Characterization of the *PDA*1 Promoter of *Nectria haematococca* and Identification of a Region That Binds a Pisatin-Responsive DNA Binding Factor

David C. Straney[1,2] and Hans D. VanEtten[1,3]

[1]Department of Plant Pathology, Cornell University, Ithaca, NY, 14853 U.S.A.; [2]Department of Botany and the Maryland Agriculture Experiment Station, University of Maryland, College Park, MD 20742 U.S.A.; [3]Department of Plant Pathology, University of Arizona, Tucson, AZ 85721 U.S.A.

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Isolates of *Nectria haematococca* (anamorph: *Fusarium solani*) are able to detoxify the pea phytoalexin pisatin through expression of pisatin demethylase (pda). This enzyme is a substrate-inducible cytochrome P450 monooxyenase that is encoded by the *PDA* gene family. In the current study, *PDA*1, a highly inducible PDA gene, was cloned and the 5′ untranslated region was sequenced. The PDA mRNA levels were measured in pisatin-treated mycelium and found to increase by 20-fold over untreated control. Gel shift assays identified a 35-bp region, –514 to –480 bp relative to the first mRNA start site, that binds a factor found in extracts of pisatin-treated mycelium and absent in untreated mycelium. The function of the binding site in pisatin regulation of the *PDA*1 gene was tested in an *in vivo* competition assay by introduction of multiple ectopic copies of the binding site into *N. haematococca* through transformation. In such transformants, induction of pda activity by pisatin was delayed and reduced, consistent with the titration of a *trans*-acting factor which responds to pisatin. These results suggest the 35-bp region is functioning as a pisatin-responsive activator binding site for *PDA*1. Additional controls were characterized that act on *PDA*1 expression. Induction of pda by pisatin was suppressed by the addition of 0.8% Casamino Acids or 5% glucose to the suspended mycelium. A unique DNA binding factor was detected only in extracts from mycelia treated with the Casamino Acids that bind to the same 35-bp region of the *PDA*1 gene as the pisatin-responsive factor.

Additional keywords: cytochrome P450, transcription factor.

Nectria haematococca Berk. & Br. mating population (MP) VI (anamorph: *Fusarium solani*) is a fungal pathogen of many plants including garden pea. One trait that all naturally occurring isolates that are pathogenic on pea share is the ability to detoxify pisatin, the isoflavanoid phytoalexin of pea (Tegtmeier and VanEtten 1982; VanEtten and Matthews 1984). The detoxification of pisatin is performed by a cytochrome P450 monooxygenase, pisatin demethylase (pda) (Desjardins *et al.* 1984; Desjardins and VanEtten 1986) and the genes (*PDA*) encoding pda represent a unique family of cytochrome P450s (CYP57) (Maloney and VanEtten 1993). Six loci in *N. haematococca* encoding *PDA* genes have been identified and one of three different phenotypes of whole-cell pda activities have been associated with each of the *PDA* genes (VanEtten *et al.* 1989). These Pda phenotypes can be distinguished by different pda expression in response to pisatin treatment, in terms of the lag period preceding induction of pda activity by pisatin and the resulting peak amount of enzyme activity induced under nonrepressive conditions: Pda^{SH} = short lag, high activity; Pda^{SM} = short lag, moderate activity; Pda^{LL} = long lag, low activity. In previous genetic analyses, only isolates with *PDA* genes encoding the Pda^{SH} or the Pda^{SM} phenotypes are highly virulent on pea (Macintosh *et al.* 1989). Preliminary studies suggested that the pisatin induction of Pda^{SH} occurs at the level of mRNA accumulation (Weltring *et al.* 1988). These findings, along with the previous observations that pda activity is specifically induced by pisatin and repressed by nutritional status (VanEtten and Barz 1981), two signals that would be expected to vary during infection on pea by this fungus, imply that specific regulation of pda *in situ* could be occurring.

Several enzymatic activities in other plant pathogenic fungi are known to be induced by signals derived from interaction with the plant. Kievitone hydratase (Turbek *et al.* 1990), pectolytic enzymes (Dean and Timberlake 1989, Gonzáles-Candelas and Kolattukudy 1992), and cutinase (Lin and Kolattukudy 1978) are induced by their substrate or a monomeric derivative of their substrate. Nutritional signals can also regulate expression of these activities, as the latter two have been shown to be glucose repressible. The pisatin demethylase system presents a well-defined system in which to study the underlying molecular mechanisms whereby signals from the plant, particularly a host-specific compound, regulate the expression of a fungal gene. The purpose of the present study was to begin a characterization of the molecular basis of pda regulation by examining the promoter of a Pda^{SH} gene for sequence elements that play a role in the pisatin-induced expression of this gene.

Corresponding author: David Straney.

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RESULTS

Cloning and sequencing *PDA*1.

The *PDA*1 gene encodes a Pda^{SH} phenotype (Kistler and VanEtten 1984) and is contained in a 3.4-kb *Xho*I/*Bam*HI DNA fragment in *N. haematococca* strain 77-2-3 (Miao *et al.* 1991). This fragment was cloned from a partial genomic library of *Xho*I/*Bam*HI fragments of strain 77-2-3 by colony hybridization using *PDA*T9 as a probe. *PDA*T9 is a genetically uncharacterized *PDA* gene that was isolated from a Pda^{SH} isolate (Weltring *et al.* 1988), and it hybridizes to all known *PDA* genes of *N. haematococca* (Miao *et al.* 1991).

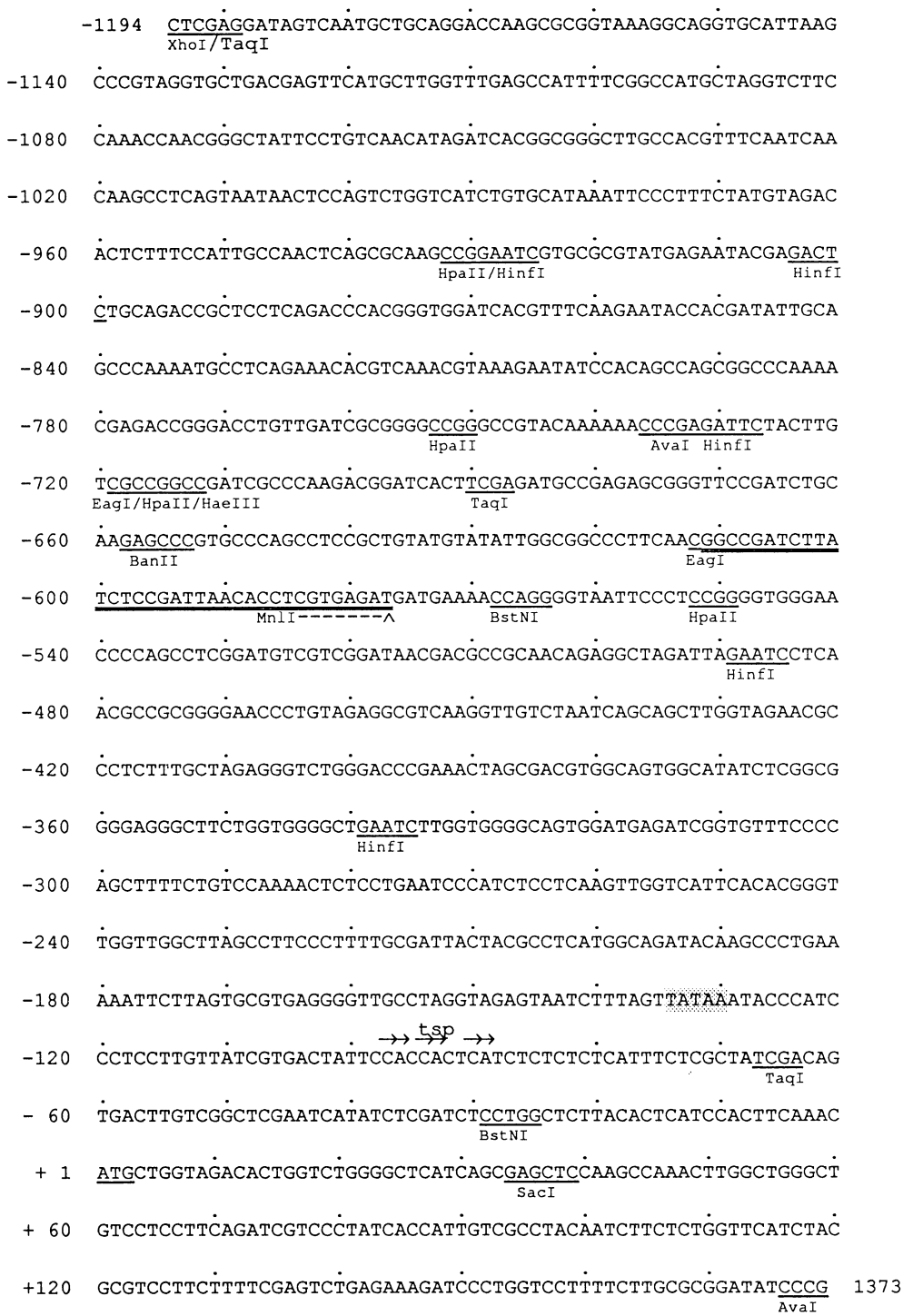

Fig. 1. Sequence of the *Xho*I/*Ava*I region of the *PDA*1 gene. The nucleotides in *PDA*1 gene are numbered relative to the translation start site at +1. Only those restriction sites mentioned in the text are shown. The transcription initiation sites are marked with arrows (→). The 35-bp region that binds to the pisatin-responsive and amino acid-responsive factors is shown with double underline. The *Mnl*I restriction enzyme cleaves at the site indicated by the arrow(^), reflecting the separation of recognition site from the cleavage site with this enzyme.

A 1.3-kb *Xho*I/*Ava*I fragment of *PDA*1, which contains the promoter and a large portion of the 5′ untranslated region of the gene was analyzed by DNA sequencing (Fig. 1). The sequence was 99% identical to the same *Xho*I/*Ava*I region of *PDA*T9 and previous studies have identified the transcription and translation start sites of this gene (Maloney and VanEtten 1994). Transcriptional start sites were identified on *PDA*1 by primer extension (results not shown) at CpA dinucleotide positions –97/–96, –94/–93, and –90/–89, and match those seen on the *PDA*T9 gene (Maloney and VanEtten 1993). A TATA-like element is located 37 bp upstream of the first mRNA start site at position –134.

Induction of pda activity and *PDA*1-specific RNA by pisatin.

The addition of pisatin to mycelium of 77-2-3 suspended in phosphate buffer induced a 20- to 25-fold increase in pda activity which peaked at 8 hr (Fig. 2). *PDA*1-specific RNA, as measured by slot blot hybridization, also increased in this fungus after exposure to pisatin. The amount of *PDA*1-specific RNA peaked at 6–7 hr after exposure to pisatin at a level about 20-fold higher than before exposure to pisatin. Mycelium, which did not receive pisatin, maintained a low level of both pda enzyme activity and *PDA*1-specific RNA. The induction of pda activity by pisatin was therefore preceded by a similar increase of *PDA*1-specific RNA in this fungus.

Repression of induction of pda activity by glucose and amino acids.

Glucose-containing media have previously been reported (VanEtten and Barz 1981) to repress induction of pda activity by pisatin. Addition of either glucose (5% w/v) or Bacto-Casamino Acids (0.8% w/v), supplemented with those amino acids (0.01% Trp, 0.032% Ser, 0.009% Thr, 0.052% Gln, 0.032% Asn) destroyed during the acid treatment used to produce this hydrolysate, in the phosphate buffer prevented the induction of pda activity by pisatin in strain 77-2-3 (Fig. 3). Enzymatically hydrolyzed casein also repressed induction of pda activity but a 1-hr preincubation was required to achieve full inhibition with some batches. To determine if this amino acid repression was due to the nitrogen content of the Casamino Acids, induction was assayed in the presence of ammonium nitrate (0.15%, w/v) at a nitrogen level equivalent to the molar amount of nitrogen in Casamino Acids. In contrast to the Casamino Acids, this nitrogen source did not inhibit the pisatin induction of pda activity. The amino acid inhibition or repression of pisatin induction therefore does not seem to be directly related to the presence of a nitrogen source but instead due to the presence of the amino acid mixture.

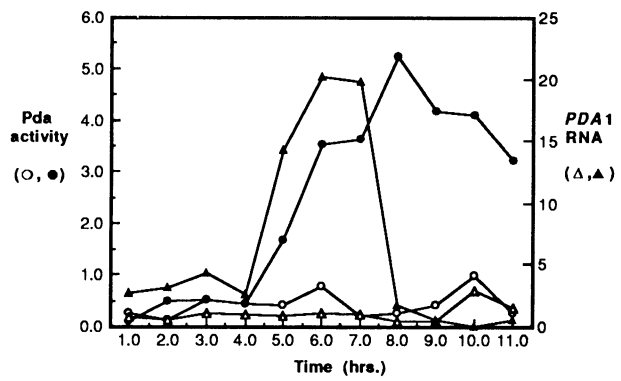

Fig. 2. Induction of pisatin demethylase activity and *PDA*-specific RNA by pisatin in *Nectria haematococca*. Mycelium was suspended in 50 mM phosphate buffer (pH 6.5), and pisatin in DMSO (●, Δ) or DMSO only (○, Δ) was added at time 0 (final concentration of 0.1 mM pisatin and 0.5% DMSO). Pisatin demethylase enzyme activity (left axis) is expressed as picomoles of pisatin demethylated per minute per microgram dry weight of mycelium. *PDA*-specific RNA (right axis) is expressed as a ratio of RNA that hybridizes to a *PDA*-specific probe (SacB) relative to the amount of DNA in the sample that hybridizes to this probe.

Protein-DNA complexes formed with mycelial extracts.

To identify putative *cis*-acting regulatory regions of *PDA*1 and putative transcriptional regulatory proteins that bind to these regions, we used the gel mobility shift assay to detect factors in mycelial extracts that bind to the cloned 1.2 kb of sequence upstream of the *PDA*1 coding region. Mycelial extracts were added to ^{32}P-labeled DNA restriction fragments of *PDA*1 and electrophoresed on a nondenaturing polyacrylamide gel (Fig. 4). The position of the labeled DNA on the gel is visualized by autoradiography. To reduce the association of nonspecific DNA binding proteins with the labeled DNA, sheared double-stranded calf thymus DNA was added to the reaction prior to addition of the extract.

In the initial assays, the 1.2-kb *Xho*I/*Sac*I fragment was digested with *Hpa*II, and the resulting five fragments were labeled with ^{32}P. The restriction digest made the upstream DNA appropriate lengths for the binding assay. The labeled DNA fragments were added to mycelial extracts prepared from mycelial suspensions treated for 3.5 hr in the following manner: in phosphate buffer (uninduced control); in phosphate buffer and 0.1 mM pisatin (pisatin induced); in phosphate buffer and 0.8% Bacto Casamino Acids; in phosphate buffer and 5% glucose. Neither the amino acid nor glucose supplemented treatments included pisatin so as to simplify analysis. Several

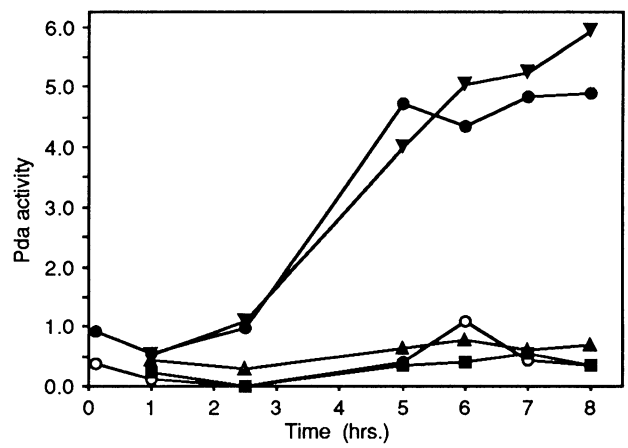

Fig. 3. Effect of amino acid supplements, ammonium nitrate, and glucose on induction of pisatin demethylase (pda) activity by pisatin. *Nectria haematococca* grown for 22 hr on a glucose, casein hydrolysate, yeast extract medium was collected and resuspended in 50 mM phosphate buffer (pH6.8) containing 0.5% DMSO and the following additions: ○, none; ●, 0.1 mM pisatin; ■, 0.1 mM pisatin and 5% glucose; Δ, 0.1 mM pisatin and 0.8% Bacto Casamino Acids; and ▽, 0.1 mM pisatin and 0.15% ammonium nitrate. At the indicated time intervals, aliquots of mycelium were withdrawn and pda activity was determined as picomoles of pisatin demethylated per minute per mg dry weight of mycelium.

DNA complexes that migrated at a slower mobility were formed at a similar yield with mycelial extracts from all four treatments (Fig. 4, left four lanes). Since regulatory proteins would be expected to appear differentially with the different treatments, we did not pursue characterization of these common complexes further. A unique complex was observed with the extract from pisatin-treated mycelium; this pisatin-responsive complex is lacking or greatly reduced when the DNA was treated with extracts from mycelium that was treated identically except for omission of pisatin (Fig. 4, lane 1 vs. lane 2). The appearance of a protein(s) after exposure to pisatin that forms such a complex would be expected for a protein involved in the pisatin-responsive activation of *PDA*1 expression.

A similar specific complex is formed by the Casamino Acid-treated mycelial extract which is absent in the control (phosphate buffer alone) extract (Fig. 4, lane 3 vs. lane 2). At this resolution, the mobility of the pisatin-responsive and amino acid-responsive complexes appear similar. Glucose treatment did not produce any unique complexes compared to the control treatment (Fig. 4, lane 4 vs. lane 2).

To assess the site-specificity of the binding factors, a 10-fold molar excess of unlabeled 1.2-kb *Xho*I/*Sac*I fragment of *PDA*1 was added along with the calf thymus DNA to the above mixtures and the gel shift assays repeated. The presence of *PDA*1 DNA should compete with protein-DNA complexes that have sequence-specific binding to the *PDA*1 DNA, but not affect nonspecific complexes at this concentration. Under these conditions, the label associated with the pisatin- and amino acid-responsive complexes disappeared (Fig. 4, right lanes), indicating that the complexes are sequence specific.

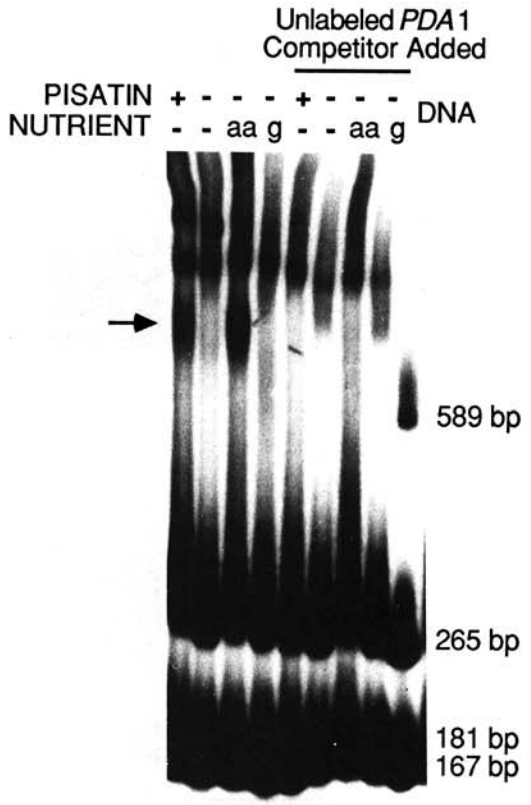

Fig. 4. Complexes formed in the gel mobility shift assay between DNA from the 5′ region of *PDA*1 and mycelial extracts of *Nectria haematococca*. The 1.2-kb *Xho*I/*Sac*I upstream region of *PDA*1 was restricted with *Hpa*II and the resulting five fragments labeled with ^{32}P. The mobility of these fragments before addition of mycelial extract is shown in the far right lane with the smallest *Hpa*II fragment (38 bp) migrating off the gel. The other lanes show the mobility of these fragments and putative protein complexes using mycelial extracts from the following treatments: lane 1, mycelium suspended for 3.5 hr in phosphate buffer with 0.1 mM pisatin; lane 2, mycelium suspended for 3.5 hr in phosphate buffer; lane 3, mycelium suspended for 3.5 hr in phosphate buffer and 0.8% Casamino Acids; lane 4, mycelium suspended for 3.5 hr in phosphate buffer and 5% glucose. A complexes unique to pisatin-treated and amino acid supplemented-mycelial extracts is indicated (→). Lanes 5 through 8 are the same extracts as lanes 1 through 4, respectively, except a 10-fold molar excess of unlabelled 1.2-kb *Xho*I/*Sac*I fragment of *PDA*1 was added before addition of the mycelial extracts. In all lanes, a 100-fold weight excess of nonspecific DNA (calf thymus) was present.

Partial characterization and purification of the binding factors.

Mycelial extracts from pisatin-induced, uninduced or amino acid-supplemented mycelia were chromatographed on a Biogel A 1.5 M (Bio-Rad) column (separation range 1,500 to 10 kDa). Column fractions were mixed with a ^{32}P-labeled *Hpa*II digest of the 1.2-kb *Xho*I/*Sac*I *PDA*1 fragment and assayed for shifts in the mobility of the *Hpa*II fragments (Fig. 5). A

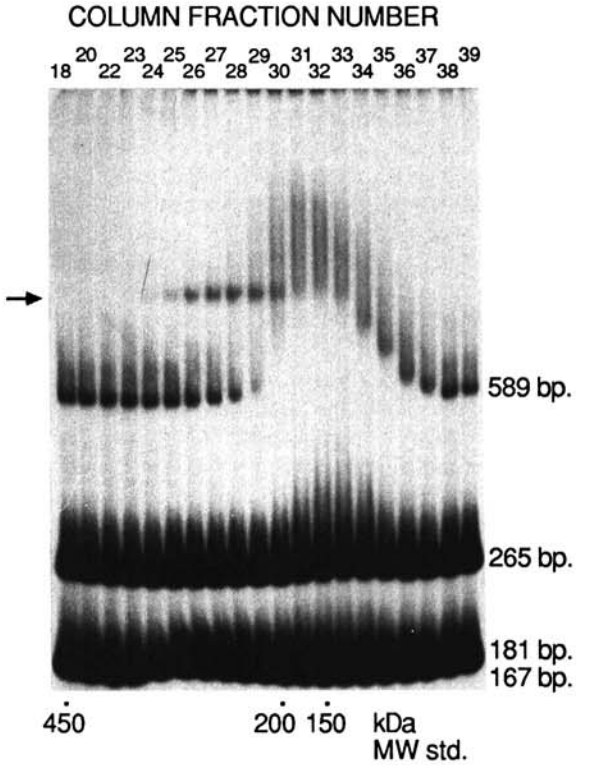

Fig. 5. Partial characterization and purification of the binding factor from pisatin induced mycelium by gel exclusion column chromatography. Mycelial extract from pisatin induced mycelium was chromatographed on a Biogel A 1.5M (Bio-Rad) column. An aliquot of each column fraction (top) was mixed with ^{32}P-labeled *Hpa*II restricted fragments of the 1.2-kb *Xho*I/*Sac*I *PDA*1 fragment and assayed for shifts in the mobility of the *Hpa*II fragments. Column purified factor is a pool of fractions 25–29 that produce the specific complex (→). Molecular weight standards eluting at the noted fractions are: apoferritin (443,000 Da), α amylase (200,000 Da), and alcohol dehydrogenase (150,000 Da).

factor estimated to be approximately 220 kDa (fractions 25–30), which formed a specific complex with a *Hpa*II fragment was readily apparent in extracts from pisatin-treated mycelium. Analysis of the DNA fragment contained in this complex, as described below, revealed that this factor was binding to the 167-bp *Hpa*II restriction fragment. Some nonspecific binding activity, binding primarily to the 589-bp *Hpa*II fragment, was observed in fractions 30–36. Similar analysis of chromatographed extracts from the uninduced and amino acid supplemented mycelia produced similar results, except extracts from the uninduced mycelium had a much lower amount of specific binding activity.

Localization and characterization of a specific region of binding on *PDA*1.

The binding site of the pisatin-responsive and amino acid-responsive binding activities were localized by identifying restriction fragments within the 1.2-kb *Xho*I/*Sac*I fragment of the *PDA*1 gene which produced the specific complexes. The determination of which DNA fragment in a mixture was bound in the complexes was accomplished by locating the complex by autoradiography, excising the complex from the gel, treating it with SDS to dissociate the protein, collecting the DNA by electroelution, and sizing the DNA by electrophoresis on a polyacrylamide gel alongside the original mixture of restriction fragments. This analysis identified the 167-bp *Hpa*II fragment (positions –716 to –550) as the *Hpa*II fragment which was forming the specific complexes with the pisatin induced and amino acid supplemented mycelial extracts shown in Figures 4 and 5. Similar analyses with other restriction digests identified a 243-bp *Hin*fI fragment (–729 to –487) and a 170-bp *Ava*I/*Bst*N1 fragment (positions –735 to –566) as forming these complexes. To further localize the binding sites, the 24-bp *Hin*fI fragment was cloned and digested with *Taq*I, *Bst*NI, *Hae*III, *Mnl*I, *Eag*I, or *Ban*II restriction enzymes. The digests were electrophoresed on a polyacrylamide gel, the restriction fragments isolated from the gel, labeled with ^{32}P and separately tested for binding activity in the gel shift assay. Consistently, only one subfragment of each restriction digest formed a complex with the pisatin-responsive and the amino acid-responsive factors. Using this strategy, we localized the binding to a 35-bp *Eag*I-*Mnl*I fragment (positions –611 to –577), 480 bp upstream of the first mRNA start site. The pisatin-responsive and amino acid-responsive factors bound this 35-bp DNA fragment with sufficient affinity that all labeled DNA could be bound in the specific complex and the binding was stable to high salt concentrations (125 mM potassium glutamate) (Fig. 6). Only a trace amount of complex was formed with uninduced extract and this was very salt sensitive. The gel mobility of the pisatin-responsive complex was different from the amino acid-responsive complex when run on such higher resolution gels. This mobility difference suggests that there may be different protein species bound in each of these complexes.

Although the 35-bp *Eag*I-*Mnl*I fragment is the smallest restriction fragment that forms these complexes, there may be sequences beyond the boundaries of this fragment which influence binding. In a competition assay using a labeled 203-bp *Taq*I/*Hin*fI fragment (positions –689 to –487), an unlabeled 46-bp *Eag*I/*Bst*NI fragment (positions –611 to –566) competed threefold less well than an equal amount of the unlabeled 203-bp fragment. Therefore, although each fragment could bind the pisatin-responsive factor, the fragment containing more flanking sequence beyond the 35-bp minimal region had stronger binding.

When a large amount of calf-thymus DNA was added as nonspecific competitor DNA in the binding reaction, the complex described above is not formed but instead a faster migrating complex was observed. The effect of calf thymus DNA on the binding complexes in *N. haematococca* may be unique, as *Escherichia coli* (DH5α) genomic DNA can be added to a 300-fold excess over labeled *PDA*1 fragments without loss of the high-yield slower complex (J. He and D. Straney, unpublished).

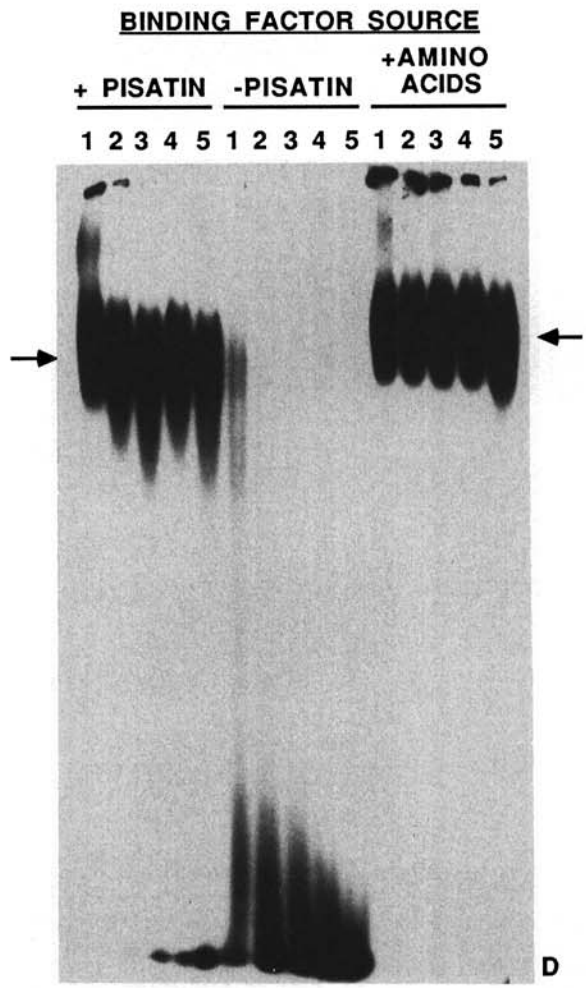

Fig. 6. Effect of salt concentration on the binding of the 35-bp *Eag*I/*Mnl*I *PDA*1 fragment to partially purified binding factors from *Nectria haematococca*. Binding factors partially purified by chromatography on Biogel A1.5M (Fig. 5) were added to the ^{32}P-labeled 35-bp *Eag*I/*Mnl*I *PDA*1 fragment in the presence of a 50-fold excess of nonspecific (calf thymus) DNA and different concentrations of potassium glutamate. Binding extracts were from: (left to right) pisatin- induced (+ pisatin), control uninduced (-pisatin), or uninduced but amino acid-supplemented (+ amino acids) mycelia in phosphate buffer. The potassium glutamate concentration were: 1, 25 mM; 2, 50 mM; 3, 75 mM; 4, 100 mM; and 5, 125 mM. The gel mobilities of the unbound 35-bp fragment (D) and the specific complexes are indicated (arrows).

Functional assay of the specific binding site.

The above binding results suggest that the 35-bp *Eag*I-*Mnl*I region of *PDA*1 may be or be part of a pisatin-responsive activator region. To test this possibility, *PDA*1 DNA fragments were transformed into *N. haematococca*; due to the multiple copies of each DNA fragment on the plasmid and the multiple integrations of the plasmid common to fungal transformation, many ectopic copies of each fragment were introduced. If a specific DNA fragment contains a binding site for a factor that is important for the induction of *PDA*1, then the multiple introduced copies should titrate the binding factor from the binding site on the native *PDA*1 gene, inhibiting pisatin induction of pda.

Transformants that contained the introduced transformation vector (pUCHI) alone, or 18 copies of the 505-bp *Taq*I/*Taq*I region (positions –1192 to –688, in plasmid pDS106) upstream of the putative activator region responded similar to untransformed 77-2-3 to induction of pda by pisatin (Fig. 7). In contrast, transformants that contained 24 copies of the 35-bp *Eag*I/*Mnl*I fragment (positions –611 to –577, in pDS104) or eight copies of the 140-bp *Taq*I/*Hpa*II fragment (positions –689 to –550, in pDS109) demonstrated a reduced and delayed pisatin induction of pda activity (Fig. 7). These latter two fragments bind the pisatin-responsive factor *in vitro* and the significant inhibition of pda induction in transformants containing multiple copies of these fragments are consistent with their binding a factor involved in pisatin induction *in vivo*. Four independent pDS104 transformants (containing 12–24 copies of the 35-bp binding region) showed similar inhibition of pda induction with an average peak pda activity of 1.4 pmoles min^{-1} mg^{-1} (standard error = 0.07). Five single spore isolates of transformant tx7-2G, the pDS104 transformant shown in Figure 7, were tested separately and similarly displayed an average peak pda activity of 1.4 pmoles min^{-1} mg^{-1} (standard error = 0.08). Three independent pDS109 transformants and three single-spored isolates of tx7-3A (containing eight copies of the 140-bp binding region) displayed an average peak pda activity of 1.4 pmoles min^{-1} mg^{-1} (standard error = 0.09). In contrast, two independent pUCHI transformants (containing vector alone) displayed pisatin induction of pda levels to an average of 3.17 pmoles min^{-1} mg^{-1} (standard error = 0.10). Such variation between replicates indicates that the small differences between controls are likely not significant, but the decrease in pda expression in binding-fragment transformants do represent a significant reduction. The transformant with the larger 140-bp fragment showed slower induction of pda activity even though there were fewer copies of the fragment in this transformant compared to the transformant with the 35-bp fragment, which may be due to the greater *in vitro* binding strength of the larger fragments observed above. Transformants that contained 21 copies of the 487-bp *Hpa*II/*Taq*I fragment (position –551 to –65, in pDS107) downstream of the putative activator region and containing the transcription initiation site displayed somewhat reduced induction compared to the untransformed control, however the effect was not as strong as that of the 35- or 140-bp binding fragments (Fig. 7) and possibly not significant given the replicate variability described above.

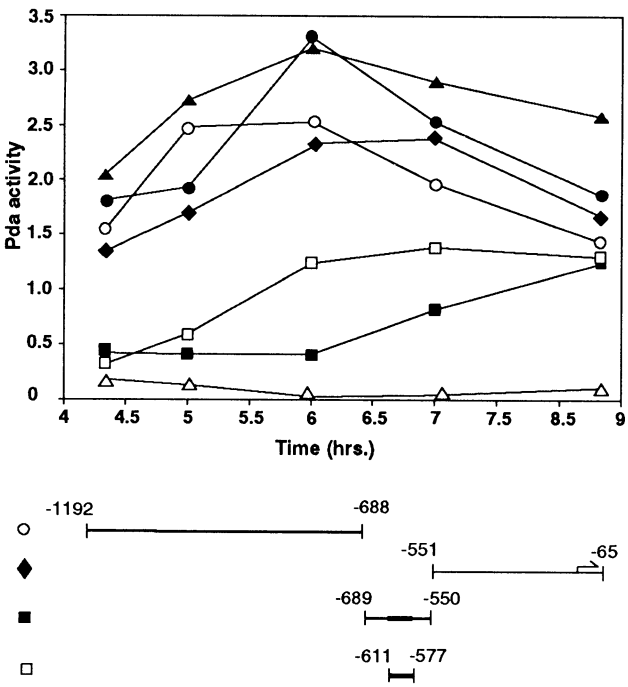

Fig. 7. Induction of pda activity in transformants of *Nectria haematococca* containing multiple copies of portions of the 5′ region of *PDA*1. Transformants of *N. haematococca* and the recipient strain 77-2-3 were induced with pisatin and assayed for pda activity as described in Figure 2: Δ, noninduced 77-2-3; ▲, induced 77-2-3; ●, induced tx7-6A which contained two copies of the transformation vector pUCHI; ○, induced tx7-4B which contains 18 copies of the 505-bp *Taq*I/*Taq*I upstream flanking fragment; ◆, induced tx7-5D which contains 21 copies of the 487 bp downstream flanking fragment; □, induced tx7-2G which contains 24 copies of the 35-bp *Eag*I/*Mnl*I fragment; ■, induced tx7-3A which contains eight copies of the 140-bp *Taq*I/*Hpa*II fragment. The 35- and 140-bp fragments bind the pisatin-responsive factor *in vitro*. The relative positions of these restriction fragments in *PDA*1 are shown.

To assure that the lower pda activity in the transformants was not due to integration of the plasmid into the native *PDA*1 gene, Southern analysis was performed on the transformants. *Xho*I/*Bam*HI digests of transformants' DNA were run on an agarose gel, blotted onto nitrocellulose, and probed with a cloned 1.4-kb *Sac*I fragment containing the coding region of the *PDA*1 gene but does not overlap with the upstream DNA. In all transformants, the only hybridizing band was at 3.4 kb, the same as in the recipient strain 77-2-3, indicating that there was no integration within the native *PDA*1 gene.

DISCUSSION

Previous results (Weltring *et al.* 1988) suggested that regulation of the *PDA*T9 gene by pisatin occurs at the level of *PDA*-specific RNA accumulation. Our measurements of pda activity and accumulation of *PDA*1-specific mRNA has quantitatively demonstrated the sequential production of *PDA*-specific mRNA and pda consistent with pisatin regulation acting at the level of *PDA*1 mRNA accumulation. We have therefore focused on molecular events that might produce pisatin-responsive transcriptional regulation of this promoter. Our strategy for detection of regulatory elements was to use an *in vitro* DNA-binding assay to find *N. haematococca* factors that bind upstream of the *PDA*1 promoter. Other methods of analysis have not been amenable for studying regulation in this fungus. For example, traditional genetic analysis has not de-

fined regulatory loci for the highly inducible PDA^{SH} genes (Kistler and VanEtten 1984; Mackintosh *et al.* 1990) and the difficulty of obtaining specific mutants in this fungus (H. VanEtten, unpublished results) discourages a mutational approach. Transformation of this fungus (Stahl and Schäfer 1992) has only recently become efficient enough to allow rapid identification of *cis*-acting regions in the promoter by deletion analysis.

The *in vitro* DNA-binding assays have identified a factor in the pisatin-induced extracts that has the properties expected of a pisatin-responsive activator of the *PDA*1 gene. First, this DNA-binding activity is present in extracts from pisatin-treated mycelium, while absent or present in very low amounts in extracts from mycelium that was similarly manipulated but not treated with pisatin. Second, this pisatin-responsive factor displays sequence-specific DNA binding activity to a 35-bp region between –514 to –480 relative to the first mRNA start site of *PDA*1. The specificity of this binding was indicated by the titration of the binding with excess specific fragment in the presence of a larger excess of nonspecific DNA, and supported by the lack of factor binding to many other flanking DNA regions in the *PDA*1 promoter. Sequences flanking the 35-bp minimum size binding fragment, however, seem to play a role in interacting with the pisatin-responsive factor since larger restriction fragments show significantly stronger binding than the minimum size fragment. The contacts between this factor and the nucleotide positions within the 35-bp fragment have not been mapped. Methylation interference has failed to identify contacts with the N7 of guanosine residues in the pisatin-responsive or amino acid-responsive complexes but has identified two residues on the lower strand (positions –596 and –597) which inhibit formation of the faster mobility pisatin-responsive complex formed under high calf thymus DNA conditions (D. Straney, unpublished). This behavior indicates that more than one component may be interacting to form these various complexes and is consistent with the relatively large size of the DNA binding activities. Further experiments with other probes of protein-DNA interactions are needed to more clearly define the extent of interactions involved in binding.

The function of this 35-bp region as a *cis*-acting site for pisatin regulation was tested in an *in vivo* competition assay (Kelly and Hynes 1987). *N. haematococca* transformants with multiple ectopic copies (8-24) of this region displayed reduced pisatin induction of the native *PDA*1 gene. The simplest explanation for this inhibition is that the 35-bp region contains a binding site for a *trans*-acting factor essential to pisatin induction of transcription, such as a pisatin-responsive activator. The result does not rule out the possibility that the site binds an accessory *trans*-acting factor needed for full pisatin response; however, one would expect the cellular concentration of such more general transcription factor to be high enough to prevent the titration we observed *in vivo*. Also, the appearance of a factor that binds specifically to this isolated region only in the presence of pisatin would not be consistent with such accessory factor. Inhibition in the *in vivo* competition assay was not complete, potentially due to an abundance of binding factor in the cell beyond the number of introduced binding sites. The ability to detect a reasonable amount of binding factor in crude extracts would suggest that this is possible. However, the inability to fully inhibit pisatin induction may instead be due to other independent regulatory sequences, elsewhere on the *PDA*1 gene, which provide additional pisatin regulation. Our results cannot determine the origin of this residual activity and further experiments are needed to test if the 35-bp region is a sole determinant in pisatin activation.

The pisatin demethylase enzyme is a cytochrome P450 monooxygenase, a class of enzymes that are responsible for initial detoxification of xenobiotic chemicals in mammalian liver. Aromatic hydrocarbons induce several mammalian cytochrome P450 genes, including the P450IA1 gene. This response is mediated by the 3-methylcholanthrene or 2,3,7,8-tetrachlorodibenzopyparadioxin (TCDD) binding to the Ah receptor protein, which induces this protein's DNA binding activity and function as a transcriptional activator (reviewed in Gonzales 1989). The pisatin demethylase system parallels this mammalian cytochrome P450 in that both are substrate induced and function to detoxify xenobiotics. Further, pisatin is a small lipophilic compound, similar to the Ah inducers and steroid hormones that readily enter the cell to induce specific genes through this mechanism (Weinberger and Bradley 1990). Pisatin is also readily partitioned into *N. haematococca* (Denny *et al.* 1987). An attractive model is that pisatin similarly interacts with a receptor/activator. In such a model, the pisatin-responsive factor identified in this study would be a candidate for testing receptor activity. Further studies will take advantage of the binding assay to test if pisatin is bound in this pisatin-responsive complex or induces the formation of this complex in extract from uninduced mycelium.

In parallel with the comparison between pisatin-induced and uninduced extracts, we also compared nutritional conditions that alter the ability of pisatin to induce pda activity. Earlier studies found that a rich culture medium (5% glucose, 0.8% Casamino Acids, 0.05% yeast extract, phosphate buffer and essential salts) prevented the pisatin-induction of the *PDA*1 activity (VanEtten and Barz 1981). In this study we found that two components of this culture medium, glucose and amino acids, act separately to produce this inhibition. The repression by amino acids does not seem to be caused by nitrogen source repression, such as the response to ammonium through the regulatory proteins AREA and NIT2 in *Aspergillus nidulans* (Eidam) Winter and *Neurospora crassa* Shear et Dodge, respectively (reviewed in Wiame *et al.* 1985), since ammonium nitrate did not repress pisatin induction of pda activity. Although amino acids have not been reported to regulate fungal virulence genes, they have been found to regulate the hypersensitive response/pathogenicity (*HRP*) gene cluster of *Pseudomonas syringae* pv. *syringae* 61 (Xiao *et al.* 1992) and the *avr*B gene of *P. s.* pv. *glycinea* (Huynh *et al.* 1989).

Control of gene expression by starvation for amino acids has been studied in fungi for the coordinate regulation of amino acid biosynthetic genes by the transcription activator GCN4 in *S. cerivisiae* Hansen (Hinnebusch 1988) and CPC1, its homolog, in the filamentous fungus *N. crassa* (Ebbole *et al.* 1991). GCN4 is translationally regulated so as to be present under amino acid starvation. No consensus binding sequence for GCN4 (TGATCT) was found in the 1.2-kb of upstream region in the *PDA*1 gene. However, two sites were found that match weaker nonconsensus GCN4 binding sites in *Saccharomyces cerivisiae* genes: TGACGA in *HIS*3.1 at –1129 in *PDA*1, and TGACTA in *HIS*4.4 at –106 in *PDA*1

(Arndt and Fink 1986). It is possible that these weaker sites may act together to regulate the *PDA*1 gene in response to amino acid starvation by a GCN4-homolog in *N. haematococca*. Alternatively, another mechanism may be regulating the *PDA*1 gene, as *GCN*4 and *CPC*1-independent amino acid regulation in *S. cerevisiae* and *N. crassa* has been described (Hinnebusch 1986; Flint 1985).

Our studies identified a DNA-binding factor that appears in extracts from mycelia treated with amino acid supplements in phosphate buffer for 3.5 hr that was absent or at low levels in extracts from mycelia treated with phosphate buffer alone. The region of *PDA*1 this factor bound to does not overlap the identified potential GCN4 binding sequences. Since this DNA-binding factor appeared only during amino acid repressing conditions, this could play a role as an amino acid-responsive repressor of the *PDA*1 gene. Such a role for this binding factor would be of interest since it overlaps the binding region of the putative pisatin-responsive activator and so could act by occlusion of that factor's binding. Such overlapping sites for a gene-specific activator and nutrition-responsive repressor were described for ALCR and CREA on the *alc*A promoter of *A. nidulans* (Kulmberg *et al.* 1993). Alternately, this amino acid-responsive binding factor could be related to the pisatin-responsive factor, perhaps as an inactive form where the lack of amino acid starvation, but not glucose starvation, has stabilized the binding factor in the cell. The gel mobility of the complexes formed with pisatin-responsive binding factor is different from those formed with the amino acid-responsive binding factor, signifying some difference in protein size, charge, or conformation. The function of this factor remains to be elucidated but may prove to be an interesting part of the pisatin regulation. Glucose-responsive regulatory factors could not be identified by this method since we did not detect differences in binding activities between extracts from glucose-treated mycelium and control mycelium. We did not test if the presence of glucose alters the formation of the pisatin-responsive complex.

The signal pathway by which fungi respond to molecular cues from the host plant have yet to be elucidated. Among other fungal genes identified to be induced by such plant signals, only the cutinase gene has been studied at the level of transcriptional regulators; a 135-bp cutin-responsive region, upstream of the cutinase promoter, and a cutin monomer-induced phosphoprotein have been identified in *N. haematococca* (Bajar *et al.* 1991). The present study identified potential gene regulatory proteins and *cis*-acting regions which may play a role in regulation of the pisatin demethylase gene in the same fungus. The regulation of *PDA* expression is unique in being induced by a host-specific compound, the isoflavanoid pisatin, and so offers a eukaryotic regulatory parallel to the induction of *nod* genes in symbiotic rhizobial bacteria by host-specific flavonoids (reviewed in Phillips 1992). This characterization of *PDA*1 provides us with specific molecular targets with which to further elucidate this regulatory process both *in vitro* and *in situ*.

MATERIALS AND METHODS

Strains, plasmids, and chemicals.

N. haematococca MPVI isolate 77-2-3, which contains a single active PDA allele, *PDA*1, that encodes a Pda^{SH} phenotype, was used for all experiments (Kistler and VanEtten 1984). The plasmid pBS^{+} (Stratagene, La Jolla, CA) was used for all constructions, which were grown in the DH5α strain of *E. coli*. The plasmid containing *PDA*T9 (pDM1) was described previously (Weltring *et al.* 1988; Maloney and VanEtten 1994). The vector pUCHI was kindly provided by Gillian Turgeon and described previously (Turgeon *et al.* 1987). Cloning, restriction digestion, preparative gel electrophoresis, and RNA and DNA hybridization was performed as described by Sambrook *et al.* (1989). The *PDA*1 sequence was determined by dideoxy sequencing (Sequenase kit, United States Biochemical, Cleveland, OH). Compressions were resolved on 40% formamide gels.

Induction of pisatin demethylase.

Minor modifications of a previous described procedure (Kistler and VanEtten 1984, method 1) were used for the induction of pda. The conidia from a culture of isolate 77-2-3 grown for 6 days on a V8 media slant culture (18 mm) was used to inoculate 250 ml of a glucose, casein hydrolysate, yeast extract (GCY) medium (VanEtten and Barz 1981) contained in a 1-L Erlenmeyer flask. After incubating the culture at 29° C for 22 hr on a gyratory shaker (180 rpm), the mycelia was collected on a cloth filter, washed with 300 ml of water, and resuspended (30 mg/ml) in 50 mM potassium phosphate buffer (pH 6.5), with or without additional amendments. The mycelial suspension was then incubated at 25° C in a flask approximately 10-fold larger than the volume of the suspension and shaken at 100 rpm. To induce pda activity, pisatin in dimethyl sulfoxide (DMSO) was added to a final concentration of 0.1 mM. Other cultures received DMSO without pisatin to serve as the uninduced controls. The final DMSO concentration was 0.5% all cases.

RNA isolation.

Mycelium was collected on Whatman GF/A filters at various times after treatment with pisatin, washed thoroughly with water, and stored at –80° C until lyophilized. Lyophilized mycelia (20 mg) was ground with 100 mg of sand in a microcentrifuge tube and the nucleic acids extracted by the procedure of Kurtz and Lundquist (1984). The contents of each microcentrifuge tube was extracted with 0.5 ml volume of their Tris, lithium chloride, SDS buffer. The resulting samples contained both RNA and DNA in 100 µl volume of Tris-EDTA. *PDA*1-specific RNA was quantitated by treating 6 µl with 1.2 µl volume of formaldehyde and 1.8 µl of 20× SSPE buffer (Sambrook *et al.* 1989), heating to 65° C for 15 min, and bound to a nitrocellulose filter using a slot blot apparatus. *PDA*1-specific DNA in the samples was measured by treatment of 6 µl volume of the samples with 1 µg of RNAse A and 15 units RNAse T1 for 45 min, 2 µg of proteinase K for 1 hr, boiling for 10 min, addition of NaOH to 0.5 M and incubating at 65° C for 40 min followed by neutralization and transfer by filtration to nitrocellulose using a slot blot apparatus. The filters containing RNA or DNA were hybridized with the 1.4-kb internal *Sac*I fragment of *PDA*T9 labeled with ^{32}P by Klenow extension from random hexameric primers (Sambrook *et al.* 1989) and purified from unincorporated label by chromatography on Sephadex G50. Hybridization was at 42° C in 50% formamide hybridization buffer. The amount of radiolabel bound to the filters was measured by

dissolving the nitrocellulose in Aquascint (ICN Radiochemicals, Irvine, CA) scintillation fluid and measuring ^{32}P in a scintillation counter. The *PDA*1-specific RNA is expressed as a ratio of radiolabel bound to the RNA containing filters relative to that bound to the DNA containing filters for each sample, in order to normalize the RNA to a constant number of fungal nuclei.

Pisatin demethylase assay.

The pisatin demethylase assay, which measures the rate of demethylation of ^{14}C pisatin in a mycelial suspension over a 20-min period, has been described previously (method 1, Kistler and VanEtten 1984). The pisatin demethylase enzyme activity is expressed as picomoles pisatin demethylated per minute per milligram dry weight of mycelium.

Extraction and partial purification of DNA binding factors.

Mycelia was incubated for 3.5 hr in phosphate buffer containing the specified amendments and collected on a cloth filter, frozen, lyophilized, and ground in liquid nitrogen with a mortar and pestle. The powdered mycelium (1.2 g) was added to a centrifuge tube with sufficient suspension buffer (0.1 M KCl, 50 mM Tris, pH 7.5, 2 mM EDTA, 10% glycerol, 5 mM β-mercaptoethanol) supplemented with phenylmethylsulfonyl fluoride (PMSF) to 1 mM concentration, to make a final volume of 12 ml. This suspension was mixed with 12 ml of 0.8 M KCl suspension buffer and the mixture allowed to sit on ice for 30 min. The suspension was centrifuged at 6,000 *g* for 5 min and the supernate centrifuged at 99,000 *g* for 60 min. The supernate was collected and one-tenth volume of 1% polyethyleneamine (pH 7.5) was slowly added to precipitate the nucleic acids. After stirring 30 min on ice, the extract was centrifuged at 6,000 *g* for 15 min, and the supernate was collected. Ammonium sulfate was then added to 50% of saturation and, after 30 min on ice, the mixture was centrifuged at 6,000 *g* for 15 min. The pellet was collected, resuspended in, and dialyzed against 1 L volume of suspension buffer. This extract is referred to as the "mycelial extract" and DNA-binding activity in the extract was measured after adjusting the extracts to equal absorbance at OD_{280}. This was equivalent to approximately 35 μg of protein per microliter as determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA) using BSA as a standard.

Further purification of the binding factor(s) involved gel exclusion column chromatography in 0.4 M KCl suspension buffer on a Biogel A 1.5M resin (Bio-Rad). The KCl concentration of the mycelial extract was increased to 0.4 M and 0.7 ml was run on a 27 × 1.6 cm column and 0.4-ml fractions collected. Column fractions were assayed for DNA binding factor by the gel mobility shift assay as described below, using 1.25 μl of each fraction and 0.5 μg of calf thymus DNA in a 10 μl volume binding reaction mixture with no additional KCl added. The molecular weight of the DNA-binding factors was estimated by comparing the elution profiles of binding activity to molecular weight standards. The active fractions were pooled, concentrated with ammonium sulfate (80% saturation) precipitation, and dialyzed against suspension buffer. Final protein concentrations of the column purified factor preparations were approximately 14 μg/μl. The column was standardized for molecular weight estimation by plotting the elution volumes of apoferritin (443,000 Da), α amylase (200,000 Da), alcohol dehydrogenase (150,000 Da), and bovine serum albumin (66,000 Da), relative to blue dextran, for the column used to purify the mycelial extracts.

Gel mobility shift assay for DNA-binding activity.

Restriction fragments of cloned DNA representing upstream sequences of the *PDA*1 gene were labeled by incorporation of $\alpha^{32}P$-deoxynucleotides into their 3′ termini (at a 5′ overhang) by the Klenow fragment of DNA polymerase (Sambrook *et al.* 1989). The labeled restriction fragments were purified from free label by either ethanol precipitation or, in the case of single restriction fragments, by polyacrylamide gel electrophoresis followed by electroelution of the fragment from the gel. DNA was generally labeled to at least 1×10^6 dpm/μg of DNA.

The binding reaction mixture contained 0.2 pmoles of labeled *PDA*1 specific DNA fragment, 1 μl of test extract in suspension buffer, 0.25–10 μg of nonspecific competitor DNA (calf thymus DNA, sheared, treated with S-1 nuclease, phenol extracted, and ethanol precipitated), 4 μg of BSA, and binding buffer (20 mM Tris, pH 7.5, 1 mM EDTA, 5% glycerol, 1 mM DTT, 0.01% Triton X-100, and 50 mM KCl) to a total volume of 10 μl. The reaction mixture was incubated for 45 min at 25° C, added to 1 μl of gel loading dye (60% sucrose, 0.001% xylene cyanol dye) and loaded on a 5% polyacrylamide gel (40:1 acrylamide:bis-acrylamide) in 0.5× Tris-borate EDTA electrophoresis buffer (Sambrook *et al.* 1989), electrophoresed at 100 V until the xylene cyanol dye had run 10 cm, and then wrapped and autoradiographed to visualize the location of the labeled *PDA*-specific DNA. Quantitation of the amount of DNA in the complex was achieved by excising the band from the gel and measuring the relative ^{32}P content by Cerenkov counting. The relative binding strengths were calculated from the ratio of bound to free DNA.

Transformation of *N. haematococca*.

Protoplasts from isolate 77-2-3 were obtained as described by Stahl and Schäfer (1992). Protoplasts were isolated by filtration through 0.2 μM nylon mesh followed by centrifugation of protoplasts in the osmotic medium under a layer of trapping buffer (1.2 M sorbitol, 10 mM Tris, pH 7). The protoplasts were collected from the junction of the two layers and subjected to two washes of 10 ml of SE (1.2 M sorbitol, 1 mM EDTA) and two washes of 10 ml of STC (1.2 M sorbitol, 10 mM Tris, pH 7, 50 mM $CaCl_2$), collecting by centrifugation in each wash. Each transformation experiment combined 1×10^7 protoplasts with 30 μg of salmon sperm DNA, 10 μg of plasmid DNA, and 10 mM spermine in 200 μl of STC buffer. Two volumes of 60% PEG 6000, 10 mM Tris (pH 7) and 50 mM $CaCl_2$ were mixed with the protoplasts and then immediately plated with 10 ml of molten (55° C) regeneration medium (34.2% sucrose, 0.1% casein hydrolysate, 0.1% yeast extract) agar. After 24 hr at 28° C, the plates were overlaid with 10 ml 1% water agar containing 100 μg of hygromycin B (Calbiochem, San Diego, CA) per milliliter.

Competition plasmids.

Plasmids containing multiple copies of specific portions of the *PDA*1 upstream region were constructed by cloning mul-

tiple copies of an isolated restriction fragment into the *Acc*I site of the transformation vector pUCHI. This vector contains the hygromycin phosphotransferase gene driven by the *Cochliobolus heterostrophus* promoter 1 and can be used to transform *N. haematococca* by selecting for resistance to hygromycin B (Stahl and Schäfer 1992). The specific plasmids constructed and used are the following: Plasmid pDS104 contained four copies of the cloned 35-bp *Eag*I (–611)/*Mnl*I (–577) fragment. Plasmid pDS109 contained four copies of the 140-bp *Taq*I(–689)/*Hpa*II(–550) fragment. Plasmid pDS106 contained three copies of the 505-bp *Taq*I (–1192)/*Taq*I(–688) fragment. Plasmid pDS107 contained four copies of the 487-bp *Hpa*II(–551)/*Taq*I(–65) fragment. These plasmids, or a control of pUCHI alone were used to transform 77-2-3. Transformation frequency varied with pUCHI vector alone, pDS104, pDS109, and pDS107 producing between three and six transformants per microgram of plasmid used, while pDS106 produced only 0.5 transformants per microgram of plasmid DNA. The transformants were cultured on regeneration media agar with 40 μg/ml hygromycin and grown for the pisatin demethylase assays in CGY media with 10 μg/ml hygromycin.

The number of copies of plasmids integrated in the transformants was determined from fungal DNA isolated from the same batch of mycelium used for the pda enzyme assay, using slot blot hybridization method previously described (Schäfer *et al.* 1989). The vector pUCHI was used as a probe and did not hybridize to the DNA from untransformed 77-2-3. The hybridization was quantitated by Cerenkov counting and copy number calculated by the number of microgram equivalents to the pUCHI plasmid on the blot, assuming the genome size of *Nectria* to be similar to *Neurospora crassa* (2.6×10^4 kb from Timberlake 1978). The number of copies of cloned insert in each transformant was calculated by multiplying the number of copies of integrated plasmid, measured by hybridization, by the number of copies of cloned fragment per plasmid.

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