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The Y123H substitution perturbs FvCYP51B function and confers prochloraz resistance in laboratory mutants of *Fusarium verticillioides*

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Fusarium verticillioides reduces corn yield and contaminates infected kernels with the toxin fumonisin, which is harmful to humans and animals. Previous research has demonstrated that *F. verticillioides* can be controlled by the azole fungicide prochloraz. Currently, prochloraz is used as a foliar spray to control maize disease in China, which will increase the risk of resistance. Although *F. verticillioides* resistance to prochloraz has not been reported in the field, possible resistance risk and mechanisms resulting in prochloraz resistance were explored in the laboratory. Four prochloraz-resistant strains of *F. verticillioides* were generated by successive selection on fungicide-amended media. The mycelial growth rates of the mutants were inversely related to the level of resistance. All four mutants were cross-resistant to the triazole fungicides triadimefon, tebuconazole and difenoconazole, but not to the multisite fungicide chlorothalonil or to the MAP/histidine-kinase inhibitor fungicide fludioxonil. Based on the Y123H mutation in FvCYP51B, the four resistant mutants were subdivided into two genotypes: PCZ-R1 mutants with wildtype FvCYP51B and PCZ-R2 mutants with substitution Y123H in FvCYP51B. Wildtype FvCYP51B complemented the function of native ScCYP51 in *Saccharomyces cerevisiae* YUG37::*erg11*, whereas Y123H-mutated FvCYP51B did not. For the PCZ-R1 mutants, induced expression of *FvCYP51A* increased resistance to prochloraz. For the PCZ-R2 mutants, disruption of FvCYP51B function by the Y123H substitution caused constitutive up-regulation of *FvCYP51A* expression and thus resistance to prochloraz.

Keywords: azole fungicide, *CYP51*, resistance mechanism, site mutation, yeast expression

Introduction

Fusarium verticillioides (teleomorph *Gibberella fujikuroi* (synonym, *Gibberella fujikuroi*)) is a widely distributed plant pathogen that causes many important diseases of corn including seedling blight, root rot, stalk rot, and kernel or ear rot (Foley, 1962; Kommedahl & Windels, 1981). Toxins produced by *F. verticillioides* such as fumonisin B_1 can be detected in plant tissues (especially in kernels) with and without symptoms, and are harmful to humans and animals (Marasas *et al.*, 1984; Howard *et al.*, 2001). This fungal pathogen primarily penetrates seeds and then moves to the root, mesocotyl and crown, and ultimately to the kernels (Munkvold *et al.*, 1997; Murillo-Williams & Munkvold, 2008). Seed treatment with fungicides, such as prochloraz and carbendazim, are commonly used to protect against fungal infection after planting or to inhibit the growth of seedborne fungal pathogens.

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The azole fungicide prochloraz inhibits fungal growth by interacting with the heme-iron of the cytochrome P450 sterol 14α-demethylase (CYP51), an essential enzyme for biosynthesis of ergosterol which is the predominant sterol in fungal membranes. Prochloraz is effective against a wide range of fungal plant pathogens and is widely used as a seed treatment to control *Fusarium* spp. attacking rice and corn, as a foliar spray to control *Magnaporthe oryzae* on rice, *Rhynchosporium secalis* on barley, and *Colletotrichum gloeosporioides* on citrus and litchi, and as a postharvest treatment to control decay of citrus caused by *Penicillium italicum* (Kendall *et al.*, 1993; Bateman *et al.*, 1995; He, 2007). Because of the high biological activity against *F. verticillioides*, prochloraz is used as a foliar spray to control maize diseases in major corn producing regions in China, probably increasing the risk of resistance.

Resistance is the primary constraint on azole (triazole and imidazole) fungicide efficacy. To date, three mechanisms of resistance to azole fungicides have been reported in ascomycete plant pathogens: (i) single or multiple mutations in the *CYP51* gene resulting in amino acid alterations that decrease the affinity of the protein for inhibitors (Loffler *et al.*, 1997; Sanglard *et al.*, 1998;

Plant Pathology

Wyand & Brown, 2005; Cools *et al.*, 2010); (ii) over-expression of the *CYP51* gene caused by insertions in the predicted promoter regions (Ma *et al.*, 2006; Luo & Schnabel, 2008; Cools *et al.*, 2012); and (iii) over-expression of genes encoding efflux pumps, such as ATP-binding cassette (ABC) transporters or major facilitator superfamily (MFS) transporters, which results in multi-drug resistance (MDR; Sanglard *et al.*, 1995; Hayashi *et al.*, 2002; Kretschmer *et al.*, 2009). These mechanisms can combine to further decrease azole sensitivity (Perea *et al.*, 2001; Mellado *et al.*, 2007; Cools *et al.*, 2012).

Amino acid alteration and/or over-expression of the target *CYP51* gene are the primary mechanisms of azole resistance in plant pathogens. Recently, multiple *CYP51* genes have been identified in many ascomycetes including *Penicillium digitatum* (2), *Aspergillus fumigatus* (2), *A. nidulans* (2), *A. flavus* (3), *M. oryzae* (2), *F. graminearum* (3), *F. oxysporum* f. sp. *lycopersici* (3) and *F. verticillioides* (3). *CYP51* paralogues have been classified into three phylogenetic clades designated A, B and C (Becher *et al.*, 2011), and to date, the *CYP51C* paralogue has only been found in *Fusarium* species and used as a reliable phylogenetic marker to enable species-specific detection.

CYP51A is generally considered to be responsible for variation in azole sensitivity of fungi with multiple *CYP51* genes. For example, deletion of *CYP51A* increases azole sensitivity in *A. fumigatus*, *M. oryzae* and *F. graminearum* (Mellado *et al.*, 2005; Jiang *et al.*, 2011; Yan *et al.*, 2011; Fan *et al.*, 2013). Mutations in *AfCYP51A* have been identified in *A. fumigatus* isolates resistant to azoles (Diaz-Guerra *et al.*, 2003; Mellado *et al.*, 2005), and increases in azole resistance have been reported when *AfCYP51A* mutations are combined with *AfCYP51A* over-expression (Mellado *et al.*, 2007; Snelders *et al.*, 2008). Although *PdCYP51A* over-expression is the most common mechanism of resistance in *P. digitatum* (Hamamoto *et al.*, 2000; Ghosoph *et al.*, 2007), *PdCYP51B* over-expression has been reported to be responsible for resistance to imizalil (Sun *et al.*, 2011). To date, no information on the resistance risk and molecular mechanisms of prochloraz against *F. verticillioides* have been reported.

In this study, four prochloraz-resistant mutants of *F. verticillioides* were generated and the molecular basis of the induced resistance was investigated. The resistance mechanisms were characterized by amplification and quantification of the three paralogous *FvCYP51* genes and by heterologous expression of *FvCYP51* genes in *Saccharomyces cerevisiae* strain YUG37::*erg11* to clarify the molecular mechanisms of azole resistance in *F. verticillioides*.

Materials and methods

Strains and growth conditions

Forty-six *F. verticillioides* strains were isolated from diseased corn seedlings from fields in Shandong and Jilin provinces where prochloraz has not been used. The baseline sensitivity of *F. verticillioides* to prochloraz was established (Fig. S1). Ten isolates (PS01–PS05 and PJ01–PJ05) were randomly selected to generate the prochloraz-resistant strains. Four prochloraz-resistant mutants were obtained from strain PS01 (see Results). The 10 prochloraz-sensitive isolates and the four generated prochloraz-resistant isolates in the current study were routinely cultivated on PDA (potato dextrose agar) plates. *Escherichia coli* XL-1 Blue (Stratagene) and Trans5α (TransGen) were used for routine gene subcloning. LB broth or agar amended with 100 μg mL^{-1} ampicillin was used for plasmid preparation and plating of *E. coli* transformations. *Saccharomyces cerevisiae* strain YUG37::*erg11* (*MATa ura3-52 trp1-63 LEU2::tTA tetO-CYC1::erg11*; Cools *et al.*, 2010) was used for heterologous expression of the *FvCYP51* genes. *Saccharomyces cerevisiae* transformants were grown on synthetic dropout (SD) minimal medium containing 1·6 g L^{-1} dropout medium supplement without uracil (Sigma-Aldrich), 2 g L^{-1} yeast nitrogen base without amino acids (Difco), 2% galactose and 2% raffinose (SD GAL+RAF), and 2% agar. For complementation studies, 3 μg mL^{-1} doxycycline (Sigma-Aldrich) was added to repress native *ScCYP51* expression.

Generation of prochloraz-resistant mutants

The 10 prochloraz-sensitive strains (PS01–PS05, PJ01–PJ05) of *F. verticillioides* were used to generate the prochloraz-resistant strains by successive prochloraz treatment. Plugs excised from the margins of vigorously growing 7-day-old colonies on PDA plates were placed on new PDA plates containing 0·5 μg mL^{-1} prochloraz. After 15–20 days at 25°C in the dark, mycelial plugs from growing sectors were transferred to PDA plates amended with 1 μg mL^{-1} prochloraz for 10 transfers. To confirm the stability of resistance, the growing mycelia were transferred 10 times to PDA without prochloraz, followed by transfer to PDA with 1 μg mL^{-1} prochloraz. Mycelia that continued to grow were identified as prochloraz-resistant mutants. Four prochloraz-resistant mutants were obtained from PS01.

Fungicide-sensitivity test

The fungicide sensitivities of *F. verticillioides* strains to prochloraz were assessed using an *in vitro* mycelial growth assay. Mycelial plugs (5 mm diameter) from the edges of 7-day-old colonies were placed on PDA plates amended with a series of concentrations of triadimefon, tebuconazole, difenoconazole, chlorothalonil and fludioxonil (Table 1). Like prochloraz, triadimefon, tebuconazole and difenoconazole are azole fungicides, while chlorothalonil is a multisite fungicide, and fludioxonil is a MAP/histidine-kinase inhibitor. Colony diameter was measured after 7 days at 25°C in the dark. There were three replicate plates for each combination of fungicide concentration, and isolate or strain, and the experiment was performed twice. EC_{50} values were calculated by regressing percentage growth inhibition against the log of fungicide concentration. The resistance factor (RF) was calculated as the EC_{50} value of the resistant strain divided by the EC_{50} value of the parent isolate (prochloraz-sensitive strain PS01). Hyphal growth rates of the parent isolate and resistant mutants were measured on PDA plates at 25°C by measurement of colony diameters every 24 h for six days. For each strain there were three replicate plates in two independent experiments. Data were analysed from two independent replicates using ANOVA. Following a significant *F*-test result ($P < 0·05$), means of two independent replicates were compared using least significant differences (LSD). $P < 0·05$ was

Table 1 Fungicide concentrations used to measure the sensitivity (50% effective concentration/EC_{50} values) of the parental isolate of *Fusarium verticillioides* (PS01) and the four prochloraz-resistant strains derived from PS01

Fungicide	Concentration (μg mL^{-1})	Isolate or strain
Prochloraz	0, 0·005, 0·01, 0·02, 0·05, 0·1, 0·2	PS01
	0, 0·02, 0·05, 0·1, 0·2, 0·5, 1, 2	Prochloraz-resistant strains
Tebuconazole	0, 0·02, 0·05, 0·1, 0·2, 0·5	PS01 and prochloraz-resistant strains
Triadimefon	0, 0·5, 1·0, 2·0, 5·0, 10·0	PS01 and prochloraz-resistant strains
Difenoconazole	0, 0·7, 0·8, 0·9, 1·0, 1·1, 1·2	PS01
	0, 0·02, 0·05, 0·1, 0·2, 0·5, 1·0, 2·0	Prochloraz-resistant strains
Chlorothalonil	0, 0·2, 0·5, 1, 2, 5, 10	PS01 and prochloraz-resistant strains
Fludioxonil	0, 0·02, 0·05, 0·1, 0·2, 0·5, 1	PS01 and prochloraz-resistant strains

considered to be significant. Letters were used to mark statistically significant differences ($P < 0·05$).

Extraction of DNA and RNA

Genomic DNA (gDNA) of *F. verticillioides* was extracted with a modified 2% CTAB method (Murray & Thompson, 1980). Briefly, fresh mycelia were harvested after 7 days' growth on PDA plates and homogenized to a fine powder in liquid nitrogen using a mortar and pestle. The powder was suspended in 2% CTAB extraction buffer (2% CTAB, 0·7 M NaCl, 50 mM Tris–HCl (pH 8·0), 10 mM EDTA, 1% β-mercaptoethanol) and incubated at 65°C for 30 min. The preparation was subjected to phenol:chloroform:isoamyl alcohol (v/v/v, 25:24:1) extraction and isopropanol precipitation. Genomic DNA was suspended in 50 μL distilled water.

Total RNA was extracted with TRIzol reagent (Invitrogen) treated with DNase I (TaKaRa). A 2 μg quantity of total RNA was used to synthesize the first-strand cDNA using M-MLV reverse transcriptase (Promega) and oligo$(dT)_{18}$ in a 20 μL reaction. With cDNA as template, real-time PCR was used to quantify the expression levels of the three *FvCYP51* genes.

Cloning and sequencing of *CYP51* genes and upstream sequences

The full-length genes and 1 kb upstream sequences of the three *FvCYP51* genes were amplified by PCR. The primers were designed based on the *Fusarium* comparative genome database (available at http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html). The sequence of *FvCYP51A*, *FvCYP51B* and *FvCYP51C* are provided under accession numbers FVEG_10277, FVEG_01123 and FVEG_12391, respectively. PCR was performed in a 25 μL reaction volume containing 100 ng gDNA, 1 μM primers, 0·2 mM dNTP mix, 2·5 μL 10 × buffer and 2·5 U Ex-*Taq* DNA polymerase (TaKaRa). Thermocycling began with 5 min at 95°C followed by 35 cycles of: 30 s at 95°C, 30 s at 56°C, 120 s at 72°C for primers FvcypCF1/FvcypCR1; 30 s at 60°C and 90 s at 72°C for primers FvcypAF1/FvcypAR1 and FvcypBF1/FvcypBR1; and 30 s at 60°C, 60 s at 72°C for primers FvcypAF2/FvcypAR2, FvcypBF2/FvcypBR2 and FvcypCF2/FvcypCR2. A negative control which included all PCR constituents without DNA template was included in each amplification. The PCR products were analysed by electrophoresis on 1% agarose gels stained with ethidium bromide. The amplified fragments were subsequently purified using the EasyPure Quick Gel Extraction Kit (TransGen) and cloned into pEASY-T3 plasmid using the pEASY-T3 Cloning kit (TransGen) according to the manufacturer's recommendations. To identify sequencing errors, at least three clones of each sample were sequenced (SunbioTech). Sequence alignment was performed with VectorNTI.

Quantification of expression of the three *FvCYP51* genes

The relative quantification of the three *FvCYP51* genes in prochloraz-sensitive and -resistant strains, with and without prochloraz treatment, was measured by real-time RT-PCR. Spores of each strain (1×10^8) were inoculated in 150 mL PDB media. After 24 h, mycelia were harvested, and 200 mg (fresh weight) of mycelia was added to 200 mL PDB with or without 0·5 μg mL^{-1} prochloraz. After 24 h, mycelia were harvested by vacuum filtration, frozen in liquid nitrogen, and preserved at −80°C for RNA extraction. Three independent experiments were performed.

Real-time PCR amplifications were performed with an ABI 7500 sequence detection system (Applied Biosystems) using SYBR Green I fluorescent dye detection. Amplifications were conducted in 25 μL volumes containing 12·5 μL SYBR *Premix Ex Taq* (TaKaRa), 1 μL reverse transcription product, and 1 μL each of the forward and reverse primers (5 μM). The thermal cycling conditions were 10 s at 95°C followed by 40 cycles at 95°C for 5 s and 60°C for 31 s, and finally a dissociation stage at 95°C for 15 s, 60°C for 60 s, 95°C for 15 s and 60°C for 15 s. Data were analysed with ABI 7500 Prism 7500 SDS software. The relative quantities (RQ) of products were calculated using the $2^{-\Delta\Delta C_t}$ method. The *actin* gene was used as a reference to normalize the quantification of *FvCYP51* expression. Three biological replicates were performed for statistical analysis.

Complementation of *S. cerevisiae* YUG37::*erg11* by *FvCYP51B* genes

Heterologous expression of *FvCYP51B* genes in *S. cerevisiae* YUG37::*erg11* was performed using a previously described method (Cools *et al.*, 2010). The full-length coding sequence of wildtype *FvCYP51B* was amplified using cDNA from the prochloraz-sensitive strain PS01. The mutant *FvCYP51B* (*FvCYP51B* with the Y123H mutation) was cloned using the cDNA of mutant 0109. Primers with restriction sites added to enable cloning into the yeast expression vector pYES2/CT (Invitrogen) are listed in Table 2. Reactions were carried out using a Biometra T3 thermocycler (Biotron GmbH) in a 50 μL final volume containing 1 U Phusion high-fidelity DNA polymerase (Finnzymes), 125 μM dNTP and 0·5 μM of each primer. The thermal cycling conditions were as follows: 30 s at 98°C; followed by 35 cycles at 98°C for 10 s, 58°C for 30 s and 72°C for 120 s; and a final extension at 72°C for 5 min. The amplified coding sequence of *FvCYP51B* was digested with *Kpn*I and *Xba*I. The digested sequences were cloned into the pYES2/CT vector, creating the pYES-FvCYP51s yeast expression plasmids. The full-length *FvCYP51* genes in pYES-FvCYP51s were sequenced to ensure the validity of sequences and transformed

Table 2 PCR primers used in this study

Primer	Sequence (5′–3′)[a]	Application
FvcypBF1	TTCTTGTGCTTCCTGGCGC	Amplification of the complete *CYP51B* gene
FvcypBR1	CCATTACAAAACACTCAACTCC	
FvcypAF1	AATATGGCTCTCTTCTGTCC	Amplification of the complete *CYP51A* gene
FvcypAR1	CTAAGCCTTCCTGCGTTCC	
FvcypCF1	ATCCGTATAAATTCTTCTTCG	Amplification of the complete *CYP51C* gene
FvcypCR1	CTTGCTTCCTGTTGTACTG	
FvcypBF2	GTGCTTCCAATTGAGATCATAG	Amplification of the 1-kb-upstream sequence of *CYP51B*
FvcypBR2	GGAGGATATCAGGGTTTTCG	
FvcypAF2	TCTAACATATCCTAGCCTCCC	Amplification of the 1-kb-upstream sequence of *CYP51A*
FvcypAR2	AGACAGTGAAGAGACGGCTG	
FvcypCF2	TGTCTTTGCAGTGTGTGTAGC	Amplification of the 1-kb-upstream sequence of *CYP51C*
FvcypCR2	GTCGTCGATTTACCCAGCAG	
FvcypBF3	CAGAATGGCTAACCGTGGC	Real-time quantification of *CYP51B* expression
FvcypBR3	CCACATTGCGGAACTTGAAC	
FvcypAF3	CGTTGGTGCGTAACTTCAGACT	Real-time quantification of *CYP51A* expression
FvcypAR3	CCTTCCTGCGTTCCCATTTGAT	
FvcypCF3	CAGACTGGGTAGTTCCTCC	Real-time quantification of *CYP51C* expression
FvcypCR3	AGATACGGGCTGCTGACG	
FvactF1	TGGCACGAGAACCCTGAC	Real-time quantification of *actin* gene expression
FvactR1	ACTGTTTCGTCGTTGTGGC	
FvF1F	CCGGGTACCATGGGTCTCCTCCAAGAACT	Amplification of *CYP51B* coding sequence
FvF1R	CCGTCTAGACTACTGCTGTCGTCTTTCC	
FvF2F2	CCGGGTACCATGTTTTCACTCCTCTAVTA	Amplification of *CYP51A* coding sequence
FvF2R	CCGGAGCTCCTAAGCCTTCCTGCGTTCC	
FvF3F2	CCGGGTACCATGGACGCCCTCTACACCA	Amplification of *CYP51C* coding sequence
FvF3R	CCGGAGCTCCTAACCAGTCTCCGCGTTAC	

[a]Underlined sequences indicate restriction sites.

into *S. cerevisiae* YUG37::*erg11* using the S.c. EasyComp transformation kit (Invitrogen) according to the manufacturer's instructions. The pYES2/CT vector was transformed as a negative control. The complementation efficiencies of transformants were screened on agar plates. Transformants were grown for 24 h at 30°C in SD GAL+RAF medium to induce *FvCYP51s* expression. A 5 μL volume of cell suspensions of each transformant was droplet inoculated (six 5-fold dilutions of a starting concentration of 1×10^6 cells mL^{-1}) onto SD GAL+RAF agar plates with or without 3 μg mL^{-1} doxycycline. The *ScCYP51* gene in *S. cerevisiae* YUG37::*erg11* is controlled by a doxycycline-repressible promoter, i.e. expression of *ScCYP51* is repressed by doxycycline. The plates were photographed after incubation for 6 days at 30°C.

Results

In vitro characteristics of prochloraz-resistant mutants

Resistant isolates were obtained from PS01 but not from the other nine prochloraz-sensitive field isolates. The four prochloraz-resistant mutants, which grew on 1 μg mL^{-1} prochloraz-amended PDA plates where the PS01 was completely inhibited, were designated 0104, 0109, 0113 and 0110, and their resistance factors (RFs) ranged from 8 to 46 (Table 3). The prochloraz-resistant mutants were also less sensitive to the three other azole fungicides that were tested (triadimefon, tebuconazole and difenoconazole) but with RF values ranging from about 2 to 5 (Table 3). However, there was no difference in sensitivity to the non-azole fungicides chlorothalonil and fludioxonil between the parent PS01 and the prochloraz-resistant isolates (Table 3). The growth rate of the resistant mutants was slower compared to PS01 and decreased as RF increased, i.e. increased resistance was associated with a reduced growth rate (Fig. 1). For mutants 0109 and 0110, which had RFs of 29·6 and 46, growth was reduced by almost half over the 6 days of incubation.

Sequence analysis of the three *FvCYP51* genes

The complete gDNA and cDNA sequences of the three *FvCYP51* genes of *F. verticillioides* isolate PS01 were cloned, sequenced, and deposited in GenBank (accession numbers KC527017, KC527018 and KC527019). The location of exons and introns of the three *FvCYP51* genes are shown in Figure 2. The full-length of the *FvCYP51A* gene sequence is 1574 bp with one intron, and encodes a protein of 506 amino acids. The sequence of the *FvCYP51B* gene is 1761 bp in length with two introns, and encodes a protein of 527 amino acids. The 1655 bp sequence of the *FvCYP51C* gene contains an open reading frame (ORF) of 1554 bp with two introns and encodes a protein of 517 amino acids. The data for *FvCYP51B* are the same as those in the *Fusarium* comparative genomic database; in contrast, the *FvCYP51A* is predicted to have 1694 bp genomic DNA encoding 445

Table 3 Characteristics and azole sensitivity of prochloraz-sensitive parent isolate of *Fusarium verticillioides* (PS01) and four prochloraz-resistant mutants

Isolate/mutant[a]	Sensitivity to prochloraz		Sensitivity (EC_{50}, $\mu g\ mL^{-1}$) to:					CYP51B mutation	Resistance type[c]
	EC_{50} ($\mu g\ mL^{-1}$)	RF[b]	Triadimefon	Tebuconazole	Difenoconazole	Chlorothalonil	Fludioxonil		
PS01	0·0161 ± 0·0021	–	2·2451 ± 0·3495 a[d]	0·1081 ± 0·0125 a	0·1561 ± 0·0112 a	3·4775 ± 0·1784 a	0·2030 ± 0·0201 a	–	S
0113	0·1282 ± 0·0084	8·0	4·1211 ± 0·4010 b	0·5000 ± 0·0892 b	0·5349 ± 0·0774 b	2·9988 ± 0·2175 a	0·2354 ± 0·0305 a	–	PCZ-R1
0104	0·1425 ± 0·0045	8·9	3·7517 ± 0·2052 b	0·6273 ± 0·1020 b	0·3297 ± 0·0652 bc	3·8828 ± 0·2534 a	0·2952 ± 0·0348 a	–	PCZ-R1
0109	0·4768 ± 0·0150	29·6	4·9202 ± 0·5210 b	0·4332 ± 0·0812 b	0·3643 ± 0·0251 bc	2·2082 ± 0·4042 b	0·2267 ± 0·0146 a	Y123H	PCZ-R2
0110	0·7412 ± 0·0784	46·0	3·8211 ± 0·4211 b	0·5704 ± 0·0714 b	0·4482 ± 0·0201 c	3·4091 ± 0·4530 a	0·2144 ± 0·0184 a	Y123H	PCZ-R2

[a]Mutant 0104, 0109, 0110 and 0113 were generated from PS01 by prochloraz treatment.
[b]RF (resistance factor) = EC_{50} of the resistant mutant/EC_{50} of PS01.
[c]S, sensitive; PCZ-R1, mutants with wildtype *FvCYP51B*; PCZ-R2 mutants with substitution Y123H in *FvCYP51B*.
[d]Data were analysed using ANOVA ($P < 0·05$, *F*-test). Different letters following the numbers mark statistically significant differences between strains ($P < 0·05$, least significant difference (LSD)).

amino acids, and the *FvCYP51C* gene is predicted to have an ORF of 1146 bp.

Sequence analysis of the three *FvCYP51* genes of the prochloraz-resistant strains indicated a C487T transition in *FvCYP51B* of mutants 0109 and 0110 resulting in an amino acid substitution of tyrosine with histidine at codon 123 (Y123H; GenBank accession number KC527016). Partial sequence alignment of the CYP51 protein family revealed that the tyrosine (Y123) of FvCYP51B in *F. verticillioides*, which is equivalent to Y118 in *C. albicans*, Y76 in *Mycobacteria tuberculosis*, Y111 in *Sorghum bicolor*, and Y131 in *Rattus norvegicus*, is 100% conserved in all phyla (Fig. 3). No mutation was detected in the *FvCYP51B* gene of the low-resistance mutants 0113 and 0104 or in the *FvCYP51A* and *FvCYP51C* genes of any of the mutants.

Quantification of expression of the three *FvCYP51* genes

All three *FvCYP51* genes were expressed *in vitro* (data not shown). In the absence of prochloraz, expression levels of *FvCYP51A*, *FvCYP51B* and *FvCYP51C* did not differ between PS01 and mutants 0113 or 0104 (Fig. 4). However, constitutive expression of *FvCYP51A* was about 15-fold higher in resistant mutants 0109 and 0110 than in PS01, 0113 and 0104. Constitutive expression levels of *FvCYP51B* and *FvCYP51C* did not significantly differ between 0109, 0110 and PS01, 0103 and 0104 (Fig. 4).

After treatment with prochloraz, *FvCYP51A* gene expression in PS01 increased about 10-fold but *FvCYP51B* and *FvCYP51C* gene expression in PS01 was unchanged. However, in the resistant mutants 0113 and 0104, *FvCYP51A* expression was 20- to 30-fold higher than in untreated PS01 and about 2- to 3-fold higher than in prochloraz-treated PS01. Prochloraz treatment did not change the expression of *FvCYP51A* in the resistant mutants 0109 and 0110. No insertions were detected in the 1 kb-upstream sequences of the three *FvCYP51* genes in any isolate.

Complementation of *S. cerevisiae* YUG37:*erg11* with *FvCYP51B* genes

Plasmids pYES2, pYES2-FvCYP51B and pYES2-FvCYP51BY132H were transformed into *S. cerevisiae* YUG37:*erg11*, in which the *ScCYP51* gene is controlled by a doxycycline-repressible promoter. In the absence of doxycycline, the *ScCYP51* gene was expressed in all transformants (Fig. 5, left). In the presence of doxycycline, the function of *ScCYP51* of *S. cerevisiae* was complemented by the wildtype *FvCYP51B* of *F. verticillioides* but not by the mutated *FvCYP51B* with the Y123H substitution (Fig. 5, right).

Discussion

Prochloraz has been widely used as a seed treatment fungicide against some seed-, or soilborne pathogens such as

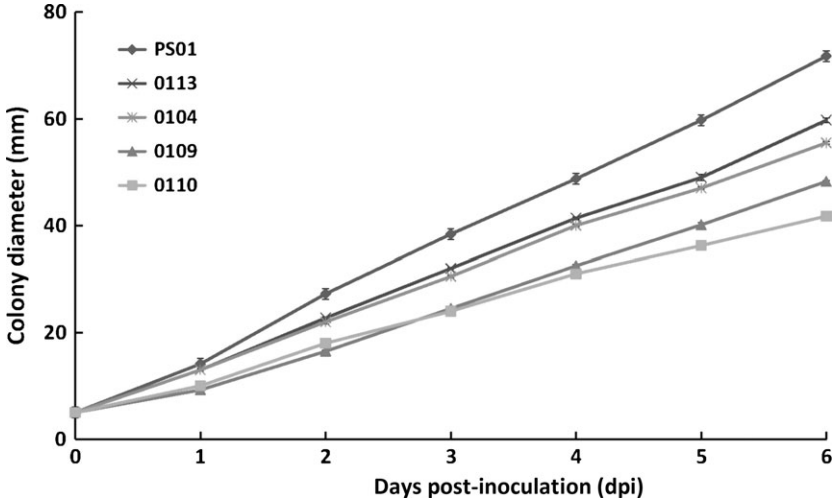

Figure 1 Growth rate of prochloraz-sensitive isolate (PS01) and prochloraz-resistant mutants of *Fusarium verticillioides*. Error bar shows ± SD. Data were analysed using ANOVA ($P < 0{\cdot}05$, *F*-test).

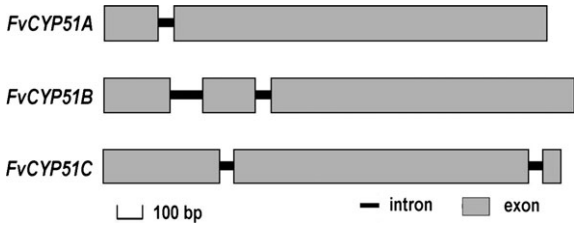

Figure 2 Location of introns and exons in three paralogous *FvCYP51* genes.

Fusarium spp. for several decades, and the incidences of resistance to prochloraz are rare. In recent years, prochloraz has been frequently applied as a foliar spray to control some *Fusarium* diseases of corn, including stalk rot, kernel and/or ear rot in China, which has increased the risk of resistance to prochloraz. The resistance mechanism of *F. verticillioides* to prochloraz is unknown. In this study, four *F. verticillioides* laboratory mutants resistant to prochloraz have been obtained by successive selection on fungicide-amended media. According to the *FvCYP51B* gene sequence, the four resistant mutants were classified into two genotypes: PCZ-R1 mutants without mutation in *FvCYP51B* (0113 and 0104), and PCZ-R2 mutants with a substitution Y123H in FvCYP51B (0109 and 0110).

In fungi with multiple *CYP51* genes, previous research demonstrated the functional variation of different paralogues. *CYP51B* encodes the enzyme primarily responsible for sterol 14α-demethylation. The *Fusarium*-specific *CYP51C* in *F. graminearum* does not encode a sterol 14α-demethylase, but is required for full virulence on host wheat ears. However, the *CYP51A* paralogue encodes an additional sterol 14α-demethylase and is responsible for intrinsic variation in azole sensitivity (Mellado *et al.*, 2005; Warrilow *et al.*, 2010; Fan *et al.*, 2013). Expression of *CYP51A* is greatly induced by tebuconazole in *F. asiaticum* and *F. graminearum* isolates resistant to azoles (Yin *et al.*, 2009). In this study, the *FvCYP51A* gene was induced by prochloraz in the parent, prochloraz-sensitive isolate PS01. Constitutive *FvCYP51A* expression was higher in PCZ-R2 mutants 0109 and 0110, which have a substitution Y123H in FvCYP51B, than in PS01 or the PCZ-R1 mutants. Unlike the wildtype gene, this mutated gene cannot complement the function of CYP51 of *S. cerevisiae*, suggesting that the substitution Y123H perturbs enzyme function. This

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FvCYP51B  112  GKLKDVSAEEIYTVLTTPVFGKDVVYDCPNAKLMEQKKFM  151
FvCYP51A   94  SRIQDANAEEIYGPLTTPVFGSDVVYDCPNSKLMEQKKFV  133
FvCYP51C  104  GKHADLNAEDVYGKLTTPVFGREVVYDCSNARFMDQKRLL  143
CaCYP51   107  AKLSDVSAEDAYKHLTTPVFGKGVIYDCPNSRLMEQKKFA  146
RnCYP51   120  SKNEDLNAEEVYGRLTTPVFGKGVAYDVPNAVFLEQKKIL  159
ScCYP51    96  APETDLSQQEVY-QFNVPTFGPGVVFDVDYTIRQEQFRFF  135
SbCYP51   100  GNEAEMSQQEVY-RFNVPTFGPGVVFDVDYSVRQEQFRFF  139
MtCYP51    65  AGDDDLDQAKAY-PFMTPIFGEGVVFDASPERRKE--MLH  104
                          ★          SRS1
                          H
```

Figure 3 Sequence alignment of the CYP51 predicted substrate recognition site 1 (SRS1) region. The predicted SRS1 region, which is based on the MtCYP51 structure, is framed. 100% conserved residues in different biological kingdoms are shaded. Fungi: FcCYP51s of *F. verticillioides* and CaCYP51 of *Candida albicans*. Animals: RnCYP51 of *Rattus norvegicus*. Plants: ScCYP51 of *Solanum chacoense* and SbCYP51 of *Sorghum bicolor*. Bacteria: MtCYP51 of *Mycobacteria tuberculosis*. The amino acid residues mutated in this study are indicated by a black star below the alignment, and the substituted amino acid is shown below the star.

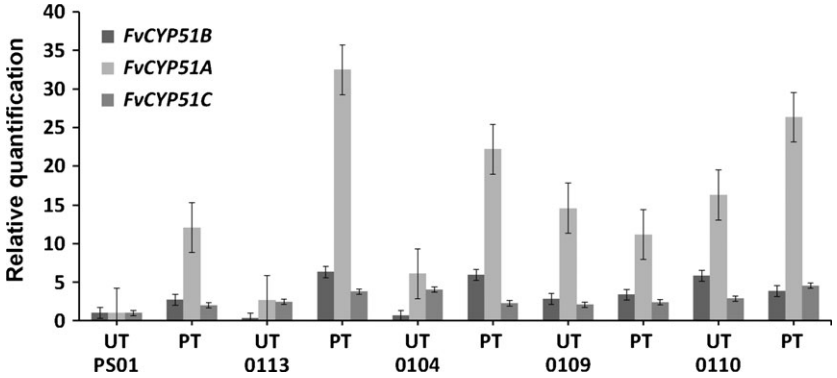

Figure 4 Constitutive and prochloraz-induced expression of three *FvCYP51* genes in *Fusarium verticillioides* strains. Relative quantification of three *FvCYP51* genes were calculated by the $2^{-\Delta\Delta C_t}$ method with the *actin* gene as the reference and untreated PS01 at 24 h incubation as a calibrator. Values are means (±SE) of three biological replicates. UT: untreated, PT: prochloraz treated. PS01 is the prochloraz-sensitive parent isolate. 0113, 0104, 0109 and 0110 are prochloraz-resistant mutants derived from PS01.

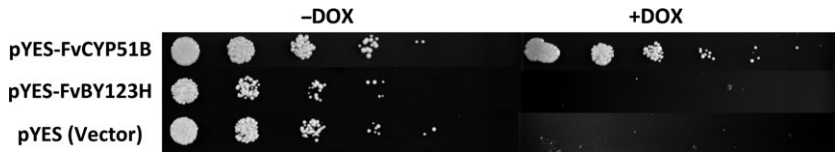

Figure 5 Complementation of *Saccharomyces cerevisiae* YUG37::*erg11* with the wildtype *FvCYP51B* and the mutant *FvCYP51B* with substitution Y123H (FvBY123H). Plates without (−DOX) and with (+DOX) doxycycline were droplet inoculated with a 5-fold dilution series starting at 1×10^6 cells mL^{-1}. The yeast expression vector pYES2/CT without the *FvCYP51* gene was transformed into the yeast strain as a negative control. The plates were photographed after 6 days.

is consistent with previous studies demonstrating that *CYP51A* gene expression increases not only after azole treatment but also as a result of *CYP51B* deletion in *F. graminearum* (Liu *et al.*, 2010; Fan *et al.*, 2013), *M. oryzae* (Yan *et al.*, 2011), *P. digitatum* (Sun *et al.*, 2011) and *A. fumigatus* (Mellado *et al.*, 2005). However, mechanisms responsible for the up-regulation of the *CYP51A* gene remain unknown.

Amino acid alteration and over-expression of *CYP51A* have been reported to be responsible for azole resistance in fungi with multiple *CYP51* genes (Ghosoph *et al.*, 2007; Mellado *et al.*, 2007). For example, mutation of *AfCYP51A* is the most common mechanism of resistance in *A. fumigatus* clinical isolates. Among them, the substitution of G54, L98 and M220 are the most frequent (Becher & Wirsel, 2012); *AfCYP51A* over-expression is responsible for multi-azole resistance in *A. fumigatus* (Mellado *et al.*, 2007; Snelders *et al.*, 2008). In this study, expression levels of *FvCYP51A* in mutants 0103, 0104 and 0109 were about 15- to 30-fold higher in the presence of prochloraz compared to untreated samples. However, no insertions were detected in the upstream sequence of *FvCYP51A* in any mutants (data not shown). The results here also document multi-azole resistance, in that four prochloraz-resistant mutants (the two PCZ-R1 strains and the two PCZ-R2 strains) were cross-resistant to the three triazole fungicides tested (triadimefon, tebuconazole and difenoconazole), with RF values ranging from about 2 to 5. Although cross-resistance between prochloraz and triazole fungicides was not strong, it would be risky to manage disease caused by *F. verticillioides* using a mixture of prochloraz and a triazole fungicide, such as triadimefon, tebuconazole or difenoconazole. A better strategy would be to alternate or mix prochloraz with non-azole fungicides such as chlorothalonil, fludioxonil, etc.

Amino acid alterations encoded by *CYP51* mutation resulting in decreased azole affinity is the most common mechanism of resistance to azoles in fungal pathogens. Moreover, both *CYP51A* and *CYP51B* determined the sensitivity to prochloraz in *F. graminearum* (Fan *et al.*, 2013) and to imazalil in *P. digitatum* (Sun *et al.*, 2011). In this study, a point mutation encoding substitution Y123H in the *FvCYP51B* gene was identified in the PCZ-R2 mutants. This is the first report of a correlation between this point mutation and azole resistance in a plant pathogen, although the corresponding alteration (Y118) in the human pathogen *C. albicans* has previously been shown to cause decreased azole susceptibility (Chen *et al.*, 2007).

However, it is unlikely that resistance is a consequence of reduced prochloraz affinity caused by Y123H. PCZ-R2 mutants with this substitution grew more slowly than the parent isolate PS01 or PCZ-R1 mutants 0113 and 0104. In addition, the tyrosine (Y123) in *F. verticillioides* is 100% conserved in all phyla, including fungi, bacteria, plants and animals (Fig. 3). Y76 in *M. tuberculosis*, the equivalent residue to Y123 in *F. verticillioides*, is the first amino acid in the B' helix/BC loop, which is predicted to form the surface of the substrate-binding activity of substrate recognition site 1 (SRS1), and is an essential amino acid for 14α-demethylation (Lepesheva *et al.*, 2003; Lepesheva & Waterman, 2011). In this study, it was observed that the Y123H mutation decreases or even

destroys FvCYP51B activity because expressing FvCYP51B with Y123H in *S. cerevisiae* failed to complement the function of the native CYP51 protein. A similar phenomenon was reported in *C. albicans*, i.e. the corresponding alteration (Y118) decreased catalytic activity and azole sensitivity in yeast (Chen *et al.*, 2007).

No cross-resistance between the demethylation inhibitor (DMI) fungicides (prochloraz, triadimefon, tebuconazole and difenocnazole) and multiple-site fungicide chlorothalonil or the MAP/histidine-kinase inhibitor fludioxonil was observed for the prochloraz-resistant mutants of *F. verticillioides*, indicating the absence of an MDR phenotype, which is normally mediated by efflux pumps, such as ABC or MFS transporters. Although all the mutants grew slower than the prochloraz-sensitive parent isolate PS01, sporulation and virulence did not differ between PS01 and the prochloraz-resistant mutants (data not shown).

In conclusion, the current study identified two categories of *F. verticillioides* mutant resistant to prochloraz. In the PCZ-R1 mutants, *FvCYP51A,* an inducible *CYP51* gene, is highly expressed after prochloraz treatment, presumably causing resistance. The molecular basis for the increased expression in the PCZ-R1 mutants remains unknown but does not involve any changes in the coding or regulatory sequences of *FvCYP51* genes. In the PCZ-R2 mutants, substitution Y123H in FvCYP51B inactivates the enzyme, reducing fungal growth rate and increasing constitutive *FvCYP51A* expression. As with the PCZ-R1 mutants, it is probable that increased expression of *FvCYP51A* in PCZ-R2 mutants causes resistance to prochloraz. Although resistance to prochloraz has not been reported in the field, the current results indicate that such resistance is possible, and appropriate precautions against resistance development should be taken.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1 Frequency distribution of prochloraz sensitivity (EC_{50} values) of 46 *Fusarium verticillioides* field isolates.