Inherent Resistance to 14 α -Demethylation Inhibitor Fungicides in *Colletotrichum truncatum* Is Likely Linked to *CYP51A* and/or *CYP51B* Gene Variants

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ABSTRACT

Anthraxnose disease, caused by *Colletotrichum truncatum*, affects marketable yield during preharvest production and postharvest storage of fruits and vegetables worldwide. Demethylation inhibitor (DMI) fungicides are among the very few chemical classes of single-site mode of action fungicides that are effective in controlling anthracnose disease. However, some species are inherently resistant to DMIs and more information is needed to understand this phenomenon. Isolates of *C. truncatum* were collected from the United States and China from peach, soybean, citrus, and begonia and sensitivity to six DMIs (difenoconazole, propiconazole, metconazole, tebuconazole, flutriafol, and fenbuconazole) was determined. Compared with DMI sensitive isolates of *C. fructicola*, *C. siamense*, and *C. fioriniae* (EC₅₀ value ranging from 0.03 to 16.2 μ g/ml to six DMIs), *C. truncatum* and *C. nymphaeae* were resistant to flutriafol and fenbuconazole (with EC₅₀ values of more 50 μ g/ml). Moreover, *C. truncatum* was resistant to tebuconazole and

metconazole (with resistance factors of 27.4 and 96.0) and displayed reduced sensitivity to difenoconazole and propiconazole (with resistance factors of 5.1 and 5.2). Analysis of the *Colletotrichum* spp. genome revealed two potential DMI targets, *CYP51A* and *CYP51B*, that putatively encode P450 sterol 14 α -demethylases. Both genes were identified and sequenced from *C. truncatum* and other species and no correlation between *CYP51* gene expression levels and fungicide sensitivity was found. Four amino acid variations L208Y, H238R, S302A, and I366L in *CYP51A*, and three variations H373 N, M376L, and S511T in *CYP51B* correlated with the DMI resistance phenotype. *CYP51A* structure model analysis suggested the four alterations may reduce azole affinity. Likewise, *CYP51B* structure analysis suggested the H373 N and M376L variants may change the conformation of the DMI binding pocket, thereby causing differential sensitivity to DMI fungicides in *C. truncatum*.

Anthraxnose disease, caused by representatives of the genus *Colletotrichum*, affects a wide variety of important fruits, vegetables, cereals, grasses, and ornamental plants. The use of molecular systematics has made it possible to differentiate at least 11 major species complexes and 23 single species belonging to the genus *Colletotrichum* (Jayawardena et al. 2016). Among them are species including *C. truncatum*, *C. acutatum*, and *C. gloeosporioides*, which have significance in tropical and nontropical fruit production.

C. truncatum has a wide host range of more than 460 documented plant species (<http://nt.ars-grin.gov/sbmlweb/fungi/databases.cfm>) (Diao et al. 2017; Huang et al. 2013; Wikee et al. 2011; Yang et al. 2009). The associated disease produces characteristic sunken, black lesions often with concentric rings of acervuli containing salmon-colored conidia. These can be observed on many plant organs,

including leaves, stems, flowers, and fruits (Damm et al. 2009). The disease can cause both pre- and postharvest fruit rots and may reduce fruit quality and marketability (Manandhar et al. 1995).

Many breeding programs make anthracnose resistance a priority, but thus far no commercially viable strawberry or peach fruit variety with complete resistance has been developed. Thus, chemical control still is the most effective strategy for management of anthracnose disease. Among the few options for anthracnose control are members of the sterol demethylation inhibitors (DMIs), which are used for their protective and curative activity (Szkolnik 1981). Most registered active ingredients for fruits and soybeans are triazole derivatives (e.g., difenoconazole, tebuconazole, and prochloraz). Monitoring studies undertaken in several countries have shown significant shifts in sensitivity of *Colletotrichum* species populations to DMIs due to resistance development (Chen et al. 2013; Deng et al. 2011). Previous studies show differential sensitivity of *C. truncatum* isolates to DMI fungicides. Isolates were resistant to tebuconazole, metconazole, flutriafol, and fenbuconazole, but less sensitive to difenoconazole and propiconazole (Chen et al. 2016). One hundred and twelve *C. truncatum* isolates collected from pepper (*Capsicum* spp.) in 13 regions of China showed significant tolerance to tebuconazole and myclobutanil (Zhang et al. 2017). *C. truncatum* can be a pathogen of immunocompromised humans (Squizzato et al. 2015) and studies in this area showed high minimum inhibitory concentration (MIC values)

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doses to voriconazole and relatively high MIC values to ketoconazole, itraconazole, and miconazole (Squizzato et al. 2015).

In plant and human fungal pathogens, several major mechanisms of resistance to DMIs have been recorded. These include the following: (i) target-site modification in the *CYP51* gene that result in a reduced affinity of DMIs for their target (Dlye et al. 1998, 1997a, b); (ii) overexpression of the *CYP51* gene (Hamamoto et al. 2000; Ma et al. 2006; Schnabel and Jones 2001); (iii) reduced accumulation of DMIs in fungal cells through up-regulation of ATP-binding cassette (ABC) or major facilitator superfamily (MFS) transporters in the membrane (Akins 2005; Hayashi et al. 2003; Nakaune et al. 1998; Palani and Lalithakumari 1999; Reimann and Deising 2005); and (iv) multiple *CYP51* genes, which can cause overexpression of encoding gene or reduce the fitness cost associated with the *CYP51* structure change (Ziogas and Malandrakis 2015).

Some ascomycete fungi have been found to possess multiple *CYP51* paralogs. *Aspergillus fumigatus* (Mellado et al. 2001), *A. nidulans* (Da Silva Ferreira et al. 2005), and *Magnaporthe oryzae* (Yan et al. 2011) contain two *CYP51* paralogs, *CYP51A* and *CYP51B*. *Fusarium graminearum* and *F. solani* possess three *CYP51* paralogs, *CYP51A*, *CYP51B*, and *CYP51C* (Becher et al. 2011; Liu et al. 2011). The number of paralogs in *Colletotrichum* spp. are not yet known.

In the present paper, we show *C. truncatum* is inherently resistant to DMIs, especially to tebuconazole, metconazole, fenbuconazole, and flutriafol. The specific objectives of our study were to (i) identify *CYP51* paralogs in *C. truncatum*; (ii) determine the role of *CYP51* gene expression levels among *Colletotrichum* species; and (iii) elucidate the affinity of DMI fungicides to variants of the *CYP51* proteins through molecular docking analysis.

MATERIALS AND METHODS

Fungal isolates and chemicals. Isolates of *Colletotrichum* species, including *C. fruticola*, *C. siamense*, *C. fioriniae*, *C. nymphaeae*, and *C. truncatum* were obtained from peach, citrus, soybean, and

begonia in the United States and China (Table 1). Six DMI fungicides, difenoconazole (Inspire EC, Syngenta Crop Protection, Research Triangle Park, Raleigh, NC), propiconazole (PropiMax EC, Dow AgroScience, Indianapolis, IN), tebuconazole (Elite 45 DF, Bayer CropScience, Morrisville, NC), metconazole (Quash 50 WDG; Valent USA, Walnut Creek, CA), flutriafol (Topguard 11.8%, Cheminova, Denmark), and fenbuconazole (Indar 75 WSP, Dow AgroSciences, Indianapolis, IN) were used for sensitivity studies.

Resistance factors. EC₅₀ values were determined as previously described (Chen et al. 2016). Briefly, the percent relative growth (RG) was calculated as (radial growth at fungicide concentration/radial growth on the nonamended check plate) × 100. The EC₅₀ value was estimated by linear regression of the probit-transformed relative inhibition (RI) value (RI = 1 – RG) on log₁₀ transformed-fungicide concentration. The EC₅₀ value for each isolate was calculated as the mean of the three replicates. Resistance factors (RF) were calculated using the formula RF = EC₅₀ for resistant isolate/mean EC₅₀ of all sensitive isolates (including isolates from *C. fruticola*, *C. siamense*, *C. fioriniae*-subgroup 1, and *C. fioriniae*-subgroup 2). Isolates were assigned as resistant when resistance factors were higher than 20, and as reduced sensitive when resistance factors were higher than 5.

DNA extraction. Genomic DNA of *Colletotrichum* spp. isolates was extracted from mycelium using a modified cetyltrimethylammonium bromide (CTAB) method (Chen et al. 2012). Single agar plugs, 6 mm in diameter, with actively growing mycelium were transferred to 40 ml of potato dextrose broth (40 ml of juice from 40 g of potato and 4 g of dextrose per liter) and incubated for 3 days. Mycelium was harvested and ground with mortar and pestle in liquid nitrogen to a powder. The powder (0.2 g) was incubated in 750 µl of extraction buffer (2% CTAB, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl) with 2 µl of RNase A (100 mg/ml, Qiagen Inc., Valencia, CA) for 30 min at 65°C with occasional mixing. After adding one volume of phenol/chloroform/isoamyl alcohol (25:24:1), DNA was precipitated with one volume of isopropyl alcohol for 10 min at room temperature. DNA was precipitated by centrifuging at 12,000 × g for 10 min. The

TABLE 1. Species complex, species, origin and year of isolation of *Colletotrichum* isolates used in this study

Species complex	Species	Isolates	Host	Origin	Year of isolation
<i>C. gloeosporioides</i> ^x	<i>C. fruticola</i>	Cg_RR12_3	Peach	Spartanburg, SC	2012
		Cg_RR12_1	Peach	Chesterfield, SC	2013
		Cg_SE12_1	Peach	Spartanburg, SC	2012
	<i>C. siamense</i>	Cg_Ey12_1	Peach	Saluda, SC	2012
		Cg_OD13_2	Peach	Saluda, SC	2012
		Cg_Ey12_10	Peach	Aiken, SC	2012
	<i>C. truncatum</i>	Ct_RR13_1 ^y	Peach	Saluda, SC	2013
		Ct_RR13_2	Peach	Saluda, SC	2013
		NJ18_1	Soybean	Jiangsu, China	2012
		NJ10-2	Soybean	Jiangsu, China	2012
		NJ13_2	Soybean	Jiangsu, China	2012
		XZ	Soybean	Jiangsu, China	2009
		ZD_1	Citrus	Yunnan, China	2008
		ZD_2	Citrus	Yunnan, China	2008
		TGBC1	Begonia	Shanxi, China	2017
		TGBC2	Begonia	Shanxi, China	2017
		TGBC3	Begonia	Shanxi, China	2017
<i>C. acutatum</i> ^z	<i>C. nymphaeae</i>	CaPH40	Peach	Spartanburg, SC	2004
		CaCO4_35	Peach	Unknown, SC	2004
		CaCO4_42	Peach	Unknown, SC	2004
	<i>C. fioriniae</i> -subgroup 2	C.2.4.2	Peach	Unknown, GA	2015
		C.2.2.2	Peach	Unknown, GA	2015
		C.2.3.2	Peach	Unknown, GA	2015
	<i>C. fioriniae</i> -subgroup 1	CaEY12_2	Peach	Spartanburg, SC	2004
		CaEY12_1	Peach	Spartanburg, SC	2004
		CaPH4	Peach	Spartanburg, SC	2004

^x Isolates refer to Hu et al. (2015b).

^y Isolates Ct_RR13_1 and Ct_RR13_2 refer to Grabke et al. (2015).

^z Isolates refer to Chen et al. (2016).

pellet was washed with 75% ethanol, air dried, and resuspended in Tris-EDTA buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0).

Amplifying the *CYP51* genes from *C. truncatum* isolates. Degenerate primers CTA_TF and CTA_TR were designed based on conserved regions from alignment of *CYP51A* sequences from *C. simmondsii* (GenBank accession no. KXH38064.1), *C. gloeosporioides* (GenBank accession no. EQB56428.1), and *C. sublineola* (GenBank accession no. KDN69988.1) (Supplementary Table S1). The initial fragment of *CYP51A* from genomic DNA of *C. truncatum* isolate CTRR13_1 was amplified by CTA_TF and CTA_TR in a 50 µl reaction volume containing 2.5 units of Easy Taq DNA polymerase (New England Biolabs, Ipswich, MA), 1× PCR reaction buffer provided by the manufacture, 200 µM of each dNTP, 0.4 µM of each primer, and 50 ng of template DNA. PCR parameters were set as follows: 94°C for 3 min, 35 cycles at 94°C for 30 s, 56°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 5 min. An amplified fragment was visualized on 1.5% agarose gel containing gel red in 1× TAE buffer (40 mM Tris acetate and 1 mM EDTA, pH 8.0) under UV light. Sequencing was conducted at the DNA core Laboratory at Arizona State University (Tempe, AZ).

Genome libraries were constructed following manufacturer's instructions (Clontech Laboratories, Inc., Mountain View, CA) with genomic DNA extracted from isolate CTRR13_1. Briefly, approximately 2.5 µg of genomic DNA was digested with restriction enzymes *Sma*I, *Eco*RV, or *Pvu*II. Uncioned genomic libraries were then constituted by ligating synthetic DNA adaptors to genomic DNA fragments. Two PCR amplification steps were performed successively. A primary PCR was conducted with an outer adaptor primer AP1 provided in the kit and an outer gene-specific primer, followed by a nested PCR with a nested adaptor AP2 from kit and a nested specific primer. To amplify the 5' end of *CYP51A*, CTA_TU_GSP1 was designed in the primary PCR and CTA_TU_GSP2 in the nested PCR. To amplify the 3' end sequence of the gene, CTA_TD_GSP1 and CTA_TD_GSP2 were used. PCR amplifications in each case were performed in a 50-µl reaction volume with 0.2 µg of digested DNA, 1 µM of each primer, 200 µM of each dNTP, 2.5 units of Easy Taq DNA polymerase (New England Biolabs, Ipswich, MA), and 1× PCR reaction buffer. Amplification reaction was done by two-step cycle parameters: seven cycles of 25 s at 94°C and 3 min at 72°C, followed by 32 cycles of 25 s at 94°C and 3 min at 67°C. The 5' end and 3' end of *CYP51A* were successfully amplified from the *Sma*I library. Amplified fragments were visualized on an agarose gel and sequenced. Resulting sequences were assembled using SeqMan software (DNASTAR, Inc., Nevada City, CA). The sequence of the final fragment was compared with the NCBI database sequences of the *CYP51* gene from other organisms via BLAST searches to determine the start and stop codons of *CYP51A*.

A similar procedure was conducted to amplify the *CYP51B* gene from *C. truncatum* isolate CTRR13_1. Briefly, *CYP51B* sequences from *C. gloeosporioides* (GenBank accession no. EQB53780.1) and *C. fioriniae* (GenBank accession no. XP_007591427.1) were aligned to design the degenerate primers CYPBF and CYPBR. Nested sets of gene-specific and allele-specific primers of GSP2_U/AP2 and GSP1_U/AP1 were used to amplify the 5' end of *CYP51B*. Primer sets of GSP2_D/AP2 and GSP1_D/AP1 were used to amplify the 3' end of *CYP51B*. Both the 5' end and 3' end were amplified from the *Eco*RV library.

RNA isolation and cDNA synthesis. *Colletotrichum* spp. isolates were grown in 40 ml of PDB at 25°C for 3 days on a 160 rpm orbital shaker. Total RNA was extracted from fresh mycelium using the TRIzol reagent (Invitrogen), according to the manufacturer's recommendations, and then digested by DNase I nuclease (New England Biolabs, Ipswich, MA) to remove DNA contamination. First-strand cDNA synthesis was carried out using 1 µg of total RNA with Revert Aid First Strand cDNA Synthesis Kit employing the oligo (dT)₁₈, according to the manufacturer's recommendation (Thermo Fisher Scientific Inc., Waltham, MA). Specific primers

DC_CTAF/DC_CTAR and CTB_CHECKF/CTB_CHECKR were used to amplify the cDNA of *CYP51A* and *CYP51B* from *C. truncatum*, respectively. Cycling parameters were 95°C for 3 min followed by 32 cycles at 58°C (for primers DC_CTAF/DC_CTAR) for 40 s and 72°C for 1.5 min, or at 53°C (for primers CTB_CHECKF/CTB_CHECKR) for 40 s and 72°C for 1.5 min, and a final extension at 72°C for 5 min. Amplified fragments were visualized and sequenced as described above. To rule out the possibility that any sequence changes identified were due to PCR-induced errors, each isolate was independently analyzed twice.

Quantification of *CYP51A* and *CYP51B* expression using real-time PCR. Real-time PCR technology was used to quantify relative expression level of the *CYP51A* and *CYP51B* genes in cDNA. Approximately 150-bp fragments of the *CYP51A* gene and *CYP51B* gene were amplified with species-specific primer pairs. Expression was normalized with expression of the *GAPDH* gene. The relative quantities of gene expression were calculated using the comparative Ct ($2^{-\Delta\Delta C_t}$) method (Wong and Medrano 2005). In order to determine whether the expression of *CYP51* genes are inducible by DMI fungicides, the expression of *CYP51* genes in isolates was investigated after exposure to flutriafol at 2 µg/ml for 6 h. Reactions were performed in a 20 µl volume containing 10 µl of SYBR Premix Ex TaqII (Takara, Dalian, China), 0.25 µM of each primers, and 1 µl of cDNA. All PCR reactions were performed in triplicates with the program: 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Specific amplification was confirmed by a single peak in the melting curve, which was performed immediately after amplification for 100 cycles of increasing temperature with 0.4°C increments starting at 55°C. RT-PCR reactions were performed with an additional reaction in which the cDNA was replaced with RNA as a negative control. The relative expression of the *CYP51B* and *CYP51A* were calibrated to the expression of *CYP51* genes from sensitive *C. fioriniae* isolate C2_4_2. For each *Colletotrichum* species, the expression level of two isolates were merged. Reactions were performed in a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories Inc.) using SYBR Green I fluorescent dye detection. Two biological replications starting with new cultures for RNA extraction were conducted.

Sequence analysis. The sequences obtained for each strain were assembled in contigs using Bioedit (Tom Hall, North Carolina State University) with the tool Clustal W. The DNA sequence was translated to six possible reading frames, and the correct one was identified on the basis of the presence of a conserved region of the sterol 14 α -demethylase from other fungi found through BLAST X (<https://www.ncbi.nlm.nih.gov/BLAST>). The presumed codons for the start and the end of the protein were determined using alignment of the same protein from other fungi. Intron regions were identified by comparing the complete genomic sequence with cDNA of the *CYP51* genes, and by comparing the complete sequence with consensus sequences in fungi: GTRNGT at the beginning of the intron and YAG at the end of the intron (Délye et al. 1997a). In order to identify mutations resulting in amino acid (aa) changes, a direct comparison of translated aa sequences from all strains were aligned by using Clustal W.

Phylogenetic analysis. Phylogenetic analysis were conducted by the maximum likelihood (ML) method with Mega 7.0 software on the basis of deduced aa sequences of *CYP51A* and *CYP51B* gene (Kumar et al. 2016). Multiple alignments were conducted using DNASTAR (DNASTAR Inc., Nevada City, CA) and CLUSTAL X v. 2.1 (Larkin et al. 2007; Thompson et al. 1997). For *CYP51A*, sequences included those from *C. truncatum* isolate CTRR13_1, *C. nymphaeae* isolate CaPH40, and other filamentous fungal species *C. gloeosporioides* (GenBank accession no. EQB56428.1), *Metarhizium anisopliae* (GenBank accession no. KJK74651.1), *Beauveria bassiana* (GenBank accession no. KGQ04950.1), *Cordyceps militaris* (GenBank accession no. XP_006668831.1), *Hirsutella minnesotensis* (GenBank accession no. KJZ76396.1),

Colletotrichum sublineola (GenBank accession no. KDN69988.1), *Diaporthe ampelina* (GenBank accession no. KKY35210.1), *Aspergillus flavus* (GenBank accession no. XP_002375123.1), *M. oryzae* (GenBank accession no. XP_003710875.1), *Neonectria ditissima* (GenBank accession no. KPM38499.1), *Colletotrichum simmondsii* (GenBank accession no. KXH38064.1), *Fusarium verticillioides* (GenBank accession no. AGK89489.1), *F. graminearum* (GenBank accession no. XP_011321548.1), *Valsa mali* (GenBank accession no. KUI72529.1), *Colletotrichum orchidophilum* (GenBank accession no. XP_022474431), *Nectria hematococca* (GenBank accession no. XP_003045204), *Colletotrichum orbiculare* (GenBank accession no. ENH81686.1), *Colletotrichum higginsianum* (GenBank accession no. CCF38358.1), *Colletotrichum fioriniae* (GenBank accession no. XP_007594880), *Colletotrichum salicis* (GenBank accession no. KXH56565.1), and *Pochonia chlamydosporia* (GenBank accession no. XP_022285666).

For *CYP51B*, sequences were obtained from *C. truncatum* isolate CtRR13_1, *C. nymphaeae* isolate CaPH40, and other filamentous fungal species, including *C. gloeosporioides* (GenBank accession no. EQB53780.1), *C. higginsianum* (GenBank accession no. CCF32640.1), *C. graminicola* (GenBank accession no. XP_008090488.1), *C. incanum* (GenBank accession no. KZL63163.1), *C. sublineola* (GenBank accession no. KDN66717.1), *C. tofieldiae* (GenBank accession no. KZL69090.1), *C. orbiculare* (GenBank accession no. ENH88478.1), *C. simmondsii* (GenBank accession no. KXH37906.1), *C. fioriniae* (GenBank accession no. XP_007591427.1), *C. salicis* (GenBank accession no. KXH56565.1), *P. chlamydosporia* (GenBank accession no. OAQ72608.1), *Metarhizium guizhouense* (GenBank accession no. KID88153.1), *Fusarium oxysporum* (GenBank accession no. EXA53151.1), *F. graminearum* (GenBank accession no. XP_011316750.1), *N. ditissima* (GenBank accession no. KPM38922.1), *Myceliophthora thermophila* (GenBank accession no. XP_003658636.1), *H. minnesotensis* (GenBank accession no. KJZ73557.1), *Madurella mycetomatis* (GenBank accession no. KXX80456.1), *Tolypocladium ophioglossoides* (GenBank accession no. KND89481.1), *Purpureocillium lilacinum* (GenBank accession no. OAQ81874.1), *Drechmeria coniospora* (GenBank accession no. KYK55628.1), *Chaetomium thermophilum* (GenBank accession no. XP_006696132.1), *Scedosporium apiospermum* (GenBank accession no. XP_0166440).

For *CYP51C*, sequence was obtained from *F. graminearum* (GenBank accession no. XP_011325340). Sequence of *Candida albicans* (GenBank accession no. AIX03614.1) was used as the out-group reference species.

For the ML method, the phylogram was inferred based on the JTT matrix-based model. The bootstrap consensus tree, inferred from 1,000 replicates, was constructed with the following parameters: the Poisson correction model, gamma distributed (five categories), and heuristic method using SPR-extensive. All positions containing gaps and missing data were eliminated.

Molecular docking analysis. To simulate the binding mode of the CYP51A protein and ligand, homolog modeling and molecular docking were conducted. The CYP51A from the *C. fruticola* isolate was selected as the sensitive model according to the EC₅₀ value. Structural modeling of the CYP51A sequence was carried out using the online SWISS-MODEL software, which scans the Blast and HHBlits for candidate homologies (Guex and Peitsch 1997; Mariani et al. 2011; Šali and Blundell 1993; Söding et al. 2005). As the kinase domain of *Aspergillus fumigatus* CYP51B shares the highest identity (55.3%) with our *Colletotrichum* isolates, the *A. fumigatus* structure (PDB, entry code 4UYM), solved with the inhibitor voriconazole was used as a template to build the kinase domain of *C. fruticola* domain of CYP51A. Based on the CYP51A structure model of *C. fruticola*, the CtCYP51A (CYP51A from *C. truncatum*) model was built with the following amino acid alterations: L213Y, H243R, S303A, and I370L (corresponding variations L208Y, H238R, S302A, and I366L in *C. truncatum* CYP51A positions).

To better understand the interactions between the inhibitors and CYP51A, molecular docking experiments were performed using Discovery Studio 3.5 (Inc 2012) with the default parameters. Six DMI fungicides (difenoconazole, propiconazole, tebuconazole, metconazole, flutriafol, and fenbuconazole) were considered for molecular docking study. The starting conformation was generated by modified CHARMM force field-based 3D structure minimization implemented in Discovery Studio ‘prepare ligands’ protocol (Brooks et al. 1983). Default settings for small molecule-protein docking were used throughout the simulations. The top 10 poses were collected for each molecule with the best docked score value and the bond conformation with the most favorable CDOCKER interaction energies was chosen.

A similar procedure was conducted to simulate the binding mode of CYP51B protein. The structure of CYP51B (PDB ID: 5FRB) was used as a template for the homolog modeling of *C. fruticola*. Amino acid alterations of H373 N, M376L, and S511T (corresponding variations H373 N, M376L, and S511T in *C. truncatum* positions) were applied to the CYP51B of *C. fruticola* to build the CYP51B structure of *C. truncatum*. Six DMI fungicides (difenoconazole, propiconazole, tebuconazole, metconazole, flutriafol, and fenbuconazole) were analyzed for the docking study.

RESULTS

Sensitivity of *Colletotrichum* spp. isolates to DMI fungicides. The EC₅₀ values for DMI fungicides of six *Colletotrichum* spp. collected from peach, including *C. fruticola*, *C. siamense*, *C. truncatum*, *C. nymphaeae*, *C. fioriniae*-subgroup 1, and *C. fioriniae*-subgroup 2 collected from the United States were determined previously (Chen et al. 2016). The current study reports the sensitivity of nine Chinese *C. truncatum* isolates collected from soybean, citrus, and begonia to six DMI fungicides (Table 2). Isolates of *C. fruticola*, *C. siamense*, *C. fioriniae*-subgroup 1, and *C. fioriniae*-subgroup 2 were sensitive to difenoconazole, propiconazole, tebuconazole, and metconazole (EC₅₀ values ranging from 0.03 to 1.2 µg/ml). The EC₅₀ value of those sensitive isolates were averaged to calculate the resistance factors for *C. truncatum* and *C. nymphaeae* isolates. Both *C. truncatum* and *C. nymphaeae* were resistant to flutriafol and fenbuconazole (EC₅₀ values > 50 µg/ml). Isolates of *C. truncatum* were also resistant to tebuconazole and metconazole, with resistance factors of 27.4 and 96.0, and displayed reduced sensitivity to difenoconazole and propiconazole, with resistance factors of 5.1 and 5.2. The EC₅₀ values of *C. nymphaeae* were slightly higher than sensitive isolates to difenoconazole, propiconazole, metconazole, and tebuconazole, with resistance factors ranging from 1.2 to 5.3.

Identifying and sequencing the CYP51 genes. To amplify the CYP51A gene from *C. truncatum* isolate CtRR13_1, an initial fragment of around 450 bp in length was PCR-amplified by the degenerate primers CTA_TF and CTA_TR. DNA from uncloned genomic libraries of *StuI* enzyme digestion was amplified by using primers CTA_TU_GSP1/AP1 and CTA_TU_GSP2/AP2 for the 5' end sequence, and by using primer pairs CTA_TD_GSP1/AP1 and CTA_TD_GSP2/AP2 for the 3' end sequence. The complete CYP51A gene amplified from genomic DNA was 1,539 bp in size.

The cDNA of the CYP51A gene was generated from RNA of CtRR13_1 using primer pair DC_CTAF/DC_CTAR to determine the arrangement of exons. The full-length cDNA was also 1,539 bp in length. The CYP51A encoded a putative polypeptide of 512 aa with no introns. The CYP51A gene sequence was deposited in GenBank (accession no. MG799553).

CYP51A gene sequences of 10 other *C. truncatum* isolates from the United States and China peach, citrus, soybeans, and begonia were amplified using primer pair DC_CTAF/DC_CTAR. There were three deduced amino acid variations in CYP51A from *C. truncatum* isolates collected from the United States and China (Fig. 1). *C. truncatum* isolates from the same country regardless of

the host had identical *CYP51A* nucleotide sequences. Compared with the *C. truncatum* isolates collected from the United States, the Chinese isolates had a 6-bp deletion at position 257, a variation at codon 375 (GAG to AAG) resulting in an amino acid alteration from glutamate to lysine (E375K), and a variation at codon 499 (ACC to GCC) resulting in an amino acid alteration from threonine to alanine (T499A; Fig. 1).

To amplify the *CYP51B* gene from *C. truncatum*, an initial fragment of roughly 1.4 kb was PCR amplified with degenerate primers CYPBF and CYPBR. This fragment served as a basis for amplifying the rest of the gene by genome walking. DNA from CtRR13_1 digested with *EcoRV* and ligated to synthetic adaptors was amplified successively by using primer pairs GSP1_U/AP1 and GSP2_U/AP2 for the 5' end sequence, and by using primer pairs GSP1_D/AP1 and GSP2_D/AP2 for the 3' end sequence. A final sequence of 1,719 bp was obtained together with the upstream 326-bp fragment and downstream 684-bp fragment.

The cDNA of the *CYP51B* gene was synthesized from CtRR13_1 RNA using primer pair CTB_CHECKF/CTB_CHECKR to determine the arrangement of exons. The full-length cDNA was 1,578 bp in length and encoded a putative polypeptide of 526 aa. Sequence comparison of the genomic DNA and cDNA revealed three exons located at positions 1 to 246, 325 to 522, and 583 to 1719 (Fig. 1B). The *CYP51B* gene sequence from *C. truncatum* isolate CtRR13_1 was deposited in GenBank (accession no. MG799552).

Using primer pair CTB_CHECKF/CTB_CHECKR, the *CYP51B* gene of *C. truncatum* isolates from the United States and China from peach, citrus, soybeans, and begonia were also amplified. There were considerable nucleotide variations between isolates, but the deduced amino acid sequences were the same regardless of the origin or host.

Phylogenetic analysis of predicted amino acid sequences.

The deduced amino acid sequence of *CtCYP51A* was 75% identical to that of *M. anisopliae* (XP_007817531), 76% identical to *Pestalotiopsis fici* (XP_007830361), 72% identical to *Ophiocordyceps sinensis* (EQL02138), 72% to *Diaporthe helianthi* (OCW41792), 70% to *Hirsutella minnesotensis* (KJZ76396), 73% to *Beauveria bassiana* (KGQ04950), 68% to *A. flavus* (KOC13200), and 66% to *F. graminearum* (ACL93387). Comparative analysis of the sequences yielded an E-value of 0.0, which confirmed *CtCYP51A* to be a member of the fungal *CYP51A* family.

Comparison of the *CtCYP51B* with those of other filamentous fungus indicated that it shared the greatest identity with the sequences of *C. gloeosporioides* (EQB53780, 86%), of *Claviceps purpurea* (CCE33417, 79%), of *P. chlamydosporia* (XP_018148691, 80%), of *M. guizhouense* (KID88153, 80%), and of *F. oxysporum* (EXA53151, 80%). The percentage identity confirmed *CtCYP51B* to be a member of the filamentous fungus *CYP51B* family.

A phylogenetic tree was constructed based on the concatenated alignment of *CYP51* homologs of *C. truncatum* with *Colletotrichum* species and other ascomycete fungi that are close to *Colletotrichum* species, including the outgroup fungus *Candida albicans* (Fig. 2). There were a total of 420 positions in the final data

set. In ML phylogenetic tree, *CYP51A*, *CYP51B*, and *CYP51C* formed three monophyletic clades. Interestingly, the *CYP51A* gene from *C. truncatum* was more similar to *M. anisopliae* than to other *Colletotrichum* species. Other *Colletotrichum* species were separated into different clades. Members of the *C. acutatum* species complex (including *C. salicis*, *C. fioriniae*, *C. simmondsii*, and *C. nymphaeae*) were closely related, together with *C. gloeosporioides*, *C. orbiculare*, *C. higginsianum*, and *V. mali* clustered in the same clade. However, *C. sublineola* and *C. orchidophilum* were more closely related to *M. oryzae* than to other *Colletotrichum* species.

For *CYP51B*, the ML tree showed that *C. truncatum* was closest related to *C. gloeosporioides* and *C. orbiculare*. All species from the *C. acutatum* complex (*C. salicis*, *C. simmondsii*, *C. fioriniae*, and *C. nymphaeae*) clustered together. Another clade was formed by *C. higginsianum*, *C. graminicola*, *C. incanum*, *C. tofieldiae*, and *C. sublineola*.

Relative expression levels of *CYP51* genes in different *Colletotrichum* species. To investigate whether overexpression of the *CtCYP51* genes was the cause of the reduced sensitivity to some DMI fungicides, the expression of the homologous genes was determined with or without fungicide exposure prior to RNA extraction. Expression level of *CYP51A* in *C. fioriniae* isolate Ca2_4_2 served for calibration.

In the absence of fungicide exposure, only the expression of *CYP51A* in *C. fructicola* was elevated ($P < 0.05$) compared with *C. fioriniae*. No correlation was observed between the *CYP51A* expression level and flutriafol sensitivity ($R^2 = 0.36$, $P = 0.40$). After flutriafol treatment, the relative expression levels of *CYP51A* was induced in all *Colletotrichum* species. For *C. fioriniae* and *C. truncatum* isolates, the relative expression level was increased by about 30-fold after treatment. The expression level was increased 18-fold in *C. nymphaeae* isolates and fourfold in *C. fructicola* isolates (Table 3).

In the absence of fungicide treatment, the expression level of *CYP51B* in all *Colletotrichum* species was higher compared with that of *CYP51A* except for *C. fructicola* isolates. There was no correlation between the *CYP51* expression level and the flutriafol sensitivity ($R^2 = 0.14$, $P = 0.62$). The sensitive isolates of *C. fioriniae* had the highest expression level of 12.76, while the resistant isolates of *C. nymphaeae* and *C. truncatum* had lower expression level of 4.37 and 6.57, respectively (Table 3).

The addition of flutriafol to mycelium prior to RNA extraction led to a statistically significant increase of *CYP51B* gene in all *Colletotrichum* isolates ($P < 0.05$). The expression of *CYP51B* in *C. fioriniae* and *C. nymphaeae* increased by around 10 times compared with untreated isolates. While *CYP51B* expression in *C. fructicola* and *C. truncatum* isolates increased 15- and 28-fold, respectively. Still, *CYP51B* expression after treatment could not be directly linked to DMI fungicide sensitivity ($R^2 = 0.09$, $P = 0.71$).

***CYP51* amino acid sequence alignments.** The amino acid sequences in the six substrate recognition sites (SRS) were investigated between *C. truncatum* isolates and other *Colletotrichum* species isolates. The amino acid residues of SRS regions

TABLE 2. Mean EC₅₀ values for demethylation inhibitor fungicides and corresponding resistance factors for *Colletotrichum* species

Species	Difenoconazole		Propiconazole		Metconazole		Tebuconazole		Flutriafol		Fenbuconazole	
	EC ₅₀ ^y	RF ^z	EC ₅₀	RF	EC ₅₀	RF	EC ₅₀	RF	EC ₅₀	RF	EC ₅₀	RF
<i>C. truncatum</i>	2.4 ± 0.3	5.1	2.5 ± 0.7	5.2	13.2 ± 0.3	27.4	62.4 ± 28.4	96	>50	–	>50	–
<i>C. nymphaeae</i>	1.3 ± 0.3	2.7	2.5 ± 0.7	5.3	1.4 ± 0.9	2.9	0.8 ± 0.4	1.2	>50	–	>50	–
<i>C. fructicola</i>	0.9 ± 0.5	–	0.2 ± 0.1	–	0.8 ± 0.4	–	0.8 ± 0.1	–	13.1 ± 12.0	–	3.3 ± 1.3	–
<i>C. siamense</i>	0.3 ± 0.3	–	0.2 ± 0.1	–	0.6 ± 0.2	–	1.1 ± 0.4	–	4.1 ± 0.6	–	3.4 ± 2.3	–
<i>C. fioriniae</i> -2	0.5 ± 0.1	–	1.2 ± 0.7	–	0.5 ± 0.4	–	0.5 ± 0.1	–	16.2 ± 8.5	–	5.2 ± 4.2	–
<i>C. fioriniae</i> -1	0.2 ± 0.2	–	0.3 ± 0.2	–	0.03 ± 0.05	–	0.2 ± 0.1	–	2.1 ± 1.3	–	0.4 ± 0.3	–

^y Mean and standard deviation from the average of three isolates for each species, except *C. truncatum*. Eleven isolates were collected for *C. truncatum*.

^z Resistance factor (RF) was calculated for each fungicide by dividing mean EC₅₀ value of each resistant species by the mean EC₅₀ value of all sensitive isolates.

are well conserved in fungi (Mellado et al. 2007). The CYP51A aa sequence of *C. truncatum* was aligned with that of *C. gloeosporioides*, *C. fioriniae*, *C. higginsianum*, *C. sublineola*, *C. orbiculare*, *C. simmondsii*, and *C. orchidophilum* acquired from GenBank and with three *Colletotrichum* species sequences generated in this study (*C. fruticola*, *C. nymphaeae*, and *C. fioriniae*). Variation in amino acid sequences were found in form of L208Y within SRS2, H238R within SRS4, S302A within SRS4, and I366L within SRS5 (Fig. 3). An alignment of the amino acid sequence from *A. fumigatus* (Fig. 3) revealed the L208Y, H238R, S302A, and I366L in *C. truncatum* to be orthologous to the archetype L206Y, H236R, S299A, and I367L mutation in *A. fumigatus*.

For the CYP51B analysis, deduced amino acid sequences from *C. truncatum* and 10 different *Colletotrichum* species acquired from GenBank (including *C. gloeosporioides*, *C. fioriniae*, *C. graminicola*, *C. higginsianum*, *C. incanum*, *C. sublineola*, *C. tofieldiae*, *C. orbiculare*, *C. simmondsii*, and *C. salicis*), and three *Colletotrichum* species from our experiment (*C. fruticola*, *C. nymphaeae*, and *C. fioriniae*) were compared. Amino acid changes were found in form of H373 N and M376L within SRS5, and S511T within SRS6 (Fig. 4). Those amino acid substitutions are orthologous to H373 N, I376L, and S505T in *A. fumigatus* CYP51B.

Molecular modeling of CYP51A and DMI fungicide interactions. The changes of the free energy calculated from the docking model suggested changes of protein conformation and protein ligand interaction may occur after the introduction of the combined amino acid alteration. The corresponding *C. truncatum* CYP51A positions L208Y, H238R, S302A, and I366L correspond to *C. fruticola* positions L213Y, H243R, S303A, and I370L, respectively. These four residues in *C. fruticola* were substituted

for the modeling of *C. truncatum*. The combined introduction of four amino acid variants resulted in enzyme conformation changes and alternation of ligand binding affinity, which may contribute to unstable drug-enzyme complexes that affect DMI fungicide efficacy.

The interaction energy score representing the favorable CDOCKER interaction energies from the six DMI fungicides docking onto CYP51A-heme complex is summarized in Table 4. The interaction energy of *C. fruticola* CYP51A to all six DMI fungicides was lower compare with that of the resistant strain *C. truncatum*, indicating stable enzyme-ligand interactions are favored for *C. fruticola* CYP51A. For example, binding affinity of fenbuconazole was -40.2 kcal/mol for *C. fruticola* CYP51A and -37.1 kcal/mol for *C. truncatum*. Among all six DMI fungicides, difenoconazole had the most binding affinity to the sensitive strain *C. fruticola* and the resistant strain *C. truncatum*, -45.9 and -44.1 kcal/mol, respectively. Flutriafol was predicted to be least effective since the binding affinity was low for both *C. fruticola* and *C. truncatum*, -30.6 and -29.4 kcal/mol, respectively.

Figures 5 and 6 show the noncovalent ligand-enzyme interactions, where key interacting residues are labeled. Difenoconazole has one extra ring structure that is further away from the Fe-N interaction pocket. The introduction of the variants causes conformation changes that interrupts the halogen bond between heme and Dif:C19 ($3.22 \text{ \AA}$). For propiconazole, the halogen bond of Ser369-Pro:Cl ($3.18 \text{ \AA}$), and electrostatic interaction Cys456-Pro ($4.96 \text{ \AA}$) were both diminished since the variant conformation prompted a longer distance that exceeded the interaction range. The variant conformation also pushed propiconazole further from heme (Fe-N coordination bond from

Fig. 1. Schematic gene structure of *Colletotrichum truncatum* CYP51A and CYP51B. CYP51A from the United States and Chinese isolates CTRR13_1 and TGBC1 are 1,539 and 1,533 bp in size, respectively. Mutations found in this study and which may confer inherent resistance to DMI fungicides are marked with a pentagram (L208Y, H238R, S302A, and I366L). Chinese *C. truncatum* isolate with amino acid differences compared with U.S. *C. truncatum* are displayed with arrows. Nucleotide deletions are indicated with Δ followed by the number of missing base pairs. CYP51B is 1,719 bp in size interrupted by two introns (with exons from 1 to 246, 325 to 522, and 583 to 1719). Mutations found in this study that may confer inherent resistance to DMI fungicides are also marked with a pentagram (H373 N, M376L, and S511T in CYP51B).

Fig. 2. Phylogenetic tree of CYP51 proteins generated by the maximum likelihood method with Mega 7.0 software. The deduced amino acid sequences of *CYP51A* for *Colletotrichum truncatum* isolate CtRR13_1, *C. nymphaeae* isolate CaCO42, and those from other fungal species *C. gloeosporioides* (GenBank accession no. EQB56428.1), *Metarhizium anisopliae* (GenBank accession no. KJK74651.1), *Beauveria bassiana* (GenBank accession no. KGQ04950.1), *Cordyceps militaris* (GenBank accession no. XP_006668831.1), *Hirsutella minnesotensis* (GenBank accession no. KJZ76396.1), *C. sublineola* (GenBank accession no. KDN69988.1), *Diaporthe ampelina* (GenBank accession no. KKY35210.1), *Aspergillus flavus* (GenBank accession no. XP_002375123.1), *Magnaporthe oryzae* (GenBank accession no. XP_003710875.1), *Neonectria ditissima* (GenBank accession no. KPM38499.1), *C. simmondsii* (GenBank accession no. KXH38064.1), *Fusarium verticillioides* (GenBank accession no. AGK89489.1), *F. graminearum* (GenBank accession no. XP_011321548.1), *Valsa mali* (GenBank accession no. KUI72529.1), *C. orchidophilum* (GenBank accession no. XP_022474431), *Nectria haematococca* (GenBank accession no. XP_003045204), *C. orbiculare* (GenBank accession no. ENH81686.1), *C. higginsianum* (GenBank accession no. CCF38358.1), *C. fioriniae* (GenBank accession no. XP_007594880), *C. salicis* (GenBank accession no. KXH56565.1), and *Pochonia chlamydosporia* (GenBank accession no. XP_022285666). The deduced amino acid sequences of *CYP51B* were from *C. truncatum* isolate CtRR13_1, *C. nymphaeae* isolates CaCO42, and those from other fungal species *C. gloeosporioides* (GenBank accession no. EQB53780.1), *C. higginsianum* (GenBank accession no. CCF32640.1), *C. graminicola* (GenBank accession no. XP_008090488.1), *C. incanum* (GenBank accession no. KZL63163.1), *C. sublineola* (GenBank accession no. KDN66717.1), *C. tofieldiae* (GenBank accession no. KZL69090.1), *C. orbiculare* (GenBank accession no. ENH88478.1), *C. simmondsii* (GenBank accession no. KXH37906.1), *C. fioriniae* (GenBank accession no. XP_007591427.1), *C. salicis* (GenBank accession no. KXH56565.1), *P. chlamydosporia* (GenBank accession no. OAQ72608.1), *Metarhizium guizhouense* (GenBank accession no. KID88153.1), *Fusarium oxysporum* (GenBank accession no. EXA53151.1), *F. graminearum* (GenBank accession no. XP_011316750.1), *N. ditissima* (GenBank accession no. KPM38922.1), *Myceliophthora thermophila* (GenBank accession no. XP_003658636.1), *H. minnesotensis* (GenBank accession no. KJZ73557.1), *Madurella mycetomatis* (GenBank accession no. KXX80456.1), *Tolypocladium ophioglossoides* (GenBank accession no. KND89481.1), *Purpureocillium lilacinum* (GenBank accession no. OAQ81874.1), *Drechmeria coniospora* (GenBank accession no. KYK55628.1), *Chaetomium thermophilum* (GenBank accession no. XP_006696132.1), and *Scedosporium apiospermum* (GenBank accession no. XP_016644011.1). The deduced amino acid sequences of *CYP51C* were from *F. graminearum* (GenBank accession no. XP_011325340). Sequence of *Candida albicans* (GenBank accession no. AIX03614.1) was used as the out-group reference species. The *CYP51A* and *CYP51B* of *C. truncatum* were marked by pentagrams.

3.08 to 3.14 Å). For metconazole, introduction of the variants shifts the binding affinity because the amino acid repositions shifted Fe–N coordination bond (2.98 Å) to a distance that Fe and N could no longer interact. The electrostatic bond (Cys456–metconazole, 4.83 Å) was also weakened. Tebuconazole forms hydrogen bond with Gly297 but the variant model favors a different hydrogen bonding (Tyr128–Teb: H43, 2.54 Å). Instead of a Fe–N coordination bond in the original model, tebuconazole and heme favored hydrogen bonding (Heme–Teb: H42, 2.35 Å) in the variant model. The overall nonbonding interaction of flutriafol–CYP51 complex was drastically in the variant model. The residues that form hydrogen and halogen bonds were

predicted to be Met497 and Thr293 in the variant instead of Tyr114 and Ser369 and heme in the original model. An electrostatic interaction between heme iron and flutriafol (3.7 Å) was diminished. For fenbuconazole, a heme–fenbuconazole hydrogen bond in the original model was replaced by Tyr128, which is in the distance of forming two hydrogen bonds with fenbuconazole H35 and H36 (3.0 Å and 2.35 Å, respectively). Hydrophobic interaction of fenbuconazole to Phe498 and Leu371 were no longer approachable.

For *CYP51B*, the corresponding *C. truncatum* CYP51B positions H373 N, M376L, and S511T were the same as *C. fructicola* positions. Residues at position 373 and 376 were in the CYP51

TABLE 3. Relative expression of *CYP51A* and *CYP51B* genes in *Colletotrichum* spp. field isolates

Species	Isolates	Flutriafol Sensitivity ^x	Relative expression level of ^y					
			<i>CYP51A</i>		<i>CYP51B</i>			
			Untreated	Flutriafol-treated	Untreated	Flutriafol-treated	Untreated	Flutriafol-treated
			Mean ^z	Mean	Mean	Mean	Mean	Mean
<i>C. truncatum</i>	CtRR13_1	R	0.55±0.06	12.84±0.8	6.14±0.33	168.19±7.69		
	CtRR13_2	R	0.15±0.01	0.34±0.20 b	7.01±0.75	6.57±0.70 b	205.77±38.73	186.99±33.66 a
<i>C. nymphaeae</i>	CaPH40	R	0.26±0.02	3.36±0.49	3.98±0.02	39.61±0.49		
	CaCO4_42	R	0.25±0.04	0.25±0.03 b	4.69±0.33	4.37±0.42 c	41.85±5.20	40.68±6.75 c
<i>C. fructicola</i>	CgRR12-3	S	7.50±0.20	28.38±3.42	3.85±0.18	62.90±1.81		
	CgSE12_1	S	7.63±0.37	7.56±0.30 a	4.15±0.33	4.00±0.31 c	61.31±4.96	62.10±8.87 c
<i>C. fioriniae</i>	Ca2_4_2	S	1.06±0.13	29.83±1.81	9.87±0.13	94.46±3.42		
	Ca2_2_2	S	1.07±0.16	1.06±0.15 b	27.96±1.33	28.90±1.84 a	15.66±1.08	12.76±3.21 a
							95.76±5.12	95.11±3.76 b

^x R = resistant, S = sensitive.

^y 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 sensitive isolate Ca2_4_2 mRNA.

^z The mean and standard deviation values indicate the average relative expression of two isolates for each *Colletotrichum* species (Ct_RR13_1 and Ct_RR13_2 for *C. truncatum*, CaPH40 and CaCO4_42 for *C. nymphaeae*, CgRR12-3 and CgSE12_1 for *C. fructicola*, and Ca2_4_2 and Ca2_2_2 for *C. fioriniae*) and between two independent biological experiments. Relative expression was determined in isolates subjected to water or flutriafol at 2 µg/ml for 6 h before mRNA isolation. Mean values within columns followed by the same letter are equivalent based on Fisher's protected least significant difference ($P = 0.05$).

Fig. 3. Alignments of CYP51A substrate recognition sites (SRS) amino acid sequences from *Aspergillus fumigatus* and various *Colletotrichum* spp. Translated amino acid sequences of various *Colletotrichum* spp. CYP51A aligned with CYP51A from the reference isolates *A. fumigatus* (GenBank accession no. AGH55423). SRS are indicated by the black boxes. Mutations in *C. truncatum* isolate Ct_RR13_1 were marked with the symbol ▲. The second to the fifth row sequences are the CYP51A sequences from isolates Ct_RR13_1, CaCO42, Cg_SE12_1, and C_2_4_2, respectively. Other sequences are from *C. gloeosporioides* (GenBank accession no. EQB56428.1), *C. fioriniae* (GenBank accession no. XP_007594880.1), *C. higginsianum* (GenBank accession no. CCF38358.1), *C. sublineola* (GenBank accession no. KDN69988.1), *C. orbiculare* (GenBank accession no. ENH81686.1), *C. simmondsii* (GenBank accession no. KXH38064.1), and *C. orchidophilum* (GenBank accession no. OHE97276.1). *C. truncatum* differs from other *Colletotrichum* spp. by the amino acid substitution L208Y, H238R, S302A, and I366L, orthologous to L206Y, H236R, S302A, and I367L in *A. fumigatus*.

binding cavity to difenoconazole, propiconazole, tebuconazole, and fenbuconazole within 5 Å (data not shown).

DISCUSSION

As previously reported, *C. truncatum* isolates from peach and chili are inherently resistant to tebuconazole, metconazole, fenbuconazole, flutriafol, and myclobutanil (Chen et al. 2016; Zhang et al. 2017). In this study, we established that *C. truncatum* isolates from soybean and citrus exhibited similar resistance to multiple triazoles, including flutriafol, tebuconazole, metconazole, and fenbuconazole. Taken together, these findings point to an inherent resistance of *C. truncatum* to many triazoles. Remarkable differences in azole efficacy due to differences in intrinsic activity were reported between yeast species of the same genus. For example, *Candida krusei* was inherently less sensitive than *Candida albicans* to both voriconazole and fluconazole (Fukuoka et al. 2003).

Species-specific responses to fungicides are not new to *Colletotrichum* species. *C. acutatum* has inherent resistance to benomyl, based on enhanced expression of the β-tubulin 1 gene (Hu et al. 2015a; Nakaune and Nakano 2007). Similar variability in sensitivity was also reported between *Colletotrichum* species to various succinate dehydrogenase inhibitor (SDHI) fungicides (Ishii et al. 2016), and between *Colletotrichum* species to polyoxin-D fungicides (Hao et al. 2017).

The molecular basis of inherent resistance to DMI fungicides in *C. truncatum* was investigated. Analysis of the *Colletotrichum* species genome revealed two *CYP51* genes, *CYP51A* and *CYP51B*. Paralogous *CYP51* genes are not uncommon and have been reported already in filamentous fungi. Some species, such as *F. graminearum* even possess three *CYP51* genes (A-, B-, and C-) (Liu et al. 2011). For *CYP51A* and *CYP51B*, functional analyses indicated that any protein could practically replace the other in terms of ergosterol synthesis and vegetative growth (Liu et al. 2011; Martel et al. 2010; Yan et al. 2011). For *CYP51C*, studies by Fan et al. (2013) have shown that *CYP51C* cannot function as a sterol 14α-demethylase;

Fig. 4. Alignments of CYP51B substrate recognition sites (SRS) amino acid sequences from *Aspergillus fumigatus* and various *Colletotrichum* species. Translated amino acid sequences of various *Colletotrichum* spp. CYP51B aligned with CYP51B from the reference isolates *A. fumigatus* (GenBank accession no. XP749134). SRS are indicated by the black boxes. Mutations in *C. truncatum* isolate Ct_RR13_1 were marked with the symbol ▲. The second to the fifth row sequences are the CYP51B amino acid sequences of isolate CtRR13_1 from *C. truncatum* (*C. trun_CtRR13_1*), isolate CaC042 from *C. nymphaeae* (*C. nymph_CaC042*), isolate Cg_SE12_1 from *C. fructicola* (*C. fruc_Cg_SE12_1*), and isolate C_2_4_2 from *C. fiorinae* (*C.fior_C_2_4_2*). Other sequences were from *C. gloeosporioides* (GenBank accession no. EQB53780.1), *C. fiorinae* (GenBank accession no. XP_007591427.1), *C. higginsianum* (GenBank accession no. CCF32640.1), *C. simmondsii* (GenBank accession no. KXH37906.1), *C. graminicola* (GenBank accession no. XP_008090488.1), *C. sublineola* (GenBank accession no. KDN66717.1), *C. incanum* (GenBank accession no. KZL63163.1), *C. tofieldiae* (GenBank accession no. KZL69090.1), *C. salicis* (GenBank accession no. KXH56565.1), and *C. orbiculare* (GenBank accession no. ENH88478.1). *C. truncatum* differs from other *Colletotrichum* spp. by the amino acid substitution H373 N, M376L, and S511T, orthologous to H373 N, I376L, and S505T in *A. fumigatus*.

TABLE 4. CDOCKER interaction energy of six demethylation inhibitor fungicides docked to the CYP51A binding site of *Colletotrichum fructicola* and *C. truncatum* models

Species	-CDOCKER interaction energy (kcal/mol)					
	Flutriafol	Fenbuconazole	Tebuconazole	Metconazole	Propiconazole	Difenoconazole
<i>C. fructicola</i> ^y	30.6	40.2	41.9	42.4	43.3	45.9
<i>C. truncatum</i> ^z	29.4	37.1	38.9	41.2	42.1	44.1

^y The modeling of the sensitive isolate *C. fructicola* was obtained with the template of 4uym.
^z The structure of *C. truncatum* was modeled by mutations at residues L213Y (L208Y), H243R (H238R), S303A (S302A), and I370L (I366L) based on the modeling of *C. fructicola*.

et al. 2007; Mullins et al. 2011). The same area was also investigated for *CYP51A* and *CYP51B* of *C. truncatum*. The amino acid sequence in this area of both *CtCYP51A* and *CtCYP51B* were the same as in other *Colletotrichum* spp. and in *M. graminicola* (data not shown).

The constitutive overexpression of *CYP51* genes is another mechanism that can lead to DMI fungicide resistance. However, in our study, differences in constitutive expression of either paralog did not correlate with azole sensitivity. Thus, the inherent resistance to DMI fungicides in *C. truncatum* may solely be caused by the secondary structure of the *CYP51* protein. In this study, we show the *CYP51A* in *Colletotrichum* species was induced after exposure to azole, indicating that *CYP51A* may be able to maintain *CYP51* function when *CYP51B* is inhibited by azoles. The same phenomenon was also found in *R. commune* (Hawkins et al. 2014). Unlike in *R. commune*, where *CYP51A* expression is more inducible in response to the effects of azoles than *CYP51B* (Hawkins et al. 2014), our results showed similar results in both *CYP51A* and *CYP51B*. *C. nymphaeae* was also resistant to flutriafol and fenbuconazole, but no mutations were found in either *CYP51s*. This suggests that as yet unknown mechanisms other than point mutations may confer resistance to DMIs in *Colletotrichum* species.

In our study, DMI fungicides affected *C. truncatum* isolates differently, which might because DMIs may target different *CYP51* enzymes. Study in *F. graminearum* showed the *CYP51A* gene deletion mutant was highly sensitive to triadimefon and propiconazole, while the *CYP51C* gene deletion mutant increased in sensitivity to tebuconazole, diniconazole, difenoconazole, flutriafol, and prochloraz, but not to triadimefon and propiconazole. The sensitivity of *CYP51B* gene deletion mutant to all the DMI fungicides did not alter. Since *Colletotrichum* spp. lack *CYP51C* paralog, DMI fungicides might have different *CYP51* enzymes target in *Colletotrichum* spp. compared with that in *Fusarium* spp. (Liu et al. 2011). In our study, difenoconazole and propiconazole may target the same *CYP51* enzyme based on similar EC₅₀ values, while another four DMI fungicides may target the other. Further knock-out experiment in *Colletotrichum* spp. will be needed to elucidate the patterns of DMIs targeting different *CYP51* paralogs.

In summary, inherent resistance to DMIs of *C. truncatum* could be a result of *CYP51A* and *CYP51B* gene variations. The main changes were found at amino acid codon positions 208, 238, 302, and 366 in *CYP51A*, and in 373, 376, and 511 in *CYP51B*. Further investigations may shed light as to which *CYP51* paralog is associated with DMI resistance.

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LITERATURE CITED

- Akins, R. A. 2005. An update on antifungal targets and mechanisms of resistance in *Candida albicans*. *Med. Mycol.* 43:285-318.
- Becher, R., Weihmann, F., Deising, H. B., and Wirsal, S. G. 2011. Development of a novel multiplex DNA microarray for *Fusarium graminearum* and analysis of azole fungicide responses. *BMC Genomics* 12:52.
- Brooks, B. R., Bruccoleri, R. E., Olafson, B. D., States, D. J., Swaminathan, S., and Karplus, M. 1983. CHARMM: A program for macromolecular energy, minimization, and dynamics calculations. *J. Comput. Chem.* 4: 187-217.
- Brunner, P. C., Stefansson, T. S., Fountaine, J., Richina, V., and McDonald, B. A. 2016. A global analysis of *CYP51* diversity and azole sensitivity in *Rhynchosporium commune*. *Phytopathology* 106:355-361.
- Carter, H. E., Fraaije, B. A., West, J. S., Kelly, S. L., Mehl, A., Shaw, M. W., and Cools, H. J. 2014. Alterations in predicted regulatory and coding regions of the sterol 14 α -demethylase gene (*CYP51*) confer decreased azole sensitivity in the oilseed rape pathogen *Pyrenopeziza brassicae*. *Mol. Plant Pathol.* 15:513-522.
- Chen, D., Shi, H., Wu, H., Xu, Z., and Zhang, C. 2013. Resistance of *Colletotrichum gloeosporioides* causing grape ripe rot to thiophanate-methyl and tebuconazole in Zhejiang. *J. Fruit Sci.* 4:27.
- Chen, F., Fan, J., Zhou, T., Liu, X., Liu, J., and Schnabel, G. 2012. Baseline sensitivity of *Monilinia fructicola* from China to the DMI fungicide SYP-Z048 and analysis of DMI-resistant mutants. *Plant Dis.* 96:416-422.
- Chen, S., Luo, C., Hu, M., and Schnabel, G. 2016. Sensitivity of *Colletotrichum* species, including *C. fioriniae* and *C. nymphaeae*, from peach to demethylation inhibitor fungicides. *Plant Dis.* 100:2434-2441.
- Cools, H. J., Mullins, J. G., Fraaije, B. A., Parker, J. E., Kelly, D. E., Lucas, J. A., and Kelly, S. L. 2011. Impact of recently emerged sterol 14 α -demethylase (*CYP51*) variants of *Mycosphaerella graminicola* on azole fungicide sensitivity. *Appl. Environ. Microbiol.* 77:3830-3837.
- Da Silva Ferreira, M., Colombo, A., Paulsen, I., Ren, Q., Wortman, J., Huang, J., Goldman, M., and Goldman, G. 2005. The ergosterol biosynthesis pathway, transporter genes, and azole resistance in *Aspergillus fumigatus*. *Med. Mycol.* 43:S313-S319.
- Damm, U., Woudenberg, J., Cannon, P., and Crous, P. 2009. *Colletotrichum* species with curved conidia from herbaceous hosts. *Fungal Divers.* 39:45.
- Délye, C., Bousset, L., and Corio-Costet, M.-F. 1998. PCR cloning and detection of point mutations in the ebuciclor 14 α -demethylase (*CYP51*) gene from *Erysiphe graminis* f. sp. *hordei*, a "recalcitrant" fungus. *Curr. Genet.* 34:399-403.
- Délye, C., Laigret, F., and Corio-Costet, M.-F. 1997a. Cloning and sequence analysis of the ebuciclor 14 α -demethylase gene of the obligate biotrophic grape powdery mildew fungus. *Gene* 195:29-33.
- Délye, C., Laigret, F., and Corio-Costet, M.-F. 1997b. A mutation in the 14 α -demethylase gene of *Uncinula necator* that correlates with resistance to a sterol biosynthesis inhibitor. *Appl. Environ. Microbiol.* 63:2966-2970.
- Deng, W., Yang, M., Du, F., Liu, Y., Zhang, L., Yan, Y., Wang, S., Yang, J., He, X., and Zhu, S. 2011. Sensitivity of *Colletotrichum gloeosporioides* causing grape anthracnose to three sterol demethylation inhibitor(DMI) fungicides. *Chin. J. Pestic. Sci.* 13:245-252.
- Diao, Y.-Z., Zhang, C., Liu, F., Wang, W.-Z., Liu, L., Cai, L., and Liu, X.-L. 2017. *Colletotrichum* species causing anthracnose disease of chili in China. *Persoonia. Mol. Phylogeny Evol. Fungi* 38:20-37.
- Fan, J., Urban, M., Parker, J. E., Brewer, H. C., and Kelly, S. L., Hammond-Kosack, K. E., Fraaije, B. A., Liu, X., and Cools, H. J. 2013. Characterization of the sterol 14 α -demethylases of *Fusarium graminearum* identifies a novel genus-specific *CYP51* function. *New Phytol.* 198: 821-835.
- Fukuoka, T., Johnston, D. A., Winslow, C. A., de Groot, M. J., Burt, C., Hitchcock, C. A., and Filler, S. G. 2003. Genetic basis for differential activities of fluconazole and voriconazole against *Candida krusei*. *Antimicrobial Agents Chemother.* 47:1213-1219.
- Grabke, A., Williamson, M., Henderson, G., and Schnabel, G. 2015. First report of anthracnose on peach fruit caused by *Colletotrichum truncatum* in South Carolina. *Plant Dis.* 99:806-814.
- Guex, N., and Peitsch, M. C. 1997. SWISS-MODEL and the Swiss-Pdb Viewer: An environment for comparative protein modeling. *Electrophoresis* 18:2714-2723.
- Hamamoto, H., Hasegawa, K., Nakaune, R., Lee, Y. J., Makizumi, Y., Akutsu, K., and Hibi, T. 2000. Tandem repeat of a transcriptional enhancer upstream of the sterol 14 α -demethylase gene (*CYP51*) in *Penicillium digitatum*. *Appl. Environ. Microbiol.* 66:3421-3426.
- Han, R., Zhang, J., Li, S., Cao, S., Geng, H., Yuan, Y., Xiao, W., Liu, S., and Liu, D. 2010. Homology modeling and screening of new 14 α -demethylase inhibitor (DMI) fungicides based on optimized expression of *CYP51* from *Ustilago maydis* in *Escherichia coli*. *J. Agric. Food Chem.* 58:12810-12816.
- Hao, X., Hu, M., Chen, S., and Schnabel, G. 2017. Challenges in assessing efficacy of polyoxin-D zinc salt against *Colletotrichum* species. *J. Plant Pathol.* 99:1-12.
- Hawkins, N. J., Cools, H. J., Sierotzki, H., Shaw, M. W., Knogge, W., Kelly, S. L., Kelly, D. E., and Fraaije, B. A. 2014. Paralog re-emergence: A novel, historically contingent mechanism in the evolution of antimicrobial resistance. *Mol. Biol. Evol.* 31:1793-1802.
- Hayashi, K., Schoonbeek, H., and De Waard, M. A. 2003. Modulators of membrane drug transporters potentiate the activity of the DMI fungicide oxpoconazole against *Botrytis cinerea*. *Pest Manag. Sci.* 59:294-302.
- Hu, M.-J., Grabke, A., Dowling, M. E., Holstein, H. J., and Schnabel, G. 2015a. Resistance in *Colletotrichum siamense* from peach and blueberry to thiophanate-methyl and azoxystrobin. *Plant Dis.* 99:806-814.
- Hu, M.-J., Grabke, A., and Schnabel, G. 2015b. Investigation of the *Colletotrichum gloeosporioides* species complex causing peach anthracnose in South Carolina. *Plant Dis.* 99:797-805.
- Huang, F., Chen, G., Hou, X., Fu, Y., Cai, L., Hyde, K., and Li, H. 2013. *Colletotrichum* species associated with cultivated citrus in China. *Fungal Divers.* 61:61-74.

- Inc, A. 2012. Discovery studio modeling environment, release 3.5. Accelrys Discovery Studio. Accelrys Software Inc., San Diego.
- Ishii, H., Zhen, F., Hu, M., Li, X., and Schnabel, G. 2016. Efficacy of SDHI fungicides, including benzovindiflupyr, against *Colletotrichum* species. *Pest Manag. Sci.* 72:1844-1853.
- Jayawardena, R., Hyde, K., Damm, U., Cai, L., Liu, M., Li, X., Zhang, W., Zhao, W., and Yan, J. 2016. Notes on currently accepted species of *Colletotrichum*. *Mycosphere* 7:1192-1260.
- Kumar, S., Stecher, G., and Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33:1870-1874.
- Larkin, M. A., Blackshields, G., Brown, N., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., and Lopez, R. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* 23:2947-2948.
- Leroux, P., Albertini, C., Gautier, A., Gredt, M., and Walker, A. S. 2007. Mutations in the *CYP51* gene correlated with changes in sensitivity to sterol 14-demethylation inhibitors in field isolates of *Mycosphaerella graminicola*. *Pest Manag. Sci.* 63:688-698.
- Liu, X., Yu, F., Schnabel, G., Wu, J., Wang, Z., and Ma, Z. 2011. Paralogous *cyp51* genes in *Fusarium graminearum* mediate differential sensitivity to sterol demethylation inhibitors. *Fungal Genet. Biol.* 48:113-123.
- Ma, Z., Proffer, T. J., Jacobs, J. L., and Sundin, G. W. 2006. Overexpression of the 14 α -demethylase target gene (*CYP51*) mediates fungicide resistance in *Blumeriella jaapii*. *Appl. Environ. Microbiol.* 72:2581-2585.
- Manandhar, J., Hartman, G., and Wang, T. 1995. Anthracnose development on pepper fruits inoculated with *Colletotrichum gloeosporioides*. *Plant Dis.* 79:380-383.
- Mariani, V., Kiefer, F., Schmidt, T., Haas, J., and Schwede, T. 2011. Assessment of template based protein structure predictions in CASP9. *Proteins. Struct. Funct. Bioinform.* 79:37-58.
- Martel, C. M., Parker, J. E., Warrilow, A. G., Rolley, N. J., Kelly, S. L., and Kelly, D. E. 2010. Complementation of a *Saccharomyces cerevisiae* *ERG11/CYP51* (sterol 14 α -demethylase) doxycycline-regulated mutant and screening of the azole sensitivity of *Aspergillus fumigatus* isoenzymes *CYP51A* and *CYP51B*. *Antimicrobial Agents Chemother.* 54:4920-4923.
- Mellado, E., Diaz-Guerra, T., Cuenca-Estrella, M., and Rodriguez-Tudela, J. 2001. Identification of two different 14- α sterol demethylase-related genes (*cyp51A* and *cyp51B*) in *Aspergillus fumigatus* and other *Aspergillus* species. *J. Clin. Microbiol.* 39:2431-2438.
- Mellado, E., Garcia-Effron, G., Alcazar-Fuoli, L., Melchers, W., Verweij, P., Cuenca-Estrella, M., and Rodriguez-Tudela, J. 2007. A new *Aspergillus fumigatus* resistance mechanism conferring in vitro cross-resistance to azole antifungals involves a combination of *cyp51A* alterations. *Antimicrobial Agents Chemother.* 51:1897-1904.
- Mullins, J. G., Parker, J. E., Cools, H. J., Togawa, R. C., Lucas, J. A., Fraaije, B. A., Kelly, D. E., and Kelly, S. L. 2011. Molecular modelling of the emergence of azole resistance in *Mycosphaerella graminicola*. *PLoS One* 6: e20973.
- Nakaune, R., Adachi, K., Nawata, O., Tomiyama, M., Akutsu, K., and Hibi, T. 1998. A novel ATP-binding cassette transporter involved in multidrug resistance in the phytopathogenic fungus *Penicillium digitatum*. *Appl. Environ. Microbiol.* 64:3983-3988.
- Nakaune, R., and Nakano, M. 2007. Benomyl resistance of *Colletotrichum acutatum* is caused by enhanced expression of β -tubulin 1 gene regulated by putative leucine zipper protein CaBEN1. *Fungal Genet. Biol.* 44: 1324-1335.
- Ohno, S. 1970. *Evolution by Gene Duplication*. Springer-Verlag, Berlin, New York.
- Palani, P. V., and Lalithakumari, D. 1999. Resistance of *Venturia inaequalis* to the sterol biosynthesis-inhibiting fungicide, penconazole [1-(2-(2, 4-dichlorophenyl) pentyl)-1H-1, 2, 4-triazole]. *Mycol. Res.* 103:1157-1164.
- Rao, S., and Nandineni, M. R. 2017. Genome sequencing and comparative genomics reveal a repertoire of putative pathogenicity genes in chilli anthracnose fungus *Colletotrichum truncatum*. *PLoS One* 12:e0183567.
- Reimann, S., and Deising, H. B. 2005. Inhibition of efflux transporter-mediated fungicide resistance in *Pyrenophora tritici-repentis* by a derivative of 4'-hydroxyflavone and enhancement of fungicide activity. *Appl. Environ. Microbiol.* 71:3269-3275.
- Šali, A., and Blundell, T. L. 1993. Comparative protein modelling by satisfaction of spatial restraints. *J. Mol. Biol.* 234:779-815.
- Schnabel, G., and Jones, A. L. 2001. The 14 α -demethylase (*CYP51A1*) gene is overexpressed in *Venturia inaequalis* strains resistant to myclobutanil. *Phytopathology* 91:102-110.
- Söding, J., Biegert, A., and Lupas, A. N. 2005. The HHpred interactive server for protein homology detection and structure prediction. *Nucleic Acids Res.* 33:W244-W248.
- Squissato, V., Ucel, Y. H., Richardson, S. E., Alkhotani, A., Wong, D. T., Nijhawan, N., and Chan, C. C. 2015. *Colletotrichum truncatum* species complex: Treatment considerations and review of the literature for an unusual pathogen causing fungal keratitis and endophthalmitis. *Med. Mycol. Case Rep.* 9:1-6.
- Szkolnik, M. 1981. Physical modes of action of sterol-inhibiting fungicides against apple diseases *Venturia inaequalis*. *Plant Dis.* 65:981-985.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. 1997. The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25:4876-4882.
- Tucker, M. A., Lopez-Ruiz, F., Jayasena, K., and Oliver, R. P. 2015. Origin of fungicide-resistant barley powdery mildew in Western Australia: Lessons to be learned. Pages 329-340 in: *Fungicide Resistance in Plant Pathogens*. Springer, Japan.
- Warrilow, A. G., Martel, C. M., Parker, J. E., Melo, N., Lamb, D. C., Nes, W. D., Kelly, D. E., and Kelly, S. L. 2010. Azole binding properties of *Candida albicans* sterol 14- α demethylase (*CaCYP51*). *Antimicrob. Agents Ch.* 54:4235-4245.
- Wikee, S., Cai, L., Pairin, N., McKenzie, E. H., Su, Y.-Y., Chukeatirote, E., Thi, H. N., Bahkali, A. H., Moslem, M. A., and Abdelsalam, K. 2011. *Colletotrichum* species from jasmine (*Jasminum sambac*). *Fungal Divers.* 46:171-182.
- Wong, M. L., and Medrano, J. F. 2005. Real-time PCR for mRNA quantitation. *Biotechniques* 39:75-85.
- Yan, X., Ma, W.-B., Li, Y., Wang, H., Que, Y.-W., Ma, Z.-H., Talbot, N. J., and Wang, Z.-Y. 2011. A sterol 14 α -demethylase is required for conidiation, virulence and for mediating sensitivity to sterol demethylation inhibitors by the rice blast fungus *Magnaporthe oryzae*. *Fungal Genet. Biol.* 48:144-153.
- Yang, Y., Liu, Z., Cai, L., Hyde, K., Yu, Z., and McKenzie, E. 2009. *Colletotrichum* anthracnose of *Amaryllidaceae*. *Fungal Divers.* 39:123-146.
- Zhang, C., Diao, Y., Wang, W., Hao, J., Imran, M., Duan, H., and Liu, X. 2017. Assessing the risk for resistance and elucidating the genetics of *Colletotrichum truncatum* that is only sensitive to some DMI fungicides. *Front. Microbiol.* 8:1779.
- Ziogas, B. N., and Malandrakis, A. A. 2015. Sterol biosynthesis inhibitors: C14 demethylation (DMIs). Pages 199-216 in: *Fungicide Resistance in Plant Pathogens*. Springer, Tokyo.