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

Jeffrey J. Coleman, Gerard J. White, Marianela Rodriguez-Carres, and Hans D. VanEtten

Department of Plant Sciences, Division of Plant Pathology and Microbiology, University of Arizona, Tucson, AZ 85721, U.S.A.

Submitted 1 September 2010. Accepted 9 November 2010.

The fungal plant pathogen *Nectria haematococca* MPVI produces a cytochrome P450 that is responsible for detoxifying the phytoalexin pisatin, produced as a defense mechanism by its host, garden pea. In this study, we demonstrate that this fungus also produces a specific ATP-binding cassette (ABC) transporter, NhABC1, that enhances its tolerance to pisatin. In addition, although both mechanisms individually contribute to the tolerance of pisatin and act as host-specific virulence factors, mutations in both genes render the fungus even more sensitive to pisatin and essentially nonpathogenic on pea. NhABC1 is rapidly induced after treatment with pisatin in vitro and during infection of pea plants. Furthermore, NhABC1 was able to confer tolerance to the phytoalexin rishitin, produced by potato. NhABC1 appears to be orthologous to GpABC1 of the potato pathogen *Gibberella pulicaris* and, along with MoABC1 from *Magnaporthe oryzae*, resides in a phylogenetically related clade enriched with ABC transporters involved in virulence. We propose that NhABC1 and the cytochrome P450 may function in a sequential manner in which the energy expense from pisatin efflux by NhABC1 releases the repression of the cytochrome P450, ultimately allowing pisatin tolerance by two mechanisms. These results demonstrate that a successful pathogen has evolved multiple mechanisms to overcome these plant antimicrobial compounds.

In 1940, Müller and Börger (1940) proposed that production of phytoalexins, specific plant antibiotics produced in response to microbial infection, is a major plant resistance mechanism. It was another 20 years before the first phytoalexin, (+) pisatin (Fig. 1) from pea (*Pisum sativum* L.), was chemically identified (Cruickshank and Perrin 1960). Soon after, in a classic study evaluating the sensitivity of 45 fungal species, Cruickshank (1962) observed that only five species were tolerant of pisatin,

all of which were pathogens of pea. This remarkable correlation demonstrating a linkage between tolerance and host range firmly established the concept that tolerance to phytoalexins could be a host-specific virulence trait (Cruickshank 1965). In 1964, it was reported that two fungal pea pathogens were capable of degrading pisatin (Uehara 1964). This conclusion was disputed by Cruickshank and Perrin (1965), but they were misled in their studies because their assay for pisatin did not distinguish between pisatin and the nontoxic demethylated compound produced from pisatin by most pea pathogens (Fig. 1). The enzyme that rapidly catalyzes pisatin detoxification, pisatin demethylase (PDA), is a unique cytochrome P450 restricted primarily to pea pathogens (Delserone et al. 1999; George and VanEtten 2001). PDA has been best studied in *Nectria haematococca* mating population (MP) VI, in which it is a host-specific virulence factor on pea (Wasmann and VanEtten 1996). The gene (*PDA1*) encoding this enzyme is clustered with three other genes involved in pea pathogenicity (*PEP* genes) (Han et al. 2001) on a supernumerary chromosome, a dispensable portion of the genome of this fungus (Miao et al. 1991a). This type of supernumerary chromosome in fungi has been called conditionally dispensable (CD) because these chromosomes contain genes, such as the *PEP* genes, which allow for growth in some habitats (e.g., pea), but the chromosomes are not required for growth in other natural habitats or in culture (Covert 1998).

Prior studies of *N. haematococca* MPVI in which *PDA1* was mutated by gene disruption demonstrated that $\Delta PDA1$ isolates exhibit increased sensitivity to pisatin and reduced virulence on pea (Wasmann and VanEtten 1996). However, these mutants are still pathogenic and fairly tolerant of pisatin when compared with other fungi. Studies using *N. haematococca* MPVI isolates that lack PDA activity demonstrated that a second mechanism for tolerance to pisatin, termed “nondegradative” tolerance, exists and is induced by pisatin and several structurally similar phytoalexins (Denny and VanEtten 1983a and b; Denny et al. 1987). This tolerance mechanism has many of the biochemical features of an efflux transporter, a process that has previously been demonstrated to be involved in fungal virulence on plant hosts (Fleissner et al. 2002; Schoonbeek et al. 2001; Urban et al. 1999). A molecular mechanism responsible for nondegradative tolerance to pisatin was identified in this current study as an ATP-binding cassette (ABC) transporter. This ABC transporter and the cytochrome P450 encoded by *PDA1* are the major tolerance mechanisms to pisatin in *N. haematococca* MPVI, and each contributes to the virulence of this fungus on pea.

Current address for J. J. Coleman: Harvard Medical School, Massachusetts General Hospital, Division of Infectious Diseases, 55 Fruit Street, Gray Jackson 5, Room GRJ-504, Boston, MA 02114-2696, U.S.A.

Current address for M. Rodriguez-Carres: Department of Biology, Duke University, 125 Science Dr., Durham, NC 27708, U.S.A.

Corresponding author: H. D. VanEtten; Telephone: +1.520.621.9355; Fax: +1.520.621.7186; E-mail: vanetten@ag.arizona.edu

Nucleotide sequence data for NhABC1 is available from the GenBank database under accession number HM106507.

RESULTS

Identification and cloning of *NhABC1*.

Previous studies identifying the *N. haematococca* MPVI nondegradative tolerance to pisatin phenomenon demonstrated that this mechanism was induced by subinhibitory concentrations of the phytoalexin within 3 h (Denny and VanEtten 1983a and b). To determine if an ABC transporter might be involved in nondegradative tolerance to pisatin, total RNA was isolated from pisatin-treated mycelia of *N. haematococca* and was subjected to reverse transcription- polymerase chain reaction (RT-PCR), using degenerate primers designed to amplify transcripts for ABC transporters. Eleven cloned PCR products were selected for sequencing and seven of these sequences corresponded to a single ABC transporter, while the other four sequences were of two other ABC transporters. Quantitative PCR was used to confirm the induction of these transporters by pisatin. In vitro induction of the ABC transporter corresponding to the most common cloned sequence was rapid and evident within 10 min after treatment with pisatin (90 µg/ml), increasing approximately 220-fold within 2 h after treatment (data not shown). The gene encoding this ABC transporter was designated *NhABC1* and was selected for further analysis.

The *NhABC1* open reading frame (ORF) is 4,528 bp in length, contains a single 47-bp intron, and encodes a protein 1,493 amino acids in length (GenBank accession number HM106507), corresponding to protein 63,187 (Nh63187) in the genome of the isolate of *N. haematococca* MPVI that was recently sequenced (77-13-4). Software topology prediction programs predict 12 transmembrane helices within the protein in a (NBD-TMD₆)₂ topology, placing *NhABC1* within the pleiotropic drug resistance (PDR) family of ABC transporters (Sanwal et al. 2009). Similarity searches to characterized proteins by BLASTp indicated that *NhABC1* was closely related to GpABC1 of the potato pathogen *Gibberella pulicaris* (79.6% amino acid identity and 88.1% amino acid similarity) and ABC1 of the rice pathogen *Magnaporthe oryzae* (57.8% amino acid identity and 70.8% amino acid similarity), two ABC transporters that are essential for high virulence for each of these fungi (Fleissner et al. 2002; Urban et al. 1999).

The two genes represented by the other cloned ABC transporter sequences were Nh77470 and Nh67597 in the sequenced *N. haematococca* MPVI isolate. However, in vitro expression analysis found these genes to be either not induced by pisatin (Nh77470) or the induction was not as high or as rapid as *NhABC1*, and they were, therefore, not selected for further molecular characterization.

N. haematococca *NhABC1* mutants have increased sensitivity to pisatin.

In order to investigate the role of *NhABC1* in pisatin tolerance and virulence on pea, gene disruption mutants were generated by homologous gene recombination. At 90 µg of (+) pisatin per milliliter, the $\Delta PDA1$ mutant showed a reduction in tolerance of approximately 20%, but the $\Delta NhABC1$ mutant showed only a slight reduction in tolerance (approximately 5%),

Fig. 1. Detoxification of (+) pisatin by pisatin demethylase to the less toxic compound (+) 6a-hydroxyumaackiain.

presumably because the pisatin was removed from the medium by the PDA activity present in the mutant (Fig. 2A).

The potential redundant relationship between *PDA1* and *NhABC1* requires a double mutant to be generated to accurately access the contribution of *NhABC1* to pisatin tolerance. Mutants of both *PDA1* and *NhABC1* were generated through a series of crosses using each of the single mutants of *N. haematococca* MPVI as a parent. The progeny were confirmed for the presence of the CD chromosome carrying the disrupted *PDA1* gene as well as the functional *PEP* genes by the ability of the isolate to utilize homoserine as a carbon source, a trait conferred specifically by this chromosome (Rodriguez-Carres et al. 2008).

Four progeny (375-1, 375-9, 375-13, and 375-19) were identified that carried mutations in both *NhABC1* and *PDA1*. These double mutants were even more sensitive (collectively averaging approximately 50% reduction compared with wild type) to pisatin than the $\Delta PDA1$ mutant, thus demonstrating the effect of *NhABC1* (Fig. 2A). The increase in sensitivity to pisatin of the double mutants of *N. haematococca* MPVI is even more evident when isolates are subjected to a higher concentration (180 µg/ml) of pisatin, as growth of the double mutants was reduced by approximately 80% in comparison with the wild-type isolate with functional copies of *PDA1* and *NhABC1* (Fig. 2B). Germination of double mutant spores from 375-13 is also greatly reduced at this concentration of pisatin, in comparison with the wild-type isolate and either single mutant (Table 1). There appears to be a slight lag in spore germination of the mutant with a disrupted copy of *NhABC1* as compared with $\Delta PDA1$ spores, suggesting *NhABC1* may be more important when the fungus is initially exposed to pisatin. Interestingly, the $\Delta NhABC1$; $\Delta PDA1$ double mutants are more toler-

Fig. 2. Pisatin tolerance bioassays of *Nectria haematococca* isolates. **A**, Radial growth after 5 days of growth on 90 µg of (+) pisatin per milliliter for (left to right) wild type (77-13-7), *NhABC1* mutant (Tr8.1), *PDA1* mutant (Tr18.5), and a *PDA1*;*NhABC1* double mutant (375-13). All isolates showed the same growth as isolate 77-13-7 on medium lacking pisatin. **B**, Radial growth on 180 µg of (+) pisatin per milliliter for (left to right) wild type (77-13-7), *NhABC1* mutant (Tr8.1), *PDA1* mutant (Tr18.5), and the $\Delta PDA1$; $\Delta NhABC1$ double mutant (375-13). The *NhABC1* mutant, the *PDA1* mutant, and the *PDA1*;*NhABC1* double mutants displayed 87 ± 8 , 57 ± 15 , and $22 \pm 11\%$ of the radial growth of 77-13-7, respectively. **C**, Radial growth on 90 µg of (+) pisatin per milliliter for (left to right) wild type (77-13-7), a $\Delta PDA1$; $\Delta NhABC1$ double mutant (375-13), and *N. haematococca* MPI (*Nh MPI*; NRRL 22165), a taxonomically related fungus that is not a pea pathogen.

ant to pisatin than the closely related fungus *N. haematococca* MPI, which is not a pea pathogen (Fig. 2C) and has been shown to be particularly sensitive to pisatin (VanEtten et al. 1980). This suggests that other pisatin tolerance mechanisms, which have yet to be identified, exist in *N. haematococca* MPVI or that MPI is unusually sensitive to pisatin.

NhABC1 is a virulence factor for pea.

PDA1 was previously shown to be a virulence factor on pea, as *N. haematococca* MPVI transformants with this cytochrome P450 mutated produce smaller lesions (Wasmann and VanEtten 1996). Pathogenicity assays performed for the current study demonstrated that Δ NhABC1 mutants of *N. haematococca* MPVI were reduced in virulence on pea and produced a lesion similar in size to those produced by PDA1-disrupted mutants (Fig. 3; Table 2). However, isolates lacking both tolerance mechanisms (375-1, 375-9, 375-13, and 375-19) are even further reduced in virulence than either single mutant (Fig. 3; Table 2).

Table 1. Percentage of *Nectria haematococca* MPVI spores germinating in (+) pisatin (180 µg/ml)^a

Time (h)	Wild type (77-13-7)	Δ PDA1 (Tr18.5)	Δ NhABC1 (Tr8.1)	Δ PDA1; Δ NhABC1 (375-13)
5	10	2	1	0
8	72	77	68	4
10	94	92	88	12
12	>95	>95	93	23
14			91	32
17			>95	58

^a Isolates not receiving pisatin treatment had germinated >95% after 10 h.

Fig. 3. Lesions produced on garden pea 6 days postinoculation for (left to right) a nonpathogenic isolate (44-100), wild type (77-13-7), Δ PDA1 mutant (Tr18.5), Δ NhABC1 mutant (Tr8.1), and a Δ PDA1; Δ NhABC1 double mutant (375-13). Arrow indicates site of inoculation.

Table 2. Virulence (lesion lengths) of *Nectria haematococca* MPVI isolates on pea^a

Experiment	Wild type (77-13-7)	Negative control (44-100)	Δ PDA1 (Tr18.5)	Δ NhABC1 (Tr8.1)	Δ PDA1, Δ NhABC1			
					375-1	375-9	375-13	375-19
1	14.1 ± 7.2	2.5 ± 0.8*	6.1 ± 2.4*	6.5 ± 1.7*	4.6 ± 0.6* [§]	4.7 ± 0.6* [§]	3.7 ± 0.8* [§]	3.4 ± 0.6* [§]
2	18.8 ± 2.9	3.0 ± 1.3*	13.4 ± 2.3*	12.1 ± 4.5*	6.7 ± 3.0* [§]	4.7 ± 1.0* [§]	6.1 ± 2.3* [§]	7.1 ± 2.3* [§]
3	13.9 ± 4.0	3.2 ± 0.9*	8.5 ± 3.0*	5.3 ± 1.3*	3.7 ± 1.8* [§]	4.0 ± 1.0* [§]	3.1 ± 1.5* [§]	3.4 ± 1.3* [§]
4	13.1 ± 4.2	3.8 ± 0.8*	6.4 ± 2.2*	10.2 ± 3.1*			3.8 ± 0.8* [§]	5.4 ± 0.8* [#]

^a Lesion sizes are in millimeters and are based on eight to 10 plants per treatment per experiment. Superscript symbols indicate lesion size is significantly different (*P* ≤ 0.05) compared with 77-13-7 (*), Tr8.1 (Δ NhABC1) (#), or both single mutants (§).

In planta expression analysis using quantitative PCR shows the expression of both *NhABC1* and *PDA1* increase as pisatin accumulates in the plant tissue (Fig. 4). Expression of *NhABC1* is initially induced more rapidly than *PDA1*, suggesting it may be more important in the early stages of tolerance to pisatin than *PDA1*, but both genes clearly demonstrate enhanced expression during pathogenesis.

Host-specific virulence is conferred by NhABC1.

N. haematococca MPVI is also pathogenic on carrot, and prior studies showed that the lack of PDA does not affect the

Fig. 4. In planta induction of pisatin tolerance genes in *Nectria haematococca* MPVI during infection of pea. Fold induction of *NhABC1* and *PDA1* are depicted. Concentration of pisatin (milligrams per gram of pea tissue dry weight) produced in infected pea pods is also shown.

virulence of this fungus on that host (Funnell and VanEtten 2002; Wasmann and VanEtten 1996). Pathogenicity assays again demonstrate that a $\Delta PDA1$ mutant is not reduced in virulence on carrot, and the $\Delta NhABC1$ mutant and the $\Delta PDA1$; $\Delta NhABC1$ double mutant also were not reduced in virulence, indicating that the virulence provided by $PDA1$ and $NhABC1$ are each specific for pathogenicity on garden pea (Fig. 5).

To determine if $NhABC1$ is found only in *N. haematococca* MPVI isolates that are pathogenic on pea, Southern hybridizations were conducted to assess the distribution of the ABC transporter among various fungi. All twelve *N. haematococca* MPVI isolates included in the analysis had genomic fragments hybridizing to $NhABC1$, including isolates with different degrees of virulence on pea from laboratory crosses (34-18, 212-5-1, 212-1-4, 312-2-5, 352-B-10, 230-30-6, and 44-100) and four field isolates from geographically diverse locations (T-23 from New York, T-110 from Mississippi, T-288 from South Carolina, and T-406 from Taiwan), including two field isolates that are not pathogenic on pea (T-110 from cottonwood and T-288 from soil) (Miao et al. 1991b) (Fig. 6).

Analysis of the distribution of $NhABC1$ was further extended to fungi besides *N. haematococca* MPVI. Southern hybridization revealed that homologs of $NhABC1$ exist in the phylogenetically related fungi *Neocosmospora vasinfecta*, *Neocosmospora boniensis* [*Neocosmospora boninensis*], and *N. haematococca* MPI. However, there was no hybridization to genomic DNA from other pea pathogens, including *Ascochyta pisi*, *Colletotrichum pisi*, *Mycosphaerella pinodes*, *Phoma pi-*

Fig. 5. Virulence of *Nectria haematococca* MPVI isolates on carrot, 7 days postinoculation with the following isolates. 1, *N. haematococca* MPI (non-pathogenic isolate, indicated by arrow), 2, wild type (77-13-7), 3, $PDA1$ mutant (Tr18.5), 4, $NhABC1$ mutant (Tr8.1), and 5, $PDA1$; $NhABC1$ double mutant (375-13).

nodella, or the phylogenetically related pea pathogen included in this study, *Fusarium oxysporum* f. sp. *pisi*. Homologs of $NhABC1$ were also not identified in fungi pathogenic on plants other than pea, such as the corn pathogen *Cochliobolus heterostrophus*, the rice pathogen *M. oryzae*, the sorghum pathogens *Gloeocerospora sorghi* and *Colletotrichum graminicola*, the wheat pathogen *Gaeumannomyces graminis* var. *tritici*, or the saprophytic fungi *Neurospora crassa* and *Aspergillus nidulans* (Fig. 6).

The specificity of the tolerance conferred to *N. haematococca* by $NhABC1$.

The $NhABC1$ mutant was tested for a change in sensitivity to 45 antimicrobial chemicals, including both natural and synthetic compounds. The $NhABC1$ mutant demonstrated an increase in sensitivity to only three of these compounds, (+) pisatin, the stereoisomer (–) pisatin, and the potato phytoalexin rishitin (Fig. 7).

$NhABC1$ is phylogenetically related to other ABC transporters involved in virulence.

$NhABC1$ is homologous (87% similar at the amino acid level) to $GpABC1$ from *G. pulicaris*, which was previously shown to

Fig. 7. Radial growth of *Nectria haematococca* MPVI on the potato phytoalexin rishitin. Left to right: wild type (77-13-7), no rishitin; wild type (77-13-7), with 200 µg of rishitin per milliliter, approximately 25% inhibition; and the $NhABC1$ mutant (Tr8.1), with 200 µg of rishitin per milliliter, approximately 45% inhibition as compared with the wild-type isolate containing no rishitin.

Fig. 6. Distribution of the $NhABC1$ homologs among various fungal isolates. A probe corresponding to $NhABC1$ from *Nectria haematococca* MPVI isolate 77-13-7 was hybridized to *EcoRI*-digested genomic DNA. Twelve *N. haematococca* MPVI isolates (77-13-7, 77-13-7 Tr18.5 [$\Delta PDA1$ transformant], 34-18, T-23, T-406, 212-5-1, 212-1-4, 312-2-5, 352-B-10, 230-30-6, T-110, T-288, and 44-100, lanes 1 to 13 respectively), seven other pea pathogenic fungi (*Fusarium oxysporum* f. sp. *pisi*, *Neocosmospora vasinfecta*, *Neocosmospora bonniensis*, *Phoma pinodella*, *Colletotrichum pisi*, *Mycosphaerella pinodes*, and *Ascochyta pisi*, lanes 14 to 20), and nine fungi not pathogenic on pea (*N. haematococca* MPI NRRL 22153, *N. haematococca* MPI NRRL 22165, *Cochliobolus heterostrophus*, *Magnaporthe oryzae*, *Colletotrichum graminicola*, *Gloeocerospora sorghi*, *Gaeumannomyces graminis* var. *tritici*, *Neurospora crassa*, and *Aspergillus nidulans*, lanes 21 to 29) were subjected to the analysis. M denotes lanes containing the DNA ladder. No hybridization fragments less than 2 kb in size were present for any isolate.

be required for tolerance to the potato phytoalexin rishitin and virulence on potato tubers (Fleissner et al. 2002). These two transporters belong to a monophyletic clade of the PDR family that includes several additional transporters from other phytopathogenic fungi (Fig. 8). The fact that NhABC1 and GpABC1 are phylogenetically within the same clade and both provide tolerance to the phytoalexin rishitin suggests they may be functionally orthologous. This ABC transporter appears to have been vertically inherited, as orthologs of *NhABC1/GpABC1* are phylogenetically conserved in the genomes of the other sequenced *Fusarium* spp. (Fig. 8). However, the role of the other orthologs in virulence on one or more of their respective host plants has not been reported.

Although ABC transporters of the PDR family usually have little phylogenetic conservation (Coleman and Mylonakis 2009; Kovalchuk and Driessen 2010), a relatively well-conserved sister clade exists that contains *M. oryzae* ABC1, a virulence factor for rice (Urban et al. 1999). The *N. haematococca* MPVI genome-sequencing project revealed two other closely related ABC transporters (Nh82055 and Nh35467) present in the genome that are homologous to *NhABC1* (Fig. 8) (Coleman et al. 2009). These two transporters are more phylogenetically related to *MoABC1* (MGG_13624.5 in the sequenced *M. oryzae* isolate 70-15) than to *NhABC1*, in which *Nh35467* appears to be orthologous to *MoABC1* (Fig. 8). However, a sequenced cDNA library constructed from pisatin-treated mycelia as part of the *N. haematococca* MPVI genome analysis contained no transcripts corresponding to the genes encoding either Nh82055 or Nh35467, suggesting they are not induced by pisatin.

DISCUSSION

The molecular arms race between plant hosts and their associated pathogens has been well documented, revealing most successful necrotrophic plant pathogens have evolved mecha-

nisms to overcome the phytoalexins produced by their host plants (Morrissey and Osbourn 1999; VanEtten et al. 2001). The two proteins responsible for the main tolerance mechanisms to pisatin in *N. haematococca* MPVI represent two different ways to achieve phytoalexin tolerance. ABC transporters confer tolerance by efflux of the compounds across the membrane, thereby preventing an increase in intracellular concentration of the toxic compound (Higgins 2007), and this appears to be the basis of the nondegradative tolerance to pisatin observed in *N. haematococca* MPVI by Denny and VanEtten (1983a and b). Thus, it is presumed that this is the function of NhABC1 in conferring tolerance, whereas the cytochrome P450 provides tolerance by detoxifying pisatin to the less-toxic compound 6a-hydroxymaackiain (Denny and VanEtten 1981).

The expression in planta of both *NhABC1* and *PDA1* and the effects on virulence on pea and carrot when each is mutated demonstrate that these are host-specific virulence factors and that these proteins act collaboratively to confer tolerance of pisatin in *N. haematococca* MPVI. However, the observed "lag" in spore germination of the *NhABC1* mutant when compared with either the wild type or the *PDA1* mutant isolates (Table 1) could mean that *NhABC1* is particularly important in conferring tolerance during the initial interaction with pisatin. As ABC transporters are primary transporters, NhABC1 hydrolyzes ATP to transport pisatin across the membrane and out of the fungal cell. However, after it is exported, pisatin is capable of reentering the fungal cell, in which it is again removed by NhABC1. This tolerance mechanism requires constant hydrolysis of ATP and is a metabolic expense for the cell. Interestingly, the expression of *PDA1* in *N. haematococca* MPVI is glucose-repressed (VanEtten and Barz 1981). Therefore, a model of sequential tolerance mechanisms conferring pisatin tolerance in *N. haematococca* MPVI can be proposed. In response to fungal infection, pisatin is synthesized by plant cells and enters the fungal cells. Once the fungus senses pisatin, *NhABC1* is induced and the corresponding protein transports the pisatin out of the fungal cell. The cycle of pisatin entering the fungal cell followed by active efflux causes a drop in ATP and, potentially, a subsequent decrease in intracellular glucose. The glucose repression of *PDA1* then ceases, allowing *PDA1* to be synthesized for enzymatic detoxification of pisatin.

Stemphylium loti, a phytopathogenic fungus of a cyanogenic plant (*Lotus corniculatus* L.), also appears to have a sequence of tolerance mechanisms that allows the fungus to overcome the toxic effects of cyanide released by its host (Rissler and Miller 1977). Upon exposure to hydrogen cyanide, the inhibition of the fungal cytochrome-based respiration activates a cyanide-insensitive respiratory system. This system allows the fungus to produce enough energy to synthesize a second tolerance mechanism, formamide hydrolyase (cyanide hydratase), which is responsible for enzymatic detoxification of cyanide to the much-less toxic compound formamide.

The enrichment of transporters involved in virulence or tolerance, or both, to phytoalexins within the *Fusarium*-specific (NhABC1 and GpABC1) clades and phylogenetically related clades containing ABC1 suggests that their function may have been retained from a common ancestor and that other transporters within these clades may be virulence factors for other phytopathogenic fungi. Hybridization studies identified homologs of *NhABC1* in all phylogenetically related members of the *Fusarium solani* species complex that were analyzed but were not present in other fungi, regardless of whether they were pathogenic on pea or not. The results indicate that taxonomic relatedness was a better predictor of the presence of a *NhABC1* homolog than was pathogenicity on pea. Furthermore, not all of the homologs detected by hybridi-

Fig. 8. Phylogenetic analysis of a select group of pleiotropic drug resistance transporters from fungi. Maximum parsimony trees were generated, and statistical support was calculated based on 1,000 bootstrap replicates. Arrows indicate ATP-binding cassette (ABC) transporters involved in pathogenicity. NhABC1 from *Nectria haematococca* MPVI isolate 77-13-7, Nh63187 from *N. haematococca* MPVI isolate 77-13-4, and GpABC1 from *Gibberella pulicaris* are evolutionarily and functionally orthologous and are part of a monophyletic cluster that appears to be present only in the genome of other fusaria (designated by the black vertical line).

zation studies conferred tolerance to pisatin, as a homolog was detected in *N. haematococca* MPI, a fungus that is very sensitive to pisatin (Fig. 2C).

The observation that *NhABC1* is on a larger chromosome (chromosome 8, 3.39 Mb) in *N. haematococca* MPVI rather than a small CD chromosome (approximately 1.6 Mb to 500 kb) (Coleman et al. 2009) is also consistent with it arising by vertical evolution rather than by horizontal gene transfer (HGT), a process postulated to have given rise to the CD chromosome. Despite *NhABC1* being vertically inherited and *PDA1* apparently being acquired by HGT, these two genes appear to have evolved in a sequential manner to function as the major mechanisms responsible for conferring pisatin tolerance in *N. haematococca* MPVI.

The best-characterized fungal efflux transporter, i.e., PDR5 of *Saccharomyces cerevisiae*, has a broad and structurally diverse substrate range, whereas *NhABC1* appears to transport only a few specific plant-produced antimicrobial compounds. Interestingly, the identified substrates of *NhABC1*—rishitin, a norsequitepene alcohol, and pisatin, a pterocarpan—have little structural similarity, although both are phytoalexins. It should be noted that the *N. haematococca* genome encodes 67 ABC transporters (Coleman et al. 2009), and it is possible that other redundant tolerance mechanisms may exist in the fungus, making the true substrate range of *NhABC1* difficult to determine. This is exemplified by the redundant function of *NhABC1* and *PDA1*, whereby the role of the ABC transporter in pisatin tolerance is not evident in the $\Delta NhABC1$ isolate but becomes apparent in the $\Delta NhABC1;\Delta PDA1$ mutant (Fig. 2). In addition to members of the PDR family of ABC transporters, members of the multidrug resistance (MDR) family and multidrug resistance-associated protein (MRP) family are also known to confer tolerance to toxic compounds (Coleman and Mylonakis 2009). The genome of *N. haematococca* MPVI encodes 22 MDR transporters, 17 MRP transporters, and 23 PDR transporters, in addition to *NhABC1* (Coleman et al. 2009).

Several other fungal plant pathogens are reported to have two phytoalexin tolerance mechanisms, although none of these pathogens have had both mechanisms molecularly characterized and evaluated as virulence traits (Morrissey and Osbourn 1999; Pedras and Ahiachoun 2005; VanEtten et al. 2001). The phytopathogen *Botrytis cinerea*, the causal agent of gray mold on grapevine, encodes an ABC transporter, *BcatrB*, and a laccase-like stilbene oxidase, which are able to provide some tolerance to the grapevine phytoalexin resveratrol (Pezet et al. 1991; Schoonbeek et al. 2001). In addition to *GpABC1*, *G. pulicaris* is capable of metabolizing rishitin, a trait that is linked to a high degree of virulence on potato tubers (Desjardins and Gardner 1989).

This additional data with *N. haematococca* MPVI clearly supports the hypothesis that fungal pathogens have evolved specific drug-resistance mechanisms and that they collectively contribute to a higher level of resistance to host toxins and higher virulence on their host. Furthermore, it shows that although phytoalexin production does provide a defense against pathogen attack in some instances, the most successful plant pathogens may have several tolerance mechanisms to overcome these antimicrobial compounds.

MATERIALS AND METHODS

Fungal strains, media, and growth conditions.

All isolates of *N. haematococca* MPI and MPVI were maintained and allowed to sporulate on V8 juice agar medium (Stevens 1974). To obtain mycelium for genomic DNA, 100 ml of potato dextrose broth (PDB) (Becton, Dickinson and Co., Sparks, MD, U.S.A.), contained in 250-ml Erlenmeyer flasks,

were inoculated with approximately 10^5 conidia per milliliter and were shaken at 180 rpm for 48 to 72 h at room temperature.

Genomic DNA was obtained from laboratory stocks for the rest of the isolates, *Neocosmospora vasinfecta* (ATCC 32302), *Neocosmospora boninensis* (NRRL 22470), *N. haematococca* MPI (NRRL 22153; NRRL 22165), *Fusarium oxysporum* f. sp. *pisi* (T415), *Ascochyta pisi* (T422), *Colletotrichum pisi* (T403), *Mycosphaerella pinodes* (T414), *Phoma pinodella* (T409), *M. oryzae* (Guy11), *Cochliobolus heterostrophus* (C5), *Colletotrichum graminicola* (T438), *Gloeocerospora sorghi* (T442), *Gaeumannomyces graminis* var. *tritici*, *Neurospora crassa* (74-OR8-1a), and *Aspergillus nidulans* (A4).

Cloning of *NhABC1*.

An overnight culture of Tr18.5, a PDA⁻ transformant of isolate 77-13-7 (Wasmann and VanEtten 1996), was grown in 100 ml of PDB under the same conditions as above and was treated with a solution of (+) pisatin in dimethyl sulfoxide (DMSO) to give a final concentration of 180 μ g of (+) pisatin per milliliter and 1.5% DMSO. The culture was incubated under the same growth conditions for 60 min, and the mycelium was collected by filtration on Whatman #4 filter paper (Whatman International Ltd., Maidstone, U.K.). Total RNA was extracted using Trizol (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.), and cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen Life Technologies) and oligo dT primer (Invitrogen Life Technologies), following the manufacturer's protocols. The cDNA was used for RT-PCR with degenerate primers designed for conserved regions of ABC transporters GAGKTT-F (5'-GGI GCN GGN AAR ACN AC-3') and TIHQPR-R (5'-GGY TGR TGD ATN GT-3'), with an annealing temperature of 48°C.

The resulting approximately 500-bp PCR product was excised from the gel and was cloned into pCR2.1, using the TA cloning kit (Invitrogen Life Technologies). The cloned 500-bp PCR fragment of *NhABC1* was used as a probe to screen a genomic cosmid library of isolate 77-13-7, and cosmid 70-F6 was shown to contain the gene of interest. A 4-kb *EcoRI/HindIII* fragment containing the 3' end of the gene was subcloned into pBluescript (Stratagene, Cedar Creek, TX, U.S.A.) and was sequenced. The remaining portion of *NhABC1* was sequenced directly from the cosmid.

Construction of disruption vector and transformation.

The approximately 2.3-kb region upstream of the coding region *NhABC1* in cosmid 70-F6 was amplified by PCR, using primers containing unique *XhoI* and *HindIII* sites (5'-AAC tcg agA AGT ACG TCC AAG CGC CAT C-3' and 5'-TTa agc ttT TTG ATC AAG ACA GAT GCT GTC C-3') and an annealing temperature of 58°C. The resulting PCR product was cloned into pBluescript that had the *SalI* site removed. The 4-kb *EcoRI/HindIII* fragment from cosmid 70-F6 was then cloned into the vector and the hygromycin-resistance cassette from pCWHyg1 (Wasmann and VanEtten 1996) was cloned into the *HindIII* site to generate p $\Delta NhABC1$.

Preparation of protoplasts was carried out as follows. Spores (10^5 ml⁻¹) were inoculated into 100 ml of PDB in a 250-ml Erlenmeyer flask and were incubated at room temperature for 16 to 18 h on a rotary shaker at 180 rpm. The mycelium was collected on Whatman #4 filter paper by vacuum filtration, and 1 g was suspended in 15 ml of 0.7 M NaCl with 6 mg of Kitase per milliliter (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 5 mg of Driselase (InterSpex Products, Inc., San Mateo, CA, U.S.A.) per milliliter. The mycelia-enzyme suspension was incubated at room temperature for 2 h on a rotary shaker at 50 rpm, and the resulting protoplasts were filtered through Nitex Nylon mesh (40 μ m, TETCO No. 3-40/26)

and were collected by centrifugation at $1,000 \times g$ for 10 min. The protoplasts were washed once in 10 ml of 0.7 M NaCl and once in 10 ml of SuTC (20% sucrose, 50 mM Tris, pH 7, 50 mM CaCl_2) and were suspended in SuTC at a final concentration of 10^7 protoplasts per milliliter.

Transformation was carried out as follows. Protoplast suspension (100 μl) was mixed with 12 μg of transforming DNA (p ΔNhABC1) and was incubated on ice for 15 min. PSTC (1 ml; 60% polyethylene glycol, molecular weight 3,350, 20% sucrose, 50 mM Tris, pH 7, 50 mM CaCl_2) was added to the protoplasts, which were mixed gently and were incubated at room temperature for 20 min. TB3 (3 ml; 20% sucrose, 1% glucose, 0.3% yeast extract, 0.3% casamino acids) were added, and the protoplasts were allowed to regenerate by gentle rocking on a platform shaker at room temperature for 18 to 20 h. The regenerated mycelium was collected by centrifugation at $3,000 \times g$, was suspended in 100 μl of SuTC, was mixed with 8 ml of regeneration top agarose (20% sucrose, 1% glucose, 1% yeast extract, 1% casamino acids, 1.5% agarose), and was poured into petri plates containing 20 ml potato dextrose agar with 60 $\mu\text{g}/\text{ml}$ hygromycin B (Calbiochem, La Jolla, CA, U.S.A.). Hygromycin-resistant colonies were visible after 4 to 7 days and were transferred to petri plates containing M-100 medium (Stevens 1974) with 60 μg of hygromycin per milliliter.

Hygromycin-resistant isolates were screened for disruption of *NhABC1* by Southern blotting. DNA was extracted as previously described (Miao et al. 1991b) and was digested with *ClaI*. The DNA was transferred to Hybond TM-N+ membrane (Amersham Pharmacia Biotech, Ltd, Buckinghamshire, U.K.) and was hybridized with a ^{32}P -labeled 668-bp *XbaI/SpeI* fragment upstream of *NhABC1*. The membrane was washed at 65°C in a series of solutions: once in $2\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS) for 20 min (low stringency), twice in $1\times$ SSC, 0.1% SDS for 20 min (medium stringency), and twice in $0.1\times$ SSC, 0.1% SDS for 20 min (high stringency). The *NhABC1* mutant (Tr8.1) was identified by the smaller size of the hybridizing DNA fragment and an absence of the corresponding wild type-size hybridizing fragment.

Crosses to generate a double mutant.

Crosses of *N. haematococca* isolates were conducted as described by Kistler and VanEtten (1984) to yield isolates with mutations in both *PDA1* and *NhABC1*. Since the transformants with disrupted genes (Tr8.1, ΔNhABC1 ; Tr18.5, ΔPDA1) were made using the same isolate (77-13-7, mat1) and this fungus is heterothallic, it was necessary to perform a cross that produced an isolate with a disrupted gene that had the opposite mating type from the initial transformants. Isolate 77-15-7 (mat2) was crossed with Tr8.1 to yield isolate 374-14, which had ΔNhABC1 and was mat2. Isolate 374-14 was crossed with Tr18.5 (ΔPDA1) to yield isolates beginning with the number 375. Tr18.5 had the disrupted *PDA1* gene but retained the CD chromosome with the *PEP* genes and the genes for homoserine utilization (Rodriguez-Carres et al. 2008). Thus, the presence of the CD chromosomes in PDA ascospores was determined by scoring for the ability to use homoserine as a carbon source. Isolates 375-1, 375-9, 375-13, and 375-19 were all $\Delta\text{NhABC1}:\Delta\text{PDA1}$ and carried the CD chromosome. These double mutants displayed similar phenotypes in the pea virulence assay (Table 2) and the radial growth pisatin-tolerance assay (Table 1; Fig. 2; data not shown), therefore limiting the possibility that the observed phenotypes (i.e., reduced virulence and enhanced pisatin sensitivity) are due to an unknown recombination event during meiosis. Since all isolates were similar, only isolate 375-13 was used in subsequent characterization assays.

Distribution studies of *NhABC1*.

Genomic DNA of fungi was extracted as described above, and 4 μg were digested with *EcoRI*. The DNA was transferred to Hybond TM-N+ membrane (Amersham Pharmacia Biotech, Ltd). Southern hybridizations were conducted using the approximately 500-bp PCR fragment of *NhABC1* described above, and washes were carried out under high stringency conditions as described above.

In vitro expression studies.

Isolate 77-13-7 was grown overnight on a rotary shaker in PDB as described above and (+) pisatin (in DMSO) was added to a final concentration of 90 $\mu\text{g}/\text{ml}$ and 1% DMSO. Over the next 2 h, 15-ml aliquots were removed at various timepoints, and the mycelium was collected by vacuum filtration and was lyophilized and ground to a powder with a mortar and pestle, using liquid nitrogen. Total RNA was extracted from the ground mycelium as described in Mandel and associates (2006) and was suspended in water. Contaminating genomic DNA was removed using DNase (GenHunter, Nashville, TN, U.S.A.), following the manufacturer's protocol. cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen Life Technologies) and oligo-dT primer (Invitrogen Life Technologies), following the manufacturer's protocol.

Quantitative PCR was carried out to measure the induction of *NhABC1* by pisatin in vitro. Transcripts of the β -actin housekeeping gene were used to normalize the level of *N. haematococca* transcripts. Primers were based on the 3' end sequences of the genes of interest. The β -actin sequence was amplified with the primers Actin-F (5'-GTG TGA TGT CGA TGT CCG AAA GG-3') and Actin-R (5'-GAA GAA GGA GCA AGA GCA GTA ATC-3') and *NhABC1* with the primers NhABC1-F (5'-CTC CAG CTA CGA CAA CCG AT-3') and NhABC1-R (5'-TGT CTA CGA CTG CTT CTG CTT CT-3').

Each reaction had a total volume of 25 μl containing 12.5 μl of Platinum quantitative PCR supermix-UDG (containing Platinum *Taq* DNA polymerase, Tris-HCl, KCl, 6 mM MgCl_2 , 400 μM dGTP, 400 μM dATP, 400 μM dCTP, 800 μM dUTP, and UDG [uracil DNA glycosylase] [Invitrogen Life Technologies] and stabilizers), 250 nM forward primer, 250 nM reverse primer, 500 nM Rhodamine X reference dye, 2 μl cDNA template, and 9 μl of sterile distilled water. The PCR parameters were an initial uracil-N-glycosylase step at 50°C for 2 min, followed by an initial denaturation and Platinum *Taq* DNA polymerase activation step at 95°C for 10 min and 40 cycles at 95°C for 15 s and 60°C for 30 s. Real-time PCR for each gene was performed at least three times. An ABI PRISM 7300 sequence detection system (Applied Biosystems, Foster City, CA, U.S.A.) was used, and the data were compared using the algorithm $x = 2^{-\Delta\Delta\text{Ct}}$ as previously described (Livak and Schmittgen 2001).

Expression of *N. haematococca* genes in infected pea pods.

Expression studies on *N. haematococca* MPVI *PDA1* and *NhABC1* were performed as described by Wagoner and associates (1982). Spores of 77-13-4 were obtained from 1- to 2-week-old V8 juice agar medium, were rinsed with sterile water, and were suspended in sterile water at a concentration of 5×10^6 spores per milliliter. Immature pea pods (Sugar Snap peas; Mann Packing Co. Inc., Salinas, CA, U.S.A.) of uniform size (approximately 4 to 5 cm long) were surface-sterilized in 70% ethanol for 5 min in a 2.5% sodium hypochlorite solution for 10 min. and were then rinsed thoroughly with sterile distilled water. Pea pods then were halved, deseeded, and placed into 60-mm diameter petri plates. The freshly exposed endocarp tissue was inoculated with 1 ml of spore suspension and was incubated at 24°C . Challenged pea endocarp tissue was re-

moved at 0, 1, 2, 3, 9, 12, 24, 36, and 48 h postinoculation by scraping off the surface layers using a metal spatula, was immediately immersed in liquid nitrogen, and was frozen at -80°C .

Total RNA was extracted using Trizol (Invitrogen Life Technologies) and was treated with DNase (GenHunter), and cDNA was synthesized as above, using Superscript II reverse transcriptase and oligo dT primer. Quantitative real-time PCR was used to measure the induction of *NhABC1* and *PDA1* in infected pea tissue, as mentioned above, with the following exceptions. *NhABC1* transcripts were amplified with the primers ABC77134-F (5'-ATC CTG TTG GCT TCC ACA AG-3') and ABC77134-R (5'-AAC TGC CAG AAC AGA AGC CA-3'). *PDA1* transcripts were amplified with the primers PDA1F (5'-CCC GAG GGT ACT GAG GTT G-3') and PDA1R (5'-GAT GCT GAT GTT CTT GCC-3') (Liu et al. 2003). *Erg* gene transcripts, used to normalize the level of *N. haematococca* transcripts, were amplified with the primers Erg1F (5'-CAG TAC AAG ATG CCC AGA GCT-3') and Erg1R (5'-TGT GCC ACA GAG TAG GAA AGT C-3').

Analysis of pisatin production in infected pea pods.

Pea-pod inoculation with *N. haematococca* isolate 77-13-4 and tissue harvesting was performed as described above. Pisatin was extracted from the tissue adjacent to the site of infection by immersing the tissue in hexanes (10 ml/g) and incubating the immersed pods on a gyratory shaker (130 rpm) at 24°C for 16 h. The tissue was removed, the hexanes were evaporated, and the residue was dissolved in 1 ml of ethanol, and pisatin was quantified as previously described (Wu and VanEtten 2004).

Pathogenicity assays.

The pea "test tube" lesion and carrot pathogenicity assays were conducted as previously described (Funnell and VanEtten 2002; Wasmann and VanEtten 1996). *Pisum sativum* cv. Alaska 2B (Carolina Biological Supply Co., Burlington, NC, U.S.A.), and organically grown carrots were used for the virulence assays.

Spore germination assay.

Macroconidia from 4-day-old V-8 juice agar medium were added to 500 μl of PDB in an Eppendorf tube to a final concentration of 10^5 spores per milliliter. Pisatin was added to a final concentration of 180 $\mu\text{g/ml}$ in 1% DMSO, and cultures were incubated at room temperature on a rotary shaker at 120 rpm. Samples were taken for microscopy, and spore germination was calculated by measuring the percentage of spores (out of at least 100) that had germinated. A spore was considered germinated if the germ-tube length was larger than the diameter of the spore (VanEtten and Stein 1978).

Antimicrobial growth assays.

Radial growth on Martin's agar medium (Stevens 1974) containing (+) pisatin or other compounds was used to measure the sensitivity to these compounds of the wild type and mutants of *N. haematococca*. The protocol used was as previously described (Delserone et al. 1999), except that one-week-old cultures were used for the inoculum and three replications were used for each treatment. Sensitivity to the different compounds is reported as percent inhibition of radial growth on a medium containing the compounds compared with the control treatment when the growth reaches the edge of the plate. The following compounds (at the concentrations shown) were dissolved in DMSO, except when H_2O is indicated, to give a final DMSO concentration of 1%: amphotericin B (35 $\mu\text{g/ml}$; Sigma, St. Louis), anoyimidol (EL-531, 20 $\mu\text{g/ml}$; Eli Lilly,

Indianapolis, IN, U.S.A.), benomyl (20 $\mu\text{g/ml}$; DuPont, Wilmington, DE, U.S.A.), benzimidazole (20 $\mu\text{g/ml}$; Sigma), benzoic acid (20 $\mu\text{g/ml}$; Sigma), berberine chloride (40 $\mu\text{g/ml}$; Sigma), bialaphos (10 $\mu\text{g/ml}$; Sigma), brefeldin A (10 $\mu\text{g/ml}$; Sigma), carbenacillin (200 $\mu\text{g/ml}$; Sigma), cefotoxin (350 $\mu\text{g/ml}$; Sigma), α -chaconine (20 $\mu\text{g/ml}$; Sigma), chloramphenicol (150 $\mu\text{g/ml}$; Sigma), coumestrol (20 $\mu\text{g/ml}$), CuSO_4 (50 $\mu\text{g/ml}$, in H_2O ; Sigma), cyclohexamide (30 $\mu\text{g/ml}$; Sigma), doxorubicin (15 $\mu\text{g/ml}$; Sigma), etaconazole (20 $\mu\text{g/ml}$), ethidium bromide (5 $\mu\text{g/ml}$, in H_2O ; Sigma), fenarimol (EL-222, 25 $\mu\text{g/ml}$; Eli Lilly), 5-fluorouracil (10 $\mu\text{g/ml}$; Sigma), gossypol (20 $\mu\text{g/ml}$), 8-hydroxyquinoline (100 $\mu\text{g/ml}$; Sigma), imazalil (5 $\mu\text{g/ml}$), ketoconazole (50 $\mu\text{g/ml}$; Sigma), kievitone (20 $\mu\text{g/ml}$), miconazole (30 $\mu\text{g/ml}$; Sigma), nuarimol (EL-228, 20 $\mu\text{g/ml}$; Eli Lilly), nystatin (20 $\mu\text{g/ml}$; Sigma), ononin (20 $\mu\text{g/ml}$, Indofine, Hillsborough, NJ, U.S.A.), phaseollin (20 $\mu\text{g/ml}$), (+) pisatin (90 and 180 $\mu\text{g/ml}$), (–) pisatin (50 $\mu\text{g/ml}$), propiconazole (20 $\mu\text{g/ml}$; Sigma), resveratrol (200 $\mu\text{g/ml}$; Sigma), rhodamine 6G (10 $\mu\text{g/ml}$; Sigma), rishitin (200 $\mu\text{g/ml}$; a gift from S. P. McCormick, United States Department of Agriculture, Peoria, IL, U.S.A.), safrole (1 mg/ml; Sigma), sesamol (50 $\mu\text{g/ml}$; Sigma), sodium azide (30 $\mu\text{g/ml}$, in H_2O ; Sigma), sodium cyanide (30 $\mu\text{g/ml}$, in H_2O ; Sigma), α -solanine (20 $\mu\text{g/ml}$; Sigma), (+) taxifolin (20 $\mu\text{g/ml}$; Sigma), tomatine (20 $\mu\text{g/ml}$; Spectrum, Gardena, CA, U.S.A.), triarimol (EL-273, 20 $\mu\text{g/ml}$; Eli Lilly), and (+) verapamil (25 $\mu\text{g/ml}$; Sigma). Several phytoalexins (coumestrol, gossypol, kievitone, phaseollin, (+) pisatin, and (–) pisatin) were isolated as previously described (George and VanEtten 2001) or were from laboratory stocks, unless noted otherwise. Antimicrobial growth assays were performed once, except for (+) pisatin, which was assayed three times at the 90- $\mu\text{g/ml}$ concentration and twice at the 180- $\mu\text{g/ml}$ concentration. Except for (+) pisatin, (–) pisatin, and rishitin, all the other compounds did not inhibit the radial growth of Tr8.1 (ΔNhABC1) more than 10%, as compared with the wild type.

Phylogenetic analysis of NhABC1 and similar proteins.

The predicted amino acid sequences of other fungal ABC transporters with high sequence similarity to NhABC1 were identified by BLASTp searches to the fungal genomes available from National Center for Biotechnology Information and the Fungal Genome Initiative of the Broad Institute. Sequences were aligned and manually edited using ClustalW 1.81 and MacClade 4.08 as previously described (Coleman et al. 2009). Phylogenetic analyses for maximum parsimony and maximum likelihood were conducted employing PAUP* 4.0 (Swafford 1999).

ACKNOWLEDGMENTS

We thank S. P. McCormick for the generous gift of rishitin and R. Celoy for quantification of extracted pisatin. J. J. Coleman would especially like to thank E. Temporini and C. Wasmann for experimental advice. Funding was provided by the National Science Foundation (grant number MCB-0641808 to H. D. VanEtten).

LITERATURE CITED

- Coleman, J. J., and Mylonakis E. 2009. Efflux in fungi: La pièce de résistance. *PLoS Pathog.* 5: e1000486. Published online.
- Coleman, J. J., Rounsley, S. D., Rodriguez-Carres, M., Kuo, A., Wasmann, C. C., Grimwood, J., Schmutz, J., Taga, M., White, G. J., Zhou, S., Schwartz, D. C., Freitag, M., Ma, L.-J., Danchin, E. G., Henrissat, B., Coutinho, P. M., Nelson, D. R., Straney, D., Napoli, C. A., Barker, B. M., Gribskov, M., Rep, M., Kroken, S., Molnár, I., Rensing, C., Kennell, J. C., Zamora, J., Farman, M. L., Selker, E. U., Salamov, A., Shapiro, H., Pangilinan, J., Lindquist, E., Lamers, C., Grigoriev, I., Geiser, D. M., Covert, S. F., Temporini, E., and VanEtten, H. D. 2009. The genome

- of *Nectria haematococca*: Contribution of supernumerary chromosomes to gene expansion. *PLoS Genet.* 5:e1000618. Published online.
- Covert, S. F. 1998. Supernumerary chromosomes in filamentous fungi. *Curr. Genet.* 33:311-319.
- Cruickshank, I. A. M. 1962. Studies on phytoalexins. IV. The antimicrobial spectrum of pisatin. *Aust. J. Biol. Sci.* 15:147-159.
- Cruickshank, I. A. M. 1965. Phytoalexins in the leguminosae with special reference to their selective toxicity. *Tagungsber. Dtsch. Akad. Landwirtschaftswiss Berlin* 74:313-332.
- Cruickshank, I. A. M., and Perrin, D. R. 1960. Isolation of a phytoalexin from *Pisum-sativum* L. *Nature* 187:799-800.
- Cruickshank, I. A. M., and Perrin, D. R. 1965. Studies on phytoalexins. VIII. The effect of some further factors on the formation stability and localization of pisatin *in vivo*. *Aust. J. Biol. Sci.* 18:817-828.
- 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.
- Denny, T. P., and VanEtten, H. D. 1981. Tolerance by *Nectria haematococca* MPVI of the chickpea (*Cicer arietinum*) phytoalexins medicarpin and maackiain. *Physiol. Plant Pathol.* 19:419-437.
- Denny, T. P., and VanEtten, H. D. 1983a. Tolerance of *Nectria haematococca* MP VI to the phytoalexin pisatin in the absence of detoxification. *J. Gen. Microbiol.* 129:2893-2901.
- Denny, T. P., and VanEtten, H. D. 1983b. Characterization of an inducible, nondegradative tolerance of *Nectria haematococca* MP VI to phytoalexins. *J. Gen. Microbiol.* 129:2903-2913.
- Denny, T. P., Matthews, P. S., and VanEtten, H. D. 1987. A possible mechanism of nondegradative tolerance of pisatin in *Nectria haematococca* MPVI. *Physiol. Mol. Plant Pathol.* 30:93-107.
- Desjardins, A. E., and Gardner, H. W. 1989. Genetic analysis in *Gibberella pulicaris*: Rishitin tolerance, rishitin metabolism, and virulence on potato tubers. *Mol. Plant-Microbe Interact.* 2:26-34.
- Fleissner, A., Sopalla, C., and Weltring, K. M. 2002. An ATP-binding cassette multidrug-resistance transporter is necessary for tolerance of *Gibberella pulicaris* to phytoalexins and virulence on potato tubers. *Mol. Plant-Microbe Interact.* 15:102-108.
- 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.
- 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.
- Han, Y., Liu, X. G., 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.
- Higgins, C. F. 2007. Multiple molecular mechanisms for multidrug resistance transporters. *Nature* 446:749-757.
- Kistler, H. C., and VanEtten, H. D. 1984. Three non-allelic genes for pisatin demethylation in the fungus *Nectria haematococca*. *J. Gen. Microbiol.* 130:2595-2603.
- Kovalchuk, A., and Driessen, A. J. M. 2010. Phylogenetic analysis of fungal ABC transporters. *BMC Genomics* 11:177.
- 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 $2^{-\Delta\Delta Ct}$ method. *Methods* 25:402-408.
- Mandel, M. A., Galgiani, J. N., Kroken, S., and Orbach, M. J. 2006. *Coccidioides posadasii* contains single chitin synthase genes corresponding to classes I to VII. *Fungal Genet. Biol.* 43:775-788.
- Miao, V. P. W., Covert, S. F., and VanEtten, H. D. 1991a. A fungal gene for antibiotic resistance is on a dispensable ("B") chromosome. *Science* 254:1773-1776.
- Miao, V. P. W., Matthews, D. E., and VanEtten, H. D. 1991b. 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.
- 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.
- Müller, K. O., and Börger, H. 1940. Experimentelle Untersuchungen über die *Phytophthora-Kesistenz* der Kartoffel. Zugleich ein Beitrag zum Problem der "erworbenen Resistenz" im Pflanzenreich. *Arbeiten Biol. Reichsanstalt Land und Forstwirtschaft* 23:189-231.
- Pedras, M. S. C., and Ahiachoun, P. W. K. 2005. Metabolism and detoxification of phytoalexins and analogs by phytopathogenic fungi. *Phytochemistry* 66:391-411.
- Pezet, R., Pont, V., and Hoang-Van, K. 1991. Evidence for oxidative detoxification of pterostilbene and resveratrol by a laccase-like stilbene oxidase produced by *Botrytis cinerea*. *Physiol. Mol. Plant Pathol.* 39:441-450.
- Rissler, J. F., and Miller, R. L. 1977. Contribution of a cyanide-insensitive alternative respiratory system to increases in formamide hydro-lyase activity and to growth in *Stemphylium loti in vitro*. *Plant Physiol.* 60:857-861.
- Rodriguez-Carres, M., White, G., Tsuchiya, D., Taga, M., and VanEtten, H. D. 2008. The supernumerary chromosome of *Nectria haematococca* that carries pea-pathogenicity-related genes also carries a trait for pea rhizosphere competitiveness. *Appl. Environ. Microbiol.* 74:3849-3856.
- Sanwal, H., Panwar, S. L., and Prasad, R. 2009. ATP-binding cassette (ABC) transporters in yeasts: Their role in multidrug resistance and survival. Pages 149-179 in: *ABC Transporters in Microorganisms: Research innovation and value as targets against drug resistance*. A. Ponte-Sucre, ed. Academic Press, Norfolk, U.K.
- Schoonbeek, H., Del Sorbo, G., and De Waard, M. A. 2001. The ABC transporter BcatrB affects the sensitivity of *Botrytis cinerea* to the phytoalexin resveratrol and the fungicide fenpiclonil. *Mol. Plant-Microbe Interact.* 14:562-571.
- Stevens, R. B. 1974. *Mycology Guidebook*. University of Washington Press, Seattle, WA, U.S.A.
- Swafford, D. L. 1999. *PAUP* Phylogenetic Analysis Using Parsimony (*and other methods)*. 4th ed. Sinauer Associates, Inc., Sunderland, MA, U.S.A.
- Uehara, K. 1964. Relationship between host specificity of pathogens and phytoalexin. *Ann. Phytopathol. Soc. Jpn.* 29:103-110.
- Urban, M., Bhargava, T., and Hamer, J. E. 1999. An ATP-driven efflux pump is a novel pathogenicity factor in rice blast disease. *EMBO (Eur. Mol. Biol. Organ.) J.* 18:512-521.
- VanEtten, H. D., and Barz, W. 1981. Expression of pisatin demethylating ability in *Nectria haematococca*. *Arch. Microbiol.* 129:56-60.
- VanEtten, H. D., and Stein, J. I. 1978. Differential response of *Fusarium solani* isolates to pisatin and phaseollin. *Phytopathol.* 68:1276-1283.
- 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.* 16:257-268.
- VanEtten, H., 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.
- Wagoner, W., Loschke, D. C., and Hadwiger, L. A. 1982. Two-dimensional electrophoretic analysis of *in vivo* and *in vitro* synthesis of proteins in peas inoculated with compatible and incompatible *Fusarium solani*. *Physiol. Plant Pathol.* 20:99-107.
- 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.
- Wu, Q., and VanEtten, H. D. 2004. Introduction of plant and fungal genes into pea (*Pisum sativum* L.) hairy roots reduces their ability to produce pisatin and affects their response to a fungal pathogen. *Mol. Plant-Microbe Interact.* 17:798-804.