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## Heterologous expression of a pleiotropic drug resistance transporter from *Phytophthora sojae* in yeast transporter mutants

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**Abstract** A system for the expression of an ATP binding cassette (ABC) transporter from the soybean pathogen *Phytophthora sojae* is described. *Pdr1*, an ABC transporter with homology to the pleiotropic drug resistance (PDR) family of transporters, was cloned by primer walking from a *P. sojae* genomic library. Reverse transcriptase PCR assays showed that the transcript disappeared after encystment of zoospores and was not detected in hyphal germlings in dilute salts, in hyphae growing in liquid V8 media, or in tissue extracts from infected hypocotyls. BLAST analysis of *Pdr1* against the *P. sojae* EST database also revealed that this gene was present only in zoospore libraries. Comparison of the number of hits to *Pdr1* with that of a set of house-keeping genes revealed that *Pdr1* was expressed at rates two- to threefold higher than other transcripts. To test the hypothesis that Pdr1p functions as a broad substrate membrane transporter, *Pdr1* was transformed into yeast mutants deficient in several drug resistance transporters. Yeast mutants transformed with *Pdr1* possessed partial drug resistance against only 5 of 17 chemically distinct compounds. Thus, when expressed in yeast, this trans-

porter has a significantly narrower substrate specificity in comparison to the yeast transporters, Pdr5p, Yorlp, and Snq2p.

**Keywords** ABC transporter · Zoospores · Oomycetes

### Introduction

*Phytophthora* species are oomycetes grouped with other heterokonts (having two different flagella) in the Kingdom Stramenopila (Van de Peer and De Wachter 1997; Baldauf 2003). Oomycetes grow in the soil and infect plants as hyphae, but are distinct from fungi in having diploid nuclei and coenocytic cells with cellulose rather than chitinaceous cell walls (Erwin and Ribeiro 1996). Oomycete pathogens attack a wide variety of plant species, some causing significant economic damage to multiple agricultural species, while others have a narrow host range (Erwin and Ribeiro 1996). *Phytophthora sojae* is a soil-borne pathogen of soybeans that has a significant economic impact in the United States (Wrather et al. 2001; Dorrance et al. 2003) and Canada (Anderson and Buzzell 1992).

Under flooding conditions, vegetative hyphae produce vertical branches with terminal sporangia. Each sporangia release 20–30 single-celled asexual zoospores. In agricultural fields, the zoospore is a particularly effective dispersal agent (Carlile 1983). Zoospores use two flagella to swim at 125–150  $\mu\text{m s}^{-1}$  (Ho and Hickman 1967) through the surface films of water in the soil, and are rapidly dispersed by water movements in soils (Carlile 1983). Both the zoospores and hyphae are chemotactically attracted by isoflavones released from soybean roots (Morris and Ward 1992; Morris et al. 1998). Upon reaching the root the zoospores encyst, a developmental process that results in the loss of flagella and formation of a cell wall (Carlile 1983). The cyst germinates and hyphae ramify through the host tissues. When the nutrients are exhausted, the hyphae form sexual gametangia. The fertilized oospore has a thick cell wall and enables the pathogen to remain dormant in the soil overwinter (Erwin and Ribeiro 1996).

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Plants, animals, fungi, *Dictyostelium* and oomycetes have in common many protein families (Chothia et al. 2003), one of the largest being the ATP-binding cassette (ABC) membrane transporters (Bauer et al. 1999; Dassa and Bouige 2001; Sanchez-Fernández et al. 2001; Anjard and Loomis 2002; van den Brûle and Smart 2002). These include proteins involved in the efflux of a wide range of substances (Kolaczowski et al. 1998). Interest in eukaryotic ABC transporters came as a consequence of the recognition of their role in contributing to multidrug resistance against chemotherapeutic agents (Gros et al. 1986). Examples of multidrug resistance mediated by ABC transporters have since been documented for bacterial, fungal and protozoan pathogens of humans (Litman et al. 2001; Morschäuser 2002; Klokouzas et al. 2003; Davidson and Chen 2004). These proteins are characterized by having a nucleotide binding domain (NBD) and a transmembrane region (TM). Members of the pleiotropic drug resistance (PDR) family are described as full-size transporters and typically have a (NBD-TM)<sub>2</sub> configuration. Full-size transporters are believed to function in vivo as dimers (Ferreira-Pereira et al. 2003). PDR transporters have been described in yeast (Bauer et al. 1999; Decottignies et al. 2002) and other fungi (Del Sorbo et al. 2000), *Dictyostelium* (Anjard and Loomis 2002), and plants (Sanchez-Fernández et al. 2001; van den Brûle and Smart 2002). In yeast, the PDR transporters Pdr5p and Snq2p are capable of effluxing a wide variety of structurally unrelated compounds (Kolaczowski et al. 1998; Decottignies et al. 2002). Functional analysis of yeast transporters has also revealed that these transporters have overlapping but distinct substrate specificities. Furthermore, deletions of one or more of these transporters result in a viable but hypersensitive phenotype to a large number of drugs.

In fungal–plant interactions, ABC transporters can function to protect the fungus from fungicides as well as a wide variety of plant-derived toxins (Del Sorbo et al. 1997, 2000; Nakune et al. 1998; Hayashi et al. 2001; Fleissner et al. 2002). During the infection process, some transporters may be involved in providing protection against plant defense compounds while others could be involved in the delivery of pathogenicity factors (Urban et al. 1999; Stergiopoulos et al. 2002). Correspondingly, some of the 15 plant PDR transporters (Van den Brûle and Smart 2002) are likely committed to protecting plant issues from fungal or bacterial toxins (Jasinski et al. 2001). The soil, and particularly the rhizosphere, is a very competitive environment with limited resources (Hirsch et al. 2003). Here too, ABC transporters function to protect cells from exogenous toxins (Hayashi et al. 2001; Schoonbeck et al. 2002; Zwier et al. 2003). In the fungal pathogen, *Mycosphaerella graminicola*, the ABC transporter MgATR2p plays a key role in protecting the hyphae from xenobiotics. Strains carrying knockouts of this gene displayed increased sensitivity to metabolites of *Pseudomonas fluorescens* and *Burkholderia cepacia*. In *Botrytis cinerea* the ABC transporter

BcatrBp protects hyphae from several broad spectrum antibiotics produced by *Pseudomonas* sp. (Stergiopoulos et al. 2002).

We hypothesized that constitutively expressed ABC transporters might play an important role in defensive strategies of swimming zoospores since their rapid movement relative to body size may result in their exposure to lethal concentrations of xenobiotics of bacterial (Yan et al. 2002) or plant origin (Deacon and Mitchell 1985; Islam et al. 2004). In the present work, we describe the sequencing, expression, and initial characterization of *Pdr1*, the first PDR transporter of an oomycete to be characterized. The expression of this transporter is restricted to zoospores, the primary dispersal agent for *P. sojae* in soybean fields. Data analysis of an extensive EST database suggests that this transporter has the highest expression level of all ABC transporters in *P. sojae*. Because of the availability of yeast knockout transporter mutants, we elected to use heterologous expression of the *P. sojae* transporter in yeast mutants to assess the substrate capabilities of the transporter. Since specific substrate for a zoospore-expressed transporter have not been identified, we elected to test a wide range of chemically distinct toxins that included some antibiotics produced by soil bacteria. Drug assays of yeast transformants revealed that *Pdr1*-protected cells from exogenous cycloheximide, ethidium bromide, 8-hydroxyquinoline, 4-nitroquinoline (4-NQ), and tetracycline but had no effect against several other cytotoxins. The limited substrate specificity of this transporter would suggest that its primary role is not protection against a broad range of exogenous xenobiotics.

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## Materials and methods

### Strains, media and general methods

Yeast strains YPH500 *MAT $\alpha$  ura3-52 his3- $\Delta$  200 leu2 $\Delta$ 1 trp1 $\Delta$ 63 lys2-801<sup>amb</sup> ade2-101<sup>oc</sup> YYM3 *MAT $\alpha$  ura3-52 his3- $\Delta$ 200leu2 $\Delta$ 1trp1 $\Delta$ 63 lys2-801<sup>amb</sup>ade2-101<sup>oc</sup>  $\Delta$ pdr5::TRP1  $\Delta$ snq2::hisG; AD123456789 *MAT $\alpha$ PDR1-3, ura3,  $\Delta$ yor1::hisG,  $\Delta$ snq2::hisG, pdr5- $\Delta$ 2::hisG,  $\Delta$ pdr10::hisG,  $\Delta$ pdr11::hisG,  $\Delta$ yef1::hisG, pdr3- $\Delta$ 2,  $\Delta$ pdr15::hisG, pdr1- $\Delta$ 3::his G* (Decottignies et al. 1998) and the plasmid pSK-PDR5PPUS (Nakamura et al. 2001) were a generous gift of A. Decottignies and A. Goffeau, Louvaine-la-Neuve, Belgium. Yeast strain YYM3-Pdr1 was constructed by the insertion of a *Hind*III–*Eco*R1 fragment containing *Pdr1* into pYes2. AD123456789-Pdr1 was constructed by the insertion of a *Hind*III–*Eco*R1 fragment containing *Pdr1* into pSK-PDR5PPUS. All strains were maintained on YPD (2% peptone, 1% yeast extract, 2% glucose plates). Strains that were produced with the pYes2 plasmid were identified by selection on complete synthetic media (CSM) plates supplemented with 2% galactose without uracil. Strains that were constructed with**

pSKPDR5PPUS were selected on CSM plates lacking uracil supplemented with 2% glucose.

*Phytophthora sojae* isolate P 6497 was grown on V8 juice agar and zoospores ( $200,000 \text{ ml}^{-1}$ ) were produced by repeated washing of V8 agar plates (Morris et al. 1998). Zoospores were induced to encyst by vortexing cells in a microfuge tube for 30 s.

#### Isolation and characterization of nucleic acids

Hyphal plugs from V8 agar plates were transferred to liquid-clarified V8 media, and cultures were maintained at room temperature for 5 days. Liquid cultures were filtered and rinsed with sterile  $\text{H}_2\text{O}$ . Hyphal mats were transferred to 1.5-ml microcentrifuge tubes, frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ . Genomic DNA was isolated from hyphal mats using a yeast DNA extraction protocol as described (Ausubel et al. 2003). Restriction analysis and blotting were completed using standard protocols (Sambrook et al. 1989). DNA-modifying enzymes were obtained from Promega.

RNA was isolated from hyphal extracts,  $\sim 200,000$  swimming zoospores, hyphal germlings 30 min postencystment, and lesions from infected hypocotyls 48 h after inoculation using the acid-phenol method (Wilkins and Smart 1996). Reverse transcription of isolated RNA (Zhao et al. 1998) was performed using MMLV-RT and a modified poly T primer. One microliter of the cDNA reaction from swimming zoospores was used in a PCR reaction with Herculanase polymerase (Invitrogen) and the degenerate primers 5'-ATYGTSCATSATYCAGCCBWSSTTY and 5'-CCTCCTGCGAAGC(T)<sub>14</sub>. The amplified product was cloned into pCR4Blunt-TOPO (Invitrogen) and sequenced at the Plant-Microbe Genomics Facility (PMGF), Ohio State University, Columbus, OH, USA. The 1,184 bp PCR product was identified as a fragment of an ABC-type transporter by BLASTx analysis of the sequence against the GenBank database. This gene fragment was used as a probe in Southern blot analyses as described below. Probes for Southern analysis were labeled by random priming (Fenberg and Vogelstein 1983) using [ $\alpha$ -<sup>32</sup>P]-dCTP (Amersham). After incubation for 24 h at  $60^\circ\text{C}$ , the blot was washed (Sambrook et al. 1989) and analyzed using a STORM 860 phosphor imaging system (Molecular Dynamics).

#### Screening of *P. sojae* BAC genomic library

A *P. sojae* BAC genomic library comprised of ten nylon filters (constructed in a 7.4 kb pBeloBAC11 vector by Brett Tyler, Virginia Bioinformatics Institute) was probed with [ $\alpha$ -<sup>32</sup>P]-dCTP-labeled PCR product essentially as described above, except that the hybridization was carried out for 48 h at  $62^\circ\text{C}$ . All positive BAC clones were obtained from Dr. Tyler in the form of stab cultures. BAC minipreps of individual clones were subjected to Southern analysis by using the PCR product as

a probe. Restriction fragments from selected clones were cloned into pUC18 and transformed into *E. coli* TOP10 cells (Invitrogen). Complete sequencing of restriction fragments at the PMGF was achieved using a primer walking strategy.

#### RT-PCR expression analysis of *Pdr1*

For expression of *Pdr1*, the master mix included Taq polymerase (Promega), the gene specific primers ABC-3left (5'CCCGCTGCCGTACAGTCTGTGCG3') and ABC-3right (5'TTTTTTTTTTTTTGCAG-GACATCACGTCAAATGT3') and  $10\mu\text{g}$  cDNA as template. Gene-specific amplification of a ribosomal S24e type protein (Gene ID 109323 <http://phytophthora.vbi.vt.edu/>) was assessed using 40S-RIB<sup>LEFT</sup> (5'TTCGGTGGCGCAA GTCCTC-GG3') and 40S-RIB<sup>RIGHT</sup> (5'TTTTTTTTTT TTGGGAAACCCGCAAATGGGAACG3'), and the same volume of template was used for amplification of the *Pdr1* fragment. PCR reactions included an initial melt at  $95^\circ\text{C}$  for 5 min, 40 cycles of  $95^\circ\text{C}$  for 30 s,  $62^\circ\text{C}$  for 30 s,  $72^\circ\text{C}$  for 60 s, and final extension at  $72^\circ\text{C}$  for 7 min. PCR products were analyzed by gel electrophoresis using 2% agarose-TBE gels stained with ethidium bromide. To verify sequence identity, the PCR products were cloned into the pCR.1-TOPA TA plasmid (Invitrogen) and sequenced.

#### BLAST analysis of *Pdr1* and selected housekeeping genes

The full-length sequence of *Pdr1* was used in a BLAST analysis of all *P. sojae* library sequences of the *Phytophthora* Functional Genome database (PFGD, <http://www.pfgd.org/>). Manual inspection was used to determine whether closely matched sequences were due to sequencing errors or simply the expression of a closely related gene. The full-length sequences of the putative sequences of  $\alpha$  tubulin (ID 109111),  $\beta$  tubulin (ID 109498), 40S ribosomal protein (ID 109323), and elongation factor 3 (ID 134912) were retrieved from the *P. sojae* database (<http://phytophthora.vbi.vt.edu/>) and used in a BLAST analysis of the PFGD. Manual inspection of each "hit" was used to confirm that the ESTs were representative of each protein.

#### Heterologous expression of *Pdr1*

The full-length sequence of *Pdr1* and an additional 207 bp of sequence downstream of the stop codon were amplified directly from the BAC clone 38D22 using the forward primer 5'GCAAGCTTCACCATGGTCAGT3' and the reverse primer GGAATTCTGGCAGAAAGG CAC3' which introduce *Hind*III and *Eco*RI sites at the 5' and 3' end of the amplified product. Template DNA was amplified using ThermoAce Polymerase (Invitrogen) in a PCR reaction with 30 cycles of  $94^\circ\text{C}$  for 30 s,

62°C for 40 s, 72°C for 6 min. The 4,158 bp PCR product was digested with *Hind*III and *Eco*RI and cloned into the plasmid pYes2 (Invitrogen) and pSK-PDR5PPUS (Nakamura et al. 2001) at the *Hind*III–*Eco*RI sites. To enable expression of *Pdr1* under the control of the yeast *Pdr5* promoter, the *Pdr1* fragment including *Pdr5* promoter and *Pdr5* stop was amplified from pSK-PDR5PPUS-*Pdr1* plasmid using the forward primer 5'ATCGATGGTCCGTCATATACGGTTCTC C3' and the reverse primer 5'AAAGGGAACAAAAGC TGGAGCTCCAC3'. The purified PCR product was then used to transform the yeast mutants AD123456789 (Decottignies et al. 1998). This enabled homologous integration of *Pdr1* into the yeast chromosome under the control of the *Pdr5* promoter. The *Pdr1-3* mutation in this strain ensured that *Pdr1* was overexpressed (Decottignies et al. 1998). Yeast transformants (Gietz et al. 1992) were selected by growing the isolates on complete synthetic media (CSM) plates with 2% glucose supplemented with amino acids minus uracil. Putative transformants were screened for activity using drug dilution assays as described below.

#### Growth assays

Yeast strains YPH500, YYM3, and YYM3-Pdr1 were grown overnight in CSM supplemented with 2% galactose. Cell suspensions were diluted to a standardized OD<sub>600</sub> = 0.2 in fresh medium in the presence or absence of 1.2 μM 4-nitroquinoline-N-oxide. Growth curves were generated by measuring OD<sub>600</sub>.

#### Drug susceptibility assays

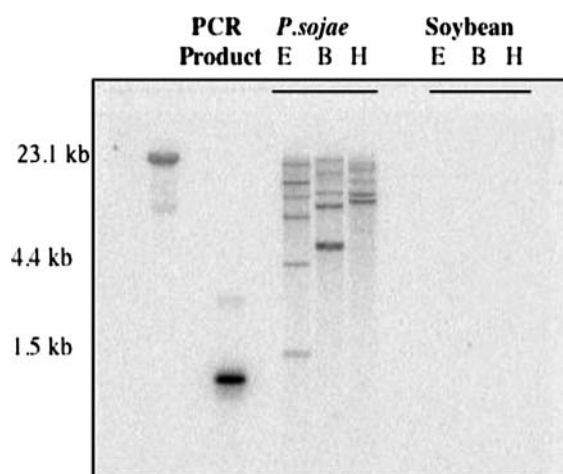
Drug susceptibility of yeast strains to different compounds was assessed on CSM media. The yeast strain AD123456789 had a distinct drug-sensitive phenotype relative to the YPH500 strain for all of the drugs tested in these assays (Kolaczowski et al. 1998; Decottignies et al. 2002). Agar plates containing different drugs at various concentrations were prepared by adding drugs from stock solutions to 5 ml of CSM equilibrated at 50°C before pouring into Petri plates. Exponentially growing cells were diluted to 0.2 A<sub>600</sub> and 3 μl of each yeast strain were plated as spots on gradient plates. Growth was monitored after incubation of the cells for 2 days at 30°C in the dark. The final concentration (μM) of the drug in the agar for each drug was as follows: 4-NQ: 0, 2, 4, 8, 16; tetracycline: 0, 240, 480, 960, 1,920; ethidium bromide: 0, 4, 8, 16, 32; cycloheximide: 0, 0.04, 0.08, 0.16, 0.32; 8-hydroxyquinoline: 0, 16, 20, 24, 28. Sources of compounds are abbreviated as follows: Aldrich (A), Eastman Kodak (E), Fisher (F), Sigma (S), and United States Biochemical (USB). The drugs benzoic acid (A), benomyl (A), chlorophenol red (A), daunorubicin (S), doxorubicin (S), 2,4-diphenoxyacetic acid (USB), miconazole nitrate (A), 4-NQ (A), quinidine (A), tetracycline (F), thiram

(A), and 2,3,5-triphenyltetrazolium chloride (F) were dissolved in dimethyl sulfoxide. The dyes malachite green (E), rhodamine 6G (S), and rhodamine123 (S); and the drugs capsaicin (A) and 8-hydroxyquinoline (A) were dissolved in ethanol.

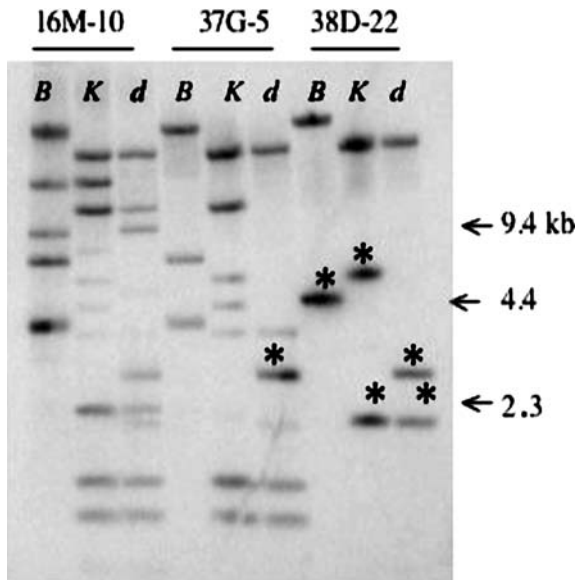
## Results

### Cloning of *Pdr1*

Degenerate primers based on a short peptide sequence of a putative chemoreceptor were used to amplify gene-specific sequences from a cDNA pool of swimming zoospores. One of the unintended PCR products was a 1,184 bp fragment with homology (37% identity) to an ABC transporter from *Nicotiana plumbaginifolia* that is thought to be involved in the secretion of antifungal terpenoids (Jasinski et al. 2001). The Southern blot of *P. sojae* genomic DNA using the PCR product as a probe revealed five or more bands for each of three restriction enzyme digests (Fig. 1), indicating that the probe probably hybridized to several closely related genes. No hybridization of the probe was seen with soybean genomic DNA. To obtain the complete sequence of the gene, the gene fragment was used to probe a *P. sojae* BAC genomic library. A total of 14 clones were identified including eight strongly hybridizing BACs and six BACs with weaker hybridization signals. Southern analysis of the BACs exhibiting the strongest hybridization signals generated several distinct hybridization patterns (Fig. 2) confirming our hypothesis that the probe was also binding closely related genes. PCR analysis of the BAC clones suggested that the gene was within BAC 38D22 (not shown). Two *Kpn*I fragments, 5.413 and 2 kb (Fig. 2), were subcloned into pUC18 and



**Fig. 1** Southern blot analysis of genomic DNA from *P. sojae* and soybean using a PCR-generated fragment of *Pdr1* as a probe. *Left lane* contains 1 μg of the PCR product. *Letters* indicate digestion of *P. sojae* and genomic DNA by *Eco*RI *E*; *Bam*HI *B*; and *Hind*III *H*. The *Pdr1* probe does not hybridize to soybean genomic DNA



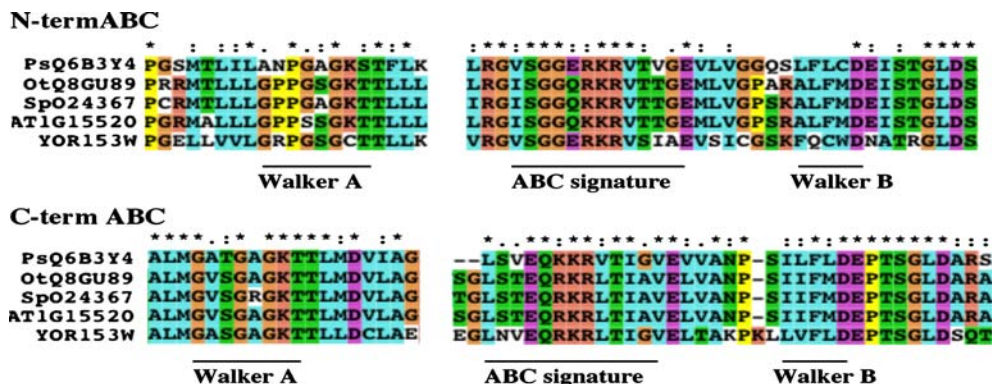
**Fig. 2** Southern blot analysis of restriction digests of selected BAC clones that probed with *Pdr1* fragment. BAC clones 16M-10, 37G-5, and 38D-22 cut with *Bam*HI *B*; *Kpn*I *K*; and a double digest (*d*) with *B* and *K*. Asterisks indicate fragments that were cloned and sequenced

sequenced by primer walking. The complete gene was found to span a single open reading frame encoding 1,310 amino acids and contained within it the original PCR sequence. The sequence of the clone and translated polypeptide can be retrieved from public databases as Genbank locus AY684168, protein id AAT8568, TrEMBL Q6B3Y4. The polyadenylation cleavage site **TGATGTGAAA** is located only 89 bp downstream of the stop codon. In the sequence 10–35 bp upstream from the polyA cleavage site, the closest sequence to a con-

sensus poly A signal is TGTA, 14 bp upstream of the cleavage site. *Pdr1* shares 89% identity with the translated sequence of CD051569, an EST from a zoospore-enriched cDNA library of *P. nicotianae* (Skalamera et al. 2004). *Pdr1* also shares a similar level of identity with Pi\_005\_35248\_Jun03, a *P. infestans* clone from a mycelial library (<http://www.pfgd.org/>).

The translated protein shares 39% identity and 57% homology with a putative transporter from *Oryza sativa* (PAC clone P0410E03; EMBL AP002844) and 38% identity and 58% homology with a *Pdr5*-like ABC transporter from *Spirodela polyrrhiza* (Smart and Fleming 1996). The sequences of the most homologous PDR transporters from *O. sativa*, *Arabidopsis thaliana*, *Spirodella polyrrhiza*, and *S. cerevisiae* were retrieved from Genbank and aligned using Clustal X with respect to *Pdr1* (Fig. 3). The nucleotide binding folds are the most highly conserved regions of ABC transporters. A comparison of the Walker A boxes, which have the consensus motif GXXXGST, shows that in the N-terminal NBD Walker A box contains an A residue in place of the first G. The C-terminal Walker A Box GATGAGKT defines a perfectly conserved consensus motif that extends to the amino acids surrounding this motif. Only the N-terminal Walker B sequences differ from a strict consensus sequence of HHHHD where H is a hydrophobic residue by having a C in the penultimate position. On the basis of its homology to other sequences, *Pdr1* can be classified in the TC3.A.1.205-PDR family of ABC transporters (Saier 2000). PDR-type transporters have been grouped in the eye pigment precursor, PDR family (Dassa et al. 2001) and are classified in the ABCG family by the Human Gene Nomenclature Committee <http://www.gene.ucl.ac.uk/users/hester/abc/html>

**Fig. 3** Amino acid alignment of the ABC domains of *Pdr1p*, its closest homologues in *O. sativa*, *S. polyrrhiza*, *A. thaliana*, and the *S. cerevisiae* transporter *Pdr5p*. The first two letters in the sequence name identify the species followed by the TrEMBL accession number. The sequences were aligned and colored by the Clustal X program as follows: yellow P, orange RKGK, pink ED, green STQNH, blue AILMFYV. The position of the Walker A, ABC signature and Walker B sequences are shown below the sequences. Asterisks denote identical amino acids in the alignment and colons are used to indicate conserved residues



*Pdr1* is only expressed in zoospores

As a first step in the characterization of *Pdr1*, a gene-specific primer was designed to determine under what conditions this transporter was expressed. Reverse transcription of isolated RNA was performed using MMLV-RT and a modified poly T primer. For expression of *Pdr1*, the master mix included an aliquot of

cDNA of swimming zoospores, hyphal germlings, hyphae grown on defined media (V8 agar plates), hyphae from infected hypocotyls or noninfected hypocotyls (as negative control). Gene-specific primers of a ribosomal protein were used as a positive control. Equal volumes of the cDNA templates were used in PCR reactions to amplify gene fragments of *Pdr1* or a 40S ribosomal protein. An analysis of the ethidium-stained gels showed that the 640 bp fragment of *Pdr1* had equivalent or greater intensity than the 420 bp fragment of a 40S ribosomal gene in cDNA prepared from swimming zoospore extracts. This PCR product was cloned and sequenced to verify that the correct product had been identified. By comparison, no expression of an amplicon was observed from cDNA extracts from hyphal germlings, hyphae grown on defined media or in *P. sojae*-infected soybean hypocotyls (Fig. 4). The excised hypocotyl tissue used in these assays included the peripheral tissues surrounding a well-developed necrotic lesion. Thus, this sample included hyphae involved in both the introgression of healthy tissues and the absorption of nutrients from necrotic regions. The presence of a RT-PCR amplicon in mRNA extracts from swimming zoospores, its absence in vegetative and infectious hyphae, and its disappearance in hyphal

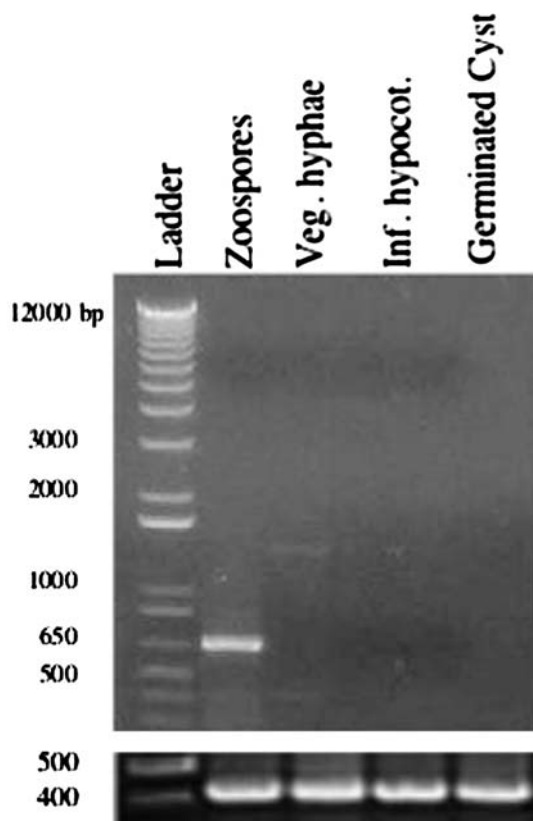
germlings (30 min postcystment) suggests a role for *Pdr1* in the swimming zoospore.

The number of EST sequences from a cDNA library that share identity with the same gene is a crude measure of gene expression in that tissue. The full-length sequence of *Pdr1* was compared by BLAST analysis to the EST database of the *Phytophthora* Functional Genome database (PFGD). A similar strategy was employed to determine the number of exact matches of ESTs to four other housekeeping genes in the PFGD database. Exact matches to the sequence of *Pdr1* were found only in the zoospore libraries (Table 1). Notably, the expression of *Pdr1* was more than twofold greater than the relative expression of selected housekeeping genes in any of the zoospore, mycelial or infected soybean hypocotyls EST libraries. *Pdr1* may be the only member of the PDR family of drug resistance transporters expressed by swimming zoospores, as no additional ESTs with homology to PDR transporters were identified using a query search with the key words. "pleiotropic" and "ABC transporters".

#### Heterologous expression of *Pdr1*

To test whether *Pdr1* functions as a drug resistance transporter in yeast, the complete gene sequence and an additional 207 bp downstream of the stop site were amplified by PCR and cloned into the yeast shuttle vector pYes2. The plasmid pYes2-PDR1 was introduced into the yeast strain YYM3 that is deficient in the PDR transporters PDR5 and SNQ2, and transformants were selected on CSM 2% galactose lacking uracil. The mutagen 4-NQ, a known substrate of the yeast transporter Snq2p, was used in liquid growth assays to determine whether heterologously expressed *Pdr1* could protect yeast cells from growth inhibition. The addition of 1.4  $\mu$ M 4-NQ substantially inhibited the growth rate of YYM3 while having minimal effects on the growth of wild-type strain YPH500 and the transformant YYM3-PDR1 (Fig. 5).

To assess whether *Pdr1* was capable of protecting yeast strains from a wider range of substrates, additional constructs were made in the yeast strain AD123456789. The complete gene sequence of *Pdr1* and an additional 207 bp downstream of the stop site was amplified by PCR and cloned into the yeast shuttle vector pSK-PDR5PPUS (Nakamura et al. 2001). The amplified *Pdr1* fragment including the *Pdr5* promoter and *Pdr5* stop codon was integrated by homologous recombination into the *Pdr5* locus of AD123456789. Transformants were selected for their ability to grow on uracil plates. Drugs that are known to be substrates for yeast transporters, Pdr5p, Snq2p, and Yor1p, were added to agar plates, and the yeast strains were added as spots (Fig. 6). Transformants were more tolerant than AD123456789 to five substrates: 4-NQ, 8-hydroxyquinoline, ethidium bromide, cycloheximide, and tetracycline. However, in all cases the transformants were more sensitive to these compounds than



**Fig. 4** RT-PCR assay of *Pdr1*. RNA was extracted as described in Materials and methods and converted to cDNA. Gene-specific primers were used to amplify a gene fragment of *Pdr1* (a) or the 40S ribosomal protein gene (b)

**Table 1** Relative expression of *Pdr1* and selected *P. sojae* housekeeping genes

Gene	EST library			
	No. of ESTs	Zoospores 4339	Mycelial 13,192	Infected hypocotyls 5,348
<i>Pdr1</i>		0.00852	0	0
$\alpha$ TubulinID 109111		0.00161	0.00023	0.000748
$\beta$ TubulinID 109498		0.000230	0	0.000934
40S ribosomalID 109323		0.0025	0.00144	0.00147
Elongation factor 3ID 134912		0.00184	0.000536	0.000187

Values are expressed as a percentage of the total EST pool. ID numbers refer to the full-length predicted transcripts (<http://phytophthora.vbi.vt.edu/>)

the yeast strain YPH500. No protection was conferred against 2,4-diphenoxyacetic acid, benomyl, benzoic acid, capsaicin, chlorophenol red, daunorubicin, doxorubicin, 2,4-diphenoxyacetic acid, malachite green, miconazole nitrate, quinidine, rhodamine 6G, rhodamine123, thiram, and 2,3,5-triphenyltetrazolium chloride. Thus, this transporter is apparently able to transport a much narrower range of chemicals in yeast than the yeast transporters PDR5 and SNQ2.

## Discussion

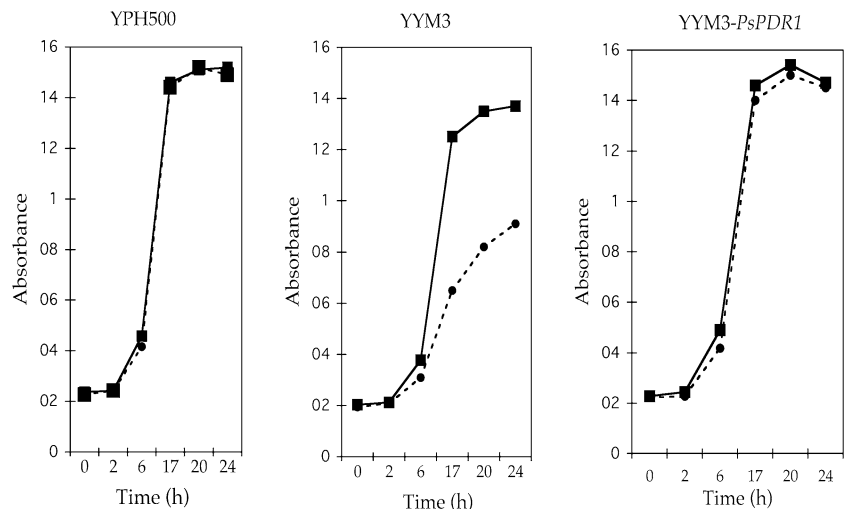
Since zoospores do not have a cell wall, any inhibition of metabolic processes or membrane transporters caused by exogenous xenobiotics may result in cell disruption due to rapid diffusion of water across the plasmalemma. Thus, it seems likely that zoospores have one or more constitutively expressed proteins to actively exclude toxins from the cytoplasm. Based only on its expression profile, *Pdr1* appears to match the expected criteria for such a function. This study shows that *Pdr1* has a higher level of expression than several housekeeping genes, and that its expression is limited to the free swimming zoospore stage. Our annotation (manuscript in preparation) of the recently released *P. sojae* genome <http://www.jgi.doe.gov/> has revealed that there are more than 40 genes encoding members of the PDR family of ABC transporters, a considerable expansion from that seen in plants (van den Br ule and Smart 2002) or dictyostomes

(Anjard and Loomis 2002). BLAST analysis of *P. sojae* PDR genes against the ESTs of the PFGD database also indicate that *Pdr1* is the only member of the PDR family known to be expressed by the motile stage of this pathogen (data not shown). Moreover, a keyword search restricted to zoospore libraries in the PFGD database using the key words such as pleiotropic, multidrug, or ABC revealed that 37 of 49 EST sequences with homology to any ABC transporter matched *Pdr1*. Thus *Pdr1* is the most frequently represented ABC transporter in all of the zoospore EST libraries.

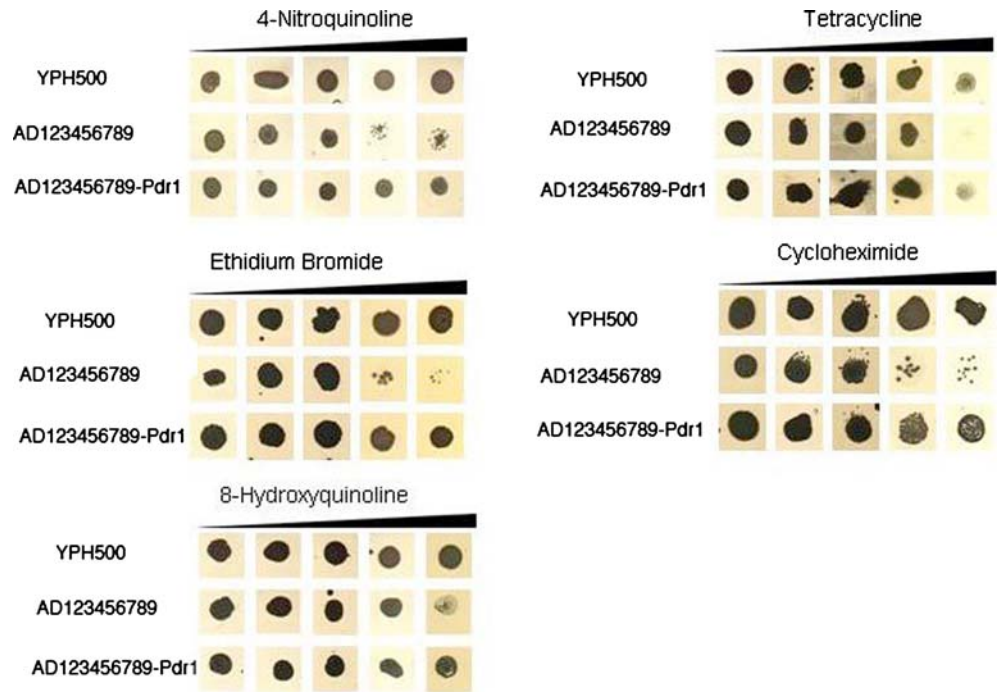
Since *Pdr1* appears to be the major ABC transporter expressed in zoospores, we hypothesized that it functioned as a broad specificity drug resistance transporter. The yeast transporter mutants are an ideal background to test the functionality of a heterologously expressed protein, since the mutants are capable of normal growth but display enhanced sensitivity to a very wide range of toxins. Admittedly, with the exception of the antibiotics, the toxins we used are unlikely to be encountered by zoospores in the soil environment. Our tests did not include glyceollins which can be excreted into the soil rhizosphere (Werner and Hohl 1990), because zoospores are in fact more sensitive to these compounds than the hyphal stages (Bhattacharyya and Ward 1985). Nonetheless, it became evident that Pdr1p does not share the characteristics of the yeast transporters Snq2p and Pdr5p

In our survey, we have been unable to identify a class of compounds for which the *P. sojae* gene fully complements

**Fig. 5** Growth of yeast strains in response to exogenous 4-nitroquinolone. Yeast strains were grown overnight, diluted to  $OD_{600} = 0.2$  and growth was monitored in the presence or absence of  $1.2 \mu\text{M}$  4-NQ. Values at each interval are the mean of three determinations. SD (not shown) did not exceed the symbol dimensions. Control (filled square), 4-NQ (filled circle)



**Fig. 6** Pdr1Pdr1p functions as a drug resistance transporter in yeast mutants. Yeast strains were grown overnight, diluted to  $OD_{600} = 0.2$  and arrayed as 3  $\mu$ l drops on plates containing drugs at varying concentrations. Growth after 36 h was determined by scanning an image of the plates on a flatbed scanner and a composite figure was assembled in Photoshop



the yeast WT genes. Partial drug resistance could be due to the fact that we have not identified the physiological substrate. In this survey we have identified five chemically distinct compounds for which the transporter provides some level of protection, and many more compounds for which no protection is mediated by the transporter. If, as we have proposed, *Pdr1* functions as a broad specificity transporter for swimming zoospores, we might expect that the transporter should mediate protection to a wider range of drugs, as is the case for the yeast transporters Pdr5p and Snq2p.

The functional analysis of PDR-type transporters indicate that their function extends beyond that of the drug resistance (Martinoia et al. 2002). Some functions of individual members include the maintenance of lipid asymmetry across the plasma membrane (Dogra et al. 1999; Voelker 2004) transport of growth factors (Martinoia et al. 2002) and the extrusion of cellular toxins. The latter role has recently been proposed for both the yeast transporter Pdr15p (Wolfger et al. 2004) as well as Pdr5p (Mamnum et al. 2004). Despite its close homology to the yeast transporter Pdr5p, Pdr15p has only limited capacity to mediate drug resistance and has never been isolated in drug-resistant screens. Pdr15p is upregulated in yeast at the end of stationary phase, and protein expression appears to be linked to the stress-induced pathway. In contrast, Pdr5p is highly expressed during the exponential growth phase and is rapidly downregulated in response to nutrient starvation. The elevated expression of Pdr5p during rapid growth could indicate a requirement for the detoxification of the cell in addition to its function as a drug efflux pump, or it could be acting to optimize membrane permeability to enable continued growth. Its disappearance at stationary phase

or under starvation conditions may be necessary to prevent the export of needed cellular metabolites. Similarly, expression of *Pdr1* in the zoospore may also function to remove a select group of cellular toxins produced as a consequence of the energy expended to sustain locomotion.

In yeast, PDR-type transporters have also been implicated in lipid trafficking to the vacuole. Could *Pdr1* contribute to the smooth operation of the water exclusion vacuole of the swimming zoospore? These questions are best addressed through gene silencing and in vivo localization experiments. Transformation of *P. sojiae* has been demonstrated (Judelson et al. 1992) but has yet to be used to inhibit gene expression in *P. sojiae*. Other techniques, such as targeting induced local lesions in genomes (TILLING), are now being applied to the *P. sojiae* genome (Kurt Lamour, personal communication), and gene silencing by RNA interference have recently been demonstrated in *P. infestans* (Whisson et al. 2005).

The functional complementation of a nucleoside transporter from *Trypanosoma brucei* in yeast suggests that proteins from even phylogenetically distant organisms can be expressed and correctly targeted in the cell (Masser et al. 1999). Nonetheless, misfolding of the protein may result in diminished expression of the expected phenotype. In eukaryotic systems, the trafficking of proteins to their correct compartment is dependent upon signals embedded within the protein (Epping and Moye-Rowley 2002; Barlowe 2003). ER-dependent export of proteins may involve signaling elements in both the N-terminal and C-terminal regions of the protein. The motif **DXE** has been reported to accelerate ER export, but this element is not present in

*Pdr1*, and it is not a feature of any of the predicted PDR transporters of *P. sojae* (<http://genome.jgi-psf.org/sojae1/sojae1.home.html>). Other elements that mediate translocation to the plasma membrane include bulky dihydrophobic motifs immediately prior to the stop codon or within ~12 amino acids from the cytoplasmic tail (Barlowe 2003). The C-terminal sequence of *Pdr1* (GLLVIVWVVLQVAIYLTFKYVSHLKR) contains two potential hydrophobic regions that could serve as transport signals. Since the YYM3-PR1 transformants grew at comparable rates to wild-type cells in liquid media containing strongly inhibitory concentrations of 4NQ, it seems likely that *Pdr1* was correctly targeted to the plasmalemma.

The release of the genome sequences of *P. sojae* and *P. ramorum* introduces a new era in the study of oomycete pathogens. The functional analysis of the membrane transporters situated at the interface between the pathogen and its host are of particular interest because they are potentially involved in the delivery of toxins to host tissues or in the protection of the pathogen from plant phytoalexins. Given the large expansion in the ABC transporter family that we have noted, heterologous expression in yeast is likely to be a valuable tool in defining the roles of specific genes.

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