Paralogous *CYP51* Genes of *Colletotrichum* spp. Mediate Differential Sensitivity to Sterol Demethylation Inhibitors

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ABSTRACT

Colletotrichum spp. isolates contain two paralogous *CYP51* genes that encode sterol 14-demethylase enzymes; however, their role in sensitivity to demethylation inhibitor (DMI) fungicides is yet to be determined. In this study, each of the two genes from *Colletotrichum fioriniae* and *C. nymphaeae* was able to rescue the function of *CYP51* in the yeast *Saccharomyces cerevisiae*, demonstrating their independent function. Deletion of *CYP51A* led to increased sensitivity to propiconazole, diniconazole, prothioconazole, cyproconazole, epoxiconazole, flutriafol, prochloraz, and difenoconazole in *C. fioriniae*, and to the same fungicides and tebuconazole in *C. nymphaeae*, with the exception of prochloraz. Deletion of *CYP51B* in *C. fioriniae* and *CYP51B* in *C. nymphaeae* made mutants increasingly sensitive to five of nine DMI fungicides tested.

The results suggest species-specific, differential binding of DMI fungicides onto the two *CYP51* enzymes. Pairing DMIs with different effects on *CYP51A* and *-B* deletion mutants resulted in synergistic effects, as determined in mycelial growth inhibition experiments. Deletion mutants showed no fitness penalty in terms of mycelial growth, sporulation, and virulence. Our study elucidates the effect of *CYP51A* and *CYP51B* of *Colletotrichum* spp. on DMI sensitivity, suggesting that using a mixture of DMIs may improve the efficacy for anthracnose management.

Keywords: *Colletotrichum* spp., *CYP51* genes, disease control and pest management, DMI fungicides, mutants

Colletotrichum spp. are economically important plant pathogens, which affect a wide range of crops including fruits and vegetables (Sutton 1996). The disease can occur on leaves, stems, and fruit of host plants. The most common symptoms include sunken necrotic lesions and erumpent, mucilaginous, pink spore masses under conducive environments. Multiple *Colletotrichum* spp. have been found to cause anthracnose in the same plant host. For example, *Colletotrichum nymphaeae* and *C. fioriniae* (both belong to the *C. acutatum* J. H. Simmonds complex) have been found on peach and blueberry in the southeastern United States (Chechi et al. 2019; Chen et al. 2016), on strawberry in the United States (Wang et al. 2019) and the United Kingdom (Baroncelli et al. 2015), on apple in Kentucky (Munir et al. 2016), and on common walnut in France (Da Lio et al. 2018).

Control of anthracnose relies heavily on fungicides, but only a few chemical classes including the sterol demethylation inhibitors (DMIs) are effective (Szkolnik 1981). DMIs specifically inhibit the demethylation of the precursor sterols by binding to *CYP51* enzymes, leading to inhibition of fungal ergosterol biosynthesis (Parker et al. 2008; Warrilow et al. 2010). With intensive use of DMIs in the past decades, reduced sensitivity or even resistance to DMI fungicides have been reported in *Colletotrichum* spp. (Chen

et al. 2013; Deng et al. 2011). However, the mechanism conferring DMI resistance in *Colletotrichum* spp. remains unclear. The amino acid variations in the substrate recognition sites (SRS) have been speculated to be associated with DMI resistance in *C. truncatum* (Chen et al. 2016; Zhang et al. 2017). However, direct genetic evidence is still lacking. In other filamentous fungi, three predominant mechanisms have been found to confer DMI resistance, including (i) target-site modification in the *CYP51* gene (Délye et al. 1997, 1998), (ii) overexpression of the *CYP51* gene (Hamamoto et al. 2000; Ma et al. 2006; Schnabel and Jones 2001), and (iii) and increased efflux pump activity (Hayashi et al. 2003; Kretschmer et al. 2009; Sanglard et al. 1995).

Several ascomycetes possess multiple *CYP51* paralogs. Although *CYP51B* is constitutively expressed in all filamentous fungi, *CYP51A* is found only in some ascomycetes, including *Aspergillus fumigatus* (Mellado et al. 2001), *A. nidulans* (Da Silva Ferreira et al. 2005), *Penicillium digitatum* (Hamamoto et al. 2000), and *Magnaporthe oryzae* (Yan et al. 2011). Thus far, *CYP51C* has only been reported in *Fusarium* spp. Previous studies have shown that paralogous *CYP51* genes mediate differential sensitivity to DMIs. In *M. oryzae*, for example, the deletion of *CYP51A* led to increased sensitivity to DMI fungicides, whereas the deletion of *CYP51B* did not alter the sensitivity (Yan et al. 2011). In *Fusarium graminearum*, the *CYP51A*-deleted mutants showed increased sensitivity to seven DMI fungicides tested, whereas sensitivity increased to some but not all DMIs tested in *CYP51C*-deleted mutants (Liu et al. 2011). No change in DMI sensitivity was observed in mutants with *CYP51B* deletion. In *F. oxysporum*, the deletion of *CYP51A* led to increased sensitivity to 11 DMI fungicides but the *CYP51B* mutants showed increased sensitivity to only 4 of them. The *CYP51C* mutants, however, did not change their DMI sensitivity (Zheng et al. 2019).

Colletotrichum spp. were found to have differential sensitivity to DMI fungicides. For example, *C. truncatum* was inherently insensitive to tebuconazole, metconazole, flutriafol, febuconazole (Chen et al. 2018), and myclobutanil (Zhang et al. 2017) but

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sensitive to difenoconazole and propiconazole (Chen et al. 2016). *C. nymphaeae* was inherently resistant to flutriafol and fenbuconazole but sensitive to difenoconazole, propiconazole, tebuconazole, and metconazole. In contrast, *C. fioriniae* was sensitive to all those DMIs (Chen et al. 2016). However, the function of the two paralogous *CYP51* genes in *Colletotrichum* spp. remains unknown, in terms of mediating sensitivity to DMI fungicides. Thus, the objective of this study was to investigate the role of *CYP51A* and *-B* paralogous genes from *C. fioriniae* and *C. nymphaeae* in the sensitivity to various DMI fungicides.

MATERIALS AND METHODS

Fungal isolates and chemicals. Isolates of *C. fioriniae* (Ca2-4-2) and *C. nymphaeae* (CaPH40) were recovered from stock cultures with silica blue gel at -20°C on dried filter paper discs (Fisher Scientific). The isolates were grown for 5 days on potato dextrose agar (PDA) before being used for further experiments. Both isolates were previously collected from peach fruit (Chen et al. 2016). Nine DMI fungicides were used for sensitivity analyses: propiconazole (95% active ingredient [a.i.]; Adama Agricultural Solutions Ltd.), tebuconazole (95% a.i.; Bayer CropScience), diniconazole (95% a.i.; Jiangsu Sevencontinent Green Chemical Co.), prothioconazole (95% a.i.; Hailir Pesticide and Chemicals Group Co., Ltd.), cyproconazole (98% a.i.; Jiangsu Sevencontinent Green Chemical Co.), epoxiconazole (97% a.i.; Jiangsu Sevencontinent Green Chemical Co.), flutriafol (97% a.i.; Jiangsu Sevencontinent Green Chemical Co.), prochloraz (97% a.i.; Adama Agricultural Solutions Ltd.), and difenoconazole (95% a.i.; Jiangsu Sevencontinent Green Chemical Co.).

Complementation of the yeast *ERG11* mutant. The complementation of yeast strains was conducted as described previously (Yang et al. 2015). The yeast strain BY4741 (wild type) and *ERG11* mutant TSA14 were kindly provided by Dr. Yang from Qingdao Agricultural University, which were purchased from the European Saccharomyces Cerevisiae Archive for Functional Analysis (EUROSCARF). The genotype of TSA14 is described as BY4741;MATa; ura3 Δ 0; leu2 Δ 0; his3 Δ 1; met15 Δ 0; erg11-td:kanMX (lethal at 37°C) under accession number Y40597. To construct the vector for yeast transformation, the linearized pYES2 plasmid was generated with *HindIII* and *XhoI* restriction enzymes (TaKaRa Biotechnology). To generate pYES2-CfCYP51A, distinctive primers CFA_YF and CFA_YR (Supplementary Table S1) were designed to amplify complementary sequences of both terminal ends of linearized pYES2 plasmid and cDNA of the *CfCYP51A* gene. The cDNA fragment was then purified by a DNA gel extraction kit (Tsingke Company) and mixed with the linearized pYES2 plasmid for ligation in an overlapping manner utilizing Soso cloning kits (Tsingke Company) at 50°C for 15 min. Similarly, cDNA of *CfCYP51B*, *CnCYP51A*, and *CnCYP51B* were amplified and ligated into a pYES2 vector. In addition, the wild-type strain BY4741 and TSA14 mutant transformed with empty pYES2 vector were used as controls. Yeast transformation with pYES2 vector was performed as described previously (Yang et al. 2015). Serial fivefold dilutions of the yeast transformants from a stock of 10^7 cell/ml were spotted on 5 μl of yeast-peptone-galactose medium (1% yeast extract, 2% peptone, 1% galactose, 1% raffinose, and 2% agar), then incubated at both 30°C and 37°C for 2 days. Three replicates for each yeast transformants were tested. The entire experiment was repeated twice, independently.

Construction of knockout fragment of *CYP51* genes. The double-joint (DJ) PCR method was applied to construct fusion PCR products for *CYP51* gene knock outs, as described previously (Chen et al. 2017). All primers are listed in Supplementary Table S1. For example, two primer pairs (FiorA_5_For/FiorA_5_Rev and FiorA_3_For/FiorA_3_Rev) were used to amplify the 5' and 3' flanking regions, respectively, of *CfCYP51A* from the *C. fioriniae* isolate Ca2-4-2. The hygromycin-resistant cassette (HYG) with the

A. nidulans TrpC promoter and terminator were amplified from the pBHT2 vector using primer pair hypF and hypR. Subsequently, the three amplicons (the 5' and 3' flanking sequences of *CfCYP51* and HYP) were fused by a second round of DJ PCR. Based on the fused fragment, the final PCR products with an overlapping part of 459 bp were amplified by the nested primer pairs FiorA_5_nest/Hyp-nest-5R and Fior3_5_nest/Hyp-nest-3F, then transformed into the *C. fioriniae* isolate Ca2-4-2. Knockout fragments of *CfCYP51B*, *CnCYP51A*, and *CnCYP51B* were constructed following the same protocols.

Transformation of *C. fioriniae* and *C. nymphaeae*. The polyethylene glycol (PEG)-mediated fungal transformation was performed as described previously (Chen et al. 2017). Briefly, mycelia of *C. fioriniae* or *C. nymphaeae* were incubated in 50 ml of potato dextrose broth (PDB) medium in a flask at 25°C for 1.5 days on a shaker at 160 rpm. To prepare protoplasts, mycelia were collected by filtration and washed with 0.7 M NaCl buffer. The suspension for digestion of the cell wall contained lysing enzymes (Sigma Aldrich) at 0.075 mg/ml in 10 ml of 0.7 M NaCl buffer as the enzyme mixture, which was filtered through a 0.22- μm MILLEX GP sterile filter membrane. A flask with 10 ml of filter-sterilized enzyme mixture and 1 g of mycelia was incubated at 30°C for 4 h with shaking at 100 rpm. The resulting protoplasts were filtered through three layers of lens cleaning tissue, collected by centrifugation at 3,000 rpm for 10 min, and washed twice in 20 ml of sorbitol-Tris-calcium (STC) buffer (1.2 M sorbitol, 10 mM Tris-HCl, and 20 mM CaCl_2 ; pH 7.5). The final concentration of protoplasts was calculated and adjusted to 10^7 CFU/ml using a hemocytometer under a microscope (Nikon).

The flanking fragments of the *CYP51* genes carrying an HYG-selectable marker fragment were added to protoplasts at 10^7 CFU/ml and incubated on ice for 15 min prior to mixing with 1.2 ml of 60% PEG 4000, 10 mM Tris-HCl, and 20 mM CaCl_2 (pH 7.5). The mixture was then incubated on ice for 15 min, and 1 ml of STC was then added prior to plating on agar plates. The pellet was added to 200 ml of regeneration medium prior to pouring to plates. After 48 h, the plates were overlaid with PDA amended with hygromycin B at 200 $\mu\text{g}/\text{ml}$. After 4 to 6 days of incubation, hygromycin-resistant colonies (transformants) were transferred onto fresh PDA medium amended with hygromycin B at 200 $\mu\text{g}/\text{ml}$ for three generations to confirm the resistance. Single germinated conidia from each transformant were transferred to PDA plates containing hygromycin B using a sterile needle to create monoconidial transformants for further experiments.

Complementation of the *CYP51* gene deletion mutants.

To generate the complementation of the *CYP51A* and *-B* mutants, the corresponding full-length of the *CYP51A* and *-B* fragments and a 1-kb upstream flanking sequence were amplified and inserted into a PCETNS4 vector. The rescue plasmid PCENTNS4-CYP51-COM was constructed using the backbone of 13-PCENTS, which contained a G418-resistant cassette (*Npt2*). For complementation of $\Delta\text{CfCYP51A}$ with *CfCYP51A*, the full-length *CfCYP51A* gene, including the 1,526-bp upstream region and 458 terminator region, was amplified from the isolate Ca2-4-2 using primers CFA-com-F and CFA-COM-R (Supplementary Table S1). The gene was then cloned into the *XhoI* and *SamI* sites of 13-PCENTS to generate the complement plasmid PC-CfCYP51A-COM. The *CfCYP51A* gene was sequenced to ensure flawlessness before being transferred into the Ca2-4-2 protoplasts. Similarly, the full-length *CfCYP51B* gene, including the 1,464-bp upstream region and 1,408-bp downstream terminator region, was amplified from the Ca2-4-2 isolate. The *CnCYP51A* gene (including the 1,407-bp upstream region and 1,386-bp terminator) and *CnCYP51B* gene (1,409-bp upstream region and 971-bp terminator) were amplified from the isolate CaPH40, using primer pairs CNA_XHO_F/CNA_SMA_R and CNB_XHO_F/ CNB_SMA_R, respectively (Supplementary Table S1). The complementation transformation was conducted as described above, except that G418 was used as a selective marker.

Sensitivity to DMI fungicides. Sensitivity to nine different active ingredients of DMI fungicides, including propiconazole, tebuconazole, diniconazole, prothioconazole, cyproconazole, epoxiconazole, flutriafol, prochloraz, and difenoconazole, was assessed on fungicide-amended PDA plates with each active ingredient at 0, 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 µg/ml. Mycelial plugs were removed with a 5-mm cork borer from the margins of 5-day-old colonies and placed upside down on the centers of 9-cm plastic Petri dishes containing the fungicide-amended or unamended PDA medium. Three replicates for each concentration were used for each isolate. Mean colony diameters were measured and expressed as percent growth inhibition after 5 to 7 days of incubation at 25°C in darkness. Mycelial growth rates were measured after 7 days of incubation on unamended medium. For each deletion genotype, the average concentration to inhibit mycelium growth by 50% (EC₅₀) value of three mutations were calculated. The EC₅₀ values were calculated by regressing percentage of mycelial growth inhibition against the log of fungicide concentrations. The entire experiment was repeated twice, independently.

Synergistic interactions between fungicides were evaluated based on the synergy factor (SF), a ratio between the expected EC₅₀ and the observed EC₅₀ for a mixture ($SF = EC_{50} \text{ expected} / EC_{50} \text{ observed}$). The expected EC₅₀ of a mixture was calculated as $EC_{50} \text{ expected} = (A + B) / (A/EC_{50} \text{ fungicide 1} + B/EC_{50} \text{ fungicide 2})$, where A and B are the proportions of fungicide 1 and fungicide 2 in a mixture, respectively, and EC₅₀ fungicide 1 or EC₅₀ fungicide 2 is the observed EC₅₀ value of fungicide 1 or fungicide 2, respectively. Typically, synergistic interactions are considered when the SF value is >1, and a higher SF value (>1) represents stronger synergy (Gisi 1996; Kosman and Cohen 1996). Ten different mixtures of DMI fungicides were selected based on their inhibitory patterns to determine the SF values using *C. fioriniae* isolates (Ca2-2-2, Ca2-3-2, and Ca2-4-2) and *C. nymphaeae* isolates (CaCO4-35, CaPH40, and CaCO4-42.)

Fitness components. Fitness components, including (i) mycelial growth rate, (ii) lesion diameter on detached fruit, and (iii) number of spores per lesion, were investigated to assess potential fitness cost associated with mutants with different genotypes. Mycelial growth rates were measured after 7 days of incubation on PDA medium. For each deletion genotype, three mutants were measured. The experiment was repeated twice.

For detached-fruit assays, peach fruit of similar size and commercial maturity were surface sterilized by immersion in 75% (vol/vol) ethanol, rinsed three times in sterile deionized water, and subsequently air dried for 4 h. Two 4-by-2-mm (diameter by depth) plugs were removed from the flesh of each fruit at equidistance location (3 cm apart) using a cork borer (4 mm in diameter) and replaced within 10 min with mycelial plugs (mycelial-side down) of the same size (4 mm in diameter) taken from the margin of 5-day-old cultures. Inoculated peach fruit were placed in boxes, covered with a cling film to keep the relative humidity at approximately 100%, and maintained at room temperature. Lesion diameters were measured after 4 days of inoculation. To evaluate sporulation ability, conidia produced on each peach fruit were collected using premoistened sterile cotton swabs after 6 days of incubation. Swabs were placed into a 50-ml tube containing 40 ml of sterile distilled water. After vortexing for 20 s, the conidial suspension was filtered through a double layer of cheesecloth. The spore concentration was measured using a hemacytometer. Three mutants of each genotype and four replicates (fruit) were used for each mutant, and the entire experiment was conducted twice, independently.

Analysis of CYP51 gene expression. Relative expression levels of CYP51 genes in the gene deletion mutants were measured by real-time PCR assays. Strains were cultured in PDB medium for 2 days and mycelia were harvested for RNA extraction. Approximately 150-bp fragments of the *CfCYP51A* and *CnCYP51A* genes were amplified with primer pairs RCFAF/RCFAR and RCNAF/RCNAR, respectively. Similarly, *CfCYP51B* and *CnCYP51B* genes

were amplified with primer pair RNFIBF/RNFIBR (Supplementary Table S1). Expression was normalized with the expression of the *GAPDH* gene from respective *Colletotrichum* spp. The relative gene expression was calculated using the comparative cycle threshold (Ct^{ΔΔ}) method (Wong and Medrano 2005). To determine whether the expression of CYP51 genes was inducible by DMI fungicides, each mutant strain was treated with or without propiconazole at 1 µg/ml for 6 h prior to RNA extraction. Real-time PCR was performed in an ABI 7500 Real-Time PCR Detection System (Bio-Rad Laboratories Inc.) using SYBR Green I fluorescent dye detection. Amplifications were conducted in a 20-µl volume containing 10 µl of SYBR Premix Ex TaqII (Takara), 0.25 µM each primer, and 1 µl of cDNA. All PCRs were performed in triplicate with a program of 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Reverse-transcription (RT)-PCRs were performed with an additional reaction in which the cDNA was replaced with RNA as a negative control. The relative expression of both CYP51A and CYP51B genes was calibrated to the expression of CYP51A genes from wild-type *C. fioriniae* isolate Ca2_4_2. Two biological replications starting with new cultures for RNA extraction were conducted independently.

Statistical analysis. Significant differences in EC₅₀ values, mycelial growth rate, sporulation, and lesion diameter between the wild type and mutants were evaluated by one-way analysis of variance with a least significant difference test in SPSS for Windows (version 13.0; SPSS Inc.).

RESULTS

Sequencing analysis of the CYP51 genes. The nucleotide sequences of CYP51A and -B from *C. fioriniae*, including exons and introns, were 1,718 and 1,720 bp in length, respectively. The full-length cDNA of *CfCYP51A* amplified from RNA of Ca2-4-2 was 1,566 bp in length, encoding a putative polypeptide of 521 amino acids (aa). Sequence comparison of the genomic DNA and cDNA revealed two introns located at nucleotide positions 223 to 293 and 729 to 809. The CYP51A gene sequences were deposited in GenBank under accession number MK990544. Similarly, two introns of 74 and 65 bp located at nucleotides 247 to 320 and 519 to 583, respectively, were predicted in the genomic sequences of *CfCYP51B* that encodes 526 aa (GenBank accession number MK990545). The identity of amino acid sequences between *CfCYP51A* and *CfCYP51B* was 50.10%.

In *C. nymphaeae*, the *CnCYP51A* gene was 1,580 bp in length interrupted by two introns spanning nucleotides 223 to 293 and 729 to 804 (GenBank accession number MK990546). The *CnCYP51B* gene was also predicted to have two introns located at nucleotide positions 247 to 320 and 519 to 583, and encode 526 aa (GenBank accession number MK990547). *CnCYP51A* and *CnCYP51B* shared 50.29% amino acid sequence identity. Interestingly, the amino acid sequence identity between *CfCYP51A* and *CnCYP51A* was 93.45%, whereas only one amino acid variation at position 391 ('A' in *CfCYP51B*, and 'V' in *CnCYP51B*, Supplementary Fig. S1) was found in CYP51B between the two species (*C. fioriniae* and *C. nymphaeae*).

Based on the alignment of the amino acid sequences of CYP51A and -B from *Colletotrichum* spp., *Saccharomyces cerevisiae*, *Mycosphaerella fijiensis*, and *A. fumigatus*, six SRS were identified (Supplementary Fig. S1). In addition, four consensus motifs responsible for enzyme functions, including heme-binding domain, two E-R-R triad domains for stabilization of the core structure, and one oxygen binding and activation domain, were identified (Supplementary Fig. S1).

Complementation of a yeast ERG11 mutant with CfCYP51 and CnCYP51 genes. Temperature-sensitive yeast ERG11 mutant TSA14 and its parental strain BY4741 were used to determine whether CYP51 genes from *Colletotrichum* spp. could rescue the function of yeast CYP51. The full-length cDNA of *CfCYP51A* and

-B and *CnCYP51A* and -B were each cloned into the pYES2 vector and transformed into TSA14. The growth defect of TSA14 at 37°C was restored in the yeast transformants expressing *CfCYP51A* and -B or *CnCYP51A* and -B, indicating that both *CYP51* genes of both species were functional 14 α -demethylases in the budding yeast (Fig. 1).

Disruption and complementation of the *CYP51* genes in *Colletotrichum* spp. Five *CfCYP51A* gene deletion mutants (Table 1) were obtained and identified by PCR analysis using the primer pairs Check_CFA_For/Check_hyg_Rev and Check_hyg_For/Check_CFA_Rev (Supplementary Table S1). These primer pairs amplified 2,675- and 1,320-bp fragments, respectively, from the *CfCYP51A* mutants (Supplementary Fig. S2). In addition, RT-PCR confirmed that the *CfCYP51* gene was unable to be transcribed in the *CYP51A* deletion mutant (Supplementary Fig. S2A). Taken together, these results indicated that the *CfCYP51A* gene was disrupted by the *HYG* gene in the *C. fioriniae* mutants. Eight *CfCYP51B* gene deletion mutants (Δ *CfCYP51B* mutants) were obtained. Primer pairs Check_CFB_For/Check_hyg_Rev and Check_hyg_For/Check_CFB_Rev amplified 2,301 and 2,289 bp, respectively, from the Δ *CfCYP51B* (Supplementary Fig. S2B). RT-PCR confirmed that the *CYP51B* gene was unable to be transcribed in the *CYP51B* deletion mutant (Supplementary Fig. S2A).

In total, 10 *CnCYP51A* and 9 *CnCYP51B* confirmed deletion mutants of *C. nymphaeae* were obtained and verified through PCR analysis. Primer pairs Check_CNA_For/Check_hyg_Rev and Check_hyg_For/Check_CNA_Rev amplified 2,460 and 1,845 bp, respectively, from Δ *CnCYP51A*, and Check_CNB_For/Check_hyg_Rev and Check_hyg_For/Check_CNB_Rev amplified 2,405 and 2,185 bp, respectively, from Δ *CnCYP51B* (Supplementary Fig. S2B). RT confirmed that both *CYP51* genes were unable to be transcribed in their corresponding deletion mutant (Supplementary Fig. S2A). Furthermore, the open reading frame sequence of *CYP51* genes containing their native promoter region was introduced into the corresponding deletion mutants to create the complemented strains. For each deletion and complemented genotype, three strains were selected for the DMI sensitivity test and phenotype test.

Effects of *CYP51* gene deletion on sensitivity of *C. fioriniae* and *C. nymphaeae* to DMIs. The different EC₅₀ values for DMIs obtained for *Colletotrichum* spp. Δ *CYP51B* and

Δ *CYP51A* suggest differential binding affinity to the *CYP51A* and *CYP51B* enzymes. In both *C. fioriniae* and *C. nymphaeae* mutants, the deletion of *CYP51A* yielded largely similar sensitivities among fungicides tested. The deletion of *CYP51B*, however, yielded noticeable variations in sensitivity to the DMIs tested (Figs. 2 and 3).

For *C. fioriniae*, the mean EC₅₀ values of Δ *CfCYP51A* exhibited significantly higher sensitivity to all DMIs compared with the parental isolate Ca2-4-2, except for tebuconazole (Fig. 2). In contrast, Δ *CfCYP51B* showed significantly higher sensitivity to tebuconazole, with the average EC₅₀ value decreasing from 0.22 to 0.04 μ g/ml (Fig. 2). A significant decrease of EC₅₀ values for Δ *CfCYP51B* was also observed for flutriafol, propiconazole, diniconazole, and epoxiconazole compared with their wild-type parent. No significant difference in EC₅₀ values to prothioconazole, cyproconazole, difenoconazole, and prochloraz were found in Δ *CfCYP51B* compared with the parental isolate Ca2-4-2 (Fig. 2).

Deletion of *CYP51A* in *C. nymphaeae* led to a significant increase of sensitivity to all the DMI fungicides tested, except for prochloraz (Fig. 3). Remarkably, EC₅₀ values in Δ *CnCYP51A* for propiconazole and epoxiconazole were substantially lower (values of 0.04 and 0.003 μ g/ml, respectively) compared with that of their parental isolate CaPH40 (1.62 and 1.17 μ g/ml, respectively). It is also noteworthy that the parental isolate CaPH40 was insensitive to

TABLE 1. *Colletotrichum* isolates and mutants used in this study

Species, isolates	Description
<i>Colletotrichum fioriniae</i>	
Ca2-4-2	Wild type
Δ <i>CfCYP51A</i>	<i>CYP51A</i> gene deletion mutant
Δ <i>CfCYP51B</i>	<i>CYP51B</i> gene deletion mutant
Δ <i>CfCYP51A</i> -Com	Complemented strain of Δ <i>CfCYP51A</i>
Δ <i>CfCYP51B</i> -Com	Complemented strain of Δ <i>CfCYP51B</i>
<i>C. nymphaeae</i>	
CaPH40	Wild type
Δ <i>CnCYP51A</i>	<i>CYP51A</i> gene deletion mutant
Δ <i>CnCYP51B</i>	<i>CYP51B</i> gene deletion mutant
Δ <i>CnCYP51A</i> -Com	Complemented strain of Δ <i>CnCYP51A</i>
Δ <i>CnCYP51B</i> -Com	Complemented strain of Δ <i>CnCYP51B</i>

Fig. 1. Complementation of *Saccharomyces cerevisiae* ERG11 mutant TSA14 with *CfCYP51A* and -B and *CnCYP51A* and -B. Controls were constructed by transforming the empty pYES2 vector to the wild-type BY4741 and the ERG11 mutant TSA14. Serial dilutions of yeast suspensions of each strain (cells per milliliter) were spotted onto yeast-peptone-galactose (1% yeast extract, 2% peptone, 1% galactose, 1% raffinose, and 2% agar). The growth of each strain on plates was examined after incubation at 30 and 37°C for 48 h.

cyproconazole and flutriafol, whereas $\Delta CnCYP51A$ isolates were found to be sensitive to both of them, with EC_{50} values declining from more than 50 $\mu\text{g/ml}$ for both fungicides to 0.03 and 1.93 $\mu\text{g/ml}$, respectively (Fig. 3). Deletion of $CnCYP51B$ in the same isolate, however, led to significantly higher sensitivity to prochloraz; the EC_{50} value was decreased from 0.07 to 0.003 $\mu\text{g/ml}$ compared with the parental isolate. The sensitivity of $\Delta CnCYP51B$ also significantly increased to prothioconazole, cyproconazole, difenoconazole, and epoxiconazole (Fig. 3).

The complemented transformants of both *C. fioriniae* and *C. nymphaeae* isolates regained the parental level of sensitivity to all DMI fungicides tested (Tables 2 and 3).

Relative expression levels of *CYP51* genes in parental isolates and gene deletion mutants. In the absence of propiconazole exposure, the expression of *CYP51A* was significantly upregulated in both $\Delta CfCYP51B$ (approximately threefold) and $\Delta CnCYP51B$ (approximately twofold) compared with the parental isolates. The expression of *CfCYP51B* was also upregulated (approximately 2.5-fold) in the $\Delta CfCYP51A$ mutant, while the expression of *CnCYP51B* was downregulated in the $\Delta CnCYP51A$ mutant. In parental isolates, the expression of *CYP51B* was approximately 10-fold (*C. fioriniae*) and 15-fold (*C. nymphaeae*) higher compared with *CYP51A* (Fig. 4).

Propiconazole treatment of mycelium prior to RNA extraction upregulated *CYP51A* and *CYP51B* expression levels in all parental isolates and mutants tested. The *CYP51A* expression was higher in response to propiconazole exposure compared with *CYP51B* genes. Compared with their parental isolates, after being propiconazole amended, the expression of *CfCYP51A* and *CnCYP51A* was induced 13-fold and 5-fold, respectively, in $\Delta CYP51B$, while the expression of *CfCYP51B* and *CnCYP51B*

was induced 2-fold and 1.5-fold, respectively, in $\Delta CYP51A$. After deletion of *CYP51B*, expression of *CYP51A* was upregulated in both $\Delta CfCYP51B$ and $\Delta CnCYP51B$ but not to the level of the parental isolates. In $\Delta CYP51A$ of both species, the expression level of *CYP51B* was comparable with or higher than the parental isolates ($P = 0.05$).

Expression of *CfCYP51A* and *CnCYP51A* was not detected in $\Delta CfCYP51A$ and $\Delta CnCYP51A$, respectively. Similarly, expression of *CfCYP51B* and *CnCYP51B* was not detected in $\Delta CfCYP51B$ and $\Delta CnCYP51B$. No correlation was found between DMI fungicide sensitivity and *CYP51* gene expression in any of the isolates tested ($R^2 < 0.50$; $P > 0.05$).

Evaluation of synergistic interactions between DMIs. DMI fungicides were grouped depending on their inhibitory pattern against *CYP51A* and *-B* deletion mutants (Tables 2 and 3). For both species, groups 1 to 3 were formed based on increased efficacy against $\Delta CYP51B$, against $\Delta CYP51A$, and against both deletion mutants, respectively. For *C. fioriniae*, tebuconazole; prothioconazole, cyproconazole, difenoconazole, and prochloraz; and flutriafol, propiconazole, diniconazole, and epoxiconazole represent Cf_group 1, 2, and 3, respectively. For *C. nymphaeae*, Cn_group 1 consisted of prochloraz; Cn_group 2 consisted of flutriafol, propiconazole, diniconazole, and tebuconazole; and Cn_group 3 consisted of prothioconazole, cyproconazole, difenoconazole, and epoxiconazole.

In both species, the EC_{50} values of DMI combinations were tested in terms of group1 with group 2, group 1 with group 3, group 2 with group 3, group 2 with group 2, and group 3 with group 3, at a mixture ratio of 1:1 (50% of each fungicide).

Based on the differential efficacy of DMIs on $\Delta CYP51A$ and $\Delta CYP51B$, we hypothesized that combinations of DMIs from

Fig. 2. The 50% effective concentration (EC_{50}) values of *Colletotrichum fioriniae* isolates for flutriafol (Flutri), propiconazole (Propi), prothioconazole (Prothio), diniconazole (Dini), cyproconazole (Cypro), difenoconazole (Difen), epoxiconazole (Epoxi), tebuconazole (Tebu), and prochloraz (Proch). For each deletion genotype, the mean and standard deviation for three mutants were calculated. Results are means of two experiments because there were no significant differences ($P > 0.05$, Fisher's least significant difference test) between the two experiments. Significant differences of values compared with that of their parental isolate Ca2-4-2 are indicated with an asterisk (*) ($P < 0.05$).

Fig. 3. The 50% effective concentration (EC₅₀) values of *Colletotrichum nymphaeae* isolates CaPH40 for flutriafol (Flutri), propiconazole (Propi), prothioconazole (Prothio), diniconazole (Dini), cyproconazole (Cypro), difenoconazole (Difen), epoxiconazole (Epoxi), tebuconazole (Tebu), and prochloraz (Proch). For each deletion genotype, the mean and standard deviation for three mutants were calculated. Results are means of two experiments because there were no significant differences ($P > 0.05$, Fisher's least significant difference test) between the two experiments. Significant differences of values compared with that of their parental isolate CaPH40 are indicated with an asterisk (*) ($P < 0.05$).

TABLE 2. Percent change of concentration to inhibit mycelium growth by 50% (EC₅₀) values for *Colletotrichum fiorinae* deletion mutants and complemented mutants compared with their parental isolate

Isolates	Change (%) ^z								
	Cf_group 1	Cf_group 2				Cf_group 3			
	Tebu	Prothio	Cypro	Difen	Proch	Flutri	Propi	Dini	Epoxi
ΔCfCYP51A	NS	53	76	87	50	55	21	41	80
ΔCfCYP51B	81	NS	NS	NS	NS	37	84	84	12
ΔCfCYP51A-Com	NS	NS	NS	NS	NS	NS	NS	NS	NS
ΔCfCYP51B-Com	NS	NS	NS	NS	NS	NS	NS	NS	NS

^z Cf_group 1 = increased sensitivity to tebuconazole (Tebu) in ΔCfCYP51B; Cf_group 2 = increased sensitivity to prothioconazole (Prothio), cyproconazole (Cypro), difenoconazole (Difen), and prochloraz (Proch) in ΔCfCYP51A; and Cf_group 3 = increased sensitivity to flutriafol (Flutri), propiconazole (Propi), diniconazole (Dini), and epoxiconazole (Epoxi) in both ΔCfCYP51A and ΔCfCYP51B. Percentage was calculated as [(1 – EC₅₀ value of each mutants)/EC₅₀ value of their parental isolate]%. NS indicates no significant differences between the EC₅₀ value of mutants and their parental isolate.

TABLE 3. Percent change of concentration to inhibit mycelium growth by 50% (EC₅₀) values for *Colletotrichum nymphaeae* deletion mutants and complemented mutants compared with their parental isolate

Isolates	Change (%) ^x								
	Cn_group 1	Cn_group 2				Cn_group 3			
	Proch	Flutri ^y	Propi	Dini	Tebu	Prothio	Cypro ^z	Difen	Epoxi
ΔCnCYP51A	NS	96	98	27	61	43	99	81	99
ΔCnCYP51B	96	NS	NS	NS	NS	36	87	35	27
ΔCnCYP51A-Com	NS	NS	NS	NS	NS	NS	NS	NS	NS
ΔCnCYP51B-Com	NS	NS	NS	NS	NS	NS	NS	NS	NS

^x Cn_group 1 = increased sensitivity to prochloraz (Proch) in ΔCnCYP51B; Cn_group 2 = increased sensitivity to flutriafol (Flutri), propiconazole (Propi), tebuconazole (Tebu), and diniconazole (Dini) in ΔCnCYP51A; and Cn_group 3 = increased sensitivity to prothioconazole (Prothio), cyproconazole (Cypro), difenoconazole (Difen), and epoxiconazole (Epoxi) in both ΔCnCYP51A and ΔCnCYP51B. Percentage was calculated as [(1 – EC₅₀ value of each mutants)/EC₅₀ value of their parental isolate]%. NS indicates no significant differences between the EC₅₀ value of mutants and their parental isolate.

^y The EC₅₀ value of *C. nymphaeae* parental isolate to flutriafol was ≥50 µg/ml, and calculated as 50 µg/ml.

^z The EC₅₀ value of *C. nymphaeae* parental isolate to cyproconazole was ≥50 µg/ml, and calculated as 50 µg/ml.

different groups may improve efficacy. For the fungicide mixtures of group 1 with group 2, group 1 with group 3, and group 2 with group 3 in both species, the SF values were > 1 in all cases tested (Figs. 5B and 6B). For example, in *C. fioriniae*, the EC₅₀ value for tebuconazole (from Cf_group1) and epoxiconazole (from Cf_group 3) was 0.38 and 0.45 µg/ml, respectively. When the two fungicides were mixed, the EC₅₀ value decreased to 0.22 µg/ml, which was significantly lower than the values for the single components (Fig. 5A). The corresponding SF value for the mixture was 1.89 (Fig. 5B). Similarly, a significantly lower EC₅₀ value of *C. nymphaeae* was found for the mixture of prochloraz and cyproconazole compared with the EC₅₀ values for the individual DMIs, with an SF value of 6.49 (Fig. 6).

Compared with group 1 and 2 fungicides, the group 3 fungicides seemed to have a better synergy effector when mixed with other groups of fungicides for both species. For *C. nymphaeae*, the SF for mixtures of Cn_group 3 and Cn_group 1 (6.49 for the mixture of prochloraz and cyproconazole, 3.16 for the mixture of prochloraz and cyproconazole), and Cn_group 3 and Cn_group 2 (4.57 for the mixture of propiconazole and epoxiconazole, 4.19 for the mixture of difenoconazole and diniconazole) were all higher than the mixtures of Cn_group 1 and Cn_group 2 (1.32 for the mixture of prochloraz and propiconazole, 1.20 for the mixture of prochloraz and diniconazole) (Fig. 6). Similar pattern was also observed for *C. fioriniae* (Fig. 5).

In contrast, if two DMI fungicides from group 2 were mixed, no synergistic effect was observed for either species. For example, the mixtures of difenoconazole and prothioconazole, and cyproconazole and difenoconazole (both belonged to Cf_group 2), showed an SF of 0.80 and 0.69, respectively, when testing with *C. fioriniae* isolates (Fig. 5B). For *C. nymphaeae*, the SF for the combinations of propiconazole and tebuconazole and the combination of propiconazole and diniconazole were also lower than 1 (0.62 and 0.77, respectively) (Fig. 6B). However, though to a lesser extent, synergy effects were observed for the mixtures of group 3 and group 3 for both species (Figs. 5 and 6).

Involvement of *CYP51* genes in the regulation of vegetative growth and virulence. The *CYP51A* and *-B* disruption mutants in *C. fioriniae* or *C. nymphaeae* were morphologically indistinguishable on PDA medium compared with their parental isolates. All mutants exhibited a growth rate similar to that of the parental isolates (Tables 4 and 5). All *CYP51* deletion mutants were able to infect peach fruit. After 4 or 6 days of incubation, no significant difference in lesion diameter or sporulation was observed between deletion mutants and their parental isolates, indicating that neither *CYP51A* nor *CYP51B* is essential for vegetative growth and virulence as well as sporulation (Tables 4 and 5).

DISCUSSION

Ergosterol is a ubiquitous component of the plasma membrane of fungi and plays an important role in fungal growth. A key enzyme for ergosterol biosynthesis is sterol 14- α -demethylase (*CYP51*). An organism may have one or multiple *CYP51* genes. For example, one *CYP51* gene was found in *Monilinia fructicola* (Chen et al. 2017), two *CYP51* genes were found in *A. fumigatus* (Mellado et al. 2001), and three were found in both *F. graminearum* and *F. solani* (Yin et al. 2009). In *Colletotrichum* spp., two *CYP51* genes have been reported (Chen et al. 2018) but their function in relation to DMI fungicide resistance has not been characterized in depth. In this study, we investigated the role of individual paralogous *CYP51* genes in conferring differential sensitivities to DMIs by heterologous expression in *S. cerevisiae* and by analyzing the sensitivities of individual *CYP51* deletions in *C. fioriniae* and *C. nymphaeae*.

Both *CYP51A* and *CYP51B* from *C. fioriniae* and *C. nymphaeae* were able to recover *ScERG* gene function in yeast. The results were comparable with those in *Fusarium* spp., where *CYP51A* and

CYP51B from *F. graminearum* and *F. oxysporum* recovered ergosterol biosynthesis function in yeast *ERG11* mutants (Liu et al. 2011; Zheng et al. 2019). However, the function of *CYP51C* in *Fusarium* spp. varied in different species, underscoring the variability in function among *CYP51* paralogs. For example, *FoCYP51C* from *F. oxysporum* complemented yeast *ERG11* function (Zheng et al. 2019) but not for the *FgCYP51C* from *F. graminearum* (Liu et al. 2011).

The *CYP51A* gene was found to be more variable than *CYP51B* between *C. fioriniae* and *C. nymphaeae*. It has been hypothesized that the two copies of *CYP51* may have evolved through a neofunctionalization process, where one daughter gene retains the ancestral function while the other copy gains novel functions (Ohno 1970). In *Rhynchosporium commune*, genetic analyses indicated that *CYP51B* was under strong purifying selection, which made it a conserved functional *CYP51* gene in the fungi. The *CYP51A* gene, in contrast, diversified following gene duplication (Brunner et al. 2016). The conserved nature of *CYP51B* may explain why mutants expressing *CYP51B* largely had similar sensitivities to all fungicides tested regardless of *Colletotrichum* spp. (Figs. 2 and 3). The slight difference in DMI fungicide sensitivities between the two species in the absence of *CYP51A* may be linked to the amino acid variation at the 391 position of *CYP51B* (Figs. 2 and 3; Supplementary Supplementary Fig. S2). In contrast, the sensitivities to the DMIs tested varied significantly between the Δ Cf*CYP51B* and Δ Cn*CYP51B* mutants. Based on our observations, the less conserved *CYP51A* seemed to have a greater impact on DMI fungicide sensitivity than *CYP51B*. Similar observations were made for *Magnaporthe oryzae* (Yan et al. 2011) and *F. graminearum* (Liu et al. 2011). In both fungi, the deletion of *CYP51A* increased

Fig. 4. Relative expression of *CYP51A* and *-B* in *Colletotrichum fioriniae* and *C. nymphaeae* isolates. **A**, Relative expression of *CYP51A* in the parental *C. fioriniae* and *C. nymphaeae* isolates and *CYP51B* deletion mutants (Δ Cf*CYP51B* and Δ Cn*CYP51B*). **B**, Relative expression of *CYP51B* in the parental *C. fioriniae* and *C. nymphaeae* isolates and *CYP51A* deletion mutants (Δ Cf*CYP51A* and Δ Cn*CYP51A*). The expression of the *CYP51A* or *CYP51B* genes was normalized using the *GAPDH* gene expression levels and then calibrated to the normalized *CYP51A* mRNA value from the Ca2_4_2 mRNA. Relative expression was determined in isolates subjected to water or propiconazole at 1 µg/ml for 6 h before mRNA isolation. Line bars in each column denote standard errors of two experiments.

sensitivity to all DMI fungicides tested, whereas the deletion of *CYP51B* did not change the sensitivity to DMIs.

Due to different chemical structures of DMI fungicides, the binding affinity of azoles to the CYP51 proteins may vary. In this study, we determined the sensitivities of *CYP51A* and *-B* mutants to nine different DMI fungicides, which allowed us to assess the binding affinity between DMIs and *CYP51* genes of *C. nymphaeae* and *C. fiorinae*. For instance, sensitivity of $\Delta CfCYP51B$ increased dramatically to tebuconazole compared with that of $\Delta CfCYP51A$, indicating higher binding affinity between tebuconazole and *CfCYP51A*. In other words, the *CYP51A* in *C. fiorinae* may be the main target for tebuconazole. Similarly, *CfCYP51B* seemed to be the main target for prothioconazole, cyproconazole, difenoconazole, and prochloraz. Other fungicides, including flutriafol, propiconazole, diniconazole, and epoxiconazole, appear to target both *CfCYP51* genes (Table 2). In *C. nymphaeae*, *CnCYP51A* was

the main target for prochloraz and *CnCYP51B* was the main target for flutriafol, propiconazole, diniconazole, and tebuconazole. Prothioconazole, cyproconazole, difenoconazole, and epoxiconazole target both *CnCYP51* genes (Table 3).

Because DMI fungicides appear to target *CYP51* paralogs with preferential affinity, DMI fungicide mixtures may increase control efficacy as compared with the individual components. Synergistic interactions between DMIs were shown in vitro in *Fusarium* spp. in which three *CYP51* paralogs were detected (Liu et al. 2011). Largely in agreement, some combinations of DMI fungicides tested in this study showed strong synergistic effects against both *C. fiorinae* and *C. nymphaeae* mycelial growth (Figs. 5 and 6). In contrast, the combinations of DMI fungicides from the same group 2, which both mainly target the *CYP51B* gene, did not show synergistic effects. Our hypothesis is that mixtures of DMIs with preferential binding affinity for both *CYP51* genes offer synergism.

Fig. 5. A, Sensitivity and **B,** synergy factors (SFs) of *Colletotrichum fiorinae* isolates to the demethylation inhibitor (DMI) fungicides and their mixtures. **A,** The 50% effective concentration (EC₅₀) values of *C. fiorinae* isolates for tebuconazole (Tebu), difenoconazole (Difen), cyproconazole (Cypro), epoxiconazole (Epoxi), diniconazole (Dini), propiconazole (Propi), and their mixtures at a ratio of 1:1. Black, white, or gray bars indicate fungicide A, B, or A + B, respectively. Significant differences of EC₅₀ values between the mixtures and their single component are indicated with an asterisk (*) ($P < 0.05$). **B,** SFs in the mixtures of DMI fungicides. SF was calculated as a ratio between the expected EC₅₀ and the observed EC₅₀ for a mixture ($SF = EC_{50} \text{ expected} / EC_{50} \text{ observed}$). The expected EC₅₀ of a mixture was calculated as $EC_{50} \text{ expected} = (A + B) / (A/EC_{50} \text{ fungicide 1} + B/EC_{50} \text{ fungicide 2})$, where A and B are the proportions of fungicide A and fungicide B, respectively. *C. fiorinae* isolates were presented by Ca2_2_2, Ca2_3_2, and Ca2_4_2 (Chen et al. 2016). The mean and standard deviation for three isolates were calculated. Results are means of two experiments because there were no significant differences ($P > 0.05$, Fisher's least significant difference test) between the two experiments.

The possible highest synergistic effect can be expected when mixing two DMI fungicides with high preferential affinity for both *CYP51* paralogs. The extent of binding affinity for *CYP51A* and *-B* could be assessed by sensitivity changes in the *CYP51B* and *-A*

deletion mutants. For example, the EC_{50} values of $\Delta CnCYP51A$ mutants decreased by 98 and 27% for propiconazole and diniconazole, respectively, suggesting that propiconazole exhibited better inhibitory activity on *CnCYP51B* compared with

Fig. 6. A, Sensitivity and **B,** synergy factors (SFs) of *Colletotrichum nymphaeae* isolates to the demethylation inhibitor (DMI) fungicides and their mixtures. **A,** The 50% effective concentration (EC_{50}) values of *C. nymphaeae* isolates for prochloraz (Proch), propiconazole (Propi), diniconazole (Dini), cyproconazole (Cypro), epoxiconazole (Epoxi), tebuconazole (Tebu), difenoconazole (Difen), prothioconazole (Prothi), and their mixtures at a ratio of 1:1. Black, white, or gray bars indicate fungicide A, B, or A + B, respectively. Significant differences of EC_{50} values between the mixtures and their single component are indicated with an asterisk (*) ($P > 0.05$). **B,** SFs in the mixtures of DMI fungicides. SF was calculated as a ratio between the expected EC_{50} and the observed EC_{50} for a mixture ($SF = EC_{50} \text{ expected} / EC_{50} \text{ observed}$). The expected EC_{50} of a mixture was calculated as $EC_{50} \text{ expected} = (A + B) / (A/EC_{50} \text{ fungicide 1} + B/EC_{50} \text{ fungicide 2})$, where A and B are the proportions of fungicide A and fungicide B, respectively, in a mixture. The *C. nymphaeae* isolates were presented by CaPH40, CaCO4_32, and CaCO4_42 (Chen et al. 2016). The EC_{50} value of cyproconazole was $\geq 50 \mu\text{g/ml}$; for calculation convenience, it was calculated as $50 \mu\text{g/ml}$. The mean and standard deviation for three isolates were calculated. Results are means of two experiments because there were no significant differences ($P > 0.05$, Fisher's least significant difference test) between the two experiments.

TABLE 4. Mycelium growth rate, sporulation, and lesion formation of *Colletotrichum fioriniae* isolate Ca2-4-2, its *CYP51A* and *-B* deletion mutants, and its complemented mutants^z

Isolates	Mycelium growth rate (mm/day)	Lesion size (mm)	Sporulation (10^6 conidia/cm ²)
Ca2-4-2	8.88 ± 0.18 a	17.14 ± 2.61 a	2.28 ± 0.58 a
$\Delta CjCYP51A$	8.92 ± 0.17 a	17.56 ± 2.23 a	2.28 ± 0.55 a
$\Delta CjCYP51B$	8.76 ± 0.24 a	18.57 ± 1.63 a	2.20 ± 0.47 a
$\Delta CjCYP51A$ -Com	8.87 ± 0.21 a	17.32 ± 1.94 a	2.21 ± 0.67 a
$\Delta CjCYP51B$ -Com	8.81 ± 0.19 a	17.92 ± 2.03 a	2.20 ± 0.54 a

^z Values are means ± standard error from the average of three isolates for each deletion and rescued genotype. Shown are combined data of two biological replicates. Numbers in each column followed by the same letter are not significantly different according to Fisher's protected least significant difference test at $P = 0.05$.

TABLE 5. Mycelium growth rate, sporulation, and lesion formation of *Colletotrichum nymphaeae* isolates^z

Isolates	Mycelium growth rate (mm/day)	Lesion size (mm)	Sporulation (10 ⁶ conidia/cm ²)
CaPH40	7.12 ± 0.40 a	17.86 ± 3.25 a	1.50 ± 0.25 a
Δ <i>CnCYP51A</i>	7.56 ± 0.51 a	17.31 ± 2.82 a	1.68 ± 0.33 a
Δ <i>CnCYP51B</i>	7.34 ± 1.00 a	17.84 ± 3.59 a	1.53 ± 0.71 a
Δ <i>CnCYP51A</i> -Com	7.28 ± 0.41 a	17.29 ± 2.83 a	1.61 ± 0.45 a
Δ <i>CnCYP51B</i> -Com	7.23 ± 0.51 a	17.93 ± 2.14 a	1.60 ± 0.35 a

^z Values are means ± standard error from the average of three isolates for each deletion and rescued genotype. Shown are combined data of two biological replicates. Numbers in each column followed by the same letter are not significantly different according to Fisher's protected least significant difference test at *P* = 0.05.

diniconazole. Therefore, it explains the better synergism observed between propiconazole and prochloraz than diniconazole and prochloraz in the study. Similarly, *Cn_group3* fungicides, including cyproconazole and epoxiconazole, showed high affinity for *CYP51B* (the EC₅₀ values decreased by 99% in Δ*CYP51A* of *C. nymphaeae*). Thus, when either of the two fungicides mixed with *CYP51A* (the main target for azole prochloraz), synergism was observed. Moreover, mixtures cyproconazole and prochloraz showed higher SF than epoxiconazole and prochloraz because cyproconazole had better inhibitory activity on *CnCYP51A* than epoxiconazole, evident by less sensitivity (i.e., EC₅₀) change for the epoxiconazole in the Δ*CnCYP51B* mutants.

A similar synergy pattern was also observed for *C. fioriniae*. For example, epoxiconazole had better inhibitory activity on *CfCYP51B* than diniconazole, with the EC₅₀ decreased by 80 and 41%, respectively, in the Δ*CYP51A* mutants. When mixing with *CYP51A* (the main target for fungicides tebuconazole and epoxiconazole), tebuconazole showed higher SF than diniconazole and tebuconazole combined. As discussed above, the synergistic effect may not be observed when mixing two DMIs with similar *CYP51* binding affinity, or have the same target preference. It is noteworthy that combinations of DMI fungicides within group 3 can provide synergism. This is because group 3 DMIs exhibited differential inhibitory activity against *CYP51A* and *CYP51B*. For example, epoxiconazole mainly inhibits *CfCYP51B* (with EC₅₀ change of 80 and 20% in Δ*CfCYP51A* and Δ*CfCYP51B*, respectively) in *C. fioriniae*, whereas propiconazole and diniconazole mainly inhibit *CfCYP51A* (with EC₅₀ change of 84% in Δ*CfCYP51B* for both fungicides and 21% and 41% for propiconazole and diniconazole, respectively, in Δ*CfCYP51A*). Thus, the mixtures of epoxiconazole and propiconazole, and epoxiconazole and diniconazole, showed synergistic interaction, as expected.

DMI mixtures have already been used in commercial formulations. The mixture of prothioconazole and tebuconazole currently marketed as Prosaro (Bayer CropScience) showed excellent field performance against Fusarium head blight of wheat compared with other single-DMI fungicide formulations (Bradley and McMullen 2008). For Septoria tritici blotch (STB), higher levels of STB control were measured for the DMI mixtures. The mixtures of triazoles (e.g., epoxiconazole, metconazole, tebuconazole, and prothioconazole) with imidazoles (prochloraz) performed better than the individual products in the mixtures (Fraaije et al. 2011).

Azole mixtures may prolong the lifespan of individual partners. If azole mixtures select for different resistance alleles, it is unlikely that a point mutation or overexpression will occur in two or more *CYP51* genes in the same fungus simultaneously (Liu et al. 2011). Thus, resistance to DMI fungicide mixtures may require a multistep process for the pathogen to develop resistance. Moreover, a mixture of azoles may differentially select specific mutations, thereby reducing the overall resistance selection compared with using individual fungicides (Dooley et al. 2016). For example, the common I381V mutation in *Mycosphaerella graminicola* is strongly selected by tebuconazole and metconazole but not by prochloraz. Similarly, the I136A mutation is selected by tebuconazole but not by prochloraz (Fraaije et al. 2007). Thus,

using mixtures of DMIs that differentially select specific mutations may delay the selection for azole resistance while maintaining disease control (Fraaije et al. 2011). Heick et al. (2017) found that the application of a mixture of propiconazole and difenoconazole kept certain point mutations at a lower level in *Zymoseptoria tritici* compared with the application of solo azoles.

For the control of anthracnose disease, the mixtures of DMI may have another advantage by expanding the fungicidal spectrum. Anthracnose is often caused by more than one *Colletotrichum* sp. (Chen et al. 2016; Xu et al. 2014). Different species of *Colletotrichum* may exhibit striking differences in their response to DMI fungicides (Chen et al. 2016). The fungicide cyproconazole, for example, was effective against *C. fioriniae* but ineffective against *C. nymphaeae* based on the EC₅₀ values of mycelial assays. Thus, the use of a single formula may cause the failure of control if *C. nymphaeae* existed. In our study, strong synergistic effects were observed if cyproconazole was mixed with prochloraz, prothioconazole, and epoxiconazole. The EC₅₀ values decreased from more than 50 μg/ml to < 2 μg/ml for *C. nymphaeae* (Fig. 6). In summary, by mixing certain DMI fungicides, the fungicidal spectrum would be expanded and the control efficacy would be improved.

Flutriafol revealed relatively low intrinsic activity for both the wild type and the *CYP51* deletion mutants in *C. fioriniae* and *C. nymphaeae* (Figs. 2 and 3). In a previous study, *C. truncatum* was also less sensitive to flutriafol, with EC₅₀ values > 50 μg/ml (Chen et al. 2016). Similarly low intrinsic activity was reported in other genera; for example, in *Cercospora beticola* (Karaoglanidis and Thanassouloupoulos 2003), *Venturia inaequalis* (Cox 2015), and *Fusarium* spp. (Cox 2015). In contrast, prochloraz was the most effective DMI against all of the wild type and *CYP51* deletion mutants in our study (Figs. 2 and 3). Prochloraz was the only imidazole among our test DMIs. Similar superior efficacy of prochloraz compared with triazoles was found for control of anthracnose of the rubber tree (Cao et al. 2017), grape and strawberry (Xu et al. 2014), and pepper (Zhang et al. 2017).

In conclusion, *CYP51* gene paralogs in *C. fioriniae* and *C. nymphaeae* appear to differentially affect DMI fungicide sensitivity, a phenomenon that could be exploited for disease management.

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