

# Analysis of Determinants of Binding and Transcriptional Activation of the Pisatin-Responsive DNA Binding Factor of *Nectria haematococca*

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Pisatin is a fungistatic isoflavonoid produced by garden pea. Field isolates of the ascomycete *Nectria haematococca* mating population VI (anamorph: *Fusarium solani*) that are highly virulent on pea have been found to possess the *PDA1* gene encoding a pisatin detoxifying activity. Expression of *PDA1* is specifically and highly induced by exposure of mycelia to pisatin. A pisatin-responsive DNA-binding activity has previously been identified with properties suggestive of a transcriptional regulator of *PDA1*. In this study, the sequence determinants for binding this pisatin-responsive factor (PRF) were localized to a 14-bp region through analysis of sequence alterations that reduced PRF binding. Using a homologous *in vitro* transcription system, a transcriptional activator of *PDA1* was shown to be present in mycelial extracts that shared the sequence specificity characteristic of the PRF, indicating function of the DNA-binding protein in transcriptional control. A 70-kDa protein was shown to be a DNA-binding component of PRF by three independent assays for DNA-binding proteins: Southwestern (DNA-protein) blotting, UV-crosslinking, and binding to immobilized DNA. These results characterize a transcriptional activator acting on the *PDA1* promoter that is responsive to a host-specific compound and provide insight into the regulation of fungal genes in response to plant flavonoids.

*Additional keywords:* fungal gene expression, pisatin demethylase.

Just as the expression of specific plant genes is induced in response to pathogen-derived elicitors, the expression of certain genes in pathogens is regulated in response to host cues during plant-microbe interactions. A number of traits and genes in plant-pathogenic fungi have been identified that are specifically induced during interaction with a plant or by plant products but not during growth in culture medium (Ehrenshaft and Upchurch 1993; Pieterse et al. 1994; Talbot et al. 1993; Van den Ackerveken et al. 1993; Van den Ackerveken et al. 1994). While plant-responsive expression of

some of these genes can be mimicked by nutritional starvation (Talbot et al. 1993; Van den Ackerveken et al. 1994), other genes appear to be induced by undefined compounds or signals encountered during interaction with the plant. Little is known about the molecular regulation of such genes. The *PDA1* gene of *Nectria haematococca* Berk. & Broome mating population (MP) VI (anamorph: *Fusarium solani*), a pea pathogen, provides a model for studying plant-induced fungal gene expression because it is induced in response to a well-characterized host-specific product. The *PDA1* gene encodes pisatin demethylase, a cytochrome P450 enzyme that detoxifies pisatin, the isoflavonoid phytoalexin produced by pea. The *PDA* genes constitute a small gene family that are commonly found on small, meiotically unstable chromosomes (Miao et al. 1991). The *PDA1* gene produces the highest level of expression among the *PDA* genes and is found in all highly virulent *N. haematococca* MP VI field isolates (Hirschi 1994; Kistler and VanEtten 1984a). Gene disruption of *PDA1* caused a reduction in the virulence of *N. haematococca* on pea (H. D. VanEtten, personal communication), to a lesser extent, however, than expected from previous genetic analysis (VanEtten et al. 1989). Furthermore, introduction of the *PDA1* gene into *Cochliobolus heterostrophus*, a corn pathogen, has been shown to increase the symptoms produced on pea (Oeser and Yoder 1994; Schäfer et al. 1989). Thus, the factors modulating expression of *PDA1* may affect virulence of the pea pathogen.

Expression of pisatin demethylase is specifically induced in cultured mycelia by the addition of pisatin (VanEtten and Barz 1981). Pisatin exposure increases the levels of both pisatin demethylase enzyme activity and *PDA1* mRNA by approximately 20-fold within 4 to 8 h (Kistler and VanEtten 1984b; Straney and VanEtten 1994), suggesting that the induction acts at the level of mRNA accumulation. This induction by pisatin in culture likely reflects gene regulation during the plant-microbe interaction since *PDA1* is induced during infection of pea at a time coinciding with pisatin biosynthesis (Hirschi 1994). Determining the intracellular signaling involved in the pisatin induction of *PDA1* transcription would provide an understanding of how this fungus is able to specifically perceive and respond to this host compound.

A previous study of the *PDA1* promoter has identified a possible pisatin-responsive regulatory factor through gel shift analysis (Straney and VanEtten 1994). A DNA binding activ-

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ity was found to specifically bind within a 35-bp region that is approximately 500 bp upstream of the first mRNA start site in the *PDA1* promoter. This DNA binding activity was present in extracts from pisatin-treated mycelia but absent in extracts from untreated mycelia, and so has been referred to as a pisatin-responsive factor (PRF) (Straney and VanEtten 1994). Its appearance only under inducing conditions suggested that this binding factor may be a pisatin-responsive transcriptional activator of *PDA1* expression. One line of evidence for such function came from the fact that introduction of multiple copies of the 35-bp binding site into *N. haematococca*, through stable transformation, decreased and delayed pisatin inducibility of the native *PDA1* gene (Straney and VanEtten 1994). This was consistent with the supposition that multiple copies were limiting the interaction of a positive-acting factor and the native promoter by competitive binding.

The purpose of this study was to better define both the DNA and protein components that contribute to binding of the PRF to this region of the *PDA1* gene. Furthermore, the function of the PRF in transcriptional control was addressed through the use of a homologous in vitro transcription system (Ruan and Straney 1994). This in vitro system accurately initiates RNA polymerase II transcription at the *PDA1* promoter and is dependent upon pisatin treatment of mycelium before

preparation of the extract (Ruan and Straney 1994). Initial analysis of the DNA recognition sequence of PRF binding in vitro provided a tool to specifically test the function of the PRF in transcriptional control and identify the DNA-binding protein components of this factor.

## RESULTS

### DNA sequence determinants of binding.

As previously described (Straney and VanEtten 1994), gel shift analysis using a 38-bp region of *PDA1*, corresponding to an *EagI/MnII* fragment, bound a factor present in extract derived from mycelium that was treated with pisatin but absent in similar extract from untreated mycelia (Fig. 1). Initial determination of the sequence requirements for PRF binding focused upon a CC dinucleotide (positions -498 and -499; Fig. 2) where methylation of the bottom-strand guanines prevented binding (Straney and VanEtten 1994). A <sup>32</sup>P-labeled 38-bp oligonucleotide that contained a CC to GG alteration (m-5 oligonucleotide; Fig. 2) displayed much weaker binding than the wild-type sequence. Since the lack of formation of a shifted band could have been caused by an instability of the protein-DNA complex during electrophoresis, competition analysis was performed in order to confirm the lack of binding by the mutated oligonucleotide (Fig. 1). The mutated oligonucleotide was found to be a poor competitor for PRF binding to the wild-type oligonucleotide, compared with that of the wild-type oligonucleotide itself. Thus the CC mutation within the 38-bp oligonucleotide appeared to greatly reduce PRF binding.

Inspection of the sequences surrounding the CC dinucleotide revealed that it was an upstream copy of a partial direct repeat of TTANCNCC. Additional double-stranded 38-bp oligonucleotides were constructed so as to alter nucleotides in the upstream and downstream partial repeats as well as between the repeats. Two or three adjacent nucleotides were altered in each oligonucleotide so as to scan the region most efficiently. Binding to the various oligonucleotides was quantitated through competition of unlabeled test oligonucleotide for PRF binding by <sup>32</sup>P-labeled wild-type oligonucleotide, shown in Figure 2. Two sets of alterations, the TTA and CC positions within the upstream partial repeat, proved to have the greatest effect upon PRF binding. Alteration of similar sequences in the downstream partial repeat reduced binding only slightly. Thus, binding of the PRF appears to be determined by sequences between position -505 and -496, and to a lesser extent by sequences within 2 bp outside these limits. Consistent with these results, cleavage of the wild-type 38-bp oligonucleotide with *MseI* (at position -493 within the downstream repeat) produced a 22-bp upstream fragment that still formed a complex with the PRF, whereas the downstream fragment did not form a complex. A 14-bp oligonucleotide representing the probable recognition sequence (positions -507 to -494) did not produce a band with mycelial extract in the gel shift assay (not shown).

### Competition analysis using homologous in vitro transcription.

A homologous in vitro transcription system was used to evaluate the function of PRF binding in modulating transcriptional activity of the *PDA1* promoter. In vitro transcription utilizes a crude extract from pisatin-treated mycelium and

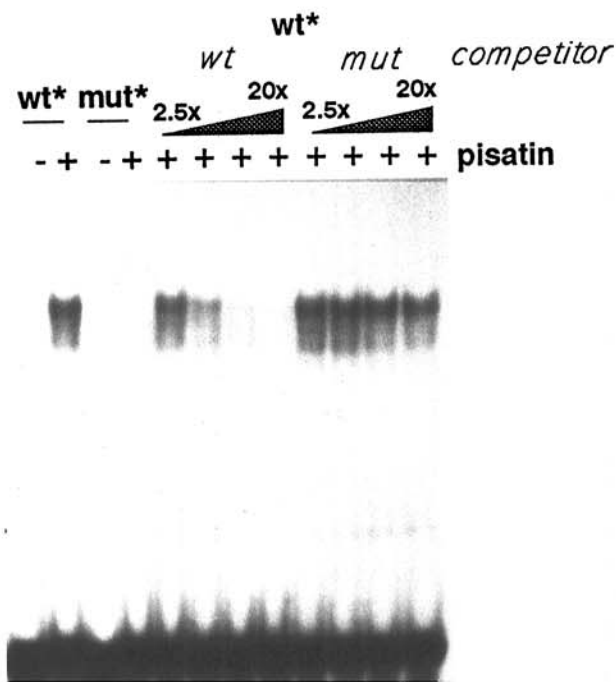


Fig. 1. Binding and competition analysis of wild-type and mutated 38-bp oligonucleotides with the pisatin-responsive factor (PRF) of *Nectria haematococca*. Cell-free extracts were prepared from mycelia grown in rich medium, then transferred to either phosphate buffer with pisatin (+) or phosphate buffer without pisatin (-) for a 4-h treatment. Extracts underwent the same fractionation by ammonium sulfate precipitation and gel exclusion chromatography. Double-stranded 38-bp oligonucleotides containing the sequence between -514 and -480 in the *PDA1* gene (wt oligo) or with a 2-bp alteration in this sequence (mut, m-5 shown in Figure 2) were used in gel shift analysis. Binding with either of these <sup>32</sup>P-labeled oligonucleotides alone is shown in left four lanes using extracts from pisatin treated (+ pisatin) and untreated (- pisatin) mycelium. Competition analysis was performed with <sup>32</sup>P-labeled wild-type oligonucleotide and increasing excess molar amounts (2.5-, 5-, 10- and 20-fold) of unlabeled wild-type (wt) or m-5 (mut) oligonucleotides.

a template containing the *PDA1* promoter (positions +25 to -1097) fused to a region of DNA that contains no guanines in the coding strand for 120 nucleotides (nt). Transcription in the presence of RNAaseT1 and 3-*O*-Me-GTP produces a 140-nt <sup>32</sup>P-labeled guanine-free RNA product initiating from the *PDA1* promoter and minimizes appearance of nonspecific transcript initiating elsewhere in the plasmid.

In parallel with the above competition experiments assaying DNA binding activity, we utilized the same wild-type and mutated oligonucleotides as competitors in the *in vitro* transcription reaction. Addition of an excess of oligonucleotide that bound a positive-acting factor should titrate this factor from the corresponding sequence on the *PDA1* promoter-containing template and decrease its transcription. The results of the competition experiment with the various oligonucleotides are shown in Figure 3. The high level of transcription in extract from pisatin-treated mycelium was greatly reduced by the addition of a 50- or 100-fold excess of the wild-type 35-bp oligonucleotide. This decrease was not observed when a 100-fold excess of restriction endonuclease-digested  $\lambda$ -DNA (nonspecific control) was added to a similar reaction. The successful competition by the wild-type oligonucleotide indicated that this oligonucleotide binds a factor present in the pisatin-induced extract that is required for high transcriptional activity. When a mutated oligonucleotide that does not bind the PRF (m-5, Fig. 3; or m-3, not shown) was used in the competition assay, transcription was only slightly reduced. In contrast, an oligonucleotide altered in another position that did not affect PRF binding (m-8, Fig. 3) was similar to the

wild-type sequence in reducing transcription. Thus, PRF binding was correlated with the function of a transcriptional activator not only through shared binding to the 35-bp region but also through a common sequence specificity within this region.

#### Characterization of protein components in binding factors.

Three complementary approaches, based upon either immobilized protein, immobilized DNA, or protein-DNA cross-linking, were used to identify the size of the protein(s) bound in the PRF-DNA complex. An initial approach utilized Southwestern (DNA-protein) blotting. This technique identifies proteins that bind a specific DNA by DNA-affinity probing of immobilized proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Partially purified mycelial extracts were subjected to SDS-PAGE. Silver staining of the SDS-PAGE gels indicated a number of proteins were present in the extract (Fig. 4A). Proteins on a duplicate SDS-PAGE gel were transferred to a polyvinylidene fluoride (PVDF) membrane, allowed to renature, and then were probed with the <sup>32</sup>P-labeled 38-bp wild-type oligonucleotide that binds the PRF in the gel shift assay. Analysis of four independent preparations of extract from pisatin-treated mycelia demonstrated specific DNA-binding by a 70-kDa protein in two preparations, a 35-kDa binding activity in a third preparation, and both 70- and 35-kDa activities in a fourth preparation (Fig. 4). The relative sizes suggested possible dimer formation. However, lengthening the heating times or increasing the concentration of  $\beta$ -mercaptoethanol

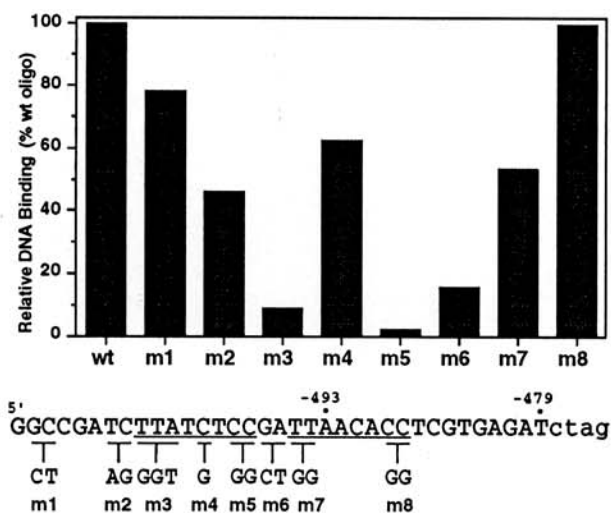


Fig. 2. Effect of sequence alterations within the 38-bp oligonucleotide on binding of the pisatin-responsive factor (PRF). Competition analysis in the gel shift assay was used to quantitate relative binding of wild-type and mutated oligonucleotides. At least four different concentrations of unlabeled competitor DNA were used for each oligonucleotide. Amount of labeled wild-type oligonucleotide remaining in the complex was quantitated by a phosphorimager and plotted against inverse of amount of competitor used. Slopes obtained with mutated oligonucleotides were compared with those obtained with wild-type oligonucleotide to provide a relative binding ratio. Top strand sequence of the 38-bp oligonucleotide is shown; numbers correspond to positions relative to first site of transcription initiation. Lowercase letters indicate *Bam*HI-compatible ends added on bottom strand for end labeling. An *Mse*I digest of the oligonucleotide followed by Klenow treatment produced an upstream fragment terminating at position -493.

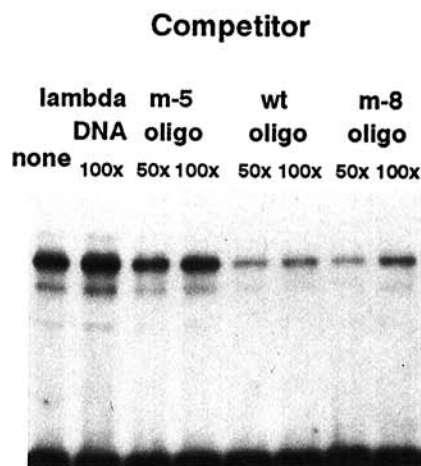


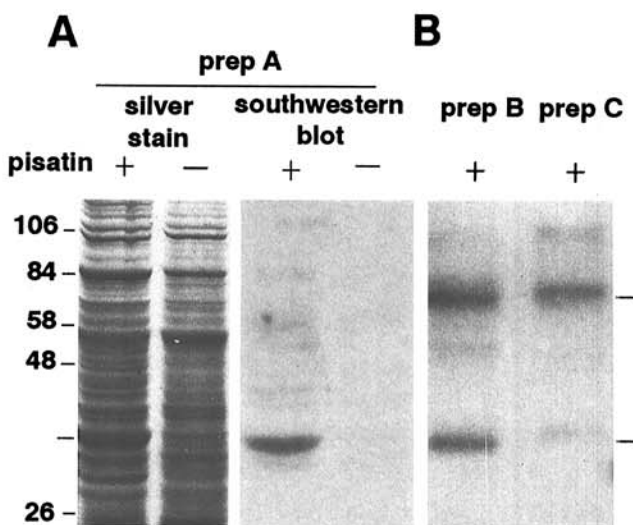
Fig. 3. *In vitro* transcription of the *PDA1* promoter using wild-type and mutated 38-bp oligonucleotide competitors. *In vitro* transcription reactions utilized a plasmid template containing a 1-kb region of the *PDA1* promoter fused to a G-free region of DNA (pDAG). Cell-free extract derived from pisatin-treated mycelia produced a 140-nucleotide-specific transcript, labeled by incorporation of <sup>32</sup>P-[ $\alpha$ ]CTP, and detected by autoradiography. Transcription in a reaction lacking competitor DNA (none) is shown at left. Transcription in the presence of a 100-fold nucleotide excess (compared with template) of lambda phage DNA demonstrates effect of additional nonspecific DNA on transcription. Remaining transcription reactions contained a 50- or 100-fold nucleotide excess of 38-bp oligonucleotide competitor: m-5, containing a 2-bp sequence alteration that greatly reduces its binding to the pisatin-responsive factor (PRF); wild type (wt) 38-bp oligonucleotide containing the PRF-binding site; m-8, containing a 2-bp sequence alteration that does not interfere with PRF binding.

during the denaturation step prior to electrophoresis did not alter the 70-kDa/35-kDa protein ratio. Both 70- and 35-kDa activities were correlated with the presence of PRF activity in several different ways: (i) they were present in extract from pisatin-treated mycelia but not in similar extracts from untreated mycelia (Fig. 4A); (ii) they eluted from Biogel A1.5m gel exclusion chromatography in the same fractions (not shown); and (iii) their binding was specific to the 38-bp oligonucleotide, in that no binding was observed with either labeled restriction fragments that flank the 35-bp region in *PDA1* (positions -592 to -514 and -480 to -390), or labeled  $\lambda$ -DNA (not shown).

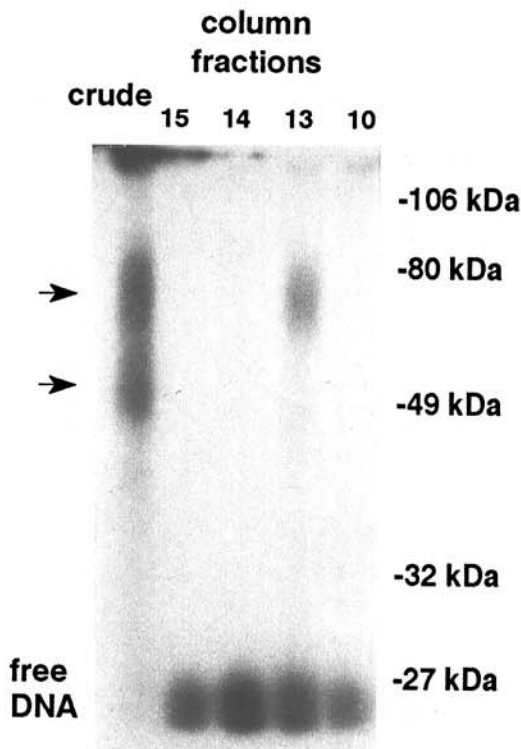
As a complementary approach, protein(s) binding the 38-bp oligonucleotide were identified by protein-DNA crosslinking. UV light-induced crosslinking of protein to  $^{32}$ P-labeled DNA results in radiolabeled proteins, which can then be identified by autoradiography after the protein-DNA conjugates are resolved by denaturing SDS-PAGE. When a crude extract from pisatin-treated mycelia was used in binding reactions with a  $^{32}$ P-labeled 38-bp wild-type oligonucleotide, UV-crosslinking produced two labeled bands on an SDS-PAGE gel with mobilities of approximately 70 and 55 kDa. Only the 70-kDa species co-eluted with the PRF binding activity when the crude extract was fractionated by gel exclusion chromatography (Fig. 5A). The 70-kDa product appeared to represent specific binding since it formed in the presence of excess nonspecific DNA and crosslinking was not observed either in subsequent column fractions that possess higher overall protein concentrations, or with the bovine serum albumin (BSA) present in the reaction buffer. The 70-kDa crosslinked product was absent when binding reactions

used a partially purified extract from mycelia that had not been treated with pisatin (not shown). Competition analysis was performed with the column-purified extract in the UV-crosslinking assay, similar to that performed with the gel shift assay above. Competition with an excess of unlabeled wild-type, m-3, m-5, and m-8 38-bp oligonucleotides in the binding reaction reduced the labeling of the UV-crosslinked band by relative amounts (wt > m-8 > m-3 > m-5, results not shown) that paralleled those observed with the PRF in the gel shift assay. The relative differences in competition for 70-kDa binding indicate that the binding specificity of the DNA-binding protein detected by UV-crosslinking possesses a nucleotide specificity similar to that of the PRF activity studied in the gel-shift assay.

A final approach utilized DNA-linked magnetic glass beads to purify the protein(s) that bind the 38-bp oligonucleotide. In this assay, biotinylated UTP was incorporated into the wild-type 38-bp oligonucleotide and the oligonucleotide was bound to avidin-coated magnetic glass beads. The DNA-coated beads were used in a large-scale protein binding reaction containing an excess of free nonspecific DNA. The beads were separated from the reaction and subjected to washes with increasing salt concentration. The supernatants containing proteins that did not bind the DNA in the original reaction, or that disassociated from the bead-linked DNA by the higher salt concentrations in later washes, were concentrated,



**Fig. 4.** Southwestern analysis of partially purified extracts of mycelia with (+) or without (-) pisatin treatment. **A**, Extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by either silver staining to visualize the proteins (silver stain) or Southwestern (DNA-protein) blotting using a  $^{32}$ P-labeled wild-type oligonucleotide (Southwestern blot). Similar pisatin-dependent appearance of a 35-kDa band after silver staining or Southwestern blotting was observed when 5% glucose (wt:vol) was included in the phosphate buffer. **B**, Southwestern analysis of independent preparations of extract from pisatin-treated mycelia. Preparations of extract from pisatin-treated mycelia (prep B and C), independent from that used above (A), were subjected to Southwestern blotting.  $^{32}$ P-labeled 38-bp wild-type oligonucleotide was used in a Southwestern blot as above. Mobilities of molecular mass size standards are shown in kilodaltons.



**Fig. 5.** DNA-binding activity detected by UV-induced crosslinking. UV light-induced crosslinking was performed on binding reactions prepared as described for gel shift assays using a  $^{32}$ P-labeled wild-type 38-bp oligonucleotide. Reactions were irradiated with UV light and then subjected to electrophoresis on denaturing polyacrylamide-sodium dodecyl sulfate gels. Crude extract (crude) or specific fractions from a Biogel A1.5m column (fraction numbers above lanes) were assayed. Pisatin-responsive factor binding activity, as assayed by gel shift analysis eluted in fractions 12 and 13. Mobilities of molecular mass standards are shown.

separated by SDS-PAGE, and then visualized by silver staining. Multiple proteins were present in the supernatant of the binding reaction and lower concentration salt washes (0.04 to 0.2 M). The 0.5 M KCl wash contained only a single visible protein band with a gel mobility of approximately 70 kDa (Fig. 6A). The higher salt required to release this protein from the bead-linked DNA indicated stronger binding of this protein relative to the proteins that eluted at lower salt concentrations. No additional proteins were eluted in subsequent washes with higher salt concentrations (0.8 and 1.5 M). The protein fractions eluted in the washes of the beads were used in Southwestern blotting to confirm that the single protein visible in the 0.5 M KCl wash was the species that bound the specific DNA. When the washes of the DNA-linked beads were run on SDS-PAGE and probed with a labeled 38-bp wild-type oligonucleotide, binding was observed only with a 70-kDa protein present only in the 0.5 M KCl wash fraction (Fig. 6B). When the concentrated and desalted wash fractions were used in the gel shift assay with <sup>32</sup>P-labeled wild-type 38-bp oligonucleotide, only the 0.5 M wash fraction demonstrated binding characteristic of the PRF (Fig. 6C). These results indicate a strong correlation between PRF activity and the affinity-purified 70-kDa protein.

## DISCUSSION

Among plant-induced fungal genes, the *PDA1* gene of *N. haematococca* is of particular interest since its expression is induced by a host-derived and host-specific compound, pisatin. Our main focus in studying the *PDA1* gene is to characterize the molecular components of this pisatin-specific induction. A putative pisatin-responsive element in the *PDA1* promoter was previously identified through a DNA-binding activity specific for this region that appeared upon pisatin treatment of the mycelium (Straney and VanEtten 1994). The results presented here further characterize the molecular determinants of this PRF by defining its binding site on the DNA, characterizing its protein components, and using nucleotide alterations to associate transcriptional activation function with PRF binding. The identification of a pisatin-responsive element in *PDA1* and the associated regulator is important in defining terminal steps in gene control in response to this host compound. The stimulation of *PDA1* expression by pisatin provides a model for studying other fungi in which isoflavonoid phytoalexins induce expression of corresponding detoxification enzymes. For example, pisatin induces pisatin demethylase activity in other pea pathogens (*Fusarium oxysporum* f. sp. *pisi*, *Ascochyta pisi*, and *Mycosphaerella pinodes*) (George 1993) and a nondegradative tolerance of pisatin in *N. haematococca* MPVI (Denny and VanEtten 1983). Isoflavonoid phytoalexins of bean induce their detoxification by *Fusarium solani* f. sp. *phaseoli* (Turbek et al. 1990), a bean pathogen. Maackiain conversion is induced in *Stemphylium botryosum* by preexposure of mycelium to this isoflavonoid phytoalexin (Higgins 1975). Beyond phytoalexin detoxification, related flavonoids exuded by legume roots induce other processes in legume-associated fungi, including spore germination (Tsai and Phillips 1991; Ruan et al. 1995), and chemotaxis (Morris and Ward 1992). Although host flavonoid-responsive transcriptional activation has been found to be mediated by the *nodD* gene product in

rhizobial bacteria (Spaink et al. 1987), no flavonoid response components have yet been identified in fungi. The identification of activator function in the pisatin-responsive DNA binding protein provides evidence for a eukaryotic flavonoid-responsive transcriptional activator and provides a tool for studying other flavonoid-regulated processes in fungi.

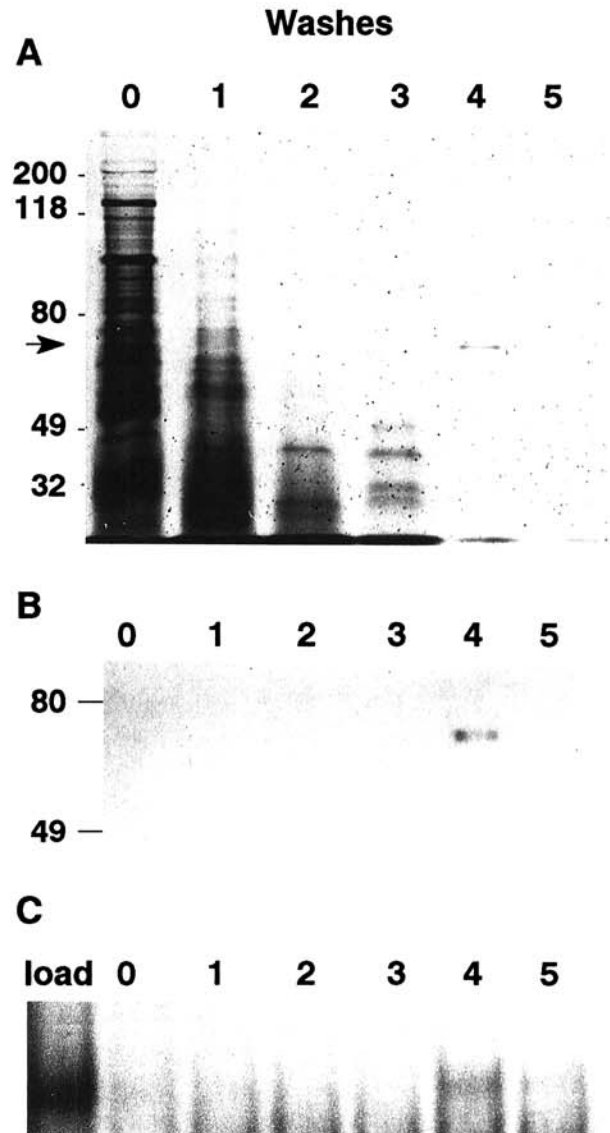


Fig. 6. Identification of bound proteins using DNA immobilized on magnetic glass beads. Scaled up binding reactions contained extract from pisatin-treated starved mycelia and a biotinylated wild-type 38-bp oligonucleotide bound to avidin-linked magnetic glass beads. Following incubation of the binding reaction for 30 min, the supernatant was removed (wash 0) and wash buffer was added to the beads. KCl concentration in the wash buffers: wash 1, 0.04 M; wash 2, 0.1 M; wash 3, 0.2 M; wash 4, 0.5 M; wash 5, 0.8 M. A, Unbound proteins present in supernatant of each wash were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were visualized with silver staining. Mobilities of molecular mass standards are shown. B, Southwestern (DNA-protein) blot of protein eluted in KCl washes. After SDS-PAGE, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane, allowed to renature, and then probed with the wild-type 38-bp oligonucleotide. C, Gel shift assay using protein eluted in washes and labeled wild-type 38-bp oligonucleotide. Binding activity of the partially purified extract loaded onto the beads (load) is shown.

The PRF binding site within the previously delineated 35-bp binding region (Straney and VanEtten 1994) was further determined by analysis of binding with a series of 38-bp oligonucleotides containing specific sequence alterations. The analysis indicated that sequence recognition for PRF binding was determined within a 14-bp region 5'-TCTTATCTCCGA. A partial repeat of this sequence occurring immediately downstream in *PDA1* did not appear to play a significant role in PRF binding or function. Although a double-stranded oligonucleotide corresponding to the 14-bp sequence recognition region could not bind the PRF, additional nonspecific contacts with the DNA outside the recognition sequence may be required for efficient binding, as commonly found with a number of DNA-binding proteins (Liu-Johnson et al. 1986; Jackson 1993). Not all nucleotides within this 14-bp region are necessary for binding since at least one internal nucleotide change did not decrease binding. Our analysis cannot, however, specify the exact nucleotide sequence required for DNA binding by the PRF since the alterations involved two, and in one case three, nucleotides at the same time. Further delineation of the sequence determinants of binding would require either more comprehensive single-site mutations in this region, or the use of in vitro genetic approaches (Szostak 1992). The current level of sequence definition, however, was useful in defining the limits of the binding site and providing a method to correlate the binding activity with transcriptional activation based upon the sequence specificity. This was important in indicating that PRF activity itself was required for a high rate of transcription rather than another protein that bound to the same 38-bp region. At the current resolution of specificity, the PRF binding sequence does not appear to be common to recognition sites of other known transcriptional regulators. In particular, the recognition sequence is different from the xenobiotic-response element of the *CYP1A1* (Watson and Hankison 1992), a mammalian gene that, similar to *PDA1*, encodes a substrate-inducible cytochrome P450 gene involved in the detoxification of xenobiotic compounds (polycyclic aromatic hydrocarbons). Although the lack of sequence similarity of the activator recognition site is not surprising, it is interesting in light of the similarity in the types of compounds that induce these two cytochrome P450 genes. The PRF binding sequence also differs significantly from the G-rich palindrome that has been reported to confer cutin-responsive expression for the cutinase gene (*CUT1*), another possible virulence gene of *N. haematococca* MP VI (Kamper et al. 1994).

Multiple lines of evidence using three different assays indicated that the PRF characterized by gel shift assays contains a 70-kDa protein with DNA-binding activity. The strongest correlation between the 70-kDa protein and PRF binding activity was their co-elution from DNA-linked beads. Not only was this protein the only species visible by silver staining, but it was the only species in the high salt fraction that displayed specific DNA-binding activity, as assayed by Southwestern blotting. This correlation was further supported by the co-appearance of a 70-kDa protein and PRF activity after pisatin treatment of mycelia, during column purification, and comparative binding to other DNA, using UV-induced protein-DNA crosslinking or Southwestern analysis of partially purified extracts. Further, competition analysis in the UV-crosslinking assay provided an indication that the nucleotide specificity of the protein binding was similar to that of the PRF ac-

tivity in the gel shift assay. These shared characters strongly suggest that a 70-kDa protein is at least one component of the PRF. However, additional proteins could be associated with the PRF complex since the assays focused upon DNA binding ability alone. In fact, the PRF activity was found to elute from gel exclusion chromatography under non-denaturing conditions with a molecular mass of approximately 220 kDa (Straney and VanEtten 1994). The difference between native and denatured molecular masses could arise from either additional proteins, or from multimerization of the DNA binding protein itself. The lack of co-purification of other proteins along with the 70-kDa protein on the DNA-linked beads would discount the presence of another species; however, associated proteins could exist that were not detected by silver staining or were eluted from the PRF complex in lower salt washes.

The appearance of an additional 35-kDa binding protein in the Southwestern assay in certain extract preparations is intriguing. Both 70- and 35-kDa proteins behave exactly the same in co-purification and binding specificity, suggesting that they may be related. The relative sizes suggest that the 70-kDa protein may represent a dimer of the 35-kDa protein that is crosslinked or bound in a manner such that  $\beta$ -mercaptoethanol treatment and SDS-PAGE could not disrupt all dimers. Alternatively, the 35-kDa band could be a proteolytic product of the 70-kDa protein that retains DNA-binding activity. However, only the 70-kDa protein was detected in the UV-crosslinking or DNA-linked bead binding assays despite the use of extracts that produced both 35- and 70-kDa proteins in the Southwestern assay. Therefore, it is possible that the DNA binding activity of the 35-kDa species requires the denaturation-renaturation step used in the Southwestern assay. The identification of the 70- and 35-kDa proteins presented here will allow further determination of their relative function and sequence relatedness.

Given the appearance of the PRF binding activity in response to pisatin treatment of mycelia and its function as a transcriptional activator, this protein would appear to be a pisatin-responsive transcriptional activator. Preliminary analysis of gene expression using stable transformation of *N. haematococca* supports this inference since fusion of the PRF-binding region upstream of a *GPD1* promoter::GUS construct confers pisatin-inducible expression of GUS activity (R. Khan and D. Straney, unpublished). The key to understanding the regulation of *PDA1* will be to determine how exposure of the mycelium to pisatin induces the appearance of this binding activity. The absence of the 35-kDa protein in extracts from mycelia not treated with pisatin, as detected by silver staining rather than binding activity, raises the possibility that synthesis of the DNA binding protein itself, and not just the DNA binding activity of the protein, is induced by pisatin. However, the extracts used for electrophoresis had been partially purified and so the absence of the 35-kDa protein in the untreated extracts could result from partition of the inactive protein into other fractions during purification.

The characterization of sequence determinants for binding, demonstration of functional activator activity associated with the PRF, and identification of protein components of PRF will allow a more detailed analysis of the activation of binding activity. These studies are important in characterization of such gene-specific regulatory elements in filamentous fungi that allow response to host-specific signals.

## MATERIALS AND METHODS

### Fungal strains and mycelial extracts.

*Nectria haematococca* MP VI strain 77-2-3 (Kistler and VanEtten 1984a) was used for making protein and transcription extracts. This strain carries one copy of the *PDA1* gene and is the source of the cloned *PDA1* gene (Straney and VanEtten 1994). Extracts from *N. haematococca* mycelia were produced as previously described (Straney and VanEtten 1994). Briefly, mycelia were grown for 24 h in a rich medium and transferred to 50 mM potassium phosphate buffer. The culture was incubated with shaking for an additional 4 h after addition of either pisatin dissolved in dimethyl sulfoxide (DMSO) (to 0.1 mM pisatin, induced), or DMSO alone (uninduced). The mycelia were lyophilized, ground in a mortar and pestle in liquid nitrogen, and suspended in extraction buffer. Large cellular components were removed by centrifugation at  $99,000 \times g$  for 60 min. Nucleic acids were removed from the supernatant by polyethyleneamine precipitation and the extract was partially purified by collecting proteins that precipitated between 25 and 50% saturation of ammonium sulfate. This extract is referred to as a crude preparation. Gel exclusion chromatography on a Biogel A1.5m (Bio-Rad Laboratories, Hercules, CA) resin produced fractions that were assayed by the gel shift assay, as described previously (Straney and VanEtten 1994). Extracts derived from uninduced mycelia, which produced only low binding in gel shift assays, were subjected to similar chromatography. Fractions were taken at an elution volume and position in protein profiles that corresponded to binding activity in pisatin-treated mycelial extracts.

### Oligonucleotides.

Oligonucleotides used in binding and competition analysis are shown in Figure. 2. Oligonucleotides were synthesized for the top strand (shown) and the complementary bottom strand, and were annealed to form double-stranded DNA. The oligonucleotide representing the minimum recognition site used a top-strand oligonucleotide of 5'-TCTTATCTCC and a bottom strand of 5'-TCGGAGATAA; fill-in of the 5' overhangs produced a 14-bp double-stranded oligonucleotide. The oligonucleotide used for attachment to magnetic beads was 5'-AAGATCTTATCTCCGATTAACACCTCTGAGAT (top strand) and 5'-GATCATCTCAGAGGTGTAATCGGAGATAAGATC (bottom strand) to allow additional biotinylated UTP incorporation at the 3' ends.

### Gel shift assay.

DNA fragments or oligonucleotides were end-labeled by the incorporation of  $^{32}\text{P}$ -[ $\alpha$ ]dATP or dCTP into the 3' ends with a fill-in reaction employing the Klenow fragment of DNA polymerase I. The labeled DNA was purified by nondenaturing PAGE. Binding reactions consisted of 0.15 to 1  $\mu\text{g}$  of nonspecific competitor DNA (sonicated *Escherichia coli* chromosomal DNA) and 1 to 2  $\mu\text{l}$  of mycelial extract (2 to 10  $\mu\text{g}$  protein) mixed in 20  $\mu\text{l}$  of binding buffer. This reaction was incubated for 5 min at room temperature, then 2 to 5 ng of  $^{32}\text{P}$ -labeled specific DNA was added. After a further 30 min incubation at room temperature, the samples were loaded on a 5% polyacrylamide (1:30 bisacrylamide/acrylamide) nondenaturing gel in 0.5 $\times$  Tris-borate-EDTA (TBE) buffer

running at 200V. The gel was transferred to Whatman 3-mm paper and dried at 80°C for 1 h without fixation. Radioactivity in gel bands was detected by autoradiography or quantitated in a phosphorimager (Molecular Dynamics, Sunnyvale, CA). When specific competitor oligonucleotide was used, this unlabeled DNA was added with the nonspecific competitor in the preincubation. The binding buffer contained 20 mM Tris-HCl pH 7.5, 2 mM  $\text{MgCl}_2$ , 50 mM KCl, 1 mM EDTA, 10% glycerol, 0.2% Nonidet P40 (NP40), 1 mM DTT (dithiothreitol), and 0.1 mg of BSA per ml.

### In vitro transcription.

Extracts for in vitro transcription were from pisatin-treated mycelia prepared as previously described (Ruan and Straney 1994). In vitro transcription reactions used conditions and procedures similar to those previously described in detail (Ruan and Straney 1994). Transcription reactions were conducted in a 25- $\mu\text{l}$  reaction that contained 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) potassium salt, pH 7.9, 5 mM  $\text{K}_2$  EGTA [ethylene glycol-bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid], pH 7.9, 40 mM K acetate, 2.5 mM Mg acetate, 2.5 mM  $\text{MgSO}_4$ , 4 mM DTT, 0.4 mM of ATP and UTP, 10  $\mu\text{Ci}$   $\alpha$ - $^{32}\text{P}$  CTP (3,000 Ci/mole), 0.2 mM 3'-*O*-Me-GTP, 12 units RNase T1, 20 units RNasin, 0.5  $\mu\text{g}$  of DNA template plasmid, and 2  $\mu\text{l}$  of dialyzed transcription extract. Reactions were incubated at 22° for 50 min, then stopped, subjected to proteinase digestion, ethanol precipitated, and electrophoresed on a 5% denaturing polyacrylamide gel (20:1 acrylamide/bisacrylamide, 8 M urea). The transcripts were detected by autoradiography. For in vitro competition experiments, 0.1 picomoles of template plasmid DNA and the stated molar excess of competitor DNA fragment were added to each reaction before the addition of extract. Lambda phage DNA digested with *AluI* to an average size of 200 bp was used as control nonspecific DNA.

### DNA immobilization on magnetic beads.

Purification of the PRF was based on procedures in Gabrielsen and Huet (1993). Biotin-21-dUTP (Sigma Chemical Co, St. Louis, MO) was incorporated into the 5' overhang of the 38-bp oligonucleotide (Fig. 2) by the Klenow fragment of DNA polymerase I. The labeled oligonucleotide was purified by Sephadex G50 chromatography. Two milligrams of magnetic streptavidin-linked glass beads (Dynabead M-280, Dynal Corp., Lake Success, NY) were washed twice in TEN buffer (10 mM Tris pH 8, 1 mM EDTA, 1 M NaCl) in a microcentrifuge tube using the Dynal magnetic apparatus to retain the beads on the side of the tube. The beads were resuspended in 1 ml of TEN buffer with 150 pmoles biotinylated oligonucleotide, incubated at room temperature for 30 min with gentle rotation, and washed three times in TEN buffer. Thirty microliters of partially purified extract (from Biogel A1.5m chromatography) and 10  $\mu\text{g}$  of nonspecific competitor DNA were added in a total volume of 1 ml of binding buffer and incubated for 5 min. The mixture was then added to 2 mg of specific DNA-linked beads. After a 15-min incubation, the supernatant was removed and the beads were washed three times with 0.5 ml of binding buffer containing 0.04 M KCl but lacking BSA. Similar washes were made with 0.1, 0.2, 0.5, 0.8, and 1.5 M KCl in binding buffer on ice. The supernatant from each wash was precipitated with 80% ammonium

sulfate, and the pellets were resuspended in mycelial extract buffer. These samples were electrophoresed on 10% polyacrylamide SDS gels (Laemmli 1970) and subjected to silver staining (Wray et al. 1981).

### Southwestern blotting.

The procedures followed were those described in Jackson (1993). Mycelial extract was diluted into an equal volume of Laemmli sample loading buffer, heated to 60°C for 5 min and electrophoresed on an SDS-polyacrylamide gel in a Bio-Rad mini Protein II system at a constant current of 30 mA. Gels consisted of a 3.75% polyacrylamide stacking and a 10% polyacrylamide separating gel. Approximately 20 to 50 µg of protein was loaded on each lane. Duplicate gels were run at the same time in the mini Protein II system. The SDS gel was either silver stained or transferred to an Immobilon-P membrane (Millipore, Bedford, MA) by electroblotting in the Protein II system at 100V for 60 min in transfer buffer (50 mM Tris, 380 mM glycine, 15% methanol). The membrane was incubated in Z' buffer (25 mM Tris-HCl pH 7.5, 12.5 mM MgCl<sub>2</sub>, 20% glycerol, 0.1% Nonidet P40, 0.1 M KCl, 10 µm of ZnSO<sub>4</sub>, and 1 mM DTT) with 5% nonfat dry milk (Carnation) for 60 min at room temperature. The filter was washed once with a small amount of binding buffer (see gel shift assay) and incubated in 5 to 10 ml of binding buffer containing approximately 100 ng of the <sup>32</sup>P end-labeled double-stranded DNA probe for 60 min at room temperature on an orbital shaker. The binding buffer was decanted and the filter was washed three times in Z' buffer for 5 min each, once with Z' buffer containing 200 mM KCl and 1 µg of nonspecific competitor (sonicated *E. coli* chromosomal DNA) per ml, and then once with Z' buffer containing 200 mM KCl. The membrane was then subjected to autoradiography.

### UV-crosslinking.

Procedures for UV-induced crosslinking followed those in Jackson (1993). Binding reactions were assembled as described for the gel shift assay, within wells of a 96-well round bottom microtitre plate. After a 20-min. incubation at room temperature, the plate was placed on top of an ice pack within a UV Stratalinker 1800 (Stratagene, La Jolla, CA) at approximately 5 cm below the light source. The samples were irradiated for 20 min. The samples were then added to an equal volume of 2× Laemmli sample loading buffer and resolved on an SDS-10% polyacrylamide gel using the Bio-Rad mini Protein II system. The gel was dried and subjected to autoradiography.

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