Mutations and Overexpression of *CYP51* Associated with DMI-Resistance in *Colletotrichum gloeosporioides* from Chili

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Abstract

Chili anthracnose caused by *Colletotrichum* spp. is an annual production concern for growers in China. Sterol C14-demethylation inhibitors (DMIs, such as tebuconazole) have been widely used to control this disease for more than three decades. In the current study, of 48 isolates collected from commercial chili farms in Jiangsu Province of China during 2018 and 2019, 8 single-spore isolates were identified as *Colletotrichum gloeosporioides* and the rest were identified as *C. acutatum*. To determine whether the DMI resistance of isolates develops in the field, mycelial growth of the 48 isolates was measured in culture medium with and without tebuconazole. In all, 6 of the 8 *C. gloeosporioides* isolates were resistant to tebuconazole, but all 40 of the *C. acutatum* isolates were sensitive to tebuconazole. The fitness cost of resistance was low based on a comparison of fitness parameters between the sensitive and resistant isolates of *C. gloeosporioides*. Positive cross-resistance was observed between tebuconazole and difenconazole or propiconazole, but not prochloraz. Alignment results of the CgCYP51 amino acid sequences from the sensitive and resistant isolates indicated that

mutations can be divided into three genotypes. Genotype I possessed four substitutions (V18F, L58V, S175P, and P341A) at the *CgCYP51A* gene but no substitutions at *CgCYP51B*, while genotype II had five substitutions (L58V, S175P, A340S, T379A, and N476T) at *CgCYP51A*, concomitant with three substitutions (D121N, T132A, and F391Y) at *CgCYP51B*. In addition, genotype III contained two substitutions (L58V and S175P) at *CgCYP51A*, concomitant with one substitution (T262A) at *CgCYP51B*. Molecular docking models illustrated that the affinity of tebuconazole to the binding site of the CgCYP51 protein from the resistant isolates was decreased when compared with binding site affinity of the sensitive isolates. Our findings provide not only novel insights into understanding the resistance mechanism to DMIs, but also some important references for resistance management of *C. gloeosporioides* on chili.

Keywords: chili, *Colletotrichum gloeosporioides*, DMI resistance, fitness, resistant mechanism

Chili is among the most popular vegetables worldwide, because it has high nutritional and economic value in both developed and developing countries (Than et al. 2008). Chili favors tropical and subtropical climates where anthracnose often occurs such as Korea, China (Cao et al. 2017; Li et al. 2005; Shin et al. 2000), and America (Harp et al. 2013). Anthracnose ranks the ninth among the most severe diseases worldwide, causing considerable yield loss worldwide (Lewis Ivey et al. 2004; Ramdial and Rampersad 2015). Furthermore, more than 167 species of *Colletotrichum* are documented to cause the disease on hundreds of plants (Zhang et al. 2017), including watermelon (Monroe and Santini 1997), grapevine (Jang et al. 2011), strawberry (Lin et al. 2016), pepper (Zhang et al. 2017), apple, pear (Ivic et al. 2013), and other economic crops (Phoulivong et al. 2010). In addition, at least four species of *Colletotrichum* have been isolated from pepper (Lamsal et al. 2012; Lewis Ivey et al. 2004; Zhang et al. 2017), including *Colletotrichum gloeosporioides*, *C. acutatum*, *C. truncatum*, and *C. coccodes*.

Because highly anthracnose-resistant varieties are unavailable, agrochemicals are relied upon to control anthracnose. Benzimidazole

derivatives (abbreviated as MBCs; e.g., carbendazim) and sterol C14-demethylation inhibitors (DMIs; e.g., tebuconazole, difenoconazole, and propiconazole) are extensively used to control this disease (Gisi and Sierotzki 2008; Sierotzki and Scalliet 2013; Xu et al. 2014). MBCs act on the assemblage of tubules from the heterodimer of α - and β -tubulins (Davidse 1986). However, DMIs interfere with fungal growth by inhibiting the cytochrome P450 sterol 14 α -demethylase (*CYP51*) in the biosynthesis of sterol for fungal membranes (Ziogas and Malandrakis 2015). Unfortunately, MBC-resistant populations of *Colletotrichum* spp. have been reported from grape, strawberry, annual bluegrass or bent grass, banana, and bean since 1973 (Chen et al. 2013; Ishii et al. 2016; Leadbeater and Thind 2012; Yang et al. 2015). Three mechanisms of resistance to DMIs alone or in combination have been documented: (i) modification at *CYP51* (Cañas-Gutiérrez et al. 2009; Chen et al. 2005; Chowdhary et al. 2012; Délye et al. 1997, 1998; Mann et al. 2003), (ii) overexpression of *CYP51* (Hamamoto et al. 2000; Rodriguez-Tudela et al. 2008), and (iii) increased drug efflux pumps, including ATP-binding cassette transporters and major facilitator systems (Perea et al. 2001; Sanglard et al. 1995; Schnabel and Jones 2001).

In China, chili anthracnose is caused mainly by *C. acutatum* and *C. gloeosporioides* (Gao et al. 2017; Liao et al. 2012). Recently, farmers have complained about the decreased efficacy of the DMIs in controlling anthracnose in commercial chili fields at Yancheng of Jiangsu Province in China. To investigate whether DMI resistance develops in the fungus, the objectives of this study were to (i) assess the sensitivity of isolates collected to tebuconazole, (ii) reveal the possible molecular resistant mechanism, (iii) determine cross-resistance patterns between tebuconazole and the other DMIs or other fungicides with different mode of action, and (iv) characterize the fitness of tebuconazole-resistant isolates.

Materials and Methods

Isolation and identification of *C. gloeosporioides*. During summer 2018 to 2019, mature fruit of chili (*Capsicum annuum*) with

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lesions typical of anthracnose were sampled from Yancheng (YC) in Jiangsu Province of China. Small pieces of tissue cut from the margin of lesions on the surface of fruit were disinfected in 1% NaClO for 3 min, dipped in 75% ethanol for 3 s, washed three times with sterile water, placed on potato dextrose agar (PDA) plates supplemented with streptomycin sulfate, and incubated at 25°C in darkness. On the fourth day, a mycelia plug of each isolate was transferred to new PDA plates. After incubation at 25°C in darkness for 7 days, a single spore of each isolate was microscopically selected, then moved to a fresh PDA plate supplemented with streptomycin sulfate. In total, 48 single-spore isolates were obtained and stored on PDA slants at 4°C. DNA was extracted from mycelia of each isolate by the cetyltrimethylammonium bromide method (Ristaino et al. 1998). All isolates were further identified by amplification with primer pairs ITS1/ITS4 and TUBF/TUBR (Table 1) (Weir et al. 2012). The internal transcribed spacer (ITS) and β -tubulin sequences of each isolate were blasted in the NCBI GenBank database to further identify the isolates.

Fungicides and culture medium. Technical-grade fungicides, including tebuconazole (95% active ingredient [a.i.]), difenoconazole (96.5% a.i.), propiconazole (98% a.i.), prochloraz (96.5% a.i.), flua-zinam (97% a.i.), fludioxonil (97.5% a.i.), and azoxystrobin (96% a.i.), were provided by Best-Reagent (Chengdu, China), and salicyl-hydroxamic acid (SHAM) (99% a.i.) was purchased from Aldrich Co. (Milwaukee, WI, U.S.A.). Tebuconazole, difenoconazole, propiconazole, prochloraz, flua-zinam, fludioxonil, and SHAM were prepared in methanol, and azoxystrobin was dissolved in acetone, as stock solutions of 10^4 μ g/ml concentration.

PDA was composed of a broth of 200 g of potato, 20 g of dextrose, and 16 g of agar powder in 1 liter of distilled water. Alkyl ester agar (AEA) was prepared with 6 g of sodium nitrate, 20 ml of glycerol, 5 g of yeast extract, 16 g of agar powder, 1.5 g of potassium dihydrogen phosphate, 0.5 g of potassium chloride, and 0.51 g of magnesium sulfate heptahydrate in 1 liter of distilled water. Water agar (WA) consisted of 15 g of agar powder per liter of distilled water. Potato dextrose broth (PDB) was prepared with the broth of 200 g of potato and 20 g of dextrose in 1 liter of distilled water.

Sensitivity of isolates to tebuconazole. To evaluate tebuconazole sensitivity, a mycelia plug (5 mm in diameter) of each isolate was transferred on the center of PDA plates supplemented with tebuconazole at 10 μ g/ml (Xu et al. 2014; Zhang et al. 2012). Petri dishes were incubated at 25°C in darkness for 5 days. If the isolate grew, it was

considered resistant to tebuconazole. If the isolate did not grow, it was considered sensitive. Each isolate was tested in triplicate and the entire experiment was repeated three times.

Mycelia growth, sporulation, and germination rates of *C. gloeosporioides*. A fresh mycelia plug of each isolate was transferred on PDA plates and incubated at 25°C in darkness for 5 days; then, colony diameters were measured twice, perpendicularly, and averaged. For determination of spore production, mycelia plugs were

Table 2. Fungicide concentrations used to determine cross-resistance

Fungicide	Concentration (μ g/ml)
Difenoconazole, propiconazole	0, 0.125, 0.25, 0.5, 1, 2
Prochloraz	0, 0.0037, 0.011, 0.033, 0.1, 0.3
Azoxystrobin	0, 0.012, 0.037, 0.11, 0.33, 1
Fluazinam	0, 0.0098, 0.030, 0.089, 0.27, 0.8
Fludioxonil	0, 0.012, 0.037, 0.11, 0.33, 1

Fig. 1. Phylogram generated from maximum parsimony analysis based on sequences of internal transcribed spacers and β -tubulin, showing the phylogenetic relationship between *Colletotrichum gloeosporioides* and the isolates tested in the study.

Table 1. Primers utilized in this study^z

Primers	Sequence (5'→3')	Reference	Description
ITS1	TCCGTAGGTGAACCTGCGG	Weir et al. 2012	Amplification of ITS to identify <i>Colletotrichum gloeosporioides</i>
ITS4	TCCTCCGCTTATTGATATGC	...	...
TUBF	AACATGCGTGAGATTGTAAGT	Weir et al. 2012	Amplification of β -tubulin to identify <i>C. gloeosporioides</i>
TUBR	TAGTGACCCTTGGCCAGTTG	...	...
AF1	ATCAGCAACAGTTCGTCCCG	...	Amplification of the 5'-UTR region of the <i>CgCYP51A</i> gene
AR1	GAAGACGGTTGGTGGTAAT	...	...
BF1	AATCATTCCTCTCTCC	...	Amplification of the 5'-UTR region of the <i>CgCYP51B</i> gene
BR1	GGCTCGTTGGCGTTCTT	...	...
AF2	ATGATGGAAGGCATAGAACTTC	...	Amplification of the <i>CgCYP51A</i> gene
AR2	TTCTTGACTTTGCGGAGGA	...	...
AF3	TTCTCTTCCCTTGGTTGC	...	...
AR3	TCAATCACGTCGGGTCCACC	...	...
BF2	ATGGGTCTCTGCAGGAGG	...	Amplification of the <i>CgCYP51B</i> gene
BR2	TGGGCGAGACGGAGCATA	...	...
BF3	TTTTCACCGCTTCCACG	...	...
BR3	TTACGCCTCACGCTTCTCCAG	...	...
RT-AF	TCACATTCACCCTCTCGGC	...	Quantitative RT-PCR primers for analysis of <i>CgCYP51A</i> expression
RT-AR	CGGGGGCAGTCTTGATGTAG	...	...
RT-BF	CATTTTCACCGCTTCCACG	...	Quantitative RT-PCR primers for analysis of <i>CgCYP51B</i> expression
RT-BR	GTGCCGTTCTGTAGGTGGA	...	...
RT-TubGF	TCTCGATGTTATCCGCCG	...	Amplification of the β -tubulin gene
RT-TubGR	TGAGCTCAGGAACACTGACG	...	...

^z ITS = internal transcribed spacer, UTR = untranslated region, and RT = reverse-transcription.

inoculated on AEA plates at 25°C in darkness for 7 days; then, spores were harvested by rinsing the colonies with 10 ml of distilled water and spore concentration in the filtered spore suspension was determined by a hemocytometer. To determine the rate of spore germination, a 20-μl spore suspension (approximately 10⁵ to 10⁶ spores/ml) was uniformly distributed onto a WA plate and, after incubation at 25°C in darkness for 4 h, 100 spores of each plate were investigated by microscopic observation. The spore was considered to be germinated when the length of the spore bud tube was equal to or longer than its short diameter. Each isolate was repeated with three plates, and the experiment was performed three times.

Pathogenicity test. Pathogenicity of the eight *C. gloeosporioides* isolates was determined by inoculation of mature fruits of chili and strawberry. Briefly, a mycelia plug of each isolate was placed on an artificial wound on the fruit surface. The lesions were measured after 5 days at 25°C with 12 h of light per day. Each treatment had three replicate fruits and the experiment was conducted three times.

Cross-resistance assay. The sensitivities of the eight *C. gloeosporioides* isolates to three DMIs (i.e., difenoconazole, propiconazole, and prochloraz), one phenylpyrrole fungicide (fludioxonil), and one oxidative phosphorylation uncoupler (fluazinam) were determined using different fungicide doses (Table 2) and estimating effective concentrations for 50% mycelia growth inhibition (EC₅₀) values of mycelia growth inhibition. Meanwhile, the sensitivity to one quinone outside inhibitor (azoxystrobin) was similarly determined for spore-germination inhibition. Each treatment had three replicate plates, and the experiment was performed three times.

Sequencing of the whole nucleotide sequences of *CgCYP51*.

The genomic DNA of each isolate was extracted as previously described. Primer pairs AF₂/AR₂, AF₃/AR₃, BF₂/BR₂, and BF₃/BR₃ (Table 1) were designed to amplify the open reading frames of *CgCYP51A* and *CgCYP51B* from resistant and sensitive isolates. To examine whether overexpression of *CgCYP51* genes could be caused by mutation at the 5' untranslated region (5'-UTR), primer pairs AF₁/AR₁ and BF₁/BR₁ were designed to amplify the 5'-UTR regions of *CgCYP51A* and *CgCYP51B*, respectively. PCR amplification was performed using the following reaction conditions: initial DNA denaturation at 95°C for 5 min; followed by 35 cycles of denaturation 95°C for 30 s, annealing at 55°C for 40 s, and extension at 72°C for 50 s; and final extension at 72°C for 7 min. All the amplification products were analyzed by electrophoresis in a 1% agarose gel with 1× Tris-acetate EDTA buffer (0.04 M Tris-acetate and 0.0001 M EDTA) + 2.5 ml of ethidium bromide. All of the PCR products were purified by the USB ExoSAP-IT PCR Product Cleanup Product kit (Affymetrix), and were sequenced by Springen Biotechnology Co., Ltd. Alignment of the sequences was conducted using the software BioEdit V7.0.1 (Manchester, U.K.).

Quantitative expression of *CgCYP51A* and *CgCYP51B*. Expression of *CgCYP51A* and *CgCYP51B* from the eight isolates was assayed according to procedures used by Wang et al. (2015). Briefly, six replicate flasks containing PDB were inoculated with each isolate. After 3 days at 25°C in a shaker, tebuconazole was added into three of the six flasks to achieve a final concentration of 10 μg/ml in the flask, and the remaining flasks were used as controls. After

Table 3. Sequences obtained from GenBank used in the analysis

Species	Strain	Reference	GenBank accession number ^z	
			ITS	TUB
<i>Colletotrichum gloeosporioides</i>	...	NCBI	LC052319	MG448605
<i>C. gloeosporioides</i>	YC11	This article	...	...
<i>C. gloeosporioides</i>	YC78	This article	...	...
<i>C. gloeosporioides</i>	YC2	This article	...	...
<i>C. gloeosporioides</i>	YC18	This article	...	...
<i>C. gloeosporioides</i>	YC22	This article	...	...
<i>C. gloeosporioides</i>	YC44	This article	...	...
<i>C. gloeosporioides</i>	YC56	This article	...	...
<i>C. gloeosporioides</i>	YC61	This article	...	...
<i>C. asianum</i>	BPD-I4 holotype	Prihastuti et al. 2009	FJ972612	FJ907439
<i>C. cordylinicola</i>	BCC 38872 holotype	Phoulivong et al. 2011	HM470246	HM470249
<i>C. fruticola</i>	BPD-II2	Prihastuti et al. 2009	FJ972611	FJ907440
<i>C. hymenocallidis</i>	CSSN2 holotype	Yang et al. 2009	GQ485600	GQ849438
<i>C. musae</i>	CBS116870 epitype	Su et al. 2011	HQ596292	HQ596280
<i>C. siamense</i>	BML-I15	Prihastuti et al. 2009	FJ972614	FJ907437
<i>C. viniferum</i>	GZAAS5.08622	Peng et al. 2013	JN412806	JN412812
<i>C. kahawae</i>	IMI319418 holotype	Prihastuti et al. 2009	FJ972608	FJ907446

^z ITS = partial or complete ribosomal DNA internal transcribed spacer region and TUB = partial β-tubulin.

Fig. 2. Mycelia colony growth of the eight isolates (YC11, YC78, YC2, YC18, YC22, YC44, YC56, and YC61) on potato dextrose agar plates with and without tebuconazole. Letters S and R indicate sensitive and resistant, respectively.

12 h, the mycelia of each isolate were harvested for RNA extraction (Fan et al. 2014). The SV Total RNA Isolation kit (Promega, Beijing, China) was used to extract total RNA, and the PrimeScrip RT reagent Kit with gDNA Eraser (Takara, Beijing, China) was conducted to synthesize cDNA in accordance with the manufacturer's protocol. Reverse-transcription (RT)-PCR was performed with an ABI7500 sequence detection system (Applied Biosystems) to analyze the expression of the two genes with the primer pairs RT-AF/RT-AR and RT-BF/RT-BR (Table 1), respectively. The SYBR Premix Dimer Eraser kit (Takara) was used to amplify the genes. The relative quantities of products were determined by the $2^{-\Delta\Delta C_t}$ method (Zhang et al. 2017). The β -tubulin gene was amplified by the primer pair RT-TubGF/RT-TubGR as a reference to normalize the quantification of *CgCYP51* expression (Fan et al. 2014). Experiments were conducted with three repetitions.

Molecular docking analysis. Bioinformatic analysis was used to investigate the molecular docking of tebuconazole with the

CgCYP51 proteins; the complete amino acid sequences of *CgCYP51A* and *CgCYP51B* from the sensitive and resistant isolates were used to search for suitable models on the online Swiss-model platform (<https://www.swissmodel.expasy.org/interactive>). The two crystal structures of 4uyl.2.A and 4uyl.1.A of CYP51B from *Aspergillus fumigatus* showed 59.70 and 55.94% identity with the amino acid sequences of *CgCYP51A* and *CgCYP51B*, respectively. The binding cavity was predicted by a ligand mode for tebuconazole complexed in CYP51 with SYBYL 2.0 software. The Tripos force field with Gasteiger-Marsili charges was used for the energy minimization. The molecular conformation of tebuconazole was established by Sketch mode, and was optimized by the Tripos force field and Gasteiger-Hückel charge. The binding cavity was set up as "ligand", and the docking total scores (DTSS) were used to assess the binding affinity between ligand and protein.

Statistical analysis. Data Processing System software (V7.05; Hangzhou, China) was used to process all data. Statistical significance was determined by the Duncan test and least significant difference test at $P = 0.05$. Cross-resistance between two fungicides was determined by Spearman's rank correlation coefficient with log-transformed EC_{50} values (Spolti et al. 2014).

Table 4. Values of EC_{50} and MIC of *Colletotrichum gloeosporioides* strains against tebuconazole via mycelial growth inhibition method^x

Strain	Origin ^y	EC_{50} (μ g/ml) ^z	MIC (μ g/ml)
YC11	Field/S	0.43 $\pm$ 0.02 d	10
YC78	Field/S	0.49 $\pm$ 0.02 d	10
YC2	Field/R	0.74 $\pm$ 0.03 b	60
YC18	Field/R	0.76 $\pm$ 0.02 b	60
YC22	Field/R	0.61 $\pm$ 0.02 c	60
YC44	Field/R	0.74 $\pm$ 0.03 b	60
YC56	Field/R	0.77 $\pm$ 0.04 b	90
YC61	Field/R	1.42 $\pm$ 0.09 a	70

^x EC_{50} = effective concentration for 50% inhibition of mycelial growth and MIC = minimum inhibitive concentration for 100% inhibition of mycelia growth.

^y S = sensitive and R = resistant.

^z Values in each column followed by the same letter for each treatment are not significantly different at $P = 0.05$.

Results

Determination the sensitivity of all isolates to tebuconazole. Of the 48 isolates collected from commercial chili fields, 8 single-spore isolates were identified as *C. gloeosporioides* by sequencing and BLAST analysis of the conserved ITS and β -tubulin (Fig. 1; Table 3). The rest of the isolates were identified as *C. acutatum* (data not show). Six of eight *C. gloeosporioides* isolates could grow on PDA plates amended with tebuconazole at 10 μ g/ml and two could not grow (Fig. 2), which indicated that six isolates were resistant to tebuconazole and the tebuconazole resistance of *C. gloeosporioides* had developed in the fields. All of the *C. acutatum* isolates were sensitive to tebuconazole based on the mycelia growth inhibition bioassay, suggesting that tebuconazole resistance of *C. acutatum* had not developed. Furthermore, the resistant mechanism of *C. gloeosporioides* to tebuconazole was studied; EC_{50} values of *C.*

Fig. 3. Virulence analysis of the eight isolates by inoculation on mature fruit of **A**, chili and **B**, strawberry. Disease symptoms were investigated at 5 days postinoculation.

gloeosporioides-resistant isolates were approximately 0.50- to 2.30-fold higher than sensitive isolates, while the minimum inhibitive concentration for 100% inhibition of mycelia growth values were approximately 5.00- to 8.00-fold higher than the sensitive isolates, indicating that the six isolates were moderately resistant to tebuconazole (Table 4).

Mycelia growth, sporulation, spore-germination rate, and pathogenicity. Mycelial growth rates of resistant isolates (except YC56) were significantly slower than the sensitive isolates ($P = 0.05$). Of the six resistant isolates, four exhibited significantly

stronger sporulation than the sensitive isolates ($P = 0.05$). Additionally, all of the resistant isolates differed significantly in spore-germination rates, and the pathogenicity on mature fruits of chili (Fig. 3A) and strawberry (Fig. 3B). In brief, not all of the resistant isolates exhibited the decreased mycelia growth rate, sporulation, and pathogenicity (Table 5).

Cross-resistance. Cross-resistance was observed between tebuconazole and difenoconazole ($r = 0.8333$, $0.005 < P < 0.01$) or propiconazole ($r = 0.7857$, $0.01 < P < 0.025$). However, there was no cross-resistance between tebuconazole and prochloraz ($r = 0.5000$,

Table 5. Comparison of mycelia growth, sporulation, virulence and conidia germination rate among the tebuconazole-resistant and -sensitive strains of *Colletotrichum gloeosporioides*^a

Strain	Origin ^w	Growth (mm) ^x	Conid ($\times 10^6$) ^y	Rate (%) ^z	Virulence (lesion diameter [mm]) ^v	
					Chili	Strawberry
YC11	Field/S	72.00 $\pm$ 2.85 b	17.62 $\pm$ 5.47 d	94.83 $\pm$ 1.03 ab	10.56 $\pm$ 1.24 cde	15.50 $\pm$ 0.89 c
YC78	Field/S	76.50 $\pm$ 0.85 a	15.40 $\pm$ 2.52 de	88.67 $\pm$ 5.75 bc	12.33 $\pm$ 1.19 bc	14.25 $\pm$ 0.29 c
YC 2	Field/R	65.00 $\pm$ 0.45 c	70.21 $\pm$ 15.42 b	81.50 $\pm$ 3.49 cd	11.67 $\pm$ 1.30 bcd	19.75 $\pm$ 0.29 ab
YC18	Field/R	65.00 $\pm$ 0.77 c	36.94 $\pm$ 13.09 c	50.33 $\pm$ 3.36 f	10.00 $\pm$ 0.60 de	19.75 $\pm$ 0.29 ab
YC22	Field/R	67.00 $\pm$ 1.55 c	47.79 $\pm$ 3.59 c	62.83 $\pm$ 1.29 e	13.83 $\pm$ 1.81 b	22.25 $\pm$ 2.02 a
YC44	Field/R	61.25 $\pm$ 1.16 d	91.10 $\pm$ 3.13 a	87.83 $\pm$ 6.27 bcd	9.00 $\pm$ 0.89 e	20.50 $\pm$ 1.15 ab
YC56	Field/R	76.00 $\pm$ 0.45 a	1.10 $\pm$ 0.92 e	100.00 $\pm$ 0 a	9.33 $\pm$ 0.30 e	19.00 $\pm$ 0.58 b
YC61	Field/R	66.83 $\pm$ 0.26 c	3.95 $\pm$ 0.25 de	80.17 $\pm$ 4.74 d	20.25 $\pm$ 0.59 a	18.50 $\pm$ 0 b

^a Values in each column followed by the same letter for each treatment are not significantly different at $P = 0.05$.

^v Lesion diameters were determined on chili and strawberry in vivo after inoculation for 5 days.

^w S = sensitive and R = resistant.

^x Mycelia growth was measured after inoculation at 25°C for 5 days on fungicide-free potato dextrose agar plates.

^y Conidiation: conidia ($\times 10^6$ conidium/ml) produced on alkyl ester agar medium 7 days after inoculation.

^z Conidia germination rate was measured after inoculation at 25°C for 4 h.

Fig. 4. Spearman rank correlation for cross-resistance in chili (pepper) anthracnose between tebuconazole and six other fungicides, including **A**, difenoconazole; **B**, propiconazole; **C**, prochloraz; **D**, fluazinam; **E**, azoxystrobin; and **F**, fludioxonil. Data points are the logarithmic values of effective concentrations for 50% mycelia growth inhibition (log EC₅₀) among the tested isolates for the indicated fungicide combinations.

$P > 0.10$), fluazinam ($r = -0.5476$, $0.05 < P < 0.1$), azoxystrobin ($r = -0.2619$, $P > 0.1$), or fludioxonil ($r = 0.3333$, $P > 0.1$) (Fig. 4).

Sequence alignment of *CgCYP51A* and *CgCYP51B* of sensitive and resistant isolates. Mutations at amino acid codons are conferred for the resistance against DMIs (Leroux et al. 2007; Mellado et al. 2007). To explore the genetic mechanism of *C. gloeosporioides* resistance to tebuconazole, all of the 5'-UTRs and the whole open reading frames of *CgCYP51A* and *CgCYP51B* from the eight isolates were analyzed. Based on the alignment, mutations occurred at *CgCYP51A* or *CgCYP51B* (Table 6). All of the mutations were categorized into three resistant genotypes. Genotype I possessed four point mutations (V18L, L58V, S175P, and P341A) at *CgCYP51A* but no mutation at *CgCYP51B*, whereas genotype II had five point mutations (L58V, S175P, A340S, T379A, and N476T) at *CgCYP51A*, concomitant with three point mutations (D121N, T132A, and F391Y) at *CgCYP51B*. Additionally, genotype III contained two point mutations (L58V and S175P) at *CgCYP51A*, concomitant with one point mutation (T262A) at *CgCYP51B*. No mutations or insertions were observed in the 5'-UTRs of *CgCYP51A* or *CgCYP51B* from the six resistant isolates.

Overexpression of *CgCYP51A* and *CgCYP51B* genes. Overexpression of fungal *CYP51* genes has been reported to be involved in the resistance of phytopathogenic fungi to DMIs (Nikou et al. 2009; Schnabel and Jones 2001). Expression of *CgCYP51* genes of the sensitive isolates was slightly increased by 0.28- to 0.50-fold in *CgCYP51A* and 0.28 to 1.00-fold in *CgCYP51B*; meanwhile, expression of *CgCYP51* genes of resistant isolates significantly increased 2.00- to 21.00-fold in *CgCYP51A* and 1.00- to 14.00-fold in *CgCYP51B* under the same conditions. Of the three resistant

genotypes, the expression of *CgCYP51* genes of the resistant genotype II was significantly higher than the other two resistant genotypes (Fig. 5).

Analysis of the affinity between *CgCYP51* proteins and tebuconazole. Models were established for the docking of tebuconazole into the *CgCYP51A* or *CgCYP51B* protein-binding pocket (Fig. 6). In addition, the *CgCYP51A* and *CgCYP51B* proteins of the sensitive isolate (YC11 or YC78) were docked as references. The docking of *CgCYP51A* protein with tebuconazole showed that five hydrogen bonds could be formed between tebuconazole and the amino acids Y128 and K139 in the wild type or resistant genotype I, while five or six hydrogen bonds were formed between tebuconazole and the amino acids Y128, K139, and H454 in the resistant genotype III or II, respectively (Fig. 6A). Meanwhile, the docking of *CgCYP51B* protein with tebuconazole indicated that three hydrogen bonds were formed between tebuconazole and amino acids R136 and H467, R110 and H309, or R136 and R377 in the wild type, resistant genotype II, or resistant genotype III, respectively (Fig. 6B). The DTSs of the three resistant genotypes were lower than that of the sensitive isolate (Table 7), indicating that the affinity between tebuconazole and *CgCYP51A* or *CgCYP51B* proteins was decreased in the resistant isolates.

Table 6. Comparison of the amino acid sequences of genes *CgCYP51A* and *CgCYP51B* among the tebuconazole-resistant and -sensitive strains of *Colletotrichum gloeosporioides*

Strain	Origin ^z	Genotype	Mutated sites	
			<i>CgCYP51A</i>	<i>CgCYP51B</i>
YC11, YC78	Field/S	...	...	...
YC2, YC18, YC22, YC44	Field/R	I	V18F, L58V, S175P, and P341A	...
YC56	Field/R	II	L58V, S175P, A340S, T379A and N476T	D121N, T132A, and F391Y
YC61	Field/R	III	L58V and S175P	T262A

^z S = sensitive and R = resistant.

Fig. 5. Expression of *CgCYP51A* and *CgCYP51B* in the sensitive and resistant isolates of *Colletotrichum gloeosporioides*. Mycelia of each isolate was treated with tebuconazole at 0 or 10 $\mu\text{g/ml}$ for 12 h after growth in potato dextrose broth for 24 h. Bars denote standard errors from three repeated experiments. An asterisk indicates significantly different from the control.

Fig. 6. Molecular docking of tebuconazole with the protein *CgCYP51* of *Colletotrichum gloeosporioides*. Modeling structures of the *CgCYP51A* (A) and *CgCYP51B* (B) protein domains from *C. gloeosporioides* were obtained by using the templates 4uyl.2.A and 4uyl.1.A, two *CYP51B* proteins from the *Aspergillus fumigatus*, and the online Swiss-model platform (<https://swissmodel.expasy.org/>). Binding cavities were predicted by a ligand mode for tebuconazole complexed in *CgCYP51* by SYBYL 2.0 software. The Tripos force field with Gasteiger-Hückel charges was used to minimize the energy. The molecular conformation of tebuconazole was constructed by Sketch mode and was optimized using the Tripos force field and Gasteiger-Hückel charge.

Discussion

Resistance of *Colletotrichum* spp. isolated from chili to benzimidazole derivatives has been documented in China since 2002 (Li et al. 2002). Owing to the specific mode of action, DMIs have become a crucial component of successful resistance management strategies to control anthracnose of chili in Jiangsu Province of China for more than three decades. However, the DMI resistance of *Colletotrichum* spp. has been reported in recent years (Chen et al. 2013; Chen et al. 2016). Although *C. gloeosporioides* and *C. acutatum* were isolated from chili fruit with typical lesions, only one of the two species was isolated from a single lesion, suggesting that one lesion is not often caused by the two combined *Colletotrichum* spp. in field. In addition, moderately resistant isolates with mutations at *CgCYP51A* or *CgCYP51B* were selected in the field, and each resistant isolate had low fitness cost, most likely due to fast selection. This scenario is similar to the mixed populations of *Fusarium graminearum* and *F. asiaticum* selected by benzimidazole derivatives in field (Chen et al. 2009; Qu et al. 2008). To our knowledge, this is the first report that mutations at *CgCYP51A* or *CgCYP51B* are involved in the resistance of *C. gloeosporioides* against DMIs.

Mutations at *CgCYP51A* or *CgCYP51B* in *C. gloeosporioides* are related to the resistance against DMIs. The first and only case related to resistance of *C. gloeosporioides* against prochloraz from mango was reported in 2003 (Gutiérrez Alonso et al. 2003). However, in the two other *Colletotrichum* spp. including *C. truncatum*, resistant to tebuconazole, metconazole, flutriafol, and fenbuconazole, and *C. nymphaeae*, resistant to flutriafol and fenbuconazole, from peach orchards in 2016, the point mutations at *CYP51A* and *CYP51B* from resistant isolates were associated with resistance to DMIs (Chen et al. 2016). *CYP51* genes are widely distributed in all biological kingdoms, including fungi, plants, bacteria, and animals (Doddapaneni et al. 2005). However, the number of *CYP51* genes varies in fungi. Unlike only a single *CYP51* gene in Hemiascomycete yeasts and basidiomycetes, Pezizomycotina, a subphylum of the phylum Ascomycota, contains many phytopathogenic and clinical fungi, and has two or three copies of the *CYP51* genes (Fan et al. 2013). Although some filamentous fungi possess more than one copy of a *CYP51* gene, mutations at *CYP51* commonly occur at *CYP51A* of phytopathogenic fungi to confer DMI resistance, and rarely occur at *CYP51B* or *CYP51A* and *B* (Délye et al. 1998). The results in clinical fungi, with an exception for *A. clavatus*, are similar to those in phytopathogenic fungi (Song et al. 2018). In *A. clavatus*, point mutations at *CYP51A* and *CYP51B* genes are involved in posaconazole and itraconazole resistance (Abastabar et al. 2019). In brief, the result in the study was similar to that in *A. clavatus*, *C. truncatum*, and *C. nymphaeae*.

Overexpression of *CgCYP51A* and *CgCYP51B* of resistant isolates was observed in response to tebuconazole. In the current study, both *CgCYP51A* and *CgCYP51B* genes from *C. gloeosporioides* overexpressed when treated with tebuconazole at 10 µg/ml for 12 h. To date, overexpression of *CYP51* genes caused by promoter insertions or retrotransposons are reported in only a few phytopathogenic fungi; for example, a duplication of a 65-bp sequence was found at a promoter of *CYP51* of *Monilinia fructicola* (Luo and Schnabel 2008), and a 2- to 5-kb retrotransposon was found at *Blumeriella jaapii* (Ma et al. 2006). However, mechanisms underlying overexpression of *CYP51* are not known in the field for DMI-resistant subpopulations

of *Cercospora beticola* (Bolton et al. 2012), *Phakopsora pachyrhizi* (Schmitz et al. 2014), *Puccinia trititica* (Stammler et al. 2009), and *Sclerotinia homeocarpa* (Hulvey et al. 2012). In current study, the molecular mechanism of *CYP51* overexpression needs further investigation.

Knowledge of fitness costs is crucial for predicting effectiveness and optimizing disease control strategies based on fungicide combinations (Mikaberidze and McDonald 2015). The results of fitness comparison indicated that the resistant genotype I exhibited decreased rates of mycelia growth and spore germination, increased sporulation, and similar pathogenicity when compared with the sensitive isolates. Because only one isolate of the resistant genotype II or III was available, we will look for more isolates with those resistant genotypes in future to characterize their specific fitness parameters. Furthermore, positive cross-resistance was observed between tebuconazole and difenoconazole but not between tebuconazole and prochloraz. The reason no cross-resistance between tebuconazole and prochloraz was observed in the current study possibly resulted from the fact that prochloraz chemically belongs to an imidazole fungicide, which is very different from tebuconazole and difenoconazole, two triazole fungicides. The lack of cross-resistance between different fungicides with different modes of action in the study such as fluazinam, azoxystrobin, and fludioxonil can be used to manage the DMI resistance of *Colletotrichum gloeosporioides*. In addition, fungicides with high or low resistance risk—such as azoxystrobin and fluazinam or fludioxonil, prochloraz, and fluazinam or fludioxonil, and so on—could be used together to delay the resistance development of *C. gloeosporioides* in fields. Furthermore, rational use of fungicides serves as an important component of an integrated antifungal management strategy.

In brief, the moderate resistance of *C. gloeosporioides* to DMIs has developed in the chili fields in Jiangsu Province of China. Mutations at *CgCYP51A* or *CgCYP51B* are involved in the resistance to tebuconazole. All of the resistant isolates possessed a low fitness penalty. Prochloraz and other fungicides with different modes of action should be integrated into disease management programs to reduce fungicide resistance development.

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Table 7. Docking total scores (DTSs) of the fungicide tebuconazole into the *Colletotrichum gloeosporioides* CYP51 protein binding pockets of sensitive or resistant mutants

Strain	Origin*	Genotype	DTSs	
			CYP51A	CYP51B
YC11, YC78	Field/S	...	3.2366	2.9772
YC2, YC18, YC22, YC44	Field/R	I	2.3081	...
YC56	Field/R	II	3.1512	1.5772
YC61	Field/R	III	1.7003	1.4701

* S = sensitive and R = resistant.

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