Characterization of the Gene Encoding Pisatin Demethylase (*FoPDA1*) in *Fusarium oxysporum*

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The pea pathogen *Fusarium oxysporum* f. sp. *pisi* is able to detoxify pisatin produced as a defense response by pea, and the gene encoding this detoxification mechanism, *FoPDA1*, was 82% identical to the cytochrome P450 pisatin demethylase *PDA1* gene in *Nectria haematococca*. A survey of *F. oxysporum* f. sp. *pisi* isolates demonstrated that, as in *N. haematococca*, the *PDA* gene of *F. oxysporum* f. sp. *pisi* is generally located on a small chromosome. In *N. haematococca*, *PDA1* is in a cluster of pea pathogenicity (*PEP*) genes. Homologs of these *PEP* genes also were found in the *F. oxysporum* f. sp. *pisi* isolates, and *PEP1* and *PEP5* were sometimes located on the same small chromosomes as the *FoPDA1* homologs. Transforming *FoPDA1* into a *pda*⁻ *F. oxysporum* f. sp. *lini* isolate conferred *pda* activity and promoted pathogenicity on pea to some transformants. Different hybridization patterns of *FoPDA1* were found in *F. oxysporum* f. sp. *pisi* but these did not correlate with the races of the fungus, suggesting that races within this forma specialis arose independently of *FoPDA1*. *FoPDA1* also was present in the formae speciales *lini*, *glycines*, and *dianthi* of *F. oxysporum* but they had mutations resulting in nonfunctional proteins. However, an active *FoPDA1* was present in *F. oxysporum* f. sp. *phaseoli* and it was virulent on pea. Despite their evolutionary distance, the amino acid sequences of *FoPDA1* of *F. oxysporum* f. sp. *pisi* and *F. oxysporum* f. sp. *phaseoli* revealed only six amino acid differences, consistent with a horizontal gene transfer event accounting for the origin of these genes.

As a disease resistance mechanism, plants synthesize a variety of small-molecular-weight toxic compounds called phytoalexins in response to attack by pathogenic microbes. However, some microbial pathogens, particularly fungi, are able to

avoid these resistance mechanisms by detoxifying the phytoalexins produced by their host plant (Morrissey and Osbourn 1999; VanEtten et al. 2001). Pea (*Pisum sativum* L.), for example, produces copious amounts of the phytoalexin (+) pisatin in response to fungal infection and, although (+) pisatin is toxic to most fungi that are not pathogenic on pea, most fungal pathogens of pea are able to detoxify this compound and are tolerant of pisatin (Cruickshank 1962; Delserone et al. 1999).

Pisatin demethylase (*pda*), the enzyme responsible for detoxifying pisatin (Fig. 1), is a substrate-inducible cytochrome P450 best characterized in the ascomycetous fungus *Nectria haematococca* MPVI (anamorph *Fusarium solani* f. sp. *pisi*). Isolates of *N. haematococca* MPVI that are highly virulent on pea have pisatin demethylating activity (*Pda*⁺) and are more tolerant of pisatin than isolates lacking this activity (*Pda*⁻) (VanEtten et al. 1980). Transformation of a functional gene (*PDA*) for *pda* into a *Pda*⁻ isolate increases both the tolerance of the isolate to pisatin and its virulence on pea (Ciufetti and VanEtten 1996). Conversely, disruption of a *PDA* gene in a *Pda*⁺ isolate reduces the virulence of the isolate and its tolerance to pisatin (Wasmann and VanEtten 1996). This relationship between *pda* and the virulence of *N. haematococca* has led to the conclusion that *pda* is a host-specific virulence factor in this fungus.

Nine *PDA* genes have been identified in *N. haematococca* MPVI by classical genetics (Funnell et al. 2002; Kistler and VanEtten 1984a; Mackintosh et al. 1989; Miao and VanEtten 1992) and four *PDA* genes have been sequenced: *PDA7* (Maloney and VanEtten 1994), *PDA6-1* (Reimann and VanEtten 1994), *PDA4* (K. Hirschi and H. D. VanEtten, unpublished), and *PDA1-1* (cDNA) (Q. D. Wu and H. D. VanEtten, unpublished). Although the *PDA* genes are highly similar (>90% amino acid identity) to each other, they are sufficiently different from other cytochrome P450s to be placed in their own family (*CYP57*) (Maloney and VanEtten 1994). *PDA* gene products' high degree of specificity and biochemical affinity for pisatin, their association with high virulence on pea, and the tolerance of pisatin associated with these enzymes have led to the hypothesis that these cytochrome P450s make up a specialized detoxification system that arose during the evolution of *N. haematococca* as a pea pathogen (VanEtten et al. 1989).

A survey of other true fungi that are pathogens found that isolates with moderate to high virulence on pea rapidly demethylated pisatin (Delserone et al. 1999). However, an attempt to identify the *PDA* genes in other pea pathogens using the *PDA* gene from *N. haematococca* as a probe in Southern hybridization showed that only DNA of the taxonomically related fungi *F. oxysporum* and *Neocosmospora boninensis* hybridized with the *Nectria haematococca* *PDA* gene probe (Delserone et al. 1999; Temporini and VanEtten 2004).

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The *PDA1* gene of *N. haematococca* MPVI is located on a 1.6-Mb conditionally dispensable (CD) chromosome and is the only member of a cluster of genes involved in pea pathogenicity (*PEP* genes) with a known biochemical function (Han et al. 2001). The four genes in this cluster shown to contribute to virulence on pea (*PDA1*, *PEP1*, *PEP2*, and *PEP5*) in *N. haematococca* also have been shown to have orthologs in *F. oxysporum* f. sp. *pisi*, and at least two of these genes (*FoPDA1* and *PEP1*) are within close proximity of one another in an isolate of *F. oxysporum* f. sp. *pisi* (Temporini and VanEtten 2004).

Whereas most pea-pathogenic fungi appear to have evolved a specific cytochrome P450 for detoxifying pisatin (George and VanEtten 2001), only in *N. haematococca* MPVI is there direct evidence that *pda* is associated with virulence (Ciuffetti and VanEtten 1996; Wasmann and VanEtten 1996). This article details the characterization of *PDA* genes in *F. oxysporum* and, in particular, from *F. oxysporum* f. sp. *pisi*. The evidence presented here confirms that pisatin detoxification is a virulence factor for pea, with a major role in conferring the ability of pea-pathogenic fungi to cause disease on this plant.

RESULTS

Identification and sequencing of *FoPDA1* and *FoPDAt*.

The gene corresponding to *FoPDA1* was isolated from a genomic lambda library of *F. oxysporum* f. sp. *pisi* isolate T415 (Table 1) by probing the library with the *PDA* gene of *N. haematococca*. The coding region of *FoPDA1* is 1,738 bp in length and contains intragenic regions at four locations (nucleotides 248 to 303, 654 to 709, 1,316 to 1,368, and 1,498 to 1,555) (Supplementary Fig. S1). The 5' and 3' boundaries of all four regions were identical to the canonical exon and intron splice sites observed for other filamentous fungi (Kupfer et al. 2004). The four predicted intragenic regions were located at the same positions as the four introns in *PDAT9* (Maloney and VanEtten 1994). *FoPDA1* is predicted to encode a protein of 505 amino acids that has >85% identity to the amino acid sequence of *PDAT9* from *N. haematococca*.

Fig. 1. Enzymatic detoxification of (+) pisatin catalyzed by pisatin demethylase.

Table 1. Fungal isolates used in this study

Fungus	Isolate	Source ^a
<i>Nectria haematococca</i> MPVI	77-13-7, T-110, 44-100	Our collection
<i>Fusarium oxysporum</i> f. sp. <i>pisi</i>		
Race 1	F28, F79, F109, F184, F206	Our collection
Race 2	F26, F40, F69, F81, F204, T415, T416	Our collection
Race 5	F23, F202, F207	Our collection
Race 6	F27, F105	Our collection
<i>F. oxysporum</i>		
f. sp. <i>aechaeae</i>	NRRL 22533	K. O'Donnell
f. sp. <i>cubense</i>	NRRL 26029	K. O'Donnell
f. sp. <i>lini</i>	NRRL 28932	K. O'Donnell
f. sp. <i>lycopersici</i>	NRRL 26383	K. O'Donnell
f. sp. <i>batatas</i>	NRRL 25594	K. O'Donnell
f. sp. <i>glycines</i>	NRRL 25598	K. O'Donnell
f. sp. <i>melonis</i>	NRRL 26406	K. O'Donnell
f. sp. <i>phaseoli</i>	NRRL 26761	K. O'Donnell
f. sp. <i>dianthi</i>	NRRL 28902	K. O'Donnell

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Previous Southern analyses using a fragment of the *N. haematococca* *PDAT9* gene suggested the presence of at least two *PDA* genes in isolate T415 (Delserone et al. 1999; Temporini and VanEtten 2004). The second hybridizing fragment was cloned and sequenced and found to contain a truncated *PDA* gene, designated *FoPDAt*, with 99% nucleotide identity to *FoPDA1*. The 5' end of the region of identity was located in the codon for residue M76 of *FoPDA1*. The similarity to *FoPDA1* ended 250 nucleotides 3' of the coding region. No evidence of DNA sequence with similarity to the promoter region of *FoPDA1* was found.

Expression of pisatin demethylating activity in culture.

The demethylation of pisatin by mycelium of *F. oxysporum* f. sp. *pisi* was characterized by a lag of approximately 1 to 2 h followed by rapid demethylation, which was complete by 4 to 6 h (Fig. 2). The addition of 1% glucose did not repress pisatin demethylation, as had been observed with *N. haematococca* (George and VanEtten 2001; Straney and VanEtten 1994; VanEtten and Barz 1981) but it did retard the onset and completion of demethylation by approximately 1 h (Fig. 2). Higher concentrations of glucose (2 and 5%) had effects similar to that of 1% glucose, in that demethylation was approximately 1 to 1.5 h slower than in the control without glucose (data not shown).

The accumulation of *FoPDA1* mRNA in response to the addition of pisatin was measured by quantitative real-time polymerase chain reaction (PCR). The maximal amount of *FoPDA1* mRNA was 670-fold of the basal level and occurred approximately 1 h after pisatin was added (Fig. 3). The induction of pisatin demethylating ability was characterized by a lag of approximately 2 h followed by a rapid rise in activity (Fig. 3) that reached a maximum of 77 pmol min⁻¹ mg⁻¹ fresh weight of mycelia approximately 6 h after the addition of pisatin and then declined. This pattern of induction of enzyme activity was similar to that observed with *PDA1* in *N. haematococca*; however, the *FoPDA1* mRNA appeared to be induced more quickly than the *PDA1* mRNA (George et al. 1998; Liu et al. 2003; Straney and VanEtten 1994).

Fig. 2. Demethylation of pisatin by *Fusarium oxysporum* f. sp. *pisi* isolate T415 in the absence or presence of 1% glucose. Mycelial suspensions of 3.5 ml were placed into 50-ml flasks and [¹⁴C] pisatin (300 dpm/μg) dissolved in dimethyl sulfoxide (DMSO) was added to a final concentration of 0.1 mM pisatin and 0.5% DMSO. At 1- to 2-h intervals, 0.5-ml aliquots were withdrawn and analyzed for pisatin content.

Four compounds, (+) maackiain, (+) methylmaackiain, methylformononetin, and 6-methoxy-1-tetralone, which are relatively effective inducers of pda activity in some fungal pathogens of pea other than *N. haematococca* (George and VanEtten, 2001) were tested for their ability to induce pda activity in isolate T415. None of the tested compounds induced pda to a greater extent than the solvent control, 0.5% dimethyl sulfoxide (DMSO) (Supplementary Table S1). The inability of these compounds to induce pda activity in *F. oxysporum* f. sp. *pisi* is similar to results from previous pda induction studies on *N. haematococca* (George and VanEtten 2001).

Characterization of *FoPDA1* as a *PDA* gene.

In order to verify that the gene identified as *FoPDA1* was responsible for the pda activity in *F. oxysporum* isolate T415, transformations to disrupt the *FoPDA1* gene were attempted. The basic approach was to disrupt *FoPDA1* by insertion of the *hph* gene (conferring resistance to hygromycin) into the coding sequence of *FoPDA1* via homologous recombination. Several different plasmid constructs were used, resulting in a total of 905 hygromycin-resistant transformants; however, all of the transformants retained pda activity. Although there was a faint hybridizing fragment in the T415 digest in addition to *FoPDA1* (Fig. 4), we found no evidence of another *PDA*-like gene that could have made gene replacement difficult. The reasons for the lack of success to create a $\Delta FoPDA1$ isolate are unknown.

As an alternative approach to demonstrate that *FoPDA1* is responsible for pda activity in *F. oxysporum* f. sp. *pisi*, the gene was transformed into 44-100, a *Pda*⁻ isolate of *N. haematococca* MPVI. The expression of *PDA1* in *N. haematococca* is repressed in the presence of glucose but not in *F. oxysporum* f. sp. *pisi*, suggesting that the two promoters may differ in their regulatory characteristics (Fig. 2). In view of these presumed differences in the promoters of these two *PDA* genes, a plasmid (pCW10071) was constructed in which *FoPDA1* was un-

der the control of the *N. haematococca* *PDA1* promoter. Using pCW10071, a 44-100 transformant containing the *NhPDA1* promoter::*FoPDA1* chimera was generated, and this transformant had pda activity (Table 2).

Distribution of homologs of *FoPDA1* in *F. oxysporum* f. sp. *pisi*.

Seventeen isolates of *F. oxysporum* f. sp. *pisi* representing four physiologic races (races 1, 2, 5, and 6) (Table 1) were examined and all had pda activity and were pathogenic but showed some variability in their ability to produce necrotic lesions on the stems of 'Little Marvel' pea, which is susceptible to all of these races (Table 3) (Haglund and Kraft 1979). *F. oxysporum* f. sp. *pisi* is known as a wilt pathogen but it also produces tissue necrosis (Kerstin et al. 2002), and measurements of the necrotic lesions on pea stems have been used previously to assess the virulence of *F. oxysporum* f. sp. *pisi* (Delserone et al. 1999). All of the isolates were subjected to Southern hybridization analysis (Fig. 4). In one gel, the fragments smaller than approximately 2 kb were run off the gel in order to resolve the larger DNA fragments (Fig. 4A), while a second gel was run for a shorter time to resolve the presence of DNA fragments of approximately 1 to 2 kb (Fig. 4B). All of the isolates surveyed contained at least one hybridizing fragment and, although common patterns of hybridization were found, they were not correlated with the race of the isolate. For example, isolates of different races (e.g., F206, F26, F23, and F27, which belong to races 1, 2, 5, and 6, respectively) had the same pattern of hybridization. Another group of isolates, which included members of race 1 (F28 and F184), race 2 (F69, F81, T415, and T416), and race 5 (F202 and F207), also displayed the same hybridization pattern. In addition, these latter isolates were the only ones that appeared to have *FoPDA1* because they had a hybridizing fragment of the same size as that for *FoPDA1* in

Fig. 3. Induction by pisatin of *FoPDA1* mRNA and pisatin demethylase (pda) activity in *Fusarium oxysporum* f. sp. *pisi* isolate T415.

Table 2. Expression of pisatin demethylase (pda) activity by *FoPDA1* in *Nectria haematococca*^a

Isolate	Pisatin remaining (%)
<i>FoPDA1</i> under <i>NhPDA1</i> promoter	
77-13-7 (positive control)	13.7
44-100 (negative control)	100
44-100 <i>NhPDA1</i> promoter: <i>FoPDA1</i>	15.4

^a Peptone-glucose agar medium (0.25 ml) supplemented with [¹⁴C] pisatin (31 μ g pisatin per 10,000 dpm per 5 μ l of dimethyl sulfoxide per 1 ml of medium) was inoculated with each isolate and percent [¹⁴C] remaining assayed after 96 h.

Fig. 4. Distribution of homologs of *FoPDA1* in *Fusarium oxysporum* f. sp. *pisi*. Genomic DNA of the indicated isolate was digested with *Xho*I and probed with a 1-kb *Eco*RI-*Cla*I fragment of the promoter and N-terminal coding region of the *FoPDA1* gene. Different gels were used to resolve **A**, the larger (>8 kb) fragments and **B**, the smaller (1 to 2 kb) fragments. Arrowheads denote the fragments that correspond to *FoPDA1* and *PDA1*, respectively.

T415. Isolates F79, F109, and F40 appeared to contain a single fragment hybridizing to the *FoPDA1* homolog.

Chromosomal location of *FoPDA1* and other *PEP* genes in *F. oxysporum* f. sp. *pisi*.

Pulsed-field gel electrophoresis (PFGE) was utilized to ascertain the size of the chromosomes in different races of *F. oxysporum* f. sp. *pisi* harboring *FoPDA1* and the homologs of the other *PEP* genes known to contribute to virulence in *N. haematococca* (*PEP1*, *PEP2*, and *PEP5*) (Fig. 5). As previously shown (Han et al. 2001), these genes were all found to be on a 1.6-Mb chromosome in the virulent isolate 77-13-7 of *N. haematococca*. Four of the seven *F. oxysporum* f. sp. *pisi* isolates (F28, F69, T415, and F202) also had *FoPDA1*, *PEP1*, and *PEP5* located on the same relatively small (approximately 2.0 Mb) chromosome. In isolate F105, *FoPDA1*, *PEP2*, and *PEP5* were located on an approximately 2.3-Mb chromosome; *PEP1* appeared to be absent in this isolate. Hybridization of *FoPDA1*, *PEP1*, and *PEP5* in isolates F79 and F109 showed that the genes are located on different chromosomes and that *FoPDA1*, in particular, was located on a large chromosome. The size of the chromosome carrying *FoPDA1* in isolates F79 and F101 is uncertain due to the lack of resolution in that portion of the PFGE analysis. The final gene, *PEP2*, may possibly

be located on the same chromosome as one of the other *PEP* genes or *FoPDA1* in isolates F79, F105, and F109.

Distribution of *FoPDA1* within the *F. oxysporum* species complex.

F. oxysporum f. sp. *pisi* belongs to a group of fungi collectively called the *Fusarium oxysporum* species complex (FOSC) which has been divided based on host range, resulting in over 120 different formae speciales (Di Pietro et al. 2003; Michielse and Rep 2009). The virulence of eight different formae speciales isolates of *F. oxysporum* was assessed on garden pea (Table 4; Fig. 6). *F. oxysporum* f. sp. *lini*, *F. oxysporum* f. sp. *glycines*, *F. oxysporum* f. sp. *lycopersici*, *F. oxysporum* f. sp. *cubense*, and *F. oxysporum* f. sp. *aechaene* were all unable to cause any significant lesion in the pea stem virulence assay. However, *F. oxysporum* f. sp. *dianthi* and *F. oxysporum* f. sp. *phaseoli* were virulent on pea, causing lesions of a size similar to those of *F. oxysporum* f. sp. *pisi* T415. However, the lesions caused by *F. oxysporum* f. sp. *dianthi* were lighter in color (tan versus brown) (Table 4; Fig. 6).

Southern analysis of representatives of 10 formae speciales representing four clades within the FOSC was undertaken to identify the distribution of *PDA* homologs. As shown before, four isolates within the same clade as *F. oxysporum* f. sp. *pisi* showed the presence of a hybridizing DNA fragment (formae speciales *glycines*, *lini*, and *dianthi*) (Fig. 7; Supplementary Fig. S2) (Temporini and VanEtten 2004). Despite the presence of an apparent homolog of *PDA* in these other members of the *F. oxysporum* f. sp. *pisi*-containing clade, only isolates of the pea pathogen *F. oxysporum* f. sp. *pisi* had *pda* activity. Previously, homologs of *PEP1*, *PEP2*, and *PEP5* were identified in

Table 3. Virulence of *Fusarium oxysporum* f. sp. *pisi* isolates on pea ('Little Marvel')^a

Isolate	Lesion length (mm)
Race 1	
F28	3.8 ± 0.7
F79	4.9 ± 2.4
F109	5.9 ± 0.9
F184	4.3 ± 0.9
F206	13.7 ± 1.9
Race 2	
F26	8.5 ± 1.4
F40	4.9 ± 2.4
F69	4.3 ± 1.5
F81	5.7 ± 1.5
F204	3.9 ± 1.8
T415	4.6 ± 1.1
T416	6.1 ± 1.7
Race 5	
F23	13.2 ± 1.4
F202	5.2 ± 1.7
F207	6.2 ± 1.0
Race 6	
F27	6.7 ± 1.4
F105	7.1 ± 1.1
<i>Nectria haematococca</i> MPVI (77-13-7)	15.3 ± 5.2

^a Lesion lengths are the averages and based on eight plants scored 8 days postinoculation.

Table 4. Virulence of various *Fusarium oxysporum* formae speciales isolates on pea ('Alaska 2B')^a

Isolate	Average lesion length (mm)	
	Experiment 1	Experiment 2
<i>Nectria haematococca</i> MPVI		
77-13-7	8.6 ± 1.0	14.7 ± 4.2
T-110/44-100	0.0	4.5 ± 1.3
<i>F. oxysporum</i>		
f. sp. <i>pisi</i> (T415)	8.2 ± 2.3	11.5 ± 3.2
f. sp. <i>dianthi</i>	7.8 ± 0.8	nd
f. sp. <i>lini</i>	0.0	0.7 ± 0.3
f. sp. <i>glycines</i>	0.0	nd
f. sp. <i>phaseoli</i>	7.8 ± 4.5	nd
f. sp. <i>lycopersici</i>	nd	1.2 ± 0.5
f. sp. <i>cubense</i>	nd	0.9 ± 1.1
f. sp. <i>aechaene</i>	nd	1.1 ± 1.0

^a Lesion lengths are the averages of lesions produced on six to 10 pea plants scored 6 days postinoculation; nd = not determined.

Fig. 5. Hybridization of *FoPDA1* and orthologs of the pea pathogenicity (*PEP*) encoding genes to chromosomes of *Fusarium oxysporum* f. sp. *pisi* separated by pulsed-field gel electrophoresis. Isolates F28, F79, and F109 are race 1; F69 and T415 are race 2; F202 is race 5; and F105 is race 6.

all the formae speciales included in the current studies (*F. oxysporum* ff. spp. *aechmeae*, *batatas*, *cubense*, *dianthi*, *glycines*, *lini*, *lycopersici*, *melonis*, and *phaseoli*) (Temporini and VanEtten 2004; E. Temporini, unpublished).

The *PDA* loci of *F. oxysporum* ff. spp. *glycines*, *lini*, and *dianthi* were sequenced to determine why they lack *pda* activity. The *PDA* locus in *F. oxysporum* f. sp. *lini* has a G to A mutation at position 182, changing the TGG tryptophan codon to a TAG stop codon. Overall, the *FoPDA1* pseudogene in *F. oxysporum* f. sp. *lini* is 92.9% identical to *FoPDA1* in *F. oxysporum* f. sp. *pisi*. The premature stop codon results in a truncated protein 60 amino acids in length that is 91.7% (55 of 60) identical to the first 60 amino acids of *FoPDA1* in *F. oxysporum* f. sp. *pisi*. The *FoPDA1* gene of *F. oxysporum* f. sp. *glycines* has an 11-bp deletion starting at nucleotide 816 of the gene, resulting in a pseudogene 90.8% identical to *FoPDA1* in *F. oxysporum* f. sp. *pisi*. This pseudogene encodes a truncated protein 266 amino acids in length with similarity to *FoPDA1* over the first 234 amino acids (94.9% identity and 96.2% amino acid similarity). The *FoPDA1* gene in *F. oxysporum* f. sp. *dianthi* is 99.8% identical to the *FoPDA1* pseudogene in *F. oxysporum* f. sp. *glycines* and contains the same 11-bp deletion found in *F. oxysporum* f. sp. *glycines*.

The only isolate outside of the clade containing *F. oxysporum* f. sp. *pisi* that had a *PDA*-hybridizing fragment is *F. oxysporum* f. sp. *phaseoli* (Fig. 7). Furthermore, *F. oxysporum* f. sp. *phaseoli* was the only isolate within this survey, besides *F. oxysporum* f. sp. *pisi*, which had *pda* activity (Fig. 7). The two *FoPDA1* genes were 98.8% identical in nucleic acid sequence (1,721 of 1,742 bp), inclusive of all four conserved introns, resulting in six amino acid differences between the two *FoPDA1* cytochrome P450 enzymes (99.0% identity and 99.2% amino acid similarity).

The presence of an *FoPDA1* gene or pseudogene within all members of the same clade as *F. oxysporum* f. sp. *pisi* supports the vertical inheritance of the gene within this clade. The high degree of identity between the *FoPDA1* pseudogenes of *F. oxysporum* f. sp. *glycines* and *F. oxysporum* f. sp. *dianthi* suggests they have only recently begun to diverge, although the 11-bp deletion resulting in the inactivation of the gene occurred prior to the separation of these formae speciales. Phylogenetic analysis of the *FoPDA1* from *F. oxysporum* f. sp. *phaseoli* indicates that the gene is most closely related to *FoPDA1* from *F. oxysporum* f. sp. *pisi* (Fig. 8).

***FoPDA1* is a virulence factor on pea.**

Since attempts to disrupt *FoPDA1* in *F. oxysporum* f. sp. *pisi* were unsuccessful, *FoPDA1* was placed in formae speciales isolates that were not pathogenic on pea to assess the role of *FoPDA1* in virulence. Two different formae speciales were chosen, one within the same clade as *F. oxysporum* f. sp. *pisi* (*F. oxysporum* f. sp. *lini*) and one from a clade more evolutionarily distant (*F. oxysporum* f. sp. *lycopersici*). As previously stated, neither of these isolates has *pda* activity; however, both of these isolates have homologs of *PEP1*, *PEP2*, and *PEP5* (Temporini and VanEtten 2004). Transformation of the *F. oxysporum* f. sp. *pisi* *FoPDA1* gene under its native promoter into either isolate resulted in transformants with *pda* activity (Sup-

Fig. 7. Phylogenetic relationship between several *Fusarium oxysporum* formae speciales. Phylogenetic analysis was based on translation elongation factor 1 α . Presence of *FoPDA1* as detected by hybridization and enzymatic activity is also denoted. *Nectria haematococca* was used as the outgroup.

Fig. 8. Phylogenetic analysis of *PDA* (pisatin demethylase) genes and pseudogenes from *Fusarium oxysporum* formae speciales. *Nectria haematococca* was used as the outgroup.

Fig. 6. Virulence of *Fusarium oxysporum* formae speciales on pea. From left to right: *Nectria haematococca* T110 (negative control), *F. oxysporum* f. sp. *pisi* (T415), *F. oxysporum* f. sp. *phaseoli*, *F. oxysporum* f. sp. *dianthi*, *F. oxysporum* f. sp. *lini*, *F. oxysporum* f. sp. *glycines*, and *N. haematococca* 77-13-7 (for comparison).

plementary Table S2). Therefore, the transcription machinery necessary to recognize pisatin and to express *pda* is present in the two Pda⁻ isolates. Several of the *F. oxysporum* f. sp. *lini* transformants not only expressed *pda* but were more virulent on pea stems than the recipient isolate, demonstrating that *FoPDA1* is a virulence factor on pea (Table 5; Supplementary Fig. S3). None of the *F. oxysporum* f. sp. *lycopersici* isolates expressing *FoPDA1* demonstrated increased virulence on pea (data not shown).

DISCUSSION

Because of the amino acid similarities between the *PDA* genes of *N. haematococca* and *F. oxysporum* f. sp. *pisi*, it would be expected that they share similar biochemical properties (substrate specificity, inhibitor sensitivity, and K_m), and preliminary experiments support this expectation (George 1998, George and VanEtten 2001). Likewise, they would be predicted to share similar regulatory properties. The two genes have similar induction specificity, because both are highly specific for pisatin as an inducer. In addition, the *FoPDA1* promoter contains a 41-bp element with significant identity (36/41 bp; 87.8%) to an element in the *N. haematococca* *PDA1* promoter that confers pisatin-responsiveness (Khan et al. 2003). However, the lack of glucose repression suggests a distinct difference between these promoters. Prior studies by Khan and Straney (1999) had identified a region between -287 and -429 bp, relative to the start of transcription, which mediated repression by glucose in culture independent of pisatin induction. This region of the promoter is different in *FoPDA1* and could explain the lack of glucose repression, although consensus binding sites for the glucose repressor CreA, similar to those observed in the promoter of *PDA1* of *N. haematococca* (Khan and Straney 1999), are present in the *FoPDA1* promoter. Since *F. oxysporum* f. sp. *pisi* is normally a wilt pathogen and proliferates in the sugar-poor xylem, it is difficult to speculate on what advantage the lack of glucose repression might have for *F. oxysporum* f. sp. *pisi*, although the persistence within this sugar-limited environment may have contributed to the lack of glucose repression.

The *FoPDA1* gene was shown to function as a virulence gene by transforming it into a Pda⁻ isolate. The variability in the degree of virulence obtained when *FoPDA1* was placed into *F. oxysporum* f. sp. *lini* might be explained by positional effects of integration of the gene. A similar variability in virulence was observed when *PDA1* was placed into a nonpathogenic isolate of *N. haematococca* MPVI (Ciuffetti and VanEtten 1996). Regardless, all of the *F. oxysporum* f. sp. *lini* *FoPDA1* transformants with *pda* activity produced a significantly larger and more necrotic lesion on pea (Table 5) than the wild-type *F. oxysporum* f. sp. *lini* isolate and the Pda⁻ transformant (Table 5). The increased virulence of *F. oxysporum* f. sp. *lini* transformants

compared with *F. oxysporum* f. sp. *lycopersici* transformants also demonstrated that other virulence factors exist, which are common to *F. oxysporum* f. sp. *lini* and *F. oxysporum* f. sp. *pisi* but absent from the more distantly related *F. oxysporum* f. sp. *lycopersici*.

In *N. haematococca* MPVI, the gene encoding the cytochrome P450 *PDA1* resides within a cluster of genes for pathogenicity on pea (the *PEP* cluster) that is located on a small CD chromosome, and all of the pea-pathogenic *N. haematococca* MPVI isolates examined have all of the *PEP* genes (Temporini and VanEtten 2002). As has been seen previously in *F. oxysporum*, there was considerable chromosome polymorphism (Ma et al. 2010) but, in *F. oxysporum* f. sp. *pisi*, *FoPDA1* often was found together with at least one *PEP* gene on a small chromosome (Fig. 5). However, it was rare for all of the *PEP* genes known to be involved in virulence (*PEP1*, *PEP2*, and *PEP5*) along with *FoPDA1* to be on the same chromosome in *F. oxysporum* f. sp. *pisi*.

The pathogenic race of some *F. oxysporum* formae speciales has been associated with more than one clonal lineage, suggesting multiple origins for a single race (Baayen et al. 2000; Gordon and Martyn 1997; O'Donnell et al. 1998, 2009). The hybridization patterns of *FoPDA1* from multiple races of *F. oxysporum* f. sp. *pisi* are consistent with the theory of multiple origins for the pathogenic races. In addition to the identical Southern hybridization pattern of eight isolates from races 1, 2, and 5, these also are the only surveyed isolates that harbor *PDA1* (Fig. 4). When a subgroup [F28 (race 1), F69 and T415 (race 2), and F202 (race 5)] of these isolates was selected for PFGE analysis, all carried *FoPDA1*, *PEP1*, and *PEP5* on an approximately 2.0-Mb chromosome (Fig. 5). Although the degree of similarity among these chromosomes from the different isolates is not known, these hybridization studies suggest that the chromosomes may share a common ancestor, despite their belonging to different pathogenic races.

The polyphyletic nature of *F. oxysporum* f. sp. *pisi* has been observed by multilocus sequence typing, where strains of *F. oxysporum* f. sp. *pisi* have been assigned into at least 10 sequence types (ST) (O'Donnell et al. 2009). Of these 10 types, *F. oxysporum* ff. spp. *pisi*, *lini*, and *dianthi* can all be found in a single group, ST 90 (O'Donnell et al. 2009), suggesting that *FoPDA1* has been retained within this group, albeit not always functional. However, the presence of a functional *FoPDA1* gene in *F. oxysporum* f. sp. *phaseoli* was unexpected. Although *FoPDA1* may have been retained from the last common ancestor of *F. oxysporum* f. sp. *phaseoli* and the clade containing *F. oxysporum* ff. spp. *pisi*, *lini*, *glycines*, and *dianthi* (followed by gene loss in the other formae speciales), a more parsimonious explanation would be that a horizontal transfer event occurred between the two lineages that retain a functional *PDA* gene. A horizontal gene transfer (HGT) event in *F. oxysporum* f. sp. *phaseoli* is supported by several other lines of evidence. As

Table 5. Virulence of *Fusarium oxysporum* f. sp. *lini* transformants harboring *FoPDA1* on pea^a

Isolate	PDA ^b	Average lesion length (mm)						Variance	<i>t</i> ^c
		Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. mean		
<i>F. oxysporum pisi</i>	+	13.0 ± 2.3	7.3 ± 2.2	10.8 ± 2.0	11.9 ± 4.3	11.5 ± 1.5	10.9 ± 2.2	...	...
<i>F. oxysporum lini</i>	-	3.4 ± 1.1	2.7 ± 1.0	5.2 ± 2.0	5.8 ± 0.8	3.0 ± 1.6	4.0 ± 1.4	4.13	...
Tr1	+	7.4 ± 1.2	5.4 ± 2.0	4.0 ± 1.7	3.4 ± 1.3	6.0 ± 1.9	5.2 ± 1.6	2.55	1.04
Tr2	+	6.2 ± 1.7	4.3 ± 2.8	6.6 ± 1.4	5.6 ± 1.0	6.3 ± 2.1	5.8 ± 0.9	0.84	1.81**
Tr3	+	7.0 ± 2.4	4.5 ± 2.0	7.2 ± 1.7	6.3 ± 2.0	5.7 ± 1.0	6.1 ± 1.1	1.20	2.05*
Tr16	-	3.1 ± 1.0	2.6 ± 1.7	5.5 ± 1.9	5.6 ± 0.7	4.3 ± 2.2	4.2 ± 1.4	1.86	0.19

^a Virulence assays were conducted on seven to 10 pea plants ('Alaska 2B'), with the exception of experiment (Exp.) 3, which was conducted on six pea plants ('Little Marvel'). Lesions were scored 6 days postinoculation.

^b PDA = pisatin demethylase.

^c *t* Values are based on a paired difference test based on the pooled estimate of the common variance compared with the *F. oxysporum* f. sp. *lini* control. Critical value for $t_{0.05} = 1.86$ and $t_{0.10} = 1.40$; * and ** indicate values statistically significant for $t_{0.05}$ and $t_{0.10}$, respectively.

mentioned previously, there were only six amino acid differences in the *F. oxysporum* f. sp. *phaseoli* and *F. oxysporum* f. sp. *pisi* *FoPDA1* genes, and the high degree of conservation between these genes suggests that they have only recently diverged (Fig. 8). HGT of small chromosomes between different *F. oxysporum* formae speciales isolates has also been observed experimentally (Ma et al. 2010). In that study, one of the transferred chromosomes contained genes involved in virulence on tomato and conferred that virulence to the recipient isolate, which was originally nonpathogenic on tomato. This observation may explain the polyphyletic nature of host virulence and the emergence of new pathogenic lineages among members of the FOSC. If the *FoPDA1* gene (and possibly the entire chromosome) from an isolate of *F. oxysporum* f. sp. *pisi* were laterally transferred into *F. oxysporum* f. sp. *phaseoli*, it would demonstrate that HGT occurs in the environment and may have facilitated the abrupt expansion of host range among isolates of the FOSC. In additional support of HGT, *F. oxysporum* f. sp. *phaseoli* has been placed into ST 51 and ST 93, two ST in which *F. oxysporum* f. sp. *pisi* have also been found (O'Donnell et al. 2009), suggesting that, if an HGT event did occur with *FoPDA1*, it was prior to the divergence of *F. oxysporum* f. sp. *pisi* and *F. oxysporum* f. sp. *phaseoli*. Alternatively, because formae speciales are assigned based on the host plant on which they cause disease, the ability of *F. oxysporum* f. sp. *phaseoli* to cause disease on pea allows speculation that *F. oxysporum* ff. spp. *phaseoli* and *pisi* are, in fact, a single forma specialis, which is supported by the similar *PDA* genes in these two fungi.

This study confirms the importance of *PDA* in detoxifying pisatin and in conferring virulence on pea in a fungal species other than *N. haematococca* MPVI. The nucleotide similarity between *PDA1* in *N. haematococca* and *FoPDA1* in *F. oxysporum* f. sp. *pisi* suggests that they share a common origin, although different regulatory mechanisms have evolved in the two pathogens. As in *N. haematococca*, *FoPDA1*, *PEP1*, and *PEP5* reside primarily on the same small chromosome in *F. oxysporum* f. sp. *pisi*. The presence of a functional *FoPDA1* gene in *F. oxysporum* f. sp. *phaseoli*, which has a high degree of similarity to *FoPDA1* in *F. oxysporum* f. sp. *pisi*, suggests that it may have been acquired recently by HGT and may be an example of host range expansion by HGT in nature; or, alternatively, that some isolates of *F. oxysporum* ff. spp. *phaseoli* and *pisi* are, in fact, a single forma specialis.

MATERIALS AND METHODS

Chemicals.

The chemicals (+) pisatin, (+) maackiain, methyl formononetin, (+) methyl maackiain, 6-methoxy-1-tetralone, and 3-*O*-methyl-¹⁴C] pisatin (¹⁴C] pisatin) were obtained as described by George and associates (1998); and 2,5-diphenyloxazole (PPO) was obtained from Research Products International (Mt. Prospect, IL, U.S.A.).

Strains and growth conditions.

F. oxysporum f. sp. *pisi* isolate JI 2 was obtained from the John Innes Institute (Norwich, U.K.) and is referred to as isolate T415 in this publication. Isolates used in this study are listed in Table 1. *N. haematococca* Berk. & Broome isolates 77-13-7 and 44-100 are the products of laboratory crosses (Kistler and VanEtten 1984a). Isolate 77-13-7 contains a single *PDA* gene (*PDA1*), which has been characterized biochemically in the closely related isolate 77-13-4 (George and VanEtten 2001; George et al. 1998). Isolate 44-100 is *Pda*⁻ (Kistler and VanEtten 1984a). Stock cultures were grown on V8 juice agar in petri plates or in tubes as slant cultures (medium 29) (Stevens

1974) at room temperature under ambient fluorescent lighting and stored at 4°C. For long-term storage, suspensions of conidia in 25 to 50% glycerol were stored at -80°C.

To obtain mycelium, 100 to 250 ml of liquid medium were inoculated with conidia (approximately 10⁵ conidia/ml) and incubated for 12 to 40 h on a gyro-rotary shaker (180 rpm) at room temperature. The mycelia were collected by vacuum filtration through Whatman number 4 filter paper (Whatman Inc., Piscataway, NJ, U.S.A.).

Escherichia coli DH5α (Invitrogen Life Technologies, Valencia, CA, U.S.A.) was used for amplifying recombinant plasmids. The Lambda library was packaged and amplified using the nonpermissive *E. coli* XL1-Blue MRA (P2) (Stratagene, La Jolla, CA, U.S.A.). For screening, the library was plated using the permissive strain XL1-Blue MRA (Stratagene). For DNA isolation, Lambda 72 (λ72) was amplified in the permissive *E. coli* LE392.

Genomic Southern hybridization analysis.

Mycelium for the extraction of genomic DNA was produced as described above by growth in glucose asparagine medium (VanEtten et al. 1980) for 3 to 4 days. The mycelia were lyophilized and DNA was extracted from the mycelia as described by Miao and associates (1991).

For Southern hybridization, equal amounts (3 μg) of genomic DNA were digested with restriction endonucleases. The DNA fragments were separated by electrophoresis in an agarose gel and transferred to nylon membranes (Hybond N⁺; GE Healthcare Bio-Sciences, Piscataway, NJ, U.S.A.) according to standard protocols (Sambrook and Russell 2001). DNA blots were probed with a 1.1-kb *EcoRI*-*Clai*I or a 1.15-kb *Sst*I-*Bam*HI fragment of *FoPDA1*. The synthesis of the probes and the detection of hybridization followed the procedure for chemiluminescent Southern hybridizations described by Wasmann and VanEtten (1996). Southern hybridization detection of *FoPDA1* in the other formae speciales of *F. oxysporum* was conducted by digesting the genomic DNA with *EcoRV* and probing with a fragment of *PDA1* from *N. haematococca* as previously described (Temporini and VanEtten 2002, 2004).

Cloning and sequence analysis of *FoPDA1* and *FoPDAt*.

FoPDA1 was isolated from a Lambda library constructed in Lambda EMBL3 using the Lambda EMBL3/*Bam*HI Vector Kit and the instructions supplied by the manufacturer (Stratagene). The library was screened with the 1.4-kb *Sac*I fragment (*Sac*B fragment) of the *PDAT9* gene of *N. haematococca* (Maloney and VanEtten 1994). One clone, λ72, was the source of the *FoPDA1* gene. A 4.5-kb *Spe*I-*Pst*I fragment of λ72 containing the entire *FoPDA1* gene was identified by Southern hybridization with the *Sac*B fragment as a probe, cloned into pBluescript II KS to give p04987, and sequenced.

FoPDAt was isolated from an ordered genomic sublibrary of isolate T415. Briefly, 25 μg of *Pst*I-digested genomic DNA was separated by electrophoresis and the portion of the gel containing fragments of 5.8 to 6.5 kb (the size range that hybridized with the 1.15-kb *Sst*I-*Bam*HI fragment of *FoPDA1* used as a probe) was excised. The DNA fragments were purified using the QAIEX Gel Extraction Kit (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's instructions and cloned into pBluescript II KS. The sublibrary was ordered in 96-well microtiter plates, blotted to nylon membranes, and probed with the 1.15-kb *Sst*I-*Bam*HI fragment of *FoPDA1*. One hybridizing clone, 39D7, was selected for sequencing.

Homologs of *FoPDA1* from the other *F. oxysporum* formae speciales were amplified from genomic DNA by PCR using the primers *FoPDA*-2F and *FoPDA*-5R (Supplementary Table S3). The PCR product was T-A cloned into pCR (Invitrogen),

transformed into DH5 α , and screened for the insert of expected size (1,873 bp in isolate T415). Plasmid inserts representing each of the *FoPDA1* homologs from the other *F. oxysporum* formae speciales were selected for sequencing.

DNA sequencing was performed by the DNA Sequencing Facility (Genetic Analysis and Technology Core of the Arizona Research Laboratories Division of Biotechnology, University of Arizona, Tucson, U.S.A.).

Determination of the effects of glucose and other compounds on the metabolism of pisatin in culture.

Mycelium was produced from cultures grown in glucose-yeast extract medium (VanEtten and Barz 1981) as described above and by George and VanEtten (2001), with the exception that cultures of isolate T415 were grown for 40 h. Mycelia were suspended in 0.05 mM potassium phosphate buffer (pH 6.5), rather than sodium phosphate buffer, at a ratio of 30 mg ml⁻¹ (fresh weight) with or without 1% glucose. Aliquots of 3.5 ml were placed into 50-ml Erlenmeyer flasks and [¹⁴C] pisatin (300 dpm/ μ g) dissolved in DMSO was added to a final concentration of 0.1 mM pisatin and 0.5% DMSO. The flasks were incubated at 25°C and 100 rpm. At 1- to 2-h intervals, 0.5-ml aliquots were withdrawn for the analysis of pisatin content.

To test the ability of different compounds to induce pda activity, suspensions of mycelia in potassium phosphate buffer were prepared as above. The test compounds in DMSO were added to a final concentration of 0.1 mM and 0.5% DMSO. A time course of pisatin demethylation induced by pisatin was performed concurrently in order to determine when to measure pda activity in the treated samples, which was normally 4.5 to 6 h after the addition of the test compound. The rate of pisatin demethylation was determined by adding [¹⁴C] pisatin (final concentration 0.1 mM) to 3.5 ml of culture, and aliquots of 0.5 ml were withdrawn at 5, 10, 15, and 20 min postinoculation and assayed for pisatin content. Each compound was tested at least twice, except for maackiain, which was tested once.

The amount of [¹⁴C] pisatin in samples was determined by adding the samples to 4.5 ml of scintillation cocktail (0.5% PPO in toluene) and using a Beckman LS6000SC Scintillation Counter (Beckman Instruments, Palo Alto, CA, U.S.A.) to record the [¹⁴C] (George and VanEtten 2001).

Measurement of *FoPDA1* expression by quantitative real-time PCR.

Conidia of isolate T415 were incubated in 100 ml of potato dextrose broth (PDB) (PhytoTechnology Laboratories, Shawnee Mission, KS, U.S.A.) for 12 h on a gyro-rotary shaker at room temperature as previously described. Mycelia were collected and suspended at a ratio of 30 mg/ml in 50 ml of PDB and dispensed into 125-ml Erlenmeyer flasks. Cultures were incubated for 30 min at room temperature on a gyro-rotary shaker and (+) pisatin in DMSO was then added to a final concentration of pisatin at 50 μ g/ml and 1% DMSO. The control culture received DMSO to a final concentration of 1%. Aliquots of the suspension were removed at 1, 2, and 4 h after the addition of (+) pisatin and lyophilized.

Total RNA was extracted from lyophilized mycelia using the RNeasy plant mini kit (Qiagen) and suspended in RNase-free water. Contaminating DNA was removed using the GenHunter MessageClean kit (GenHunter, Nashville, TN, U.S.A.), and the concentration of the total RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, U.S.A.). Complementary DNA (cDNA) was generated using SuperScript II reverse transcriptase (Invitrogen) with oligo-dT (Invitrogen) according to the manufacturer's protocol.

For PCR, the SYBR Green PCR Master mix (Invitrogen) and gene-specific primers were used. The number of transcripts

of the β -actin gene was used to normalize the number of transcripts of *FoPDA1*. The primers for β -actin and *FoPDA1* were those used by Liu and associates (2003) to analyze the expression of the *PDA1* gene of *N. haematococca*. Quantitative real-time PCR was performed in an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA, U.S.A.) and the data compared using the algorithm $X = 2^{-\Delta\Delta Ct + SPE}$ as described by Livak and Schmittgen (2001).

PFGE and Southern hybridization analysis.

Protoplasts of *F. oxysporum* were generated as previously described (Temporini and VanEtten 2002) and suspended in agarose at a density of 4×10^8 protoplasts/ml using the first method described by Orbach and associates (1988). A Bio-Rad CHEF-DRIII system (Bio-Rad, Hercules, CA, U.S.A.) was used with 0.8% of certified megabase agarose (Bio-Rad), 1 $\times$ Tris-acetate-EDTA (40 mM Tris-acetate-EDTA, pH 8.0), and the following running conditions: 3.0 V/cm for 48 h at 14°C, a switch time of 500 s, and a pulse angle of 106°. After PFGE, the agarose gel was soaked in 0.25 M HCl for 15 min and 0.4 M NaOH for 30 min. Chromosomal DNA was capillary transferred to a positively charged nylon membrane (Roche, Indianapolis, IN, U.S.A.). The DIG DNA Labeling and Detection Kit (Roche) was used for probe labeling, hybridization, and probe detection. Hybridization to DNA probes was performed at 60°C. DNA fragments used as probes for *FoPDA1*, *PEP1*, *PEP2*, and *PEP5* were described previously (Temporini and VanEtten 2004).

Plasmid for the expression of *FoPDA1* in *N. haematococca*.

The construct for the expression of *FoPDA1* under the control of the *N. haematococca* *PDA1* promoter (pCW10071) was made in the following manner. The plasmid pD1-XS (Straney and VanEtten 1994) (*unpublished*), which contains a 1.2-kb *XhoI*-*SacI* fragment with the promoter and 40 bp of the amino acid coding region of *PDA1*, was digested with *HindIII*, the ends were filled in with the large fragment of DNA Polymerase I, and the plasmid digested with *SstI*. The resulting 1.2-kb promoter fragment was ligated with pUC19 that was previously digested with *SmaI* and *SstI* to create pCW04071. To create pCW10071, the *BamHI*-*SstI* fragment from pCW04071 containing the promoter and 39 bp of the coding region of *PDA1* and an *SstI*-*NotI* fragment of pCW07071 containing the *FoPDA1* coding region and the 3' flanking region were ligated with the fungal transformation vector pCW02052 (described below) digested with *BamHI* and *NotI*.

To make the fungal transformation vector pCW02052, a *Clal* site was added 5' of the start codon of the *hph* gene in pCX62 (Zhao et al. 2004) using PCR with the primer pair CX62+*Clal*F and CX62+*Clal*R and pCX62 as the template. The 0.6-kb PCR product was cloned into the pGEM-T Easy vector (Promega Corp., Madison, WI, U.S.A.) to yield pCW02051. To make pCW02052, a 320-bp *FseI*-*Clal* fragment from pDH33 (Smith et al. 1990) containing a minimal *glal*A promoter and approximately 100 bp of additional 5' untranslated sequence (Fowler et al. 1990), a *Clal*-*SphI* fragment from pCW02051 containing 600 bp of the amino terminal region of *hph*, and a 3.4-kb *SphI*-*FseI* fragment of pCX62 containing the remainder of the coding region of the *hph* gene and the plasmid backbone were ligated together. The regions flanking the *hph* gene to target homologous recombination at the *FoPDA1* locus ranged from approximately 960 to 1,300 bp for the 5' flanking region and approximately 600 bp to 1,800 bp for the 3' flanking region.

Fungal transformation and screening for pda activity.

F. oxysporum and *N. haematococca* protoplasts for transformation were generated with Kitalase (Wako Pure Chemical In-

dustries, Ltd., Osaka, Japan) and Lytzyme (Karlan Research Products Corporation, Cottonwood, AZ, U.S.A.), as previously described (Coleman et al. 2011).

Transformation was carried out as described by Coleman and associates (2011), except that *F. oxysporum* and *N. haematococca* transformants were selected on potato dextrose agar with hygromycin B (Calbiochem, La Jolla, CA, U.S.A.) at 70 or 60 µg/ml, respectively. Hygromycin-resistant colonies were transferred to plates containing M-100 medium (Stevens 1974) with hygromycin at 100 µg/ml, or hygromycin at 70 µg/ml for *F. oxysporum* or *N. haematococca* transformants. Transformants were assessed for their ability to demethylate pisatin in a vial assay as described by Mackintosh and associates (1989) using 0.25 ml of peptone-glucose agar medium (VanEtten et al. 1980) supplemented with [¹⁴C] pisatin (31 µg of pisatin per 10,000 dpm per 5 µl of DMSO per milliliter of medium). Scintillation fluid (4 ml 0.5% PPO in toluene) was added to terminate the assay after 96 h and to extract the remaining [¹⁴C] pisatin. The amount of [¹⁴C] was measured in a Beckman Instruments model LS 6000SC scintillation counter.

Pea pathogenicity assay and statistical analysis.

Virulence was assessed using the test tube assay (VanEtten et al. 1980) as modified by Wasmann and VanEtten (1996). Virulence was quantified by measuring the lengths of lesions produced by each isolate on the hypocotyls of pea seedlings 6 to 8 days after inoculation. Unless noted otherwise, each test isolate was inoculated onto eight seedlings per experiment. In each trial, the mean lesion length was used as a measure of virulence. In addition to the test isolates, trials to assess the virulence of transformants included the Pda⁻ parent isolate. The Student's *t* test was used in pairwise comparisons across trials.

Phylogenetic analysis.

The translation elongation factor (EF)-1α of *F. oxysporum* f. sp. *phaseoli* was amplified by PCR using the primers EF-1 and EF-2 and sequenced. Phylogenetic analysis was conducted using MacVector and previously obtained EF-1α sequences (Temporini and VanEtten 2004). Analyses of the *PDA* genes and pseudogenes from the other formae speciales were performed using the full length of DNA corresponding to the coding region of the functional *FoPDA1* gene in *F. oxysporum* f. sp. *pisi*. Alignments were conducted using ClustalW, and phylogenetic neighbor-joining trees generated with 1,000 bootstraps.

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LITERATURE CITED

Baayen, R. P., O'Donnell, K., Bonants, P. J. M., Cigelnik, E., Kroon, L. P. N. M., Roebroeck, E. J. A., and Waalwijk, C. 2000. Gene genealogies and AFLP analyses in the *Fusarium oxysporum* complex identify monophyletic and nonmonophyletic formae speciales causing wilt and rot diseases. *Phytopathology* 90:891-900.

Ciuffetti, L. M., and VanEtten, H. D. 1996. Virulence of a pisatin-deficient *Nectria haematococca* MPVI isolate is increased by transformation with a pisatin demethylase gene. *Mol. Plant-Microbe Interact.* 9:787-792.

Coleman, J. J., White, G. J., Rodriguez-Carres, M., and VanEtten, H. D. 2011. An ABC transporter and a cytochrome P450 of *Nectria haematococca* MPVI are virulence factors on pea and are the major tolerance mechanisms to the phytoalexin pisatin. *Mol. Plant-Microbe Interact.* 24:368-374.

Cruickshank, I. A. M. 1962. Studies on phytoalexins. IV. The antimicrobial spectrum of pisatin. *Aust. J. Biol. Sci.* 15:147-159.

Delserone, L. M., McCluskey, K., Matthews, D. E., and VanEtten, H. D. 1999. Pisatin demethylation by fungal pathogens and nonpathogens of pea: Association with pisatin tolerance and virulence. *Physiol. Mol. Plant Pathol.* 55:317-326.

Di Pietro, A., Madrid, M. P., Caracul, Z., Delgado-Jarana, J., and Roncero, M. I. G. 2003. *Fusarium oxysporum*: Exploring the molecular arsenal of a vascular wilt fungus. *Mol. Plant Pathol.* 4:315-325.

Fowler, T., Berka, R. M., and Ward, M. 1990. Regulation of the *glaA* gene of *Aspergillus niger*. *Curr. Genet.* 18:537-545.

Funnell, D. L., and VanEtten, H. D. 2002. Pisatin demethylase genes are on dispensable chromosomes while genes for pathogenicity on carrot and ripe tomato are on other chromosomes in *Nectria haematococca*. *Mol. Plant-Microbe Interact.* 15:840-846.

Funnell, D. L., Matthews, P. S., and VanEtten, H. D. 2002. Identification of new pisatin demethylase genes (*PDA5* and *PDA7*) in *Nectria haematococca* and non-Mendelian segregation of pisatin demethylating ability and virulence on pea due to loss of chromosomal elements. *Fungal Genet. Biol.* 37:121-133.

George, H. L. 1998. Biochemical characterization of pisatin demethylases from fungal pea pathogens. Ph.D. thesis, University of Arizona.

George, H. L., and VanEtten, H. D. 2001. Characterization of pisatin-inducible cytochrome P450s in fungal pathogens of pea that detoxify the pea phytoalexin pisatin. *Fungal Genet. Biol.* 33:37-48.

George, H. L., Hirsch, K., and VanEtten, H. D. 1998. Biochemical properties of the products of cytochrome P450 genes (*PDA*) encoding pisatin demethylase activity in *Nectria haematococca*. *Arch. Microbiol.* 170:147-154.

Gordon, T. R., and Martyn, R. D. 1997. The evolutionary biology of *Fusarium oxysporum*. *Annu. Rev. Phytopathol.* 35:111-128.

Haglund, W. A., and Kraft, J. M. 1979. *Fusarium oxysporum* f. sp. *pisi*, race 6: Occurrence and distribution. *Phytopathology* 69:818-820.

Han, Y., Liu, X., Benny, U., Kistler H. C., and VanEtten, H. D. 2001. Genes determining pathogenicity to pea are clustered on a supernumerary chromosome in the fungal plant pathogen *Nectria haematococca*. *Plant J.* 25:305-314.

Kerstin, S., Bodker, L., and Rosendahl, S. 2002. Population structure and pathogenicity of members of the *Fusarium oxysporum* complex isolated from soil and root necrosis of pea (*Pisum sativum* L.). *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol.* 42:367-374.

Khan, R., and Straney, D. C. 1999. Regulatory signals influencing expression of the *PDA1* gene of *Nectria haematococca* MPVI in culture and during pathogenesis of pea. *Mol. Plant-Microbe Interact.* 12:733-742.

Khan, R., Tan, R., Mariscal, A. G., and Straney, D. 2003. A binuclear zinc transcription factor binds the host isoflavonoid-responsive element in a fungal cytochrome p450 gene responsible for detoxification. *Mol. Microbiol.* 49:117-130.

Kistler, H. C., and VanEtten, H. D. 1984a. Three non-allelic genes for pisatin demethylation in the fungus *Nectria haematococca*. *J. Gen. Microbiol.* 130:2595-2603.

Kupfer, D. M., Drabenstot, S. D., Buchanan, K. L., Lai, H., Zhu, H., Dyer, D. W., Roe, B. A., and Murphy, J. W. 2004. Introns and splicing elements of five diverse fungi. *Eukaryot. Cell* 3:1088-1100.

Liu, X. G., Inlow, M., and VanEtten, H. D. 2003. Expression profile of pea pathogenicity (*PEP*) genes *in vivo* and *in vitro*, characterization of the flanking regions of the *PEP* cluster and evidence that the *PEP* cluster region resulted from horizontal gene transfer in the fungal pathogen *Nectria haematococca*. *Curr. Genet.* 44:95-103.

Livak, K., and Schmittgen, T. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔC_T} method. *Methods* 25:402-408.

Ma, L.-J., van der Does, H. C., Borkovich, K. A., Coleman, J. J., Daboussi, M.-J., Di Pietro, A., Dufresne, M., Freitag, M., Grabherr, M., Henrissat, B., Houterman, P. M., Kang, S., Shim, W.-B., Woloshuk, C., Xie, X., Xu, J.-R., Antoniwi, J., Baker, S. E., Bluhm, B. H., Breakspear, A., Brown, D. W., Butchko, R. A. E., Chapman, S., Coulson, R., Coutinho, P. M., Danchin, E. G. J., Diener, A., Gale, L. R., Gardner, D. M., Goff, S., Hammond-Kosack, K. E., Hilburn, K., Hua-Van, A., Jonkers, W., Kazan, K., Kodira, C. D., Koehrsen, M., Kumar, L., Lee, Y.-H., Li, L., Manners, J. M., Miranda-Saavedra, D., Mukherjee, M., Park, G., Park, J., Park, S.-Y., Proctor, R. H., Regev, A., Ruiz-Roldan, M. C., Sain, D., Sakthikumar, S., Sykes, S., Schwartz, D. C., Turgeon, B. G., Wapinski, I., Yoder, O., Young, S., Zeng, Q., Zhou, S., Galagan, J., Cuomo, C. A., Kistler, H. C., and Rep, M. 2010. Comparative genomics reveals mobile pathogenicity chromosomes in *Fusarium*. *Nature* 464:367-373.

Mackintosh, S. F., Matthews, D. E., and VanEtten, H. D. 1989. Two additional genes for pisatin demethylation and their relationship to the

- pathogenicity of *Nectria haematococca* on pea. *Mol. Plant-Microbe Interact.* 4:341-349.
- Maloney, A. P., and VanEtten, H. D. 1994. A gene from the fungal plant pathogen *Nectria haematococca* that encodes the phytoalexin-detoxifying enzyme defines a new cytochrome P450 family. *Mol. Gen. Genet.* 243:506-514.
- Miao, V. P. W., and VanEtten, H. D. 1992. Three genes for metabolism of the phytoalexin maackiain in the plant pathogen *Nectria haematococca*: Meiotic instability and relationship to a new gene for pisatin demethylase. *Appl. Environ. Microbiol.* 58:801-808.
- Miao, V. P. W., Matthews, D. E., and VanEtten, H. D. 1991. Identification and chromosomal locations of a family of cytochrome P-450 genes for pisatin detoxification in the fungus *Nectria haematococca*. *Mol. Gen. Genet.* 226:214-223.
- Michielse, C. B., and Rep, M. 2009. Pathogen profile update: *Fusarium oxysporum*. *Mol. Plant Pathol.* 10:311-324.
- Morrissey, J. P., and Osbourn, A. E. 1999. Fungal resistance to plant antibiotics as a mechanism of pathogenesis. *Microbiol. Mol. Biol. Rev.* 63:708-724.
- O'Donnell, K., Kistler, H. C., Cigelnik, E., and Ploetz, R. C. 1998. Multiply evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. *Proc. Natl. Acad. Sci. U.S.A.* 95:2044-2049.
- O'Donnell, K., Gueidan, C., Sink, S., Johnston, P. R., Crous, P. W., Glenn, A., Riley, R., Zitomer, N. C., Colyer, P., Waalwijk, C., van der Lee, T., Moretti, A., Kang, S., Kim, H.-S., Geiser, D. M., Juba, J. H., Baayen, R. P., Crome, M. G., Bithell, S., Sutton, D. A., Skovgaard, K., Ploetz, R., Kistler, H. C., Elliott, M., Davis, M., and Sarver, B. A. J. 2009. A two-locus DNA sequence database for typing plant and human pathogens within the *Fusarium oxysporum* species complex. *Fungal Genet. Biol.* 46:936-948.
- Orbach, M. J., Vollrath, D., Davis, R. W., and Yanofsky, C. 1988. An electrophoretic karyotype of *Neurospora crassa*. *Mol. Cell. Biol.* 8:1469-1473.
- Reimann, C., and VanEtten, H. D. 1994. Cloning and characterization of the *PDA6-I* gene encoding a cytochrome P-450 which detoxifies the phytoalexin pisatin from garden pea. *Gene* 146:221-226.
- Sambrook, J., and Russell, D. W. 2001. *Molecular Cloning: A Laboratory Manual*, third ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A..
- Smith, T. L., Gaskell, J., Berka, R. M., Yang, M., Henner, D. J., and Cullen, D. 1990. The promoter of the glucoamylase-encoding gene of *Aspergillus niger* functions in *Ustilago maydis*. *Gene* 88:259-262.
- Stevens, R. B. 1974. *Mycology Guidebook*. University of Washington Press, Seattle.
- Straney, D. C., and VanEtten, H. D. 1994. Characterization of the *PDA1* promoter of *Nectria haematococca* and identification of a region that binds a pisatin-responsive DNA binding factor. *Mol. Plant-Microbe Interact.* 7:256-266.
- Temporini, E. D., and VanEtten, H. D. 2002. Distribution of the pea pathogenicity genes in the fungus *Nectria haematococca* mating population VI. *Curr. Genet.* 41:107-114.
- Temporini, E. D., and VanEtten, H. D. 2004. An analysis of the phylogenetic distribution of the pea pathogenicity genes of *Nectria haematococca* MPVI supports the hypothesis of their origin by horizontal transfer and uncovers a potentially new pathogen of garden pea: *Neocosmospora boniensis*. *Curr. Genet.* 46:29-36.
- VanEtten, H. D., and Barz, W. 1981. Expression of pisatin demethylating ability in *Nectria haematococca*. *Arch. Microbiol.* 129:56-60.
- VanEtten, H. D., Matthews, P. S., Tegtmeyer, K. J., Dietert, M. F., and Stein, J. I. 1980. The association of pisatin tolerance and demethylation with virulence on pea in *Nectria haematococca*. *Physiol. Plant Pathol.* 16:257-269.
- VanEtten, H. D., Matthews, D. E., and Matthews, P. S. 1989. Phytoalexin detoxification: Importance for pathogenicity and practical implications. *Annu. Rev. Phytopathol.* 27:143-164.
- VanEtten, H. D., Temporini, E., and Wasmann, C. 2001. Phytoalexin (and phytoanticipin) tolerance as a virulence trait: Why is it not required by all pathogens? *Physiol. Mol. Plant Pathol.* 59:83-93.
- Wasmann, C. C., and VanEtten, H. D. 1996. Transformation-mediated chromosome loss and disruption of a gene for pisatin demethylase decrease the virulence of *Nectria haematococca* on pea. *Mol. Plant-Microbe Interact.* 9:793-803.
- Zhao, X., Xue, C., Kim, Y., and Xu, J.-R. 2004. A ligation-PCR approach for generating gene replacement constructs in *Magnaporthe grisea*. *Fungal Genet. Newl.* 51:17-18.