Appl Microbiol Biotechnol (2011) 91:1107–1119
DOI 10.1007/s00253-011-3355-7

PdCYP51B, a new putative sterol 14α-demethylase gene of *Penicillium digitatum* involved in resistance to imazalil and other fungicides inhibiting ergosterol synthesis

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Received: 4 January 2011 / Revised: 23 April 2011 / Accepted: 25 April 2011 / Published online: 3 June 2011

Abstract *Penicillium digitatum*, causing green mold decay, is the most destructive postharvest pathogen of citrus fruits worldwide. The phenotypes and genotypes of 403 isolates of *P. digitatum*, collected from packing houses and supermarkets in Zhejiang, China, during 2000 to 2010, were characterized in terms of their imazalil sensitivity. The frequency of detected imazalil-resistant (IMZ-R) isolates increased from 2.1% in 2000 to 60–84% during 2005–2010. Only 6.5% and 4.5% of the collected IMZ-R isolates belong to the previously described IMZ-R1 and IMZ-R2 genotypes, respectively. To determine the resistance mechanism of the predominant and novel IMZ-R isolates of *P. digitatum* (termed IMZ-R3), genes *PdCYP51B* and *PdCYP51C*, homologous to the sterol 14α-demethylase encoded gene *PdCYP51*, were cloned from six IMZ-R3 and eight imazalil-sensitive (IMZ-S) isolates of *P. digitatum*. A unique 199-bp insertion was observed in the promoter region of *PdCYP51B* in all IMZ-R3 isolates examined but in none of the tested IMZ-S isolates. Further analysis by PCR confirmed that this insertion was present in all IMZ-R3 isolates but absent in IMZ-S, IMZ-R1, and IMZ-R2 isolates. Transcription levels of *PdCYP51B* in three IMZ-R3 isolates were found to be 7.5- to 13.6-fold higher than that in two IMZ-S isolates of *P. digitatum*. Introduction of another copy of $PdCYP51B^{s}$ (from IMZ-S) into an IMZ-S isolate decreased the sensitivity of *P. digitatum* to 14α-demethylation inhibitors (DMIs) only to a small extent, but introduction of a copy of $PdCYP51B^{R}$ (from IMZ-R3) dramatically increased the resistance level of *P. digitatum* to DMIs. Regarding *PdCYP51C*, no consistent changes in either nucleotide sequence or expression level were correlated with imazalil resistance among IMZ-R and IMZ-S isolates. Based on these results, we concluded that (1) the CYP51 family of *P. digitatum* contains the *PdCYP51B* and *PdCYP51C* genes, in addition to the known gene *PdCYP51A* (previously *PdCYP51*); (2) *PdCYP51B* is involved in DMI fungicide resistance; and (3) overexpression of *PdCYP51B* resulting from a 199-bp insertion mutation in the promoter region of *PdCYP51B* is responsible for the IMZ-R3 type of DMI resistance in *P. digitatum*.

Keywords *Penicillium digitatum* · CYP51 family · Gene mutation · Gene expression · Fungicide resistance · Demethylation inhibitors (DMIs)

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Introduction

China is one of the largest citrus-producing countries, ranked as the number one for the citrus-growing acreage and second to Brazil for the citrus yield in 2006. Green mold caused by *Penicillium digitatum* (Pers.: Fr.) Sacc. is the most destructive postharvest disease of citrus. It is responsible for about 90% of the losses during postharvest citrus packing, storage, transporting, and marketing (Kanetis et al. 2007; Macarisin et al. 2007). Immediate application of fungicides after harvesting is very effective and used worldwide for green mold control (Smilanick et al. 2005, 2006). In China, benzimidazoles, such as carbendazim, thiabendazole, and thiophanate methyl had

been used for green mold control before the mid-1990s. They were replaced gradually by imazalil (IMZ) and prochloraz due to the widespread emergence of benzimidazole resistance in *P. digitatum* (Jiang et al. 2010; Li et al. 2003; Zhu et al. 2006). Imazalil and prochloraz are currently the primary fungicides for postharvest green mold control of citrus in China.

Both imazalil and prochloraz belong to the demethylation inhibitors (DMIs) of ergosterol biosynthesis. They inhibit cytochrome P450-dependent sterol 14α-demethylase ($P450_{14\alpha dm}$) activity, following the stoichiometric interaction of the N-3 (imidazoles) or the N-4 (triazoles) substituents of the azole ring with the heme iron of the $P45014_{\alpha dm}$ (Yoshida and Aoyama 1987; Yoshida 1988). Because of its different mechanism of action, imazalil is effective against *P. digitatum* resistant to benzimidazole. Imazalil has been used for postharvest citrus disease control since 1976 (Harding 1976). As a consequence of continuous use, packing house imazalil-resistant isolates have occurred in China and elsewhere (Li et al. 2003; Chen et al. 2008; Rebellato and Monteiro 1984; Eckert 1987; Bus et al. 1991; Wild 1994; Sánchez-Torres and Tuset 2011), and the control efficiency has been compromised (Eckert et al. 1994; Jiang et al. 2010).

Previously, two similar types of mutations have been found to be associated with imazalil resistance of *P. digitatum*. These are: (1) the insertion of four extra tandem copies of a 126-bp transcriptional enhancer in the promoter region of the *CYP51A* gene, which resulted in the overexpression of *CYP51A* (IMZ-R1; Hamamoto et al. 2000); and (2) the insertion of a 199-bp sequence within the 126-bp transcriptional enhancer, which also resulted in the elevated expression of *CYP51A* (IMZ-R2; Ghosoph et al. 2007). In addition, overexpression of the ATP-binding cassette efflux transporters, *PMR1* and *PMR5*, was also shown to contribute to the resistance to imazalil and other fungicides in *P. digitatum* (Nakaune et al. 1998, 2002).

The resistance of *P. digitatum* to DMIs in Spain was evaluated recently (Sánchez-Torres and Tuset 2011). Regarding *CYP51* gene, only IMZ-R1 genotype was found in three of 58 IMZ-R isolates. Several mutations in promoter and the coding region of *PMR5* were found to be correlated with DMI fungicide resistance in IMZ-R isolates compared with IMZ-S ones. They also proved the *PMR1* gene might play a partial role in determining the baseline resistance to DMIs. However, more IMZ-R isolates indicated that other genes more than *CYP51* and *PMR5* could involve in DMI fungicide resistance.

In our previous study, both IMZ-R1 (Pd01) and IMZ-R2 (Pd19d) of *P. digitatum* were detected in Zhejiang province, China (Li et al. 2003; Zhu et al. 2006; Chen et al. 2008). However, further investigation indicated that the mechanism underlying imazalil resistance in many of the imazalil-resistant isolates collected in Zhejiang, especially from Quzhou, was neither IMZ-R1 nor IMR-R2, and the expression levels of *PdCYP51A* in these isolates were comparable to that of the IMZ-S ones examined. Additionally, point mutations associated with DMI resistance, as reported in other phytopathogenic fungi (Delye et al. 1997, 1998; Cools et al. 2002; Albertini et al. 2003; Fraaije et al. 2007; Leroux et al. 2007; Canas-Gutierrez et al. 2009; Stammler et al. 2009), were not detected in the promoter and the coding region of *PdCYP51A*. Thus, we concluded that a novel imazalil resistance mechanism, termed IMZ-R3, must be present in these resistant isolates.

Recently, the homologous *CYP51* genes found in some filamentous fungi were approved to contribute to the sensitivity to DMI fungicides in *Aspergillus fumigatus* (Martel et al. 2010) and *Fusarium graminearum* (Liu et al. 2011). Based on these studies, we cloned two homologous *CYP51* genes in *P. digitatum*, designated *PdCYP51B* and *PdCYP51C*. Further experiments indicated that a 199-bp insertion in the promoter region of the *PdCYP51B* gene that resulted in the overexpression of *PdCYP51B* was responsible for the observed resistance mechanism of IMZ-R3 isolates of *P. digitatum* to imazalil.

Materials and methods

Fungal isolates and cultivation

The monoconidial isolates used in this study were isolated from *P. digitatum*-infected citrus fruits collected from packing houses in Quzhou, Jinhua, Lishui, and Hangzhou of Zhejiang province, China, between 2000 and 2010, 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 conidial masses were harvested from colonies cultured on PDA at 25°C.

Determination of IMZ-S or IMZ-R phenotypes and resistance mechanisms of *P. digitatum*

The phenotype (imazalil-sensitive or imazalil-resistant) of the collected *P. digitatum* isolates was determined by growing them on imazalil-amended PDA as described previously (Ghosoph et al. 2007; Holmes and Eckert 1999). IMZ-S isolates were showing no or little growth after 5 days at 25°C on PDA amended with 0.1 μg/ml (or less) of IMZ. IMZ-R isolates were capable of growing after 5 days at 25°C on PDA with 0.5 μg/ml or more of IMZ. Each isolate was tested twice for IMZ sensitivity.

The genotypes or molecular mechanisms of imazalil resistance were determined using primer pair CYP51A1/

CYP51A2, designed specifically for the detection of IMZ-R1 (insertion of four extra tandem repeats of the 126-bp transcriptional enhancer in the promoter region of *PdCYP51A*) and IMZ-R2 (199-bp insertion mutation in the promoter region of *PdCYP51A* gene), respectively.

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 *PdCYP51B* gene are shown in Fig. 1.

Cloning of *PdCYP51B* and *PdCYP51C*

Based on *P. digitatum* transcriptome data (unpublished), we found two EST sequences, homologous to the formerly reported *CYP51* (*PdCYP51A*; GenBank, AJ439080.1). They were designated as *PdCYP51B* and *PdCYP51C*. According to the *Penicillium chrysogenum* genome sequence (NCBI genome project, NS_000201), the primer pairs CYP51B-F/CYP51B-R and CYP51C-F/CYP51C-R were used for amplifying the genomic DNA fragment, including the complete coding region and part of the upstream and downstream region sequences of *PdCYP51B* and *PdCYP51C*. The amplified fragments were cloned into the PMD18-T vector (TaKaRa Biotech. Co., Dalian, China) and sent for sequencing.

To determine the gene structure of *PdCYP51B* and *PdCYP51C*, total RNA was extracted using the Axy-PrepTM multisource total RNA miniprep Kit (Axygen, USA). First-strand cDNA was synthesized using the RNA PCR Kit (AMV) 3.0 kit (TaKaRa Biotech. Co.). The full-length *PdCYP51B* and *PdCYP51C* genes were amplified from the cDNA template, using primer pairs B-CDS-F/B-CDS-R and C-CDS-F/C-CDS-R.

Sequence analysis of *PdCYP51B* and *PdCYP51C*

PdCYP51B and *PdCYP51C* and their partial upstream and downstream regions were amplified, using primers pairs CYP51B-F/CYP51B-R and CYP51C-F/CYP51C-R, from eight IMZ-S isolates, six IMZ-R3 isolates, one IMZ-R1 isolate, and one IMZ-R2 isolate of *P. digitatum*. The obtained sequences were aligned by ClustalX 2.0 (http://www.clustal.org) for identification of DNA differences. The NSITE program (www.softberry.com) and the eukaryotic promoter predictor (Berkeley *Drosophila* Genome Project, http://www.fruitfly.org/seq_tools/promoter.html) were used to analyze the sequence of *PdCYP51B* gene. The protein

Table 1 Primers used in this study

Primer	Sequence (5′–3′)	Purpose
CYP51B-R	GAGGCTGGATGAGAGAAAGAAGGC	Amplification of *PdCYP51B*
CYP51B-F	TGGCGATCCCACCTTGTATGACAA	
CYP51C-F	AATGGAAAGACCATTGGCTCGG	Amplification of *PdCYP51C*
CYP51C-R	GCGAGCAGAGACGAGATAGATTGT	
CYP51B-EX-R	*CGAGCTCG*GAGGCTGGATGAGAGAAAGAAG	Construction of *PdCYP51B* overexpression plasmid; restriction sites for *Sac*I and *Kpn*I (underlined) were added
CYP51B-EX-F	*GGGGTACCCC*TGGCGATCCCACCTTGTATGACAA	
B1	TATAGCGACATTAGTTTGGC	Rapid identification of IMZ-R3 isolates
B2	AGGAAAGTTGCAGAGAGACCCAT	
CYP51A1	TAGCTCCAAAACAAATCGTCTGCC	Rapid identification of IMZ-R1 and IMZ-R2
CYP51A2	GGTGAAGATATTGCCGTACTAGAC	
qCYP51A-F	TCTCGTCATCGACAATGGTTCG	Quantitative analysis of *PdCYP51A* expression
qCYP51A-R	CAGGCCGTATTTGATGAACTTCTT	
qCYP51B-F	CACCCAAAGTCGTGCAAAGTAT	Quantitative analysis of *PdCYP51B* expression
qCYP51B-R	TTGACAAACTTCTTCTGCTCCA	
qCYP51C-F	TGAGAAGCTCCAGAAATTGATT	Quantitative analysis of *PdCYP51C* expression
qCYP51C-R	AAGCGACCTCATGAAGGGAAGA	
q2-actin-R	CCGCCAGACTCAAGACCAAGAAC	Quantitative analysis of actin gene expression
q2-actin-F	TCCACTACTGCCGAGCGTGAAAT	
B-CDS-F	ATGGGTCTCTCTGCAACTTTC	RT-PCR primers for amplifying the complete coding sequence of *PdCYP51B*
B-CDS-R	TTATGCCTTGACTCCACGTTTCT	
C-CDS-F	ATGCTGCAAAGCACCACTC	RT-PCR primers for amplifying the complete coding sequence of *PdCYP51C*
C-CDS-R	TCACTTCTTAACGAACTTGATTCC	
hph1	TTCGATGTAGGAGGGCGTGGAT	Amplifying partial sequence of *hph* gene
hph2	CGCGTCTGCTGCTCCATACAAG	

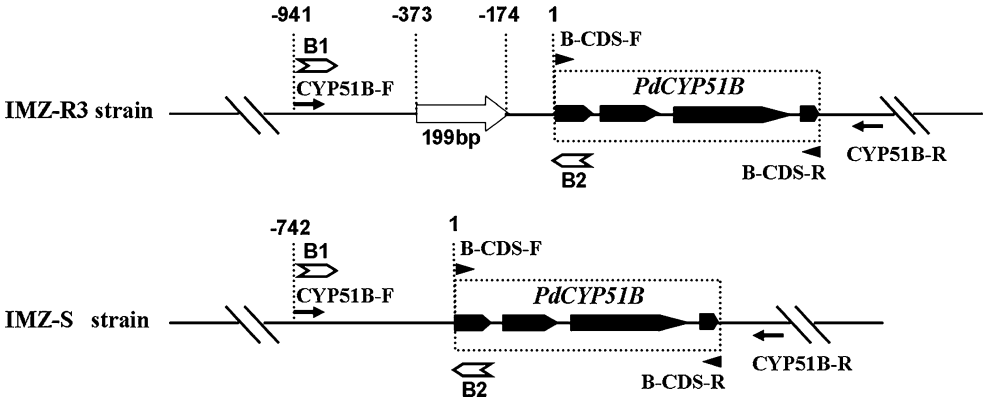

Fig. 1 Schematic diagram of the promoter and coding region of the *PdCYP51B* gene. The *blank arrow* represents the 199-bp insertion in the promoter region of the *PdCYP51B* gene. The *dotted box* represents the coding region of the *PdCYP51B* which contains four exons indicated by the *solid arrows* and three introns indicated by *solid lines* between the exons. The positions of selected primers are indicated in the diagrams

sequences obtained of genes *PdCYP51A*, *PdCYP51B*, and *PdCYP51C* and of related fungal and plant proteins obtained from the NCBI GenBank were aligned using ClustalX 2.0, and phylogenetic analysis of the protein sequences was conducted by MEGA 4.1 (http://www.megasoftware.net).

Gene expression analysis by real-time RT-PCR

The relative expression levels of *PdCYP51A*, *-B*, and *-C* of selected *P. digitatum* isolates with different levels of imazalil resistance were assayed by real-time reverse transcription PCR (RT-PCR) on a 7300 Real-Time PCR system (ABI, USA). Total RNA extraction and first-strand cDNA synthesis were described previously (Wang and Li 2008). Real-time PCR was carried out using the SYBR *Premix Ex Taq*™ (Perfect Real Time) kit (TaKaRa Biotech. Co.). The thermal cycling conditions were 95°C for 30 s, 40 cycles of 95°C for 5 s, and 60°C for 31 s. The primer pairs qCYP51A-F/qCYP51A-R, qCYP51B-F/qCYP51B-R, and qCYP51C-F/qCYP51C-R (Table 1) were designed for relative expression analysis of *PdCYP51A*, *PdCYP51B*, and *PdCYP51C*, respectively. The *P. digitatum* actin-encoding gene (GenBank, AB030227), amplified with primers q2-actin-R/q2-actin-F, was used to normalize the quantification of gene expression.

In all tests, negative controls containing no template cDNA were subjected to the same procedure to eliminate or identify any possible contamination. All genes were amplified in three separate reactions using the same cDNA template, and the mean values were obtained. Each experiment was conducted in triplicate. In the validation experiment, the log cDNA dilution of ten-fold serially diluted cDNA aliquots versus C_t, was used to calculate PCR efficiency of each gene according to the equation $E=10^{(-1/\text{slope})}$. The relative quantification of the target gene in comparison with the reference actin gene was calculated according to the formula, $\text{Ratio} = [(E_{\text{target}})^{\Delta C_t\text{target(control}-\text{sample)}}]/[(E_{\text{reference}})^{\Delta C_t\text{reference(control}-\text{sample)}}]$ (Pfaffl et al. 2002). ANOVA was applied to determine significant differences among the C_t of the examined isolates (Tang and Feng 2007).

Induced expression of *PdCYP51A*, *PdCYP51B*, and *PdCYP51C* of *P. digitatum* was studied in IMZ-S isolate PdKH8 by adding imazalil to a final concentration of 0.1 μg/ml to 4-day-old mycelial cultures of *P. digitatum* grown in liquid potato medium. After addition of imazalil, cultures were incubated at 25°C, 160 rpm for 6 h. Then, the mycelia were harvested by filtrating through a two-layer cheesecloth, washed with ddH_2O, frozen in liquid nitrogen, and stored at −80°C until RNA isolation.

Construction of the *PdCYP51B* overexpression plasmid

The full-length *PdCYP51B* as well as its upstream fragment was amplified from IMZ-R (PdW03) and IMZ-S (PdKH8) isolates using primer pair CYP51B-EX-R/CYP51B-EX-F, into which 5′-terminal *Sac*I and *Kpn*I restriction sites were introduced. The PCR products were digested with *Sac*I and *Kpn*I and cloned into the pTFCM to generate pTFCM-*PdCYP51B* (Fig. 2a).

P. digitatum transformation and analysis of transformants

The imazalil-sensitive isolate PdKH8 was used for *Agrobacterium tumefaciens*-mediated transformation (ATMT) with the *A. tumefaciens* strain AGL-1, as described previously (Wang and Li 2008), to create *PdCYP51B* overexpression mutants, called OE-*PdCYP51B*^{R} and OE-*PdCYP51B*^{S}. OE-*PdCYP51B*^{R} and OE-*PdCYP51B*^{S} were confirmed by PCR using primers B1/B2 (Table 1, Fig. 2b). Southern blot (Fig. 2c) was carried out as described previously (Wang and Li 2008). Briefly, genomic DNA (5–30 μg) of individual isolates was digested with *Kpn*I. Part of the hygromycin-resistant gene *hph* was used as a hybridization probe and labeled with digoxigenin using the DIG High Primer DNA Labeling and Detection Starter

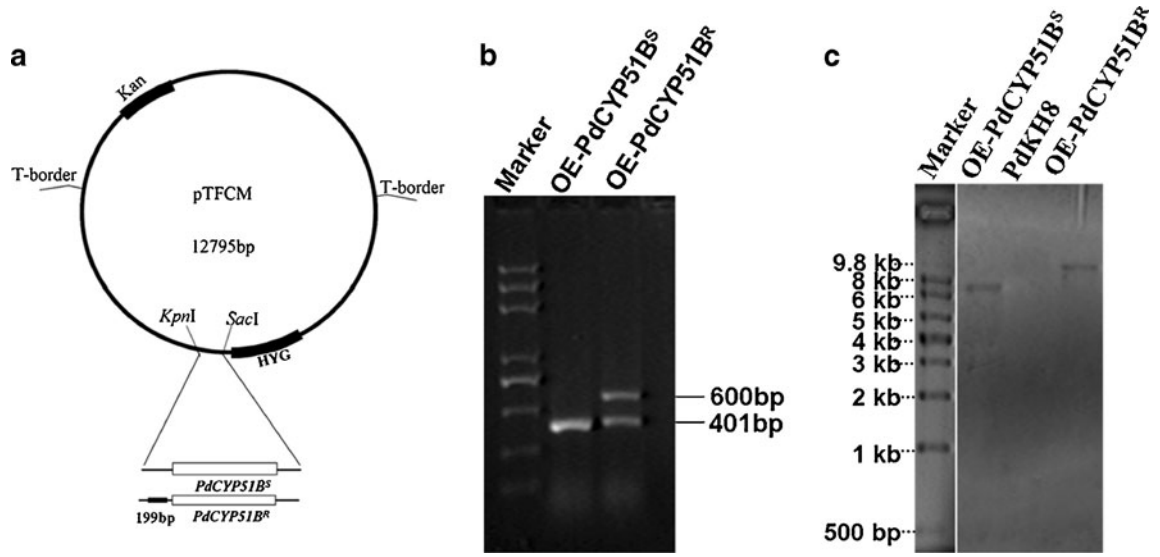

Fig. 2 Construction and identification of *PdCYP51B* overexpression mutants. **a** Diagram for construction of the *PdCYP51B* overexpression plasmid. *PdCYP51B*^{S} and *PdCYP51B*^{R} were inserted into *Sac*I and *Kpn*I sites of plasmid pTFCM, respectively, to produce the corresponding pTFCM-*PdCYP51B*^{S} and pTFCM- *PdCYP51B*^{R} constructs. **b** PCR identification with primers B1 and B2 of *PdCYP51B* overexpression mutants OE-*PdCYP51B*^{S} and OE-*PdCYP51B*^{R}. **c** Southern blot analysis of OE-*PdCYP51B*^{S} and OE-*PdCYP51B*^{R}. The fungal DNA was digested by *Kpn*I, and *hph* (hygromycin-resistant gene) was used as the probe

Kit II (Roche, Mannheim, Germany) according to the manufacturer's instructions.

DMI resistance assays

The resistant levels of mutants OE-*PdCYP51B*^{S} (its exogenous *PdCYP51B* was derived from an IMZ-S isolate), OE-*PdCYP51B*^{R} (its exogenous *PdCYP51B* derived from an IMZ-R3 isolate), and the parental isolate PdKH8 (imazalil-sensitive) to imazalil and other DMIs were assayed on PDA containing imazalil and other three DMIs, as described previously. The following fungicide concentrations were used at 0.04, 0.1, and 1 μg/ml for imazalil; 0.02, 0.05, and 0.2 μg/ml for difenoconazole; 0.2, 0.3, and 0.7 μg/ml for tebuconazole; and 0.3, 0.5, and 2.5 μg/ml for myclobutanil. The assays were conducted three times.

Table 2 Isolates of *P. digitatum* collected from Zhejiang during 2000 to 2010

Year	Number of isolates, percentage of indicated phenotype isolates				
	Subtotal	IMZ-S[a] (%)	IMZ-R (%)		
			IMZ-R1[b] (%)	IMZ-R2[c] (%)	IMZ-R3[d] (%)
2000	189	185 (98)	4 (2)	0 (0)	0 (0)
2005	25	10 (40)	0 (0)	3 (12)	12 (48)
2006	45	7 (16)	0 (0)	0 (0)	38 (84)
2007	7	2 (29)	0 (0)	0 (0)	5 (71)
2008	67	17 (25)	6 (9)	4 (6)	40 (60)
2010	70	28 (40)	0 (0)	0 (0)	42 (60)
Total	403	249 (62)	10 (2)	7 (2)	137 (34)

[a] IMZ-S, imazalil-sensitive isolates incapable of growing on PDA containing imazalil at 0.1 μg/ml; IMZ-R, imazalil-resistant isolates capable of growing on PDA containing imazalil at 0.5 μg/ml

[b] IMZ-R1, the imazalil-resistant isolates with the resistance mechanism of four additional 126-bp transcription enhancer insertions in the promoter region of *PdCYP51A*

[c] IMZ-R2, the imazalil-resistant isolates with resistance mechanism of 199-bp insertion within the 126-bp transcription enhancer in the promoter region of *PdCYP51A*

[d] IMZ-R3, imazalil-resistant isolates with the resistance mechanism of 199-bp insertion in the promoter region of *PdCYP51B*

PCR-based rapid detection of imazalil resistance in *P. digitatum*

To establish a rapid molecular method to detect imazalil resistance of *P. digitatum* and their genotypes of resistance, the primer pair B1/B2 was designed (Table 1). By combining primers CYP51A1/CYP51A2 (Table 1) that were used to distinguish the IMZ-R1 and IMZ-R2 (Chen et al. 2008), a duplex PCR was developed to detect imazalil-resistant genotypes. Rapid DNA extraction was conducted according to the method described by Chen et al. (2008). PCR was performed with a total volume of 20 μl containing 2 μl *Taq* Buffer (Mg^{2+} plus), 2 μl dNTP (2.5 mM), 1 μl of each of four primers (5 μM), 0.1–1 μg DNA template, 0.2 μl Taq polymerase (5 U/μl), and distilled water up to 20 μl. A gradient PCR with thermal cycling conditions 95°C 3 min, 32 cycles of 95°C 40 s, 55–62°C 40 s, and 72°C 1 min was conducted to confirm the optimal annealing temperature of the mixed primers.

Results

Sensitivity to imazalil and resistance mechanisms of *P. digitatum*

In total, 403 isolates of *P. digitatum* collected during 2000 to 2010 were tested for their IMZ-S or IMZ-R phenotypes (Table 2). One hundred twenty isolates were from 15 supermarkets in Hangzhou in 2000; 12 isolates were from four packing houses in Jinhua in 2005, 6 isolates from two packing houses in Lishui in 2005, and the other 265 isolates were from 22 packing houses in Quzhou during 2000 to 2010, the main citrus-producing region in Zhejiang. No sequential year sampling was taken in Quzhou. The result showed that IMZ-R1 isolate was firstly detected Quzhou and Hangzhou in 2000 (Zhu et al. 2006), then detected again in Quzhou in 2008, accounting for 6.5% of the total IMZ-R. IMZ-R2 isolates were firstly found in Jinhua in 2005, then in Quzhou in 2008, representing 4.5% of the total IMZ-R, whereas the unknown resistant genotype termed as IMZ-R3 was predominant (89%) in the IMZ-R population (Table 2).

Cloning and sequencing of *PdCYP51B* and *PdCYP51C*

To explore whether any homologous genes of *CYP51*, as reported in *A. fumigatus* (Mellado et al. 2001), were present in *P. digitatum*, we manually checked our annotated *P. digitatum* transcriptome database (unpublished) and found two EST sequences which were designated *CYP51B* and *CYP51C*. To obtain the full as well as the partial sequences upstream and downstream of the *PdCYP51B* and *PdCYP51C* genes, primer pairs of CYP51B-R/CYP51B-F and CYP51C-R/CYP51C-F were designed according to the corresponding sequences in *P. chrysogenum*. Results indicated that the cloned fragment with CYP51B-R/CYP51B-F from IMZ-S isolate PdKH8 contained 2,919 bp, encompassing a complete coding region (1,751 bp) and its upstream (742 bp) and downstream (425 bp) sequences. RT-PCR using primers B-CDS-F/B-CDS-R verified that this gene contained an opening reading frame (ORF) of 1,751 bp, with three introns of 73, 51, and 52 bp, located between positions 989–1,061; 1,260–1,310; and 2,376–2,427 bp, respectively (Fig. 1).

PdCYP51C and partial sequences of its upstream and downstream were obtained using the same method. The cloned fragment with CYP51C-R/CYP51C-F had 2295 nt, containing an ORF of 1,497 bp, with five introns located

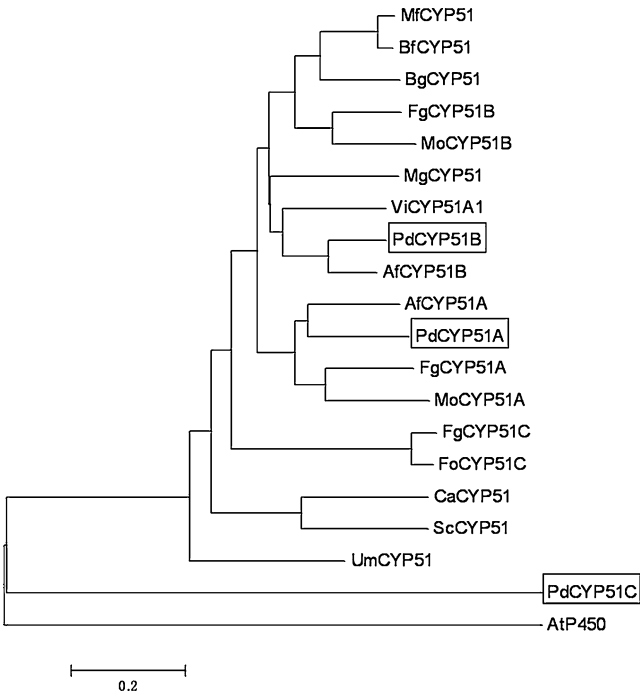

Fig. 3 Phylogenetic tree of *PdCYP51A*, *PdCYP51B*, and *PdCYP51C* with other CYP51s. Phylogenetic tree was generated by the bootstrapped neighbor-joining method with Mega 4.1 software on the basis of deduced amino acid sequences of *PdCYP51A* (BAB03658.1), *PdCYP51B* (HQ724322), and *PdCYP51C* (HQ724324) from *P. digitatum* isolate KH8 and those from fungal species *A. fumigatus* (AfCYP51A, XP_752137.1; AfCYP51B, XP_749134.1), *Monilinia fructicola* (MfCYP51, ACY41226.1), *Mycosphaerella graminicola* (MgCYP51, AAP79601.1), *F. graminearum* (FgCYP51A, FGSG_04092, FgCYP51B, FGSG_01000, FgCYP51C, FGSG_11024), *F. oxysporum* (FoCYP51C, FOXG_13138), *Blumeria graminis* (BgCYP51, CAE17515.1), *C. albicans* (CaCYP51, BAB03399.1), *Botryotinia fuckeliana* (BfCYP51, AAK26391.1), *S. cerevisiae* (ScCYP51, DAA06695.1), *Ustilago maydis* (UmCYP51, CAA88176.1), *Arabidopsis thaliana* (AtP450, AAD30262.1), *Venturia inaequalis* (ViCYP51A, AAF76464.1), and *M. oryzae* (MoCYP51A, MGG_04628.6; MoCYP51B, MGG_04432.6). The CYP51s from *P. digitatum* are indicated in *boxes*

between 541–596, 682–733, 823–885, 1,177–1,242, and 1,292–1,348 bp, respectively (data not shown). The nucleotide sequences of *PdCYP51B* and *PdCYP51C* genes were deposited in the GenBank with accession number HQ724322 and HQ724324.

Phylogenetic analysis (Fig. 3) showed that the deduced amino acid sequence of PdCYP51B was 78% and 61% identical to AfCYP51B of *A. fumigatus* (GenBank, XP_749134.1) and FgCYP51B of *F. graminearum* (Broad Institute, FGSG_01000), respectively. The PdCYP51C showed 19% identity to FgCYP51C of *F. graminearum* (FGSG_11024) and FoCYP51C of *Fusarium oxysporum* (FOXG_13138). Moreover, PdCYP51B was 59% and 22% identical to PdCYP51A and PdCYP51C, respectively, while PdCYP51C was 23% identical to PdCYP51A (data not shown). The percentage of identity at the amino acid level was high enough to consider PdCYP51B a member of the fungal CYP51 family.

Presence of a unique 199-bp insertion in the promoter region of *PdCYP51B* in IMZ-R3 of *P. digitatum*

PdCYP51B and *PdCYP51C* were amplified from eight IMZ-S isolates (PdHL5, PdHL6, PdKH1, PdJ1, PdYX1, PdYX2, PdW07, and PdKH8), one IMZ-R1 (Pd01) isolate, one IMZ-R2 (Pd19d) isolate, and six IMZ-R3 isolates (PdXJ2, PdDQ8, PdXJ3, PdW03, PdQZ15, and PdXJ8) and sequenced. Alignment of nucleotide sequences of these *PdCYP51B* genes showed that an extra 199-bp fragment insertion was present at the position −174 bp (from ATG) of the *PdCYP51B* promoter region in all six IMZ-R3 isolates but was absent in both IMZ-R1 and IMZ-R2, as well as in all eight IMZ-S isolates (Figs. 1 and 4). Apart from this difference, no point mutations that related to imazalil resistance were detected either in the encoding or in the promoter regions of *PdCYP51B* of *P. digitatum* (data not shown).

PdCYP51C was also amplified from the above 16 isolates. However, neither an insertion mutation nor a consistent point mutation was detected in the coding or the promoter regions that correlated with imazalil resistance (data not shown).

To test if the 199-bp insertion in the promoter region of *PdCYP51B* was universal in all collected IMZ-R3 isolates of *P. digitatum*, the primer pair B1/B2 (Table 2) was used for PCR amplification from the following *P. digitatum* isolates: 36 IMZ-R3, 3 IMZ-R1, 3 IMZ-R2, and 20 IMZ-S. As expected, a 600-bp fragment was obtained from all 36 IMZ-R3, while a 400-bp fragment was amplified from the other isolates of *P. digitatum*, indicating that this 199-bp insertion was universal in IMZ-R3, and it must be correlated with imazalil resistance.

Bioinformatical analysis of the 199-bp insertion sequence

Analyzing the 199-bp insertion sequence (Fig. 4), we found a 6-bp target site duplication (TSD) and the imperfect terminus inverted-repeat (TIR) sequences. Moreover, four sites for known fungal transcriptional binding factors (*Aspergillus nidulans* abaA, *Neurospora crassa* CYS-3, *Saccharomyces cerevisiae* Reb1, and *S. cerevisiae* CLN3) and 11 sites for different vertebrate binding factors (data not shown) were predicted within the 199-bp insertion by NSITE program (Fig. 4). A putative promoter region was also found by the eukaryotic promoter predictor (Fig. 4).

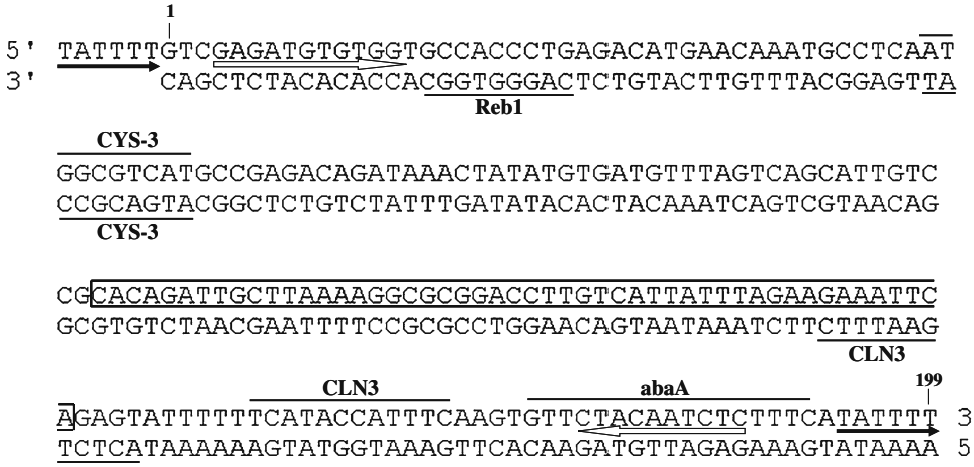

Fig. 4 Analysis of the 199-bp insert. Transcription factor binding sites within the 199-bp sequence found by means of a computer search on the regulatory element database using the NSITE program (www.softberry.com). Promoter analysis was performed by eukaryotic promoter predictor (Berkeley Drosophila Genome Project, http://www.fruitfly.org/seq_tools/promoter.html). The sites found in the *upper strand* are shown *above* the sequence, and the sites in the *lower strand* are shown *below* the sequence. The *box* shows the putative promoter region. The TSD and imperfect TIR are indicated by the *solid* and *blank arrows*, respectively

Table 3 Relative expression of the *PdCYP51A*, *PdCYP51B*, and *PdCYP51C* genes

Isolate	C_t values[a]			Fold change[b]			
	PdCYP51A[c]	*PdCYP51B*[c]	*PdCYP51C*[c]	Actin gene[c]	*PdCYP51A*	*PdCYP51B*	*PdCYP51C*
PdKH8(S)	15.10	14.90	17.45	10.65	0.009	0.013	0.004
PdKH1(S)	15.81	16.21	16.86	11.10	0.007	0.007	0.007
Mean (S)					0.008±0.001	0.010±0.004	0.0055±0.004
Pd01(R1)	10.37	14.45	19.11	11.40	0.388	0.030	0.002
Pd19d(R2)	9.98	14.12	17.15	10.93	0.370	0.027	0.005
Mean (R1+R2)					0.379±0.013	0.0285±0.002	0.0035±0.002
PdXJ8(R3)	15.35	11.52	17.48	10.25	0.006	0.098	0.003
PdDQ8(R3)	16.09	12.38	17.97	10.71	0.005	0.075	0.003
PdW03(R3)	14.26	11.51	17.91	10.72	0.017	0.136	0.003
Mean (R3)					0.0093±0.007	0.103±0.030	0.003
NTC	29.42	30.15	30.89	32.26			

[a] C_t values are the means of three experiments. Each experiment contains three replicates

[b] Fold change (±SD) is the amount of relative gene expression of *PdCYP51A*, *PdCYP51B*, and *PdCYP51C* vs. the relative expression of the actin reference gene. The average C_t values without template control (*NTC*) were normalized to the average C_t values of an endogenous housekeeping gene for actin using formula, Ratio $= \left[(E_{\text{target}})^{\Delta C_t\text{target(control}-\text{sample)}}\right] / \left[(E_{\text{actin}})^{\Delta C_t\text{actin(control}-\text{sample)}}\right]$

[c] The calculated PCR efficacy of *PdCYP51A*, *PdCYP51B*, *PdCYP51C*, and the actin gene are 1.999, 1.967, 1.954, and 1.970, respectively

Expression of *PdCYP51B* is constitutively higher in IMZ-R3 isolates of *P. digitatum*

To explore if the 199-bp insertion changed the expression of the *PdCYP51B* gene, the relative expression levels of *PdCYP51B* of three IMZ-R3 isolates (PdW03, PdXJ8, and PdDQ8), two IMZ-S isolates (PdKH8 and PdKH1), one IMZ-R1 isolate (Pd01), and one IMZ-R2 isolate (Pd19d) were determined by qPCR. The results showed that the transcription levels of *PdCYP51B* in three IMZ-R3 isolates were about 7.5–13.6 times higher than that of two IMZ-S isolates (Table 3, $P<0.01$). However, no significant expression differences of *PdCYP51A* and *PdCYP51*C were observed among these three IMZ-R3 isolates and two IMZ-S isolates tested (Table 3), indicating that constitutive overexpression of the *PdCYP51B* gene was correlated with the presence of 199 bp insertion in the promoter region of *PdCYP51B*. Additionally, the expression level of *PdCYP51A* instead of *PdCYP51B* and *PdCYP51C* in Pd01 and Pd19d was significantly higher than that of IMZ-S and IMZ-R3 of *P. digitatum* (Table 3), consistent with previous reports by Hamamoto et al. (2000) and Ghosoph et al. (2007).

PdCYP51A, *PdCYP51B*, and *PdCYP51C* are inducible by imazalil

To examine if the expression of *PdCYP51A*, *PdCYP51B*, and *PdCYP51C* was inducible by imazalil, the expression levels of these genes pre- and posttreated (6 h) with 0.1 μg/ml imazalil were compared in the imazalil-sensitive isolate PdKH8. The results showed that all of these genes were significantly upregulated after imazalil treatment (Fig. 5, $P<0.05$).

Overexpression of *PdCYP51B* leads to reduced sensitivity of *P. digitatum* to imazalil

To determine if overexpression of *PdCYP51B* could lead to reduced sensitivity of *P. digitatum* to imazalil, the full-length *PdCYP51B*, including the complete coding region and the promoter region, was amplified from PdKH8 (termed $PdCYP51B^S$) and PdW03 (termed $PdCYP51B^R$) using primers CYP51B-EX-R/CYP51B-EX-F and introduced into PdKH8 by ATMT. Selected transformants were

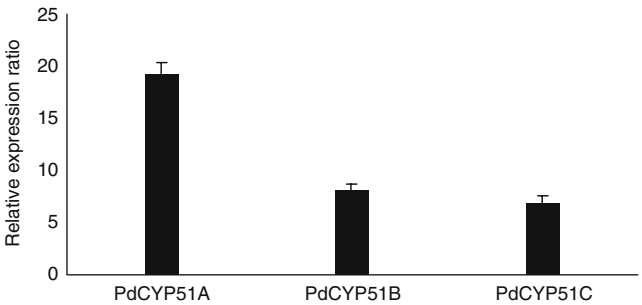

Fig. 5 Relative expression levels of *PdCYP51A*, *PdCYP51B*, and *PdCYP51C* in *P. digitatum* after treatment with imazalil. The IMZ-S isolate PdKH8 was treated with 0.1 μg/ml imazalil for 6 h. The relative expression of *PdCYP51A*, *PdCYP51B*, and *PdCYP51C* in treated and untreated PdKH8 were compared

verified by PCR with primers B1/B2. For OE-PdCYP51B^S transformants, one 401-bp fragment was amplified, whereas, in the OE-*PdCYP51B*^{R} transformant, two fragments of 401 and 600 bp were amplified (Fig. 2b). Southern blot using portion of the *hph* gene (626 bp) as a probe demonstrated that a single target gene insertion occurred in both OE-*PdCYP51B*^{S} and OE-*PdCYP51B*^{R}.

Resistance of OE-*PdCYP51B*^{S} and OE-*PdCYP51B*^{R} mutants, as well as the parental isolate PdKH8 of *P. digitatum* to DMI fungicides, was compared on PDA

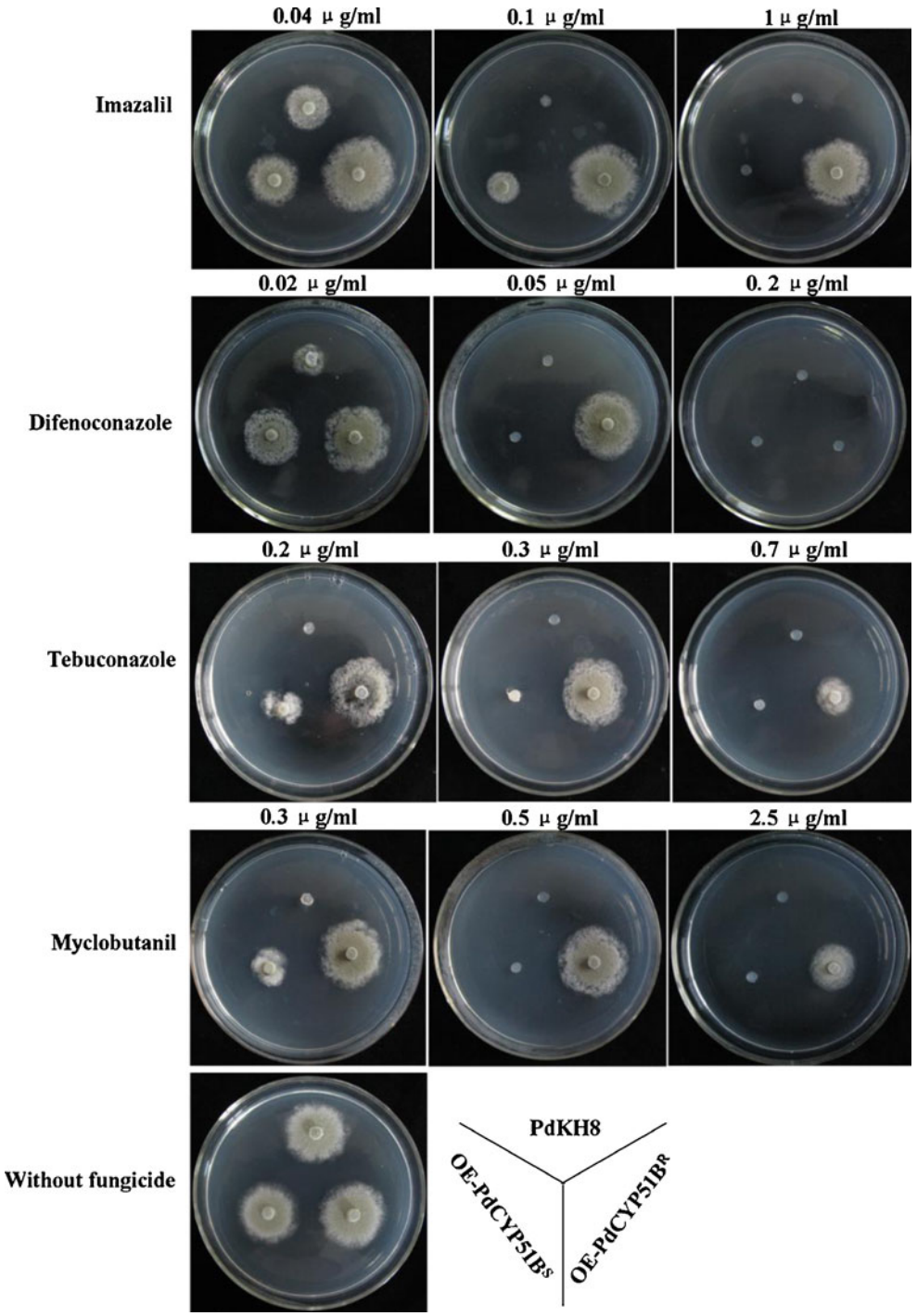

Fig. 6 Comparisons of sensitivity to different DMI fungicides among mutants of OE-*PdCYP51B* and their parental isolate PdKH8 of *P. digitatum*. OE-*PdCYP51B*^{S}, the overexpression mutant of the *PdCYP51B* gene amplified from IMZ-S *P. digitatum*; OE-*PdCYP51B*^{R}, the overexpression mutant of the *PdCYP51B* gene amplified from IMZ-R3 *P. digitatum*; *PdKH8*, the parental isolate. Concentrations of DMI fungicides are indicated on the *top* of each plate, whereas the fungicides are presented in *left* column

containing different concentrations of imazalil, difenoconazole, tebuconazole, and myclobutanil, respectively. The results showed that both *PdCYP51B*^{S} and *PdCYP51B*^{R} confer PdKH8 with increased DMI fungicide resistance; however, the effect of *PdCYP51B*^{R} was significantly higher than that of *PdCYP51B*^{S} (Fig. 6), indicating that expression levels of *PdCYP51B* had a major role in determining the sensitivity of *P. digitatum* to DMI fungicides and that the insertion of 199 bp in the promoter region of *PdCYP51B* indeed significantly increased the resistance of *P. digitatum* to imazalil and other DMI fungicides.

PCR-based rapid detection of DMI-resistant phenotypes and their resistant genotypes in *P. digitatum*

Based on the discovered molecular mechanisms of DMI resistance of *P. digitatum*, a PCR-based rapid detection for DMI-resistant genotypes was developed by using primer pair CYP51A1/CYP51A2 (Chen et al. 2008) together with B1/B2 (this study). Optimization of annealing temperature through gradient PCR indicated that 56°C was the ideal annealing temperature to obtain the best amplification in terms of specificity and efficiency. With these pairs of primers, specific fragments of 401 and 506 bp were generated from DMI-sensitive isolates (Fig. 7, lines 4 and 5); 401 and 1,010-bp fragments were amplified from IMZ-R1 isolates (Fig. 7, lines 1 and 5); 401 and 705-bp fragments were obtained from IMZ-R2 isolates (Fig. 7, lines 2 and 5); and fragments of 506 and 600 bp were amplified from IMZ-R3 isolates (Fig. 7, lines 3 and 4).

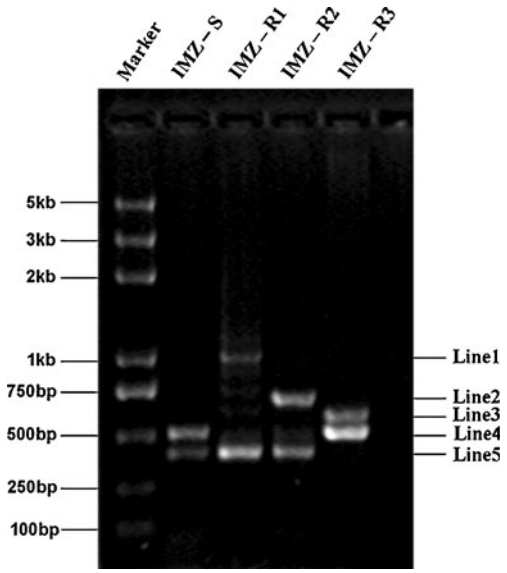

Fig. 7 PCR-based rapid detection of DMI resistance in *P. digitatum* strains. PCR was performed using primers CYP51A1/CYP51A2 and B1/B2 with the annealing temperature of 56°C

Discussion

Ergosterol is an essential component of fungal cell membranes, serving the same function as cholesterol in animal cells (Espenshade and Hughes 2007). Given the absence of ergosterol in mammals, ergosterol biosynthesis pathway is an ideal target for antifungal drugs development (Georgopapadakou and Walsh 1994). Sterol 14α-demethylase (CYP51) is a key enzyme in the sterol biosynthesis pathway in fungi (Mitropoulos et al. 1976; Trzaskos et al. 1986). Thus far, more than 400 redundant CYP51 amino acid sequences from 20 genera in fungi are available in GenBank. The copies of *CYP51* in fungi vary depending on the species. In *S. cerevisiae* and *Candida albicans*, only one *erg11*/*CYP51* gene was found (Ferreira et al. 2005). However, in filamentous fungi, more than one CYP51 genes were usually identified, e.g., two in *A. fumigatus* and *Magnaporthe oryzae* (Ferreira et al. 2005; Yan et al. 2011), three in *Aspergillus oryzae*, *F. graminearum*, and *F. oxysporum* (Ferreira et al. 2005; Yin et al. 2009; Liu et al. 2011).

Although homologous genes of *CYP51* have been found in some fungal species, the precise roles of these genes, especially the *CYP51B* gene, in mediating fungicide resistance have been poorly understood. In *A. fumigatus*, point mutations of *AfCYP51A* amino acid at different positions have been reported to be correlated with azole resistance, but resistance mechanisms related to *AfCYP51B* gene were not detected in naturally occurred azole-resistant isolates (Verweij et al. 2009). However, heterologous expression of *AfCYP51A* and *AfCYP51B* in an *S. cerevisiae* mutant (YUG37-*erg11*) resulted in a similar increase of resistance to four azole fungicides tested, indicating that both *AfCYP51A* and *AfCYP51B* are involved in azole resistance (Martel et al. 2010). Deletion of *FgCYP51A* in *F. graminearum* resulted in increased sensitivity to seven DMI fungicides (triadimefon, propiconazole, tebuconazole, diniconazole, difenocoazole, flutriafol, and prochloraz) studied, and the deletion of *FgCYP51C* resulted in increased sensitivity to five (tebuconazole, diniconazole, difenocoazole, flutriafol, and prochloraz) of the seven DMIs mentioned above. However, deletion of *FgCYP51B* did not change *F. graminearum*'s sensitivity to these DMIs, indicating that *FgCYP51B* may not have a role in DMI resistance in *F. graminearum* (Liu et al. 2011). Similar results were shown for *CYP51B* in *M. oryzae* (Yan et al. 2011).

In this study, two genes (*PdCYP51B* and *PdCYP51C*) homologous to *PdCYP51A* were cloned in *P. digitatum.* Both *PdCYP51B* and *PdCYP51C* were inducible by imazalil treatment (Fig. 5). Introduction of another copy of *PdCYP51B*^{S} did increase the resistance of *P. digitatum* to DMIs (Fig. 6). These results definitely demonstrated that

PdCYP51B is involved in DMI resistance for *P. digitatum*. Quantitative PCR showed that constitutive expression levels of *PdCYP51B* in IMZ-R3 *P. digitatum* were significantly higher than that of IMZ-S as well as of IMZ-R1 and IMZ-R2 (Table 3). A unique 199-bp insertion in the promoter region of *PdCYP51B* was found to be responsible for the increased expression of *PdCYP51B*. The positive effect of 199-bp insertion on DMI resistance was then supported by the genetic evidence since OE-*PdCYP51B*^{R} showed significantly higher resistance to DMIs than that of OE-*PdCYP51B*^{S} (Fig. 6). Thus, it could be concluded that both *PdCYP51A* and *PdCYP51B* were involved in DMI resistance, and the increased expression of *PdCYP51B* resulted from the 199-bp nucleotide-insertion mutation in the promoter region of *PdCYP51B* was responsible for IMZ-R3, the predominant IMZ-R genotype of *P. digitatum* in Zhejiang, China. To our best knowledge, this is a new mechanism of DMI resistance for fungi.

Since *PdCYP51B* and *PdCYP51C* were initially identified from the transcriptome database of *P. digitatum* (unpublished), both genes should be expressed and thus were unlikely to be pseudogenes. Sequence alignment indicated that PdCYP51B was ≥78% identical to CYP51B from *A. fumigatus* (XP_749134.1). Although PdCYP51C was only 18–23% identical to other CYP51s of fungi, it was still assigned to the CYP51 family when BLAST searching in GenBank database.

Analysis of this 199-bp insert showed that no typical signs of a transposon (e.g., transposase-encoded sequence) were found, but a 6-bp TSD and the imperfect TIR were presented at both terminus of the fragment, indicating that the 199-bp insert could be a miniature inverted-repeat transposable element (MITE, Fig. 4). A promoter located inside this 199-bp fragment was predicted by bioinformatical tools, but it was absent in the putative promoter region of *PdCYP51B* of IMZ-sensitive *P. digitatum*. In addition, four known fungal transcription factors binding sites were found in the 199-bp fragment. Thus, an unproved but reasonable inference is that this putative MITE acting as both a transcription enhancer and a promoter inserts into the upstream of *PdCYP51B* gene, and such insertion event leads to the increased expression of *PdCYP51B*, therefore the reduced sensitivity of *P. digitatum* to DMI fungicides. *P. digitatum* bearing this mutation is selected, the population of this genotype increases, then becomes predominant under sustained DMI fungicide pressure introduced by the common usage of imazalil, one of DMIs, for postharvest disease control of citrus since the middle 1990s in Zhejiang province (Li et al. 2003; Chen et al. 2008), particularly in Quzhou, where the IMZ-R3 isolates were obtained for this study.

The origin of IMZ-R1 and IMZ-R2 *P. digitatum* in California were determined by RAPD analysis (Ghosoph et al. 2007), the result indicated that imazalil resistance developed independently. Together with our study on IMZ-R *P. digitatum* in Zhejiang, it could be concluded that *P. digitatum* has simultaneously evolved multiple mechanisms to adapt to the selection pressure introduced by the application of DMI fungicides in modern agriculture.

The application of synthetic fungicides is the primary method in citrus postharvest disease control. The evolution of fungicide-resistant pathogens becomes a significant problem for citrus postharvest disease control because control systems are less effective or no longer effective (Bus et al. 1991; Eckert et al. 1994). The detection and quantification of fungicide-resistant pathogenic populations would be useful in assisting to determine whether the ineffective disease control results from fungicide resistance. The most common method to determine the fungicide resistance of a pathogen is based on the inhibition of mycelial growth sensitivities measured by either EC_{50} values or minimal inhibitory concentrations. Molecular detection and quantification of fungicide-resistant populations have been demonstrated to be an alternative and effective way in diagnosis of resistant populations (Ma et al. 2005; Chen et al. 2008; Ghosoph et al. 2007; Zhang et al. 2009). In the current study, our work not only extends the knowledge of DMI-resistant mechanism in fungi but also provides a foundation to develop a practical method for rapidly identifying and monitoring the incidence of DMI-resistant isolates among populations of conidia within citrus packing houses, an important commercial practice for citrus.

Acknowledgments Special thanks go to Dr. Xiaoguang Liu (School of Bioengineering, Tianjin University of Science and Technology, China) and Dr. Matthias Hahn (Department of Biology, University of Kaiserslautern, Germany) for their helpful suggestions and critical reviews of the manuscript. This work was supported by the National Foundation of Natural Science of China (30571236 & 31071649) and the earmarked fund for Modern Agro-industry Technology Research System (MATRS).

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