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Full Length Research Paper

PdMfs1, a major facilitator superfamily transporter from *Penicillium digitatum,* is partially involved in the imazalil-resistance and pathogenicity

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Penicillium digitatum,* causing green mold, is the most important postharvest pathogen of citrus fruits worldwide. Fungicide imazalil has been used in control of this mold for more than three decades. In the presence of imazalil pressure, imazalil resistance strain has risen worldwide. To explore the potential role of multidrug resistance (MDR) in imazalil resistance, a membrane efflux transporter *Penicillium digitatum* major facilitator surperfamily1 (*PdMfs1*) was cloned, and its functions in imazalil resistance and pathogenicity were analyzed. *PdMfs1* has an open reading frame (ORF) of 1,876 bp and 3 introns of 55, 49 and 71 bp, respectively. It encodes a protein of 566 amino acids that shares a high degree of similarity with members of the drug: H^+ antiporter efflux family of the major facilitator surperfamily (MFS) transporters of other fungi. Expression of *PdMfs1* was up-regulated by treatment with imazalil and other fungicdes in both imazalil-sensitive and –resistant *P. digitatum*. Disruption of *PdMfs1* gene rendered *P. digitatum* more sensitive to imazalil and other DMI fungicides, and the imazalil-resistance could be rescued by reintroducing the wild-type *PdMfs1* gene into the *PdMfs1* disruption mutant (Δ*PdMfs1*). Overexpression of *PdMfs1* rendered *P. digitatum* more resistant to imazalil. These results indicate that *PdMfs1* is a multidrug transporter of *P. digitatum* that could pump imazalil out of cells, thus contributing resistance to imazalil partially. Pathogenicity analysis showed that the disease on the citrus fruits inoculated with the Δ*PdMfs1* developed much slower than that induced by the parental strain PdW03, suggesting that *PdMfs1* also plays a role on the virulence of *P. digitatum.

Key words: *Penicillium digitatum*, major facilitator surperfamily (MFS) transporters, imazalil resistance, virulence.

INTRODUCTION

Phytopathogenic fungi, like all other organisms in the natural environment, have evolved various effective mechanisms to protect themselves from adverse effects caused by various toxic compounds, such as antibiotics produced by other microorganisms in their saprophytic phase, and antimicrobial compounds produced by host plants, such as phytoalexins, during their parasitic phases. In addition, due to increased application of synthetic fungicides in controlling plant fungal diseases, fungal pathogens are under a great pressure to either avoid or develop resistance to those chemicals (de Waard et al., 2006).

Multidrug resistance (MDR) or pleiotropic drug resistance (PDR) is one of the mechanisms initially identified in human pathogenic microbes and cancer cells

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Table 1. Primers used in this study.

Names	Sequence （5’ to 3’）	Restruction site	Tm (℃)
PdMFS-F	tggMgNtggtgYttYtaYatHaa	-	52
PdMFS-R	gcYtgRaaccaDatNggNAgRta	-	55
PdMFS1	gttacttctagtcgatgagcttgatg	-	56
PdMFS2	gaggcagcagtcggtaattaa	-	57
PdmfsA	aactcgaggttaacttctagtcgatgagcttgatg	*Xho* I	58
PdmfsB	aaactagtttcagaatggcctttgggcttt	*Spe* I	60
PdmfsC	aagagctcgccttttgtggtgtttggcgtcct	*Sac* I	62
PdmfsD	aaggtaccgaggcagcagtcggtaattaa	*Kpn* I	63
PdmfsE	aatctagagaggcagcagtcggtaattaa	*Xba* I	57
PdmfsF	gaggcagcagtcggtaattaa	*Sph* I	59
PdmfsG	cgctgctttctttgtcct	-	55
PdmfsH	cctgatacccgatccact	-	57
PdtubA	tgagcactccgacgagacttac	-	61
PdtubB	gacgagggaagggaaccat	-	59

(Hiller et al., 2006; Perez-Tomas, 2006; Gulshan and Moye-Rowley, 2007). MDR is mediated by energy-dependent plasma membrane efflux transporters, which can transport toxic compounds from the inner leaflet of the membranes to the outer environment of cells, thereby reducing the concentration of the toxic compounds in cells. The major types of drug efflux proteins are ATP binding cassette (ABC) and major facilitator superfamily (MFS) transporters (Gbelska et al., 2006; Sipos and Kuchler, 2006; Sá-Correia et al., 2009). In filamentous fungi, the roles of ABC transporters on resistance to anti-fungal agents have been well documented in *Aspergillus nidulans* (*atrB, atrD* and *atrG*) (Andrade et al., 2000; de Waard et al., 2006), *Botrytis cinerea* (*BcatrB, BcatrD* and *BcatrK*) (Hayashi et al., 2001, 2002b; Schoonbeek et al., 2001; Vermeulen et al., 2001), *Mycosphaerella graminicola* (*MgAtr1* and *MgAtr5*) (Zwiers et al., 2002, 2003), as well as in *Penicillium digitatum* (*PMR1* and *PRM5*) (Nakaune et al., 1998, 2002). However, the works on MFS are much more limited. Prasad and Kapoor (2005) reported that the MFS pump *CaMDR1* from the opportunistic human pathogen *Candida albicans* played a key role in resistance to azole fungicides (Prasad and Kapoor, 2005). In phytopathogenic fungi, the MFS transporters have been shown to play mainly a role on the secretion of toxins, thus providing self-protection for the toxin-producing fungi, and conferring virulence to host plants (Pitkin et al., 1996; Callahan et al., 1999; Hayashi et al., 2002a; Choquer et al., 2007; Roohparvar et al., 2007). Yet, the role on fungicide resistance is minor (Hayashi et al., 2002a; de Waard et al., 2006; Roohparvar et al., 2007).

P. digitatum (Pers.:Fr) *Sacc.* is the causal agent of green mold of citrus, being responsible for about 90% of production losses during postharvest citrus packing, storing, transportation, and marketing (Kanetis et al., 2007; Macarisin et al., 2007). Treatment with the fungicide imazalil, which belongs to the demethylation inhibitors (DMIs) of ergosterol biosynthesis, is the primary approach to control green mold of citrus. As a consequence of continuous use, imazalil resistant isolates have emerged universally (Li et al., 2003; Chen et al., 2008; Eckert, 1987; Bus et al., 1991), and the control efficiency has been compromised (Eckert et al., 1994; Jiang et al., 2010). To explore if MFS is involved in the imazalil resistance and pathogenicity, a MFS transporter gene (*PdMfs1*) of *P. digitatum* was cloned and its role in imazalil resistance and virulence was investigated by genetic approaches.

MATERIALS AND METHODS

Fungal strains and cultivation

The PdW03 (imazalil resistance) and Pd23 (imazalil sensitivity) of *P. digitatum* used in this study were isolated from *P. digitatum*-infected citrus fruits, collected from storage houses in Zhejiang, China, in 2005 and 2000, respectively, and were maintained on potato dextrose agar medium (PDA) at 4°C. Mycelium of *P. digitatum* was cultured in liquid potato dextrose on a rotary shaker (160 rpm) at 25°C, whereas the conidia were cultured on solid PDA.

Oligonucleotide primers

The oligonucleotide primers used in this study are listed in Table 1. The positions of these primers within and around the coding region of the *P. digitatum MFS1* (*PdMfs1*) gene are shown in Figure 1A.

Cloning of *PdMfs1* gene

Based on the conserved amino acid sequences of *Mfs1* genes *Bcmfs1* (*B. cinerea*, AF238225, Hayashi et al., 2002a), *ToxA*

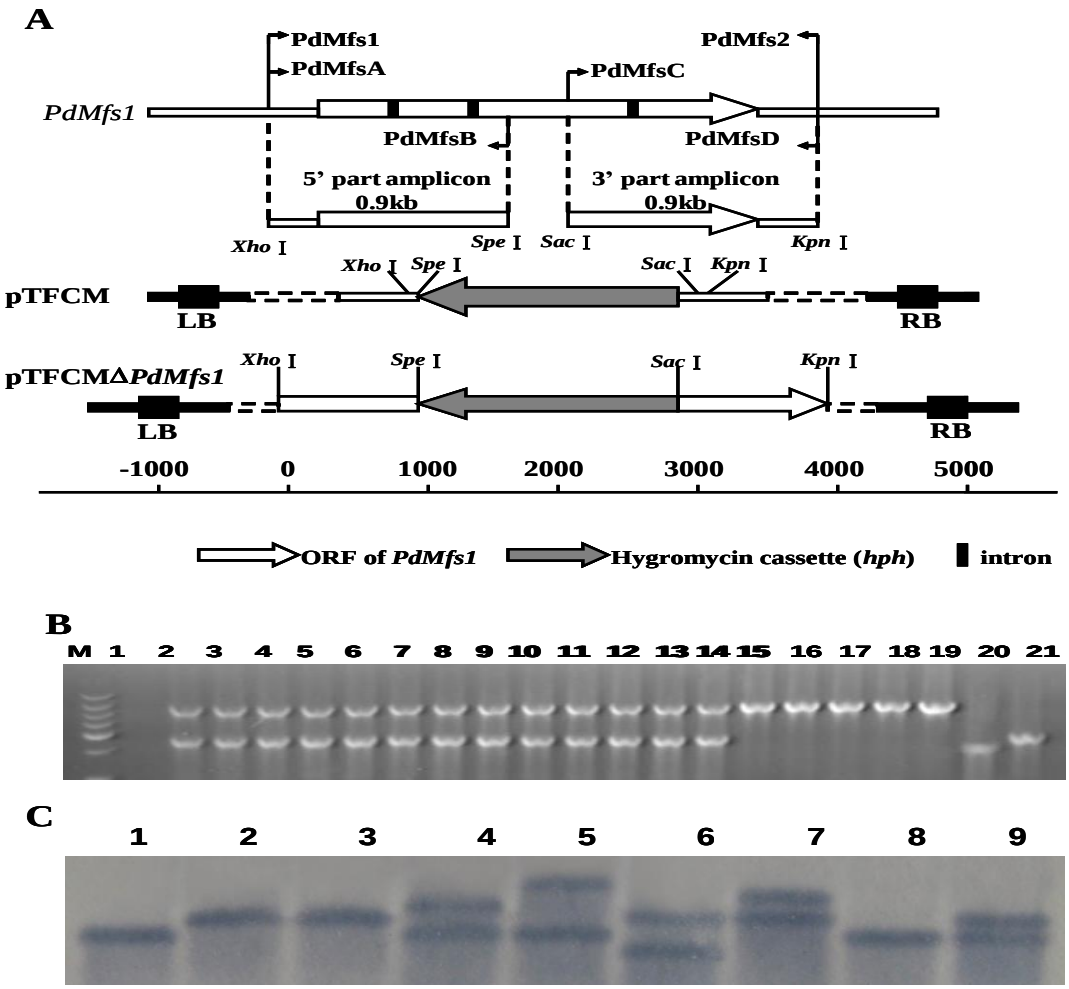


Figure 1. Diagram of construction of *PdMfs1* disruption plasmid and identification of *PdMfs1* mutants. A, Diagram of construction of *PdMfs1* disruption plasmid; 0.9kb fragments at both 5' and 3' end of the *PdMfs1* gene were amplified from genomic DNA of *P. digitatum* and inserted into the left and right sides of *hph* in vector pTFCM, respectively, to generate the *PdMfs1* disruption plasmid pTFCM-Δ*PdMfs1*. B, identification of *PdMfs1* disruption mutants by PCR. Lane 1, dd H_2O; Lane 2-14, ectopic transformants (*EctPdMfs1*); Lane15-18, disruption transformants (Δ*PdMfs1*); Lane19: plasmid pTFCM-Δ*PdMfs1;* Lane20, PdW03 cDNA; and Lane21, PdW03 DNA. C, Confirmation of *PdMfs1* mutants by Southern blot analysis. Genomic DNA (5 µg) was digested with restriction enzymes *SpeI*. The full length *PdMfs1* gene was used as the probe. PdW03, parental strain; Δ*PdMfs1*A and Δ*PdMfs1*B*, PdMfs1* disruption mutants; OE*PdMfs1*A and OE*PdMfs1*B, *PdMfs1* overexpression mutants; CP*PdMfs1*A and CP*PdMfs1*B*,* Δ*PdMfs1* complementation mutants; PdpTFCM, transformant of empty pTFCM; Ect*PdMfs1*A, ectopic transformant.

(*Cochliobolus carbonum,* L48797, Pitkin et al., 1996), and *Cfp* (*Cercospora kikuchii,* AF091042, Callahan et al., 1999), a set of degenerate primers PdMfs-F and PdMfs-R (Table 1) were designed to amplify a genomic DNA fragment encoding part of the *PdMfs1* gene. The amplified fragment was cloned into the T-Easy vector (Promega USA) and sequenced. The sequence was then used to design nested insertion-specific primers (Wang and Li, 2008) and used to amplify the 5' and 3' flanking regions of the *PdMfs1* gene. Thermal asymmetric interlaced (TAIL)-polymerase chain reaction (PCR) (Wang and Li, 2008) was performed with the Genome Walking Kit (Seegene, Inc. Seoul Korea) according to the manufacturer's instructions.

The full-length cDNA of *PdMfs1* was obtained by reverse transcription (RT)-PCR using the total RNA as template. RT reactions were performed using RNA PCR Kit (AMV) 3.0 kit (TaKaRa Biotech. Co.. PCR amplification was performed using primers PdMfs1 and PdMfs2.

Expression of *PdMfs1* after treatment of imazalil and other toxic compounds

Induced transcription of *PdMfs1* for both imazalil resistance and sensitivity *P. digitatum* was studied by adding imazalil (22.2% EC, Cerexagri, USA), and propiconazol (25% EC, Strongwill, Zhengzhou, China), mancozeb (80% EC, Halsen USA), actidione (Genview, USA), and ethidium bromide (Genmed, USA) to four-day old mycelial culture of *P. digitatum* grown in liquid potato medium. After addition of the above compounds, mycelial cultures were incubated at 25°C, 160 rpm for 1 h. Thereafter, the mycelia were

harvested by filtrating through a two-layer cheesecloth, and washed with distilled (dd) H_2O, then were frozen in liquid nitrogen and stored at -80°C until RNA isolation.

The relative expression levels of *PdMfs1* between pre- and post-toxin treatments were assayed by real-time RT-PCR. Total RNA was extracted as described previously. First strand cDNA was synthesized using RNA PCR Kit (AMV) 3.0 kit (TaKaRa Biotech. Co.). Real time PCR was carried out using the SYBR Premix Ex TaqTM (Perfect Real Time) kit (TaKaRa) on 7300 Real time PCR system (ABI, USA). A final volume of 25 µL mixture, containing 12.5 µL of 2×SYBR Premix Ex TaqTM Buffer, 0.5 µL of each primer (10 µM), 0.5 µL 50×ROXReference Dye II, 2 µL cDNA template, 9 µL dd H_2O, was set up in 0.2 mL thin wall clear 8-strip tubes (Axygen, USA). The thermal cycling conditions were 95°C 30 s, 40 cycles of 95°C 5 s, 55°C 20 s and 72°C 20 s. The sense primer *PdMfsF* and antisense primer *PdMfsG* (Table 1) were designed in expression analysis of *PdMfs1*. The β-tubulin gene amplified using primers PdtubA and PdtubB was used as a reference to normalize the quantification of *PdMfs1* gene expression.

In all tests, appropriate negative controls containing no template cDNA were subjected to the same procedure to eliminate or identify any possible contamination. Both *PdMfs1* and β-tubulin gene were amplified in four separate reactions from the same cDNA preparation and the mean value was obtained. Both *PdMfs1* and β-tubulin gene melting curve (Y=slop×log(X)+N) were established by real-time PCR, using serially diluted cDNA of *P. digitatum*. The real-time PCR efficiencies were calculated according to the equation: E=10(−1/slope). The relative quantification of the target gene *PdMfs1* in comparison to the reference β-tubulin gene was calculated according to the formula Ratio = [(Etarget) ΔCt target (control-sample)]/[(Ereference) ΔCt reference (control-sample)] (Pfaffl et al., 2002). For each strain, the Ct value was determined by subtracting the average β-tubulin gene Ct from the average *PdMfs1* Ct value. Analysis of variance (ANOVA) was applied to determine significant differences (P< 0.05) among the Ct of the strains (Data Processing System (DPS) 9.50, Tang, 2007).

Construction of *PdMfs1* disruption plasmid

A PCR strategy was used to construct *PdMfs1* disruption plasmid and the schematic procedure was shown in Figure 1. Briefly, 0.9kb DNA fragments at both 5' and 3' end of *PdMfs1* gene were amplified from genomic DNA of *P. digitatum* using a set of primers PdMfsA (containing *Xho*I) and PdMfsB (containing *Spe*I), and PdMfsC (containing *Sac*I) and PdMfsD (containing *Kpn*I) (Table 1), respectively. The fragments were inserted into the left and right sides of *hph* in theplasmid pTFCM, respectively, to generate the *PdMfs1* disruption plasmid pTFCM□*PdMfs1* (Figure 1A).

Constructions of *PdMfs1* over-expression and complementation plasmids

The *PdMfs1* open reading frame (ORF) was amplified using primer pairs PdMfsA and PdMfsD, in which the *Xho*I and *Kpn*I restriction sites were added. The PCR product was digested with *Xho*I and *Kpn*I and cloned into pSilent-1 to obtain pSilent-*PdMfs1* (supplement). The full-length *PdMfs1* ORF containing *trpC* promoter and *trpC* terminator was obtained by digestion of the plasmid pSilent-*PdMfs1* with *Spe*I, then ligated into the vector pTFCM to obtain the *PdMfs1* over-expression plasmid pTFCM-*PdMfs1.*

The *PdMfs1* complementation plasmid pTBG-*PdMfs1* was constructed using a similar approach, except that the plasmid pTBG contains herbicide glufosinate resistant gene *bar* as a selection marker instead of the hygromycin B resistant gene *hph*as in the pTFCM.

P. digitatum transformation and analysis of transformants

Agrobacterium tumefaciens-mediated transformation (ATMT) was performed using *A. tumefaciens* strain AGL-1 as described previously (Wang and Li, 2008) to create a *PdMfs1* mutant (Δ*PdMfs1*) and an over-expression strain (OE*PdMfs1*) in the genetic background of *P. digitatum* strain PdW03.

The *PdMfs1* complementation transformants were obtained by a similar approach except using Δ*PdMfs1* as a recipient strain and the herbicide glufosinate as a selection agent. Mitotic stable Δ*PdMfs1* mutants, over-expression mutants (OE*PdMfs1*) and complementation transformants (CP*PdMfs1*) were obtained by repetitive sub-culturing on non-hygromycin B PDA for five times, then transferred back to hygromycin B-containing PDA.

Δ*PdMfs1*, OE*PdMfs1*, and CP*PdMfs1* were confirmed by PCR using primers PdMfs1 and PdMfs2 (Table 1 and Figure 1B) and Southern blot (Figure 1C) as described previously (Wang and Li, 2008). Briefly, genomic DNA (5 µg) of individual strains to be evaluated was digested with restriction enzymes *Sph*I. The full length *PdMfs1* gene was used as a hybridization probe, and labeled with digoxigenin using the DIG High Primer DNA Labeling and Detection Starter Kit Ⅱ (Roche, Mannheim Germany) according to the manufacturer's instructions.

Growth, conidiation and fungicide resistance assays

The growth rates of the wild-type strain PdW03, Δ*PdMfs1*Amutant, OE*PdMfs1*A and CP*PdMfs1*A strains were compared on PDA. A mycelial plug of 0.5 cm in diameter prepared as described previous (Zhang et al., 2008) was placed on the center of a PDA plate. The plates were incubated at 25°C, the diameters of colonies were measured and the conidiation was evaluated with naked eye after five days. Three plates were used for each strain, and the experiment was conducted twice.

Commercial products of imazalil (22.2% EC, Cerexagri, USA) were tested in this study. The resistance of above mutants, as well as the parental strain PdW03 and field imazalil-sensitive strain Pd23 against imazalil were performed by the similar method described previously, except for imazalil was added in the media to obtain a series of concentrations (0, 0.001, 0.01, 0.1, 0.2, 0.4, 0.8, 1.0, 2.0, 4.0 and 8.0 $\mu g \cdot mL^{-1}$) imazalil-containing media. The diameters of colonies were recorded five days after incubation. The half maximal effective concentration (EC_{50}, $\mu g \cdot mL^{-1}$) was determined by using DPS 9.50 (Tang, 2007). Three replicates were used for each concentration. Mean values with standard deviations are presented. ANOVA was applied to determine significant differences (P< 0.05) among the EC_{50} of the strains/mutants by using DPS 9.50 (Tang, 2007). The experiment was conducted twice.

The resistance of the wild-type strain PdW03, Δ*PdMfs1*A, OE*PdMfs1*A and CP*PdMfs1*A mutants to other DMI fungicides prochloraz and tebuconazole (25 and 43%, respectively, Bayer Crop Sciences, Monheim, Germany), propiconazole (25% EC, Strongwill, Zhengzhou, China), myclobutanil (40% EC, Noposion UK), difenoconazole (10% EC, Synegenta, Switzerland) were compared on fungicide amended-PDA. The concentrations of these fungicides were indicated on Figure 3.

Pathogenicity assays

Mature citrus fruits (*Citrus reticulata* Blanco) were harvested from an orchard in Quzhou, Zhejiang. Before inoculation, fruits were washed with tap water and dried at room temperature. Two-microliters (2 µl) of fresh conidial suspension at a concentration of 10^6 ml^{-1} of *P. digitatum* were spotted onto a wounded site near by

the navel of the fruit, punctured by a bunch of 5-needles (1 mm in depth). The inoculated fruits were incubated at 25°C with 16 h of daylight. Disease development was observed daily and the lesion area of each fruit was determined by measuring lesion's diameter at five days after inoculation, three fruits were used for each strain/ mutant inoculation. Mean values with standard deviations were presented. ANOVA was applied to determine significant differences (P< 0.05) among the lesion area of strain (DPS 9.50). The experiment was conducted triplets.

RESULTS

Amplification of *PdMfs1* gene and its homology to other fungal *Mfs1s*

Based on the conserved amino acid sequences of *Mfs1* transporters from *Botryotinia fuckeliana* (AF238225, Hayashi et al., 2002a), *A. flavus* (AF515601, Chang et al., 2004), *C. carbonum* (L48797, Pitkin et al., 1996) and *C. kikuchii* (AF091042, Callahan et al., 1999), the degenerate oligonucleotide primers PdMfs-F and PdMfs-R was designed to amplify a partial *PdMfs1* gene. With these primers, a fragment of 526 bp DNA was amplified from genomic DNA of *P. digitatum* PdW03. The deduced amino acid sequence is highly similar to *Mfs1s* from other fungi. Thereafter, a TAIL-PCR was used to amplify the 5' and 3' ends of this genomic region, respectively, and fragments of 1,088 bp of 5' TAIL and 987 bp of 3' TAIL were amplified and sequenced. Sequence analysis revealed that this genomic region contained an open reading frame of 1,876 bp, with 3 introns of 55, 49 and 71 bp, located between positions 247-301 bp, 682-730 bp and 1,158-1,228 bp, respectively. We designated this cloned DNA fragment as the *PdMfs1* gene, being predicted to encode a major facilitator superfamily transporter 1 in *P. digitatum*. The structure of the*PdMfs1* gene was confirmed by sequencing the full-length cDNA, obtained by RT-PCR. The nucleotide sequence of *PdMfs1* gene was deposited in the GenBankas accession number AM412556.

The deduced amino acid sequence of the *PdMfs1* gene was aligned with several *Mfs1* genes from other fungi. It was found that *PdMfs1* shared 60, 60, 39, and 37% identity to the its homologous genes from *B. fuckeliana* (AF238225), *M. graminicola* (DQ661911), *C. kikuchii* (AF091042) and *C. carbonum* (L48797), respectively, indicating that the *PdMfs1* gene belongs to the MFS. Hydropathy analysis using the software/TMPRED (the Swiss Institute of Bioinformatics) revealed that *PdMfs1* has 14 putative trans-membrane domains.

Expression of *PdMfs1* after treated with imazalil and other toxic compounds

To examine if the expression of *PdMfs1* is inducible by imazalil and other toxic compounds, the expressions of *PdMfs1* pre- and post-treatments (1 h) of fungicides imazalil, propiconazole, mancozeb, and actidione, as well as toxic compound EB for both imazalil-resistant (Pd23, Figure 2A) and imazalil-sensitive (PdW03, Figure 3B) strains were compared. The results showed that *PdMfs1* expression in both strains were up regulated with a similar pattern by each of the five compounds (Figures 2A and B). The data also showed that levels of induced *PdMfs1* expression were associated with the species of compounds, but not with compound concentrations. Actidione had the strongest inducing effect, followed by imazalil and propiconazole (Figures 2A and B).

Creation and analysis of *PdMfs1* mutants

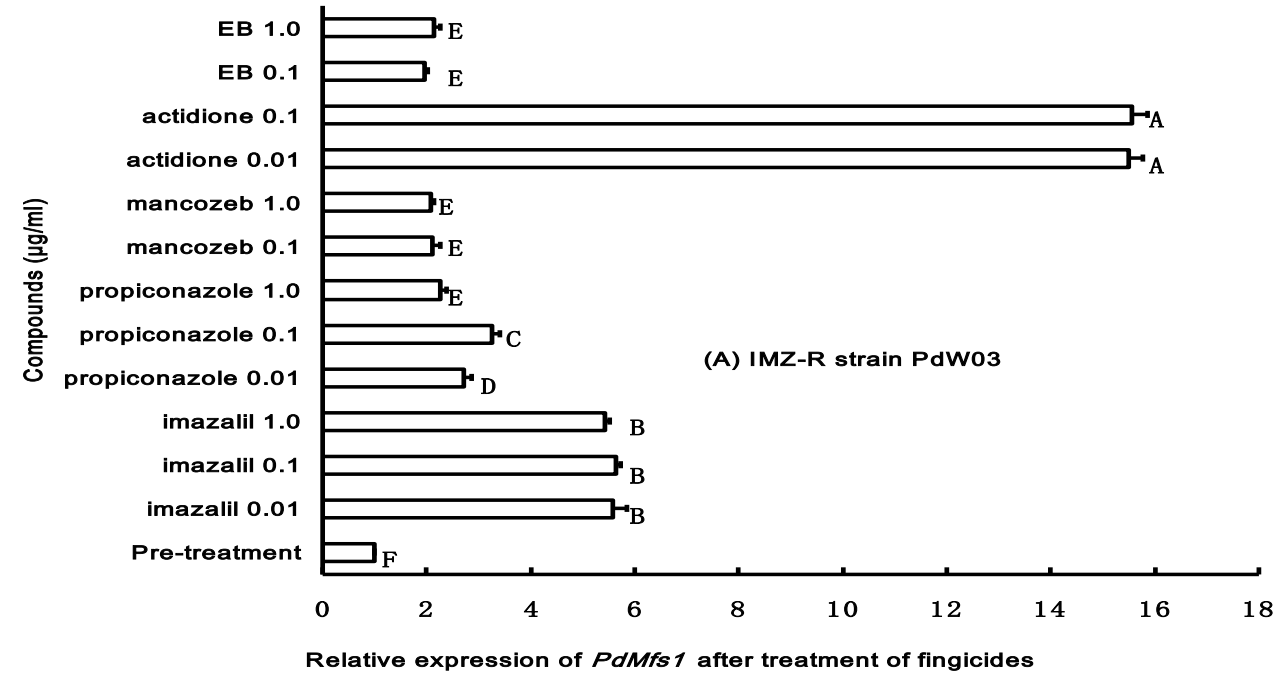
To evaluate the potential role of *PdMfs1* in resistance to imazalil, four *PdMfs1* disrupted mutants (Δ*PdMfs1*) and six over-expression mutants (OE*PdMfs1*), as well as three complement mutants (CP*PdMfs1*) of Δ*PdMfs1* were obtained by ATMT method. PutativeΔ*PdMfs1s* were identified with PCR using primers *PdMfs1* and *PdMfs2* (Figure 1A). In Δ*PdMfs1,* a fragment of 4 kb was amplified (Figure 1B, line 15-18), whereas in ectopic transformants (Ect*PdMfs1*), two fragments of 1.9 kb and 4kb, respectively, were obtained (lane 2-14). The fragments of 4, 1.7 and 1.9 kb were amplified from plasmid pTFCMΔ*PdMfs1* (lane 19), genomic cDNA (lane 20) and genomic DNA (lane 21) of parental isolate PdW03, respectively.

A disruption mutant Δ*PdMfs1*A was used to create the Δ*PdMfs1* complementation mutant. The full *PdMfs1* cDNA driven by P*trpC* and terminated by T*trpC* (plasmid pTBG-*PdMfs1*) was introduced into Δ*PdMfs1*A by ATMT mentioned previously. *PdMfs1* over-expression strains (OE*PdMfs1*) were created by introducing the plasmid pTFCM-*PdMfs1* into PdW03 by the same method. These mutants were initially confirmed by PCR (data not shown).

The correction of disrupted mutants (Δ*PdMfs1*A and Δ*PdMfs1*B), over-expression (OE*PdMfs1*A and OE*PdMfs1*B), complemented mutants (CP*PdMfs1*A and CP*PdMfs1*B), and ectopic transformant (Ect*PdMfs1*A) were further verified by southern blot analysis using *PdMfs1* gene as probe. The plasmid pTFCM transformants (PdpTFCM) of *P. digitatum*and PdW03 were used as control (Fig. 1C).

Role of *PdMfs1* on resistance to imazalil and other DMI fungicides

The resistance levels to imazalil of Δ*PdMfs1*A, OE*PdMfs1*A, CP*PdMfs1*A and Ect*PdMfs1*A mutant were compared with that of their parental strain PdW03, as well as imazalil-sensitive strain Pd23 of *P. digitatum* in imazalil-amended PDA. Our data showed that disruption of *PdMfs1* resulted in decreased resistance to imazalil,



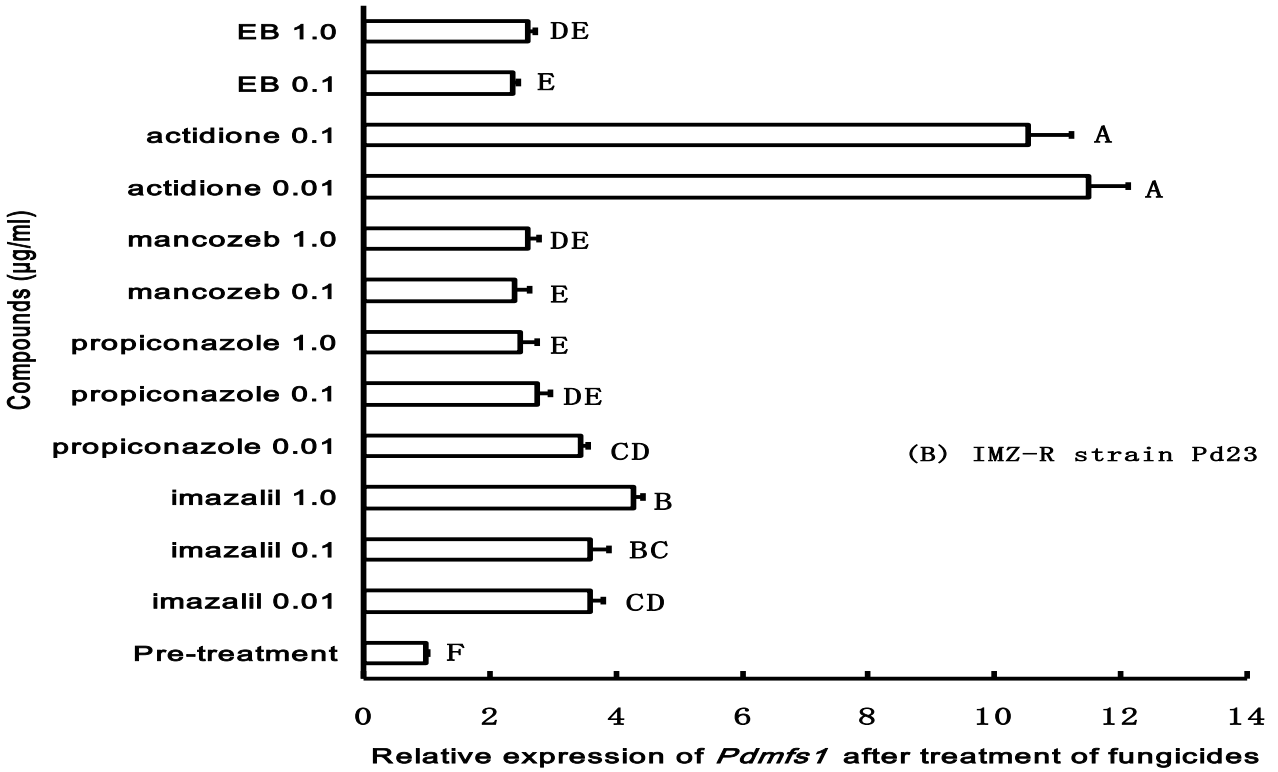


Figure 2. Expression of *PdMfs1* by treatment of imazalil and other toxic compounds. A, imazalil-sensitive strain Pd23; B, imazalil-resistant strain PdW03. Four-day mycelial cultures of *P. digitatum* were treated by adding indicated compounds at indicated concentrations, and incubated for 1 h. Total RNAs were extracted, Real-time RT-PCR was preformed to evaluate the relative expressions of *PdMfs1.* Bars represent the standard deviations of three replicates. Different letters show significant differences ($P<0.05$).

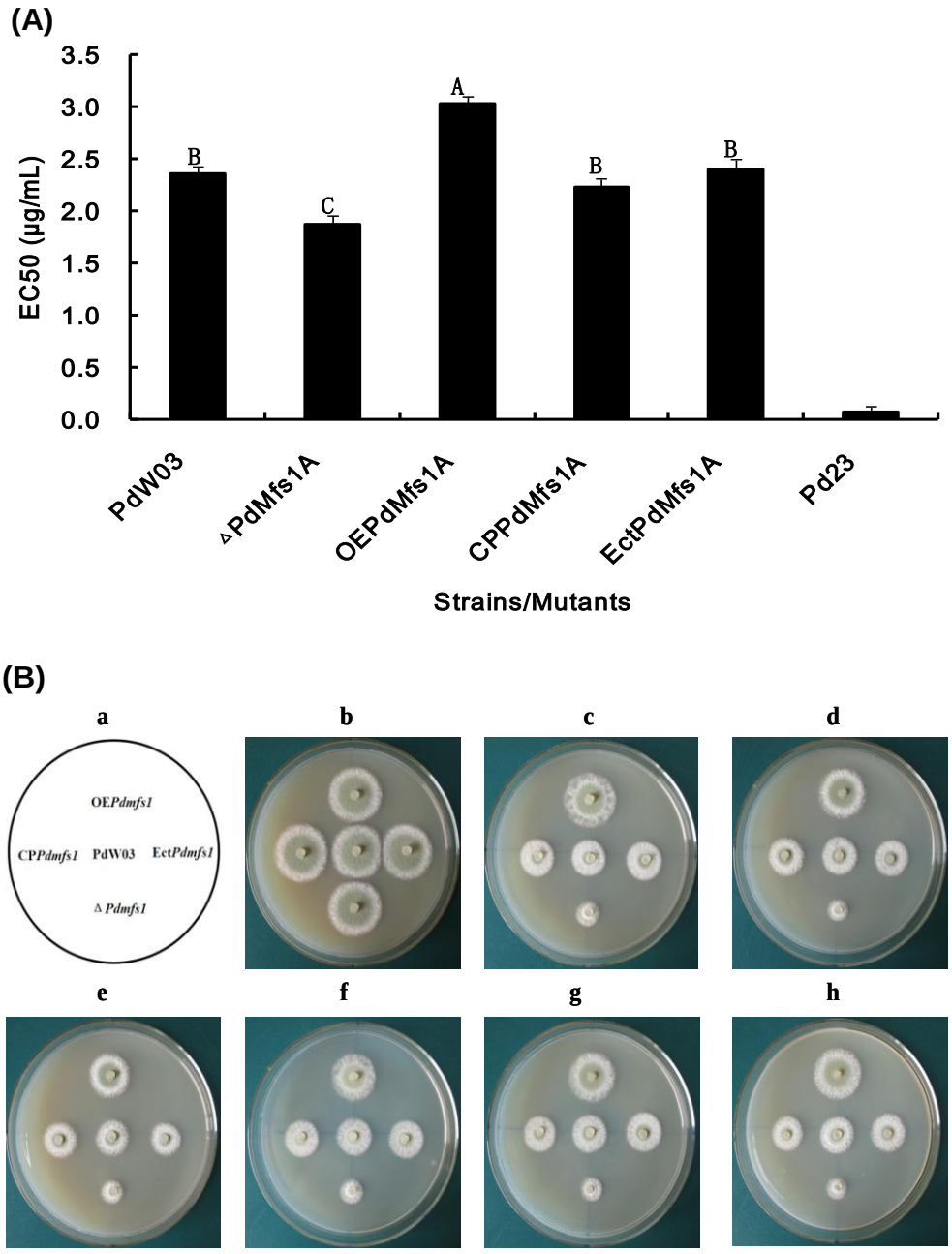


Figure 3. Resistance assay of *PdMfs1* mutants to imazalil and other DMIs. A, EC_{50} of *PdMfs1* mutants, PdW03 and Pd23. PdW03, parental strain; Δ*PdMfs1*A, *PdMfs1* disruption mutant; CP*PdMfs1*A, complementation mutant of Δ*PdMfs1*A; OE*PdMfs1*A, *PdMfs1*-overexpression strain; Ect*PdMfs1*A, ectopic mutant; PdpTFCM, transformant of the empty vector pTFCM; Pd23, imazalil-sensitive strain. B, illustration for resistance assay of imazalil and other DMIs; a, diagram of the layout of strains; b, control (fungicide-free medium); c, imazalil-amended (1.5 $\mu g \cdot mL^{-1}$), d, prochloraz-amended (0.15 $\mu g.mL^{-1}$); e, propiconazole-amended (0.5 $\mu g.mL^{-1}$); f, myclobutanil (2.0 $\mu g \cdot mL^{-1}$); g, ebuconazole (0.5 $\mu g.mL^{-1}$); h, difenoconazole (0.15, $\mu g.mL^{-1}$). Pictures were taken after three days of incubation

whereas the over-expression of *PdMfs1* had the opposite effect as indicated by change of their EC_{50} values (Figure 3 A) and their colonies formed on imazalil-amended (1.5 $\mu g \cdot mL^{-1}$) PDA (Figure 3B-b). The EC_{50} value of complemented mutant CP*PdMfs1*A against imazalil was similar to parental strain PdW03, indicating that decreased resistance for *PdMfs1* disruption mutant could be complemented by the introduction of *PdMfs1* gene

(Figure 3 A and B-b). These results indicate that *PdMfs1* is definitely involved in imazalil resistance in *P. digitatum*. However, the EC_{50} of Δ*PdMfs1*A was still much higher than that of imazalil-sensitivity strain Pd23 (Figure 3A), strongly suggesting that other resistant mechanism (s) must be involved the imazalil resistance in PdW03.

Similar to imazalil, the role of *PdMfs1* on resistance to other DMIs, including prochloraz, propiconazole, myclobutanil, ebuconazole, and difenoconazole were also confirmed (Figure 3B-d to B-h).

Role of *PdMfs1* on mycelial growth and conidiation

The role of *PdMfs1* on the mycelial growth and conidiation were evaluated by growing Δ*PdMfs1*A, OE*PdMfs1*A, CP*PdMfs1*A and Ect*PdMfs1*A, as well as PdW03 on PDA for five days, then measuring their diameters of colonies and estimating their conidiation. The results indicated that there was no significant difference found among the colony diameters and conidiation among these strains (Figure 3B), suggesting that the *PdMfs1* does not participate in the mycelial growth and conidiation of *P. digitatum*.

Role of *PdMfs1* on pathogenicity

The involvement of *PdMfs1* in virulence was evaluated by inoculating conidial suspensions of Δ*PdMfs1*A, OE*PdMfs1*A, CP*PdMfs1*A and Ect*PdMfs1*Amutants, as well as PdW03 strain on citrus fruits. The data showed that the disease developments on Δ*PdMfs1*-inoculated citrus fruits were slower than those inoculated with PdW03, OE*PdMfs1*A, and Ect*PdMfs1*A mutants (Figures 4A and B). The reduced virulence in Δ*PdMfs1* could be was rescued by reintroducing *PdMfs1* into Δ*PdMfs1*A, as shown in sizes of lesions induced by CP*PdMfs1*A (Figures 4A and B). This result indicates that *PdMfs1* plays a role on pathogenicity of *P. digitatum*.

DISCUSSION

The current investigation was aimed to understand if the MFS transporters Mfs1p of *P. digitatum* was involved in imazalil resistance as well as in virulence. To approach this purpose, *PdMfs1* was cloned, and a serials mutants containing *PdMfs1* disruption, complement, over-expression mutants were constructed for comparing their sensitivity to imazalil and other DMIs. The results demonstrated that *PdMfs1* contributes partial resistance to imazalil. Pathogenicity analysis indicated that *PdMfs1* also involved in the virulence of *P. digitatum*. To our best knowledge, this is the first gene in MFS transporter family cloned and functionally analyzed in *P. digitatum.*

In *M. graminicola,* the *MgMfs1* deletion mutant only displayed decreased resistance to strobilurin fungicides and to the host-specific mycotoxin cercosporin, but not to any other fungicides tested (Roohparvar et al., 2007). A similar result was observed in *B. cinerea.* The disruptants of *BcMfs1* also only exhibited reduced resistance to the alkaloid camptothecin and the perylene quinone cercosporin, but not to some fungicides (Hayashi et al., 2002a). The role of *BcMfs1* in transport of DMI fungicides only became obvious after functional inactivation of *BcatrD,* one of ABC transporters (Hayashi et al., 2002a), suggesting the functional redundancy of various transporters with an overlapping substrate specificity (Hayashi et al., 2001; Hayashi et al., 2002a). However, *BcMfs1*-mediated transport of DMIs could be supported by the fact that the *BcMfs1* over-expressing strains showed a significant increase in DMI resistance (Hayashi et al., 2002a). In *P. digitatum*, the *PdMfs1* disruption mutant decreased partial resistance to imazalil, and the decreased resistance could be rescued by reintroducing *PdMfs1* into Δ*PdMfs1*, while over-expression of *PdMfs1*would increase resistance (Figure 3), definitely supporting the role of *PdMfs1* on imazalil resistance. The functional variation among the gene family members might reflect the complexity of MFS transporters mediating the transportation of toxic compounds among organisms. As indicated in Figure 2, *PdMfs1* was up regulated by toxicants in both imazalil-resistant and sensitive strains (Figures 2A and 2B), suggesting that *PdMfs1*-based toxicant efflux mechanism was present in both in imazalil-resistant and sensitive strains of *P. digitatum*, and *PdMfs1* might only play a role on determining the baseline resistance to DMIs, as that summarized for ABC-based MDR (de Waard et al., 2006) and for *PMR1*, an ABC transporter gene of *P. digitatum*, but it might not act as a major determinant in DMI resistance (Hamamoto et al., 2001).

Although the resistance of Δ*PdMfs1* to imazalil was lower than that of the parental strain PdW03 (Figure 4), it was still much more resistant than that of the imazalil-sensitive strain Pd23 (Figure 4). Given that targeted disruption of *PdMfs1* resulted in only partial reduction in resistance of PdW03 strain to imazalil. It could be concluded that *PdMfs1*-based toxicant efflux is only a partial resistance mechanism responsible for imazalil-resistance in PdW03. So far, over-expression of the *PdCYP51* gene, which encodes 14-α-demethylase, the target enzyme of DMIs including imazalil, resulted from the insertion mutation in the promoter region of *PdCYP51* was reported to be responsible for imazalil resistance of field *P. digitatum* strains (Hamamoto et al., 2000; Li et al., 2003; Ghosoph et al., 2007; Chen et al., 2008). Moreover, the point mutation of *CYP51* gene was reported to confer DMI resistance in several other plant pathogens (Delye et al., 1997).

For *P. digitatum* PdW03, both point mutations and the insertion mutation in the promoter region of *CYP51* were not detected (Sun et al., 2011), suggesting that an unknown imazali resistance mechanism must be operate in this

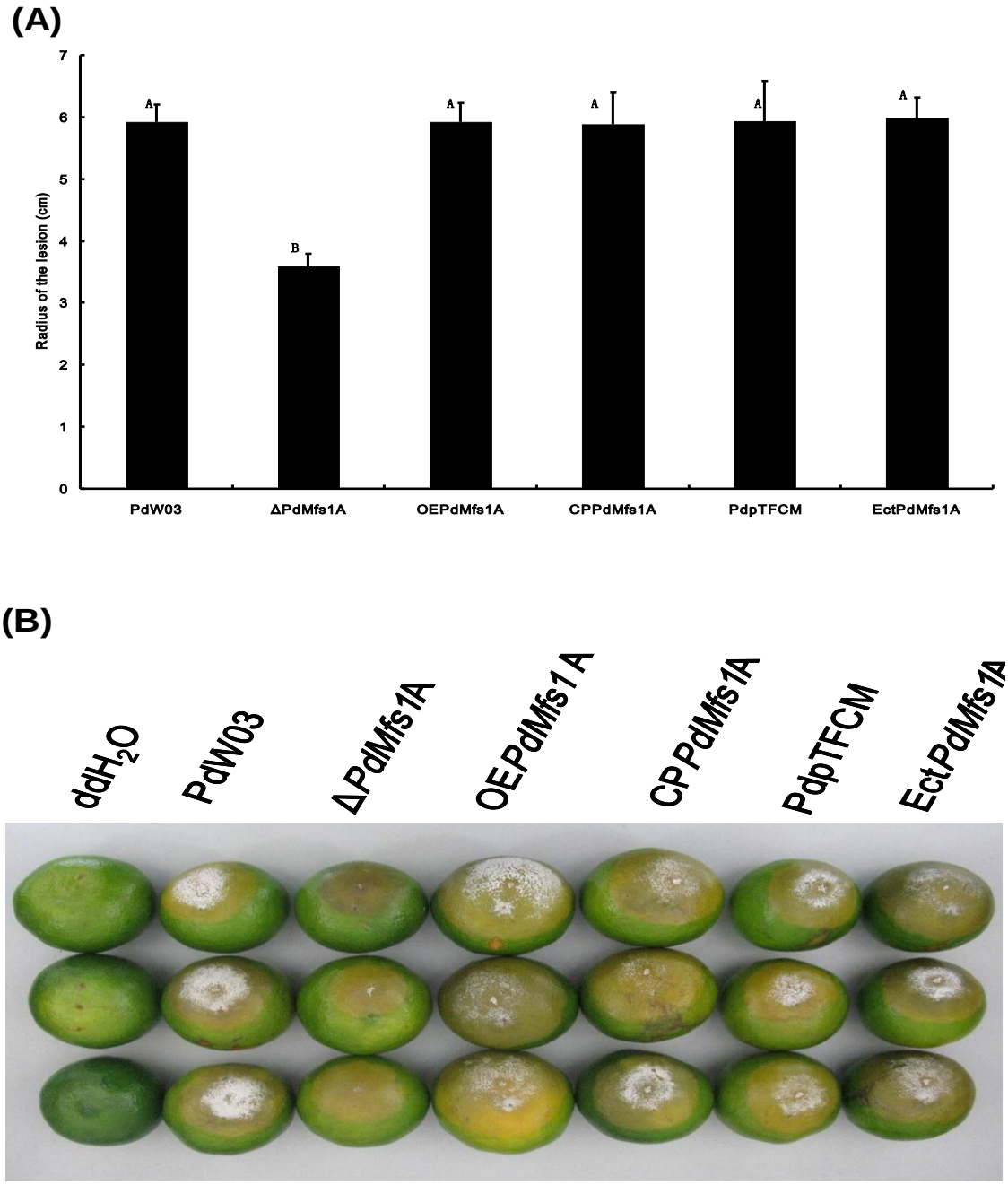


Figure 4. Virulence assay of *PdMfs1* mutants on citrus fruits. A, Radius of lesions at six days post-inoculation; B, illustration for virulence assay, picture taken at three days post-inoculation; PdW03, parental strain of *P. digitatum*; Δ*PdMfs1*A, *PdMfs1* disruption mutant; CP*PdMfs1*A, complementation mutant of Δ*PdMfs1*A; OE*PdMfs1*A, *PdMfs1*-overexpression mutant; Ect*PdMfs1*A, ectopic mutant.

strain. Recently, two new members of *CYP51* genes (assigned as *CYP51B* and *CYP51C*) were found from the EST library of *P. digitatum* completed recently by our lab (unpublished). We have found that over-expression of *CYP51B* gene resulted from the insertion mutation of a 199 bp in promoter region of CYP51B gene was account for the major mechanism of imazalil resistance for PdW03 (Sun et al., 2011).

A virulence assay showed that disease development on Δ*PdMfs1* inoculated citrus fruits was significantly slower than that on citrus fruits inoculated with parental strain PdW03, and full virulence could be restored by reintroducing the *PdMfs1* gene into Δ*PdMfs1*A (Figure 4), suggesting that *PdMfs1* plays a role on pathogencity. The

involvements of MFS transporters in virulence have been demonstrated for *Cfp* in *C. kikuchii* (Callahan et al., 1999), *Ctb4* in *C. nicotianae* (Choquer et al., 2007), *ToxA* in *C. carbonum* (Pitkin et al., 1996), but not for *BcMfs1* in *B. cinerea* (Hayashi et al., 2002a) and *MgMfs1* in *M. graminicola* (Roohparvar et al., 2007). *Cfp* and *Ctb4* encode a cercosporin efflux pump, while *ToxA* encodes an HC-toxin efflux pump that contributes to self-protection from cercosporin and HC-toxin by actively exporting cercosporin and HC-toxin, respectively, subsequently rendering these pathogens virulence on their host plants. Disruption of these pump genes drastically reduce the accumulation of related toxins and pathogenicity (Pitkin et al., 1996; Callahan et al., 1999; Choquer et al., 2007). However, there is little knowledge concerning the virulent mechanism of *P. digitatum* (Macarisin et al., 2007; Prusky et al., 2004). It is worthwhile to explore whether there is some kind of toxin secreted that acts as a virulence factor, as well as how *PdMfs1* plays a role in infection of *P. digitatum.*

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REFERENCES

Andrade AC, Del Sorbo G, Van Nistelrooy JG, Waard MA (2000). The ABC transporter *AtrB* from *Aspergillus nidulans* mediates resistance to all major classes of fungicides and some natural toxic compounds. Microbiology, 146: 1987-1997.

Callahan TM, Rose MS, Meade MJ, Ehrenshaft M, Upchurch RG (1999). CFP, the putative cercosporin transporter of *Cercospora kikuchii*, is required for wild type cercosporin production, resistance, and virulence on soybean. Mol. Plant Microbe Interact., 12: 901-910.

Chen GJ, Zhang ZF, Jiang LY, Xu FS, H, MZ, Li HY (2008). Real-time PCR assay for detection of the frequency of imazalil-resistance of *Penicillium digitatum*. Acta Phytopathologica Sinica 38, 561-569.

Choquer M, Lee MH, Bau HJ, Chung KR (2007). Deletion of a MFS transporter-like gene in *Cercospora nicotianae* reduces cercosporin toxin accumulation and fungal virulence. FEBS Lett., 581: 489-494.

de Waard MA, Andrade AC, Hayashi K, Schoonbeek HJ, Stergiopoulos I, Zwiers LH (2006). Impact of fungal drug transporters on fungicide sensitivity, multidrug resistance and virulence. Pest. Manage. Sci, 62: 195-207.

Delye C, Laigret F, CorioCostet MF (1997). A mutation in the 14 alpha-demethylase gene of *Uncinula necator* that correlates with resistance to a sterol biosynthesis inhibitor. Appl. Environ. Microbiol., 63, 2966-2970

Eckert JW (1987). *Penicillium digitatum* biotypes with reduced sensitivity to imazalil. *Phytopathol*., 77, 1728-1728.

Eckert JW, Sievert JR, Ratnayake M (1994). Reduction of imazalil effectiveness against citrus green mold in california packinghouses by resistant biotypes of *penicillium digitatum*. Plant Dis., 78, 971-974.

Gbelska Y, Krijger JJ, Breunig KD (2006). Evolution of gene families: the multidrug resistance transporter genes in five related yeast species. Fems Yeast Res., 6: 345-355.

Ghosoph JM, Schmidt LS, Margosan DA, Smilanick JL (2007). Imazalil resistance linked to a unique insertion sequence in the *PdCYP51* promoter region of *Penicillium digitatum*. Postharvest Biol. Technol., 44: 9-18.

Gulshan K, Moye-Rowley WS (2007). Multidrug resistance in fungi. Eukaryot Cell, 6: 1933-1942.

Hamamoto H, Hasegawa K, Nakaune R, Lee YJ, Makizumi Y, Akutsu K (2000). Tandem repeat of a transcriptional enhancer upstream of the sterol 14alpha-demethylase gene (*CYP51*) in *Penicillium digitatum*. Appl. Environ. Microbiol. 66: 3421-3426.

Hayashi K, Schoonbeek HJ, De Waard MA (2002a). *Bcmfs1*, a novel major facilitator superfamily transporter from *Botrytis cinerea*, provides tolerance towards the natural toxic compounds camptothecin and cercosporin and towards fungicides. Appl. Environ. Microbiol., 68: 4996-5004.

Hayashi K, Schoonbeek HJ, De Waard MA (2002b). Expression of the ABC transporter *BcatrD* from *Botrytis cinerea* reduces sensitivity to sterol demethylation inhibitor fungicides. Pest. Biochem. Physiol., 73: 110-121.

Hayashi K, Schoonbeek HJ, Sugiura H, De Waard MA (2001). Multidrug resistance in *Botrytis cinerea* associated with decreased accumulation of the azole fungicide oxpoconazole and increased transcription of the ABC transporter gene BcatrD. Pest. Biochem. Physiol., 70: 168-179.

Hiller D, Sanglard D, Morschhauser J (2006). Overexpression of the *MDR1* gene is sufficient to confer increased resistance to toxic compounds in Candida albicans. Antimicrob. Agents Chemother, 50: 1365-1371.

Kanetis L, Forster H, Adaskaveg JE (2007). Comparative efficacy of the new postharvest fungicides azoxystrobin, fludioxonil, and pyrimethanil for managing citrus green mold. Plant Dis. 91:1502-1511.

Li H, Xie Q, Song A (2003). Sequence comparison of CYP51 genes between imazalil-sensitive and imazalil-resistant strains of *Penicillium digitatum.* Mycosystema 22, 153–156

Macarisin D, Cohen L, Eick A, Rafael G, Belausov E, Wisniewski M (2007). *Penicillium digitatum* suppresses production of hydrogen peroxide in host tissue during infection of citrus fruit. Phytopathology, 97: 1491-1500.

Nakaune R, Adachi K, Nawata O, Tomiyama M, Akutsu K, Hibi T (1998). A novel ATP-binding cassette transporter involved in multidrug resistance in the phytopathogenic fungus *Penicillium digitatum*. Appl. Environ. Microbiol., 64: 3983-3988.

Nakaune R, Hamamoto H, Imada J, Akutsu K, Hibi T (2002). A novel ABC transporter gene, *PMR5*, is involved in multidrug resistance in the phytopathogenic fungus *Penicillium digitatum*. Mol. Genet. Genomics, 267: 179-185.

Perez-Tomas R (2006). Multidrug resistance: retrospect and prospects in anti-cancer drug treatment. Curr. Med. Chem., 13: 859-1876.

Pfaffl MW, Horgan GW, Dempfle L (2002). Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res., 30: 9 e36.

Pitkin JW, Panaccione DG, Walton JD (1996). A putative cyclic peptide efflux pump encoded by the *TOXA* gene of the plant-pathogenic fungus *Cochliobolus carbonum*. Microbiology, 142: 1557-1565.

Prasad R, Kapoor K (2005). Multidrug resistance in yeast Candida. Int. Rev. Cytol., 242: 215-248.

Roohparvar R, De Waard MA, Kema GH, Zwiers LH (2007). *MgMfs1*, a major facilitator superfamily transporter from the fungal wheat pathogen *Mycosphaerella graminicola*, is a strong protectant against natural toxic compounds and fungicides. Fungal Genet. Biol., 44: 378-388.

Sa-Correia I, dos Santos SC, Teixeira MC, Cabrito TR, Mira NP (2009). Drug:H+ antiporters in chemical stress response in yeast. Trends Microbiol., 17: 22-31.

Schoonbeek H, Del Sorbo G, De Waard MA (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.

Sipos G, Kuchler K (2006). Fungal ATP -binding cassette (ABC)

transporters in drug resistance & detoxification. Curr. Drug Targets, 7: 471-481.
Sun XP, Wang JY, Feng D, Li HY (2011). PdCYP51B, a new CYP51 gene of *Penicillium digitatum* involved in DMI fungicide resistance. Appl. Microbiol. Biotechnol., 91(4): 1107-1119
Vermeulen T, Schoonbeek H, De Waard MA (2001). The ABC transporter BcatrB from *Botrytis cinerea* is a determinant of the activity of the phenylpyrrole fungicide fludioxonil. Pest. Manage. Sci., 57: 393-402.
Wang X, Tryndyak V, Apostolov EO,Yin X, Shah SV, Pogribny IP (2008). Sensitivity of human prostate cancer cells to chemotherapeutic drugs depends on EndoG expression regulated by promoter methylation. Cancer Lett., 270: 132-143.
Zhang ZF, Wu KJ, Li HY (2008). Sensitivisty of Penicillium digitatum in Zhejiang to the strobilurin fungicide azoxystrobin. Chinese J. Pest. Sci., 3: 375-378.
Zwiers LH, Stergiopoulos I, Gielkens MM, Goodall SD, De Waard MA (2003). ABC transporters of the wheat pathogen *Mycosphaerella graminicola* function as protectants against biotic and xenobiotic toxic compounds. Mol. Genet. Genomics, 269:499-507.
Zwiers LH, Stergiopoulos I, Van Nistelrooy JG, De Waard MA (2002). ABC transporters and azole susceptibility in laboratory strains of the wheat pathogen *Mycosphaerella graminicola*. Antimicrob. Agents Chemother., 46:3900-3906.