA.

Isolates of *N. haematococca* MP VI used in this study have a single active *PDA* gene except for isolate T-2 which has two *PDA* genes (*PDA*1, PDA^{SH}, and *PDA*2, PDA^{LL}) (Kistler and VanEtten 1984; Miao and VanEtten 1991), 156-2-1 which has no *PDA* genes and is Pda^{-} (Miao and VanEtten 1992) and T-236 which is Pda^{-} but has *PDA* hybridizing DNA (*Phda*) (Miao and VanEtten 1991). Isolates with a single *PDA* gene are 77-2-3 (*PDA*1, PDA^{SH}; Kistler and VanEtten 1984), 196-10-7 (*PDA*4, PDA^{SM}; Mackintosh et al. 1989), T-12 (*PDA*3/6, PDA^{LL}; K. Hirschi, unpublished) and T-200 (*PDA*6-2, PDA^{LL}; Miao et al. 1991). All of the *PDA* genes confer the Pda phenotype indicated and all were originally identified by conventional genetic analysis except for the *PDA* gene in T-12. RFLP analysis does not distinguish *PDA*3 from *PDA*6-1 and *PDA*6-2 and thus this PDA^{LL} gene in T-12 is listed as *PDA*3/6. *PDA*T9 was also not identified by conventional genetic analysis but restriction site analysis and partial sequence analysis indicate that it is a homolog of *PDA*1 (Maloney and VanEtten 1994; Straney and VanEtten 1994). Isolates designated with the prefix "T" were conidial descendants of field isolates. Those with only a numerical label are descendants of laboratory crosses. Strain 77-2-3 transformant (Trn) 1.2 (C. Wassman and H. D. VanEtten, unpublished) was used to inoculate peas for the temporal expression of *PDA* and *IFR* in planta. This strain contains the *E. coli* hygromycin β-phosphotransferase gene (*hph*) and a single copy of *PDA*1. *N. haematococca* isolates were grown and maintained as described previously (VanEtten and Kistler 1988; Miao et al. 1991).

Aspergillus nidulans strain UCD1 (pabaA1, yA2, biA1, argB2, metG, trpC801) was used as the recipient for cotransformation (Welting et al. 1988) to test if the chimeric *PDA* gene constructs were functional. The cosmid pKBY2 (Yelton et al. 1985) carrying the *trpC* gene from *A. nidulans* was used as the selection marker in cotransformation of *A. nidulans*. The plasmid pDH33 (Smith et al. 1990) contains the promoter region of the *A. niger* glucoamylase-encoding gene (*glaA*) linked to the *hph* and was used as the selection marker in cotransfromation experiments of *N. haematococca*. The plasmid pUCHPda, called pUP1 in Schäfer et al. 1989, was used to directly transform *N. haematococca* isolate T-12. The plasmids containing subclones from *PDA*T9, (pSacA, pSacB, and pSacC) and *PDA*6-1 (pCR31 and pCR37) have been described previously (Maloney and VanEtten 1994; Reimann and VanEtten 1994). pBT3 was obtained from M. Orbach, and pK0.1 was a gift from C. Wasmann, both of the Department of Plant Pathology, University of Arizona.

Pea genomic DNA was a gift from E. Vierling, Department of Biochemistry, University of Arizona.

Pdm assay and induction of *PDA* and pdm.

Induction of *PDA* and pdm by pisatin was as described by Straney and VanEtten (1994). The assay for calculating the rate of pisatin demethylation was performed as described previously (VanEtten and Matthews 1984). Briefly, a 4.0-ml sample was withdrawn from the pisatin-treated or control mycelial suspension at the times indicated and 3-*O*-Methyl-^{14}C pisatin was added to give a ^{14}C-pisatin concentration of 0.1 mM (specific activity 1.1×10^5 $min^{-1}\mu mole^{-1}$). The flasks were incubated on a reciprocal shaker (90 strokes min^{-1}) at 25°C, and 4 or 5 samples (0.5 ml) were withdrawn at equal time intervals over the following 20 or 25 min and the content of ^{14}C -pisatin in each sample measured (Matthews and VanEtten 1983).

Pisatin demethylation rates were determined by calculating the linear regression of pisatin concentration v. time, and were expressed as pmoles of pisatin demethylated min^{-1} mg^{-1} (fresh wt) of mycelium.

Chimeric gene constructions.

The upstream regulatory regions of *PDA*T9 and *PDA*6-1 were exchanged by subcloning the structural genes and adding the presumptive regulatory region to the other gene. The regulatory region of *PDA*T9 was cloned by ligating the 190-bp *Avr*II to *Sst*I fragment 5′0 to the PDAT9 ORF from pK0.1 (C. Wasmann and H. D. VanEtten, unpublished) into *Hin*dIII/*Sst*I digested pBluescript SK+, resulting in plasmid pdH.1. The 1.35-kb *Sst*I fragment of *PDA*T9 was cloned into the *Sst*I site of pdH.1 resulting in pdH.2. Then the 1.4-kb *Xho*I/*Sph*I fragment of pdH.2 was cloned into the *Xho*I/*Sph*I sites of p*PDA*T9 (Maloney and VanEtten 1994) to obtain pdH. Finally, the region 5′ of the structural gene of *PDA*6-1 gene from –170 to –770 termed pCR30 (Reimann and VanEtten, 1994) was digested with *Sma*I and *Hin*dIII and this fragment was cloned as a *Sma*I and *Hin*dIII fragment into the *Xho*I blunted and *Hin*dIII site of pdH to obtain pLH.

The *PDA*6-1 structural gene was subcloned by combining the 1.1-kb *Hin*dIII to *Eco*RI fragment of pCR38 and the 1.5-kb *Eco*RI fragment of pCR40 in pBluescript SK+ to form pdL (Reimann and VanEtten 1994). To obtain the 5′ regulatory region of *PDA*T9, first the 780 bp *Sst*II/*Hin*dIII fragment of pSacA (Maloney and VanEtten 1994) which contains the region of DNA from –460 to –1240 was cloned into the *Sst*II/*Hin*dIII fragment of pBluescript SK+ resulting in pPSH1. The 780-bp *Sst*I blunted/*Xho*I fragment was then ligated into the *Cla*I blunted/*Xho*I fragment of pdL, resulting in pSL.

Two reconstructed *PDA* genes served as controls to assess if the cloning manipulations had altered the phenotype of these genes. The *PDA*T9 5′ region of pPSH1, was subcloned as a *Cla*I blunted *Sst*I blunted fragment into the *Xho*I blunted pdSH1 site to form pSH. As a complementary control, the 600-bp pCR30 *Kpn*I/*Bam*HI fragment was ligated into *Kpn*I/*Bam*HI sites of pUC19 to obtain pCR30.1 which was then digested with *Hin*dIII, blunted, and ligated into the *Cla*I blunted site of pdL to form pLL. All 5′ regulatory region constructs were checked by restriction analysis to confirm the proper orientation.

Transformation of *Aspergillus nidulans*.

Plasmid DNA lacking suitable markers for direct selection into *A. nidulans* can be introduced at varying efficiencies into *A. nidulans* via cotransformation (Timberlake et al. 1985). Plasmids with the chimeric *PDA* genes (pdH, pLH, pdL, and pSL) were mixed in a 1:1 ratio (approximately 2 µg each) with plasmid DNA of pKBY2 and transformants selected by growth on medium lacking tryptophan. Since pKBY2 carries a trpC gene complementing the tryptophan deficiency in *A. nidulans* strain UCD-1, it provides the selection for *A. nidulans* transformation. These transformants were then tested for their ability to demethylate pisatin and in all cases where plasmids with chimeric *PDA* genes were mixed with pKBY2, some (5 to 40%) of the transformants were Pda^+, indicating that the chimeras formed functional *PDA* genes.

***Nectria* transformation.**

The transformation protocol is based in part on the procedures of Stahl and Schäffer (1992) as adapted by S. Covert (unpublished). One-hundred milliliters of glucose-asparagine medium (Kistler and VanEtten 1984) was inoculated with a spore suspension from one culture grown for 3 to 5 days on a plate of V8 medium (200 ml of V8 juice, 3 g of $CaCO_3$, 25 g of agar per liter). This liquid culture was grown overnight (9 to 14 h) at 28°C, 200 rpm, harvested and rinsed with 25 ml of 0.6 M $MgSO_4$. The mycelium was then transferred to a sterile 250-ml flask containing 24 ml of osmolarity medium (1.2 M $MgSO_4$ $7H_2O$ in 10 mM NaH_2PO_4, pH 5.8). Filter sterilized Novozym (25 mg/ml obtained from Novo Industries, Copenhagen, Denmark) in a 6-ml volume was added and the cultures were gently swirled (40 rpm) for 2 h. Cultures were then filtered through miracloth (Calbiochem, San Diego, CA.) into two 30-ml Corex tubes. The solutions were gently layered with 10 ml of trapping buffer (0.6 M sorbitol in 100 mM Tris-HCl, pH 7.0) centrifuged at 5,000 rpm for 5 min and the protoplasts collected at the interface. Protoplasts were washed twice with 10 ml of 1 M sorbitol, 50 mM EDTA, pH 8.0, and once with 10 ml of STC (1.2 M sorbitol, 10 mM Tris-HCl, 50 mM $CaCl_2$, pH 8.0). The protoplasts were then counted and the solution adjusted with STC to 5×10^7 protoplasts/ml. For cotransformation, 5 µg of pDH33 to provide selection of transformants and 5 µg of the plasmid DNA with chimeric *PDA* genes were added to 100 µl of protoplasts and incubated for 30 min on ice. The DNA/protoplast mixture was then brought to a volume of 1 ml with 20% PEG 4000 (in 25 mM Tris-HCl and 25 mM $CaCl_2$, pH 8.0). The mixture was then immediately mixed with 4 ml of molten (48° C) medium (1% agar containing 410 g of sucrose, 1 g of yeast extract, and 1 g of casein hydrolysate per liter). The 5-ml solution was then poured onto 1.5 % agar plates (1.0 g of yeast extract, 1.0 g of casein hydrolysate, 342 g of sucrose per litre). Plates were incubated at 28°C and overlaid after 18 h with 5 ml of molten water agar containing hygromycin B (final hygromycin B concentration 50 µg/ml). Routinely, after 3 to 4 days, 1 to 5 transformants/µg of DNA were observed. As with the *A. nidulans* transformation, approximately 5 to 20% of pDH33 transformants also contained functional *PDA* constructs. Transformants were purified by propagating colonies from isolated conidia.

Production of diseased pea tissue.

The previously described "test-tube assay" (VanEtten et al. 1980) was used to obtain pea tissue infected with *N. haematococca* for the *PDA* RNA accumulation analysis. Each pea plant was inoculated at several (one to four) locations on the epicotyl (starting 2 to 4 cm above the lip of the test tube) and after 3 days each lesion was excised with a scalpel, lyophilized, and weighed. Each sample contained between 75 and 100 lesions (from approximately 30 plants), depending on the size of the lesion produced. The total dry weight of each sample was approximately 200 mg.

Preparation of infected tissue for transcript analysis by PCR.

Pea seeds were sterilized as previously described (Pueppke and VanEtten 1974) and germinated on water agar for 3 to 5 days. When root length was approximately 5 cm the germinated seeds were placed on the surface of sterile moist vermiculite with roots exposed in 20 × 13 × 8 cm autoclaved

pans. The vermiculite was kept moist with water, and plants were kept in the dark at 22°C for 1 to 2 days before inoculating. Inoculum was prepared by growing strains 77-2-3 Trn 1.2, T-2 or T-200 at 18°C in diffuse light on 1% agar medium of M-100 (Stevens 1974). These isolates produced approximately 90% macroconidia and 10% microconidia on this medium under these conditions. After 5 days, spores were collected from the plates, washed twice in 10 ml of water, and diluted to approximately 4×10^4 spores/ml in 0.5% Tween-20 (BioRad). Ten-microliter aliquots were applied directly to the root surface approximately 5 cm from the cotyledons. Each plant was inoculated at up to three sites and the location of the inoculation was identified by placing a toothpick in the vermiculite adjacent to the site. The plants were maintained at 22°C in the dark with occasional addition of water. After 6 days, approximately 50% of the peas inoculated with virulent isolates of *N. haematococca* produced visible lesions.

Prior to 2 days postinoculation, no lesion could be seen and thus the area adjacent to the toothpick corresponding to the site of inoculation was excised. Approximately 10 inoculated pea plants were used for each time point to obtain a total biomass of 17.5 to 22.5 mg fresh weight.

RNA extraction and gel blot analysis.

RNA was isolated from mycelium and infected pea tissue as described previously (Straney and VanEtten 1994). To obtain mRNA for the polymerase chain reaction (PCR) analysis, mRNA was isolated from inoculated pea tissue using the RNAgents total RNA isolation kit (Promega, Madison, WI) and the PolyATract system 1000 (Promega, Madison, WI) according to manufacturer's protocols.

For slot blot analysis, equal amounts (as determined spectrophotometrically) of RNA from the mycelia were treated as previously described (Straney and VanEtten 1994). RNA hybridization intensities were quanitated using a scanning laser densitometer (Molecular Dynamics Computing Densitometer, using Image Quant software). The relative increase of *PDA*-specific RNA was defined as the level of increase above the first detectable transcript.

An internal 1.35-kb *Sac*I fragment (SacB) of *PDA*T9 was used as a general probe for *PDA* (Maloney and VanEtten 1994). A 1.65-kb *Hin*dIII-*Bgl*II fragment of the *PDA*6-1 structural gene was used as a probe for *PDA*6-1-specific transcripts in the slot blot assay (Reimann and VanEtten 1994). For RNA blots, the PDA^{SH}- specific probe (prSH) was the 70-bp *Taq*I-*Hpa*II fragment (prSH) from the 3′ region of *PDA*T9 that is absent from *PDA*6-1 (Maloney and VanEtten 1994). The PDA^{LL}-specific probe was constructed from the 45-bp *Ava*I-*Hin*fI fragment (prLL) that occurs in the 3′ region of *PDA*6-1 but not in *PDA*T9 (Reimann and VanEtten 1994).

A 2.5-kb fragment of the *Neurospora crassa* tubulin gene obtained by digesting plasmid pBT3 with *Sal*I and *Hin*dIII (Orbach et al. 1986) was used as a probe for *N. haematococca* tubulin transcripts.

Each probe was labeled to a specific activity $\geq 1 \times 10^8$ dpm/μg, and membranes hybridized and washed as previously described (Miao et al. 1991).

In Southern analysis, prSH hybridizes only to *PDA*1 homologs and prLL hybridizes only to *PDA*2 and *PDA*3/6 homologs under conditions of high stringency. In Southern and Northern analysis, the *N. crassa* tubulin probe hybridized to each *N. haematococca* isolate with equal intensity and did not hybridize to pea genomic DNA at high stringency.

Detection of *PDA* and *IFR* transcripts in inoculated pea tissue by PCR.

After mRNA isolation, cDNA was synthesized using Stratagene's (La Jolla, CA) first-stand synthesis kit according to the manufacturer's protocol. The cDNA pool (4 μl) was diluted with 95 μl of 50 mM KCl, 50 mM Tris chloride, 2.5 mM $MgCl_2$, pH 8.3, 1.5 mM of each dNTP, and 50 pmol of each primer by heating for 5 min, at 95°C and cooled to 72°C. Then 2.5 units of *Thermus aquaticus* DNA polymerase (Perkin-Elmer-Cetus, Norwalk, CT) was added and the mixture was overlaid with 100 μl of mineral oil. A DNA Thermal cycler (Perkin-Elmer-Cetus), was used to perform 35 cycles of amplification of the following thermal profile: 94°C, 1 min; 52 to 58°C, 1.5 min; 72°C, 1 min); this was followed by a final 5-min extension at 72°C.

The cDNA from infected pea tissue was typed by PCR analysis with a set of *IFR* primers (5′-TTGGACTAGATGTGGATCGTCACGATGC-3′ and 5′CAGGGTCAATCTCATACACTGCATCTCC-3′, 526-bp cDNA PCR fragment and 700-bp genomic PCR fragment), a set of *PDA*T9 primers (5′-TGCCTTACCTACAGGCGGTTATCCAAGG-3′ and 5′-TGTTCTTGCCAATACAGGATCGGGAACC-3′, 300-bp cDNA PCR fragment and 410 bp genomic PCR fragment), a set of *hph* primers (5′-CATGTGTATCACTGGCAAACTGTGATGG-3′ and 5′-TGGTCAAGACCAATGCGGAGCATATACG-3′, 340-bp cDNA PCR fragment and 340-bp genomic PCR fragment); and in some cases *PDA*6-1 primers (5′-CTGGAGACAAAGGGGAACCTG-3′ and 5′-AATGGGCGGTATCTAAGCCGC-3′, 238-bp cDNA PCR fragment and 298 bp genomic PCR fragment).

ACKNOWLEDGMENTS

We thank Greg DiCenzo for help with figure preparation, Cathy Wasmann and Scott Soby for numerous helpful discussions, and comments on the manuscript and the entire VanEtten lab for their input. We would also like to thank Nancy Paiva of the Samuel Roberts Noble Foundation for the *IFR* primers. This research was supported by grant No. DE-FG62-89ER14038 from the DOE and grant No. MCB9319733 from NSF.

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