The Cytochrome P450 Lanosterol 14 α -Demethylase Gene Is a Demethylation Inhibitor Fungicide Resistance Determinant in *Monilinia fructicola* Field Isolates from Georgia[†]

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Resistance in *Monilinia fructicola* to demethylation inhibitor (DMI) fungicides is beginning to emerge in North America, but its molecular basis is unknown. Two potential genetic determinants of DMI fungicide resistance including the 14 α -demethylase gene (MfCYP51) and the ATP-binding cassette transporter gene MfABC1, were investigated in six resistant (DMI-R) and six sensitive (DMI-S) field isolates. No point mutations leading to an amino acid change were found in the MfCYP51 gene. The constitutive expression of the MfCYP51 gene in DMI-R isolates was significantly higher compared to DMI-S isolates. Gene expression was not induced in mycelium of DMI-R or DMI-S isolates treated with 0.3 μ g of propiconazole/ml. A slightly higher average MfCYP51 copy number value was detected in DMI-R isolates (1.35) compared to DMI-S isolates (1.13); however, this difference could not be verified in Southern hybridization experiments or explain the up to 11-fold-increased MfCYP51 mRNA levels in DMI-R isolates. Analysis of the upstream nucleotide sequence of the MfCYP51 gene revealed a unique 65-bp repetitive element at base pair position –117 from the translational start site in DMI-R isolates but not in DMI-S isolates. This repetitive element contained a putative promoter and was named Mona. The link between Mona and the DMI resistance phenotype became even more apparent after studying the genetic diversity between the isolates. In contrast to DMI-S isolates, DMI-R isolates contained an MfCYP51 gene of identical nucleotide sequence associated with Mona. Still, DMI-R isolates were not genetically identical as revealed by Microsatellite-PCR analysis. Also, real-time PCR analysis of genomic DNA indicated that the relative copy number of Mona among DMI-S and DMI-R isolates varied, suggesting its potential for mobility. Interestingly, constitutive expression of the MfABC1 gene in DMI-R isolates was slightly lower than that of DMI-S isolates, but expression of the MfABC1 gene in DMI-R isolates was induced in mycelium after propiconazole treatment. Therefore, the MfABC1 gene may play a minor role in DMI fungicide resistance in *M. fructicola*. Our results strongly suggest that overexpression of the MfCYP51 gene is an important mechanism in conferring DMI fungicide resistance in *M. fructicola* field isolates from Georgia and that this overexpression is correlated with Mona located upstream of the MfCYP51 gene.

Brown rot of peach is caused by the ascomycete fungus *Monilinia fructicola* (G Wint) Honey. The disease is primarily controlled by preharvest applications of fungicides. Among the most effective are the sterol demethylation inhibitor (DMI) fungicides, which are widely used for controlling brown rot in the United States. The first evidence that *M. fructicola* was able to develop resistance to DMI fungicides was found in laboratory studies (19). However, it took more than two decades after the introduction of DMI fungicides to detect DMI resistance in *M. fructicola* field populations, and thus far such populations have only been reported in Georgia and New York (21, 27). Isolates with documented in vitro resistance from Georgia were harder to control in detached fruit assays (27) compared to sensitive isolates, and follow-up studies showed that label rates of propiconazole cannot control such isolates in the field (2). Knowledge of the mechanism of DMI resistance in *M. fructicola* may help in designing antiresistance strategies and

may be useful for identifying molecular markers for DMI fungicide resistance.

DMI fungicides specifically bind to the cytochrome P450 lanosterol 14 α -demethylase (CYP51), thereby inhibiting the biosynthesis of ergosterol (32), the primary fungal cell membrane sterol that is responsible for maintaining membrane fluidity and stability (22, 25). Molecular mechanisms leading to DMI resistance have been studied in several important plant fungal pathogens. Common mechanisms include mutations in the target gene CYP51 (6, 7), overexpression of the CYP51 gene (10, 14, 30), and energy-dependent drug efflux mechanisms (11, 18, 20, 24).

We recently cloned and sequenced potential genetic determinants for DMI fungicide resistance in *M. fructicola*. These included the 14 α -demethylase gene (designated MfCYP51) and the ATP-binding cassette (ABC) transporter gene 1 (MfABC1) (28, 29). When the MfCYP51 gene was introduced into PDR5::TN5 *Saccharomyces cerevisiae*, transformants revealed reduced sensitivity to the DMI fungicide myclobutanil (28). This result indicated that overexpression of the MfCYP51 gene is a potential mechanism of DMI fungicide resistance in *M. fructicola*. In a different study, the expression of the ABC transporter gene MfABC1 was induced by the addition of sublethal concentrations of propiconazole to the mycelium prior

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TABLE 1. Characteristics of *M. fructicola* isolates used in this study

Isolate	Origin	Yr isolated	Sensitivity to propiconazole ^a
Bmpc7	Byron, GA	2006	R
Bmpc10	Byron, GA	2006	R
Bmpc13	Byron, GA	2006	R
GADL129	Fort Valley, GA	2004	R
GADL193	Fort Valley, GA	2004	R
GAAP5	Macon, GA	2003	R
GAAP6	Macon, GA	2003	R
BGA25	Byron, GA	2004	S
GAAP11	Macon, GA	2003	S
GAAP12	Macon, GA	2003	S
GADL3	Fort Valley, GA	2004	S
GADL161	Fort Valley, GA	2004	S
SCDL72	Anderson, SC	2004	S
SC99A3	Edgefield, SC	2004	S
SC99A4	Edgefield, SC	2004	S
Kac-18	Fresno, CA	2005	S

^a An isolate was considered resistant (R) when the relative mycelial growth was $\geq 20\%$ at the discriminatory dose of 0.3 μg of propiconazole/ml; all other isolates were considered sensitive (S).

to RNA extraction, indicating a possible involvement of this gene in DMI fungicide resistance as well (29). Furthermore, *MfABC1* revealed high amino acid homology with *atrB* gene from *Aspergillus nidulans*, an ABC transporter gene conferring resistance to different classes of fungicides, including DMI fungicides.

Based on the above-mentioned references, the *MfCYP51*

and *MfABC1* genes were thought to be determinants for DMI fungicide resistance in *M. fructicola*. The goal of the present study was to substantiate the potential role of the *MfCYP51* and *MfABC1* genes in conferring DMI fungicide resistance in field isolates with documented resistance to propiconazole. Our specific objectives were to sequence the *MfCYP51* gene and its upstream flanking sequence from DMI-resistant (DMI-R) and DMI-sensitive (DMI-S) isolates and to conduct expression analyses of both genes.

MATERIALS AND METHODS

***M. fructicola* isolates.** Seven DMI-R and nine DMI-S single-spore field isolates of *M. fructicola* were randomly chosen from our collection for the present study. Their origin and other characteristics are listed in Table 1. All of these isolates were stored at -25°C permanently.

Determination of propiconazole sensitivity. The sensitivity of the isolates to DMI fungicide propiconazole (PropiMax EC; Syngenta Crop Protection, Greensboro, NC) was determined by measuring relative mycelium growth on fungicide-amended medium. Briefly, each isolate was grown for 4 days on potato dextrose agar (PDA; EMD Chemicals, Gibbstown, NJ) and mycelium-containing plugs (5 by 5 mm) from the periphery of these cultures served as inoculum. Plugs from the same inoculum plate and from visibly identical areas were placed in triplicates upside down on nonamended or propiconazole-amended PDA at a discriminatory dose of 0.3 $\mu\text{g}/\text{ml}$ in petri dishes (90 mm in diameter). At this dose, sensitive isolates are arrested in growth, whereas resistant isolates typically grow between 20 and 70% (3). Colony diameters were measured after 4 days of incubation in the dark at 22°C . Relative growth was calculated by expressing the growth of isolates on amended medium as a percentage of the growth on nonamended medium.

Extraction of genomic DNA. Each fungal isolate was grown in 40 ml of potato dextrose broth (PDB; Difco Laboratories, Sparks, MD) for 5 days at 22°C on an

TABLE 2. Nucleotide sequence and characteristics of primers used in this study

Primer	Sequence (5'→3')	Description	Source or reference
MfCYP51-F	GTTTCGCCATGGGTGTTCT	Primer for amplification and partial sequencing of <i>MfCYP51</i>	28
MfCYP51-R	TCGTCTCTCCCATGCCACAA	Same as for MfCYP51-F	28
MfCYP51-5'7	TTGCAGGGAAAGGAAGTC	Sequencing primer for <i>MfCYP51</i>	28
MfCYP51-3'1	CGGAATGATCAGCAAAGGTGC	Same as for MfCYP51-5'7	28
MfCYP51-5'2	GAGAAGAGCGATCATCATGTTAGCGA TCTCT	Same as for MfCYP51-5'7; also used as a gene-specific primer for amplifying the region upstream of <i>MfCYP51</i> in nested PCR assays	28
MfCYP51-5'5	GGGCAGTCGTAAACCACAT	Same as for MfCYP51-5'2	28
Micro 1	GACAGACGGACGGACG	Microsatellite primer for MP-PCR analysis	15
Micro 4	AGAGAGAGAGAGAGAGC	Same as for Micro 1	15
M13	GAGGGTGGCGGTTCT	Same as for Micro 1	15
RealCYP-F2	TCTTGGGCGCCGACCTTC	Real-time PCR primer for amplifying a 120-bp fragment of <i>MfCYP51</i>	This study
RealCYP-R2	CTTTGCGCATGATAGAGTGGAT	Same as for RealCYP-F2	This study
MfABC5-F	CCGATGAAACAAGCTCGACCTTT	Real-time PCR primer for amplifying a 260-bp fragment of <i>MfABC1</i>	29
MfABC5-R	CGGATTTGATTGGGTGTTAT	Same as for MfABC5-F	29
MfActin-F	GTGTTGATATGGCCGGTCGTGATT	Real-time PCR primer for amplifying a 85-bp fragment of <i>MfActin</i> , the endogenous control	28
MfActin-R	TCGGCAGTGTTGGAGAAAGTGTA	Same as for MfActin-F	28
AP1	GGATCCTAATACGACTCACTATAGGGC	Adaptor-specific primer for amplifying the upstream region of <i>MfCYP51</i> in nested PCR	31
AP2	AATAGGGCTCGAGCGGC	Same as for AP1	31
UpCYP-1F	AGAGCTACCAACCCACGAGGAA	Sequencing primer for the upstream region of <i>MfCYP51</i>	This study
UpCYP-2F	GCCATACCGCAGACCATTGAA	Same as for UpCYP-1F	This study
UpCYP-3F	CCATGCACCTGCCACTGACTA	Same as for UpCYP-1F	This study
UpCYP-1R	GACCGTGCAGAAATCTCTTGA	Same as for UpCYP-1F	This study
UpCYP-2R	GGGAATCCAATCACGAGTTT	Same as for UpCYP-1F	This study
UpCYP-3R	CCCTTGCAGCCCTCAGTA	Same as for UpCYP-1F	This study
Insert-1F	CACACCGCTCTTTTAGT	Real-time PCR primer for amplifying a 56-bp fragment of <i>Mona</i>	This study
Insert-2R	CTCTGCTCCTACTACGGA	Same as for Insert-1F	This study

TABLE 3. Relative expression of the MfCYP51 and MfABC1 genes in *M. fructicola* field isolates

Isolate	Relative growth (%) ^a	Propiconazole sensitivity phenotype ^b	Relative expression of ^c :							
			MfCYP51				MfABC1			
			Untreated		Propiconazole treated		Untreated		Propiconazole treated	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD
Bmpc7	29.7	R	11.2	0.0	10.8	1.4	1.6	1.0	1.7	0.7
Bmpc10	30.8	R	7.3	1.0	7.6	1.6	1.5	1.1	2.2	0.1
Bmpc13	29.5	R	10.1	0.8	9.5	1.4	1.6	0.1	2.2	0.9
GADL193	35.0	R	9.1	1.6	7.5	0.9	1.2	0.1	2.1	0.0
GAAP5	36.3	R	10.3	1.5	10.0	0.7	0.6	0.3	1.9	0.9
GAAP6	21.8	R	5.2	0.8	4.6	0.4	1.4	0.5	1.8	0.5
GAAP12	0	S	1.9	0.5	1.3	0.3	2.6	0.7	1.5	0.0
GADL3	0	S	3.4	0.2	1.9	0.4	2.8	0.3	1.1	0.6
SCDL72	3.1	S	1.2	0.1	1.8	0.3	2.0	0.4	1.1	0.1
SC99A3	5.0	S	2.3	1.0	1.8	0.0	1.0	0.0	1.6	0.9
SC99A4	1.3	S	1.0	0.1	1.6	0.5	2.0	0.4	2.5	0.4
Kac-18	0.4	S	1.0	0.2	0.9	0.4	2.2	0.1	2.5	0.4

^a Relative growth was calculated by expressing the growth of isolates on medium amended with 0.3 µg of propiconazole/ml as a percentage of the growth on nonamended medium.

^b An isolate was considered resistant (R) when the relative mycelial growth on medium amended with 0.3 µg of propiconazole/ml was ≥20%; all other isolates were considered sensitive (S).

^c The expression of the MfCYP51 or MfABC1 genes was normalized using the MfActin gene expression levels and then calibrated to the normalized MfCYP51 or MfABC1 mRNA value from the isolate with the lowest amount of MfCYP51 or MfABC1 mRNA. The mean and standard deviation values indicate the average relative expression between two independent biological experiments. Relative expression was determined in isolates subjected to water or 0.3 µg of propiconazole/ml 1 h before mRNA isolation.

orbital shaker (120 rpm). Mycelium was separated from the liquid culture by centrifugation at 10,000 rpm for 5 min. Mycelial mats were frozen in liquid nitrogen and ground into fine powder with mortar and pestle. Genomic DNA was isolated by using a DNeasy plant minikit (Qiagen, Inc., Valencia, CA) according to the manufacturer's manual. DNA quality was assessed by electrophoresis on 1.0% agarose gels (Promega Corp., Madison, WI) including ethidium bromide (1 µg/ml) in 0.5× TBE buffer (44.5 mM Tris-borate, 1 mM EDTA [pH 8.0]).

Amplification and sequencing of the MfCYP51 gene. Gene sequencing analysis was restricted to the MfCYP51 gene because point mutations in ABC transporters are not known to cause fungicide resistance. The primers used to amplify and sequence the MfCYP51 gene are listed in Table 2. The complete MfCYP51 gene was amplified with the primer pair MfCYP51-F and MfCYP51-R designed by Schnabel and Dai (28). PCR volumes were 50 µl containing 1× PCR buffer provided by the manufacturer, 20 ng of genomic DNA, 0.5 µM concentrations of each primer, 200 µM concentrations of each deoxynucleoside triphosphate, 1.25 U of *Taq* DNA polymerase (New England Biolabs, Ipswich, MA). Amplifications were performed in an iCycler thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA) programmed for 3 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, followed by a final extension step of 5 min at 72°C. The PCR products were separated on 1.0% agarose gels in 0.5× TBE buffer at 100 V for 1 h. Gels were photographed with an electrophoresis documentation and analysis system (Kodak EDAS 290; Eastman Kodak Company, New Haven, CT).

PCR products were purified by using the QiaQuick gel extraction kit (Qiagen) and sequenced with BigDye Terminator v3.1 kit (Applied Biosystems, Inc., Foster City, CA). The sequencing primers were the forward primers MfCYP51-F, MfCYP51-5'7, and MfCYP51-3'1 and the reverse primers MfCYP51-R, MfCYP51-5'2, and MfCYP51-5'5. Sequencing was performed at the Clemson University Genomics Institute. The software of DNASTAR (DNASTAR, Inc., Nevada City, CA) was used to assemble and align the MfCYP51 nucleotide sequences.

Investigation of genetic diversity between *M. fructicola* isolates using MP-PCR. Three microsatellite primers (MPs), Micro 1, Micro 4, and M13, which have been successfully used to characterize *M. fructicola* isolates by Ma et al. (15), were selected to investigate genetic diversity between isolates used in the present study. PCR was largely performed as described for the amplification of the MfCYP51 gene, except that a single MP primer was used at 1 µM in each reaction and the annealing temperature was 50°C. For each isolate, the presence or absence of polymorphic bands was scored as "1" or "0," respectively. Dice coefficients were calculated for all pairwise comparisons by using the Windist program (Winboot; International Rice Research Institute, Manila, Philippines)

as $F = 2N_{xy}/(N_x + N_y)$, where N_{xy} is the number of bands shared by a given pair of isolates, and N_x and N_y are the number of bands found in isolates x and y , respectively. A phenogram was generated based on similarity coefficients by using UPGMA (unweighted pair-group method with arithmetic averages) and the SAHN program of the software package NTSYS-pc 2.1 (Department of Ecology and Evolution, State University of New York).

RNA isolation, cDNA synthesis, and quantification of MfCYP51 and MfABC1 mRNAs. Mycelia of *M. fructicola* isolates were transferred into 250-ml flasks containing 40 ml of PDB and nine glass beads (10 mm in diameter) each. The liquid cultures were incubated for 5 days at 22°C and shaken vigorously every 8 h to encourage uniform growth of mycelium in the liquid medium. After 5 days, water or propiconazole (0.3 µg/ml) formulated as PropiMax EC (Syngenta) was added to the flasks. One hour later, total RNA was isolated using an RNeasy plant minikit according to the manufacturer's manual. cDNA was synthesized by using iScript cDNA synthesis kit (Bio-Rad). The absence of genomic DNA contamination was verified by PCR using the primers MfCYP51-F and MfCYP51-5'2, which span introns of the genomic sequence. Genomic DNA would have generated an ~1,000-bp PCR product, 100 bp longer than from cDNA.

Real-time PCR technology was used to quantify mRNA levels of the MfCYP51 and MfABC1 genes in cDNA. Fragments of the MfCYP51 gene (120 bp) and MfABC1 gene (260 bp) were amplified with the primer pairs RealCYP-F2–RealCYP-R2 and MfABC5-F–MfABC5-R, respectively. The expression of the MfCYP51 and MfABC1 genes was normalized with the expression of the MfActin gene by using the primer pair MfActin-F–MfActin-R. Reactions were performed in a 30-µl volume containing 1× iQ SYBR Green Supermix (Bio-Rad), 0.3 µM concentrations of each primer, and 0.5 µl of cDNA. All PCRs were amplified in triplicates with the program: 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 55°C for 45 s and one cycle of 95°C for 1 min and 55°C for 1 min. To confirm that products were amplified correctly in these reactions, a melting curve analysis was performed immediately after amplification for 100 cycles of increasing temperature with 0.4°C increments starting at 55°C. Reactions were performed in an iCycler thermal cycler (Bio-Rad Laboratories). Two biological replications starting with new cultures for RNA extraction were conducted.

The comparative C_T method (separate tubes) (1) was used to determine MfCYP51 expression, because efficiencies of target and reference amplifications were nearly equal in validation experiments (slope = 0.048). The relative expression of the MfCYP51 gene from *M. fructicola* isolates was calibrated to the lowest expressor (isolate Kac-18). The validation experiment yielded unequal efficiencies of the target and reference amplifications (slope = 0.147) for the MfABC1 gene expression analysis; therefore, the "relative standard curve method" (1) was used to analyze the data. Standard curves were made by using

TABLE 4. Silent nucleotide variations in the coding region of the *MfCYP51* gene from seven DMI-R and nine DMI-S isolates

Isolate(s)	Sensitivity to propiconazole	Nucleotide at position ^a :														
		105	108	132	177	312	387	540	546	576	588	675	690	702	735	
Bmpc7, -10, -13, GAAP5, -6, GADL129, -193	R	T	T	G	T	C	G	C	C	C	C	G	T	C	T	
SCDL72	S	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
BGA25	S	C	C	—	C	—	A	—	—	T	T	C	—	T	—	
GAAP11	S	C	C	—	C	—	A	—	—	G	—	C	C	T	—	
GAAP12	S	C	C	—	C	—	—	T	—	T	T	C	—	—	C	
GADL3	S	C	C	—	C	—	A	—	T	T	—	C	—	T	—	
GADL161	S	C	C	—	C	A	A	—	—	—	—	—	—	—	—	
SC99A3	S	C	C	C	C	—	—	—	—	T	—	—	—	—	—	
SC99A4	S	C	C	C	C	—	—	—	—	—	—	—	—	—	—	
Kac-18	S	C	C	C	C	—	A	—	—	T	—	C	—	T	—	

^a Dashes (—) indicate nucleotide sequences identical to the corresponding positions in the DMI-R isolates.

the fivefold dilution series of genomic DNA from isolate SC99A3 as templates. The DNA concentration of isolate SC99A3 was determined by using GeneQuantPro (Biochrom, Cambridge, United Kingdom). The relative expression of *MfABC1* gene was calculated for each isolate by dividing the amount of normalized *MfABC1* mRNA by the amount of normalized *MfABC1* mRNA of isolate SC99A3, which had the lowest amount of *MfABC1* mRNA among DMI-S isolates.

Determination of relative *MfCYP51* gene copy numbers. The relative copy number of the *MfCYP51* gene was determined by using real-time PCR technology for each isolate as described above except genomic DNA was used as a template. The relative copy number ($2^{-\Delta\Delta C_T}$) was calculated by the equation:

$$2^{-\Delta\Delta C_T} = 2^{-(C_T, \text{MfCYP51} - C_T, \text{MfActin gene})_x - (C_T, \text{MfCYP51} - C_T, \text{MfActin gene})_y}$$

where *x* is one of the isolate samples, and *y* is SCDL72, which was chosen as a calibrator because it had the highest ΔC_T value thus the lowest number *MfCYP51* gene copies in the genome compared to all other isolates.

Cloning of the region upstream the *MfCYP51* gene. Nested PCR with gene-specific and adaptor-specific primers was used to amplify the upstream region of the *MfCYP51* gene from PCR libraries (31). PmlI, SmaI, EcoRV, and XmnI PCR libraries were created previously to isolate the *MfCYP51* and *MfABC1* genes (28, 29). The first PCR amplification was conducted with the adaptor-specific primer AP1 combined with the gene-specific primer *MfCYP51*-5'2. Nested PCR was then conducted with the adaptor-specific primer AP2 combined with the gene-specific primer *MfCYP51*-5'5 using a 1:50 dilution of the first PCR product as a template. PCRs were conducted in 50- μ l volumes containing 1 μ l of template, 1 $\times$ *Ext* PCR buffer including 1.5 mM MgCl₂, 200 μ M concentrations of each deoxynucleoside triphosphate, 0.4 μ M concentrations of each of adaptor-specific and gene-specific primer, and 2 μ l of *Ext* polymerase (1 U/ μ l) (Roche Applied Sciences, Indianapolis, IN). The cycle parameters were as described previously (31). PCR products were examined on a 1.0% agarose-ethidium bromide gel and sequenced as described above. Based on the resulting sequence, the primer pair UpCYP-1F and UpCYP-1R was designed to amplify the upstream regions of the *MfCYP51* gene from the isolates used in the present study. Sequences were compared to database sequences by using BLAST searches, aligned with DNASTAR software, and analyzed with the promoter predictor software NNPP (Neural Network Promoter Prediction, version 2.2, March 1999; http://www.fruitfly.org/seq_tools/promoter.html).

Determination of the relative copy number of the 65-bp repetitive element (Mona) in *M. fructicola* field isolates. The relative copy number of Mona was determined by using primer pair Insert-1F and Insert-2R and the real-time PCR protocol described above, except that the annealing and extension temperature was 50°C. The primer pair amplified a 56-bp internal fragment of Mona.

The relative copy number ($2^{-\Delta\Delta C_T}$) of Mona was calculated by the equation:

$$2^{-\Delta\Delta C_T} = 2^{-(C_T, \text{Mona} - C_T, \text{MfActin gene})_x - (C_T, \text{Mona} - C_T, \text{MfActin gene})_y}$$

where *x* is one of the field isolates, and *y* is SCDL72, which was chosen as calibrator because it had the highest ΔC_T value and thus the lowest Mona copy number in the genome compared to all other isolates.

RESULTS

Sensitivity of *M. fructicola* isolates to propiconazole. Relative growth of DMI-S isolates subjected to 0.3 μ g of propiconazole/ml ranged from 0 to 5%. On the other hand, DMI-R isolates revealed relative growth values greater than 20% ranging from 21.8 to 36.3% (Table 3). These data were consistent with the observation that DMI-R isolates typically grew with relative growth ranging from 20 to 70% on 0.3 μ g of propiconazole/ml (3).

Amplification and sequencing of the *MfCYP51* gene. The entire *MfCYP51* gene was amplified with primer pair *MfCYP51*-F and *MfCYP51*-R. PCR amplifications yielded a specific product of the expected size (1,685 bp) from all isolates (data not shown). Comparison of the 1,569-bp coding region of the *MfCYP51* gene from DMI-R and DMI-S isolates revealed several nucleotide variations at 30 positions (Table 4). All variations were found among eight DMI-S isolates (Table 4). The DMI-R isolates and one DMI-S isolate (SCDL72) had identical coding regions (Table 4). None of these nucleotide sequence variations resulted in a predictable amino acid change.

Genetic diversity between *M. fructicola* isolates. Three MPs generated 14 polymorphic bands from 16 isolates of *M. fructicola*. Analysis of the MP-PCR data revealed a phenogram with two groups of isolates and two ungrouped isolates. DMI-R isolates did not cluster independently from the DMI-S isolates (Fig. 1). Three DMI-S isolates were grouped into group 1 together with five DMI-R isolates. One DMI-R isolate (GAAP6) was grouped together with five DMI-S isolates into group 2. Two isolates, the DMI-R isolate GADL129 and the DMI-S isolate GAAP12, were not part of either group (Fig. 1). Based on the MP-PCR profiles, six DMI-R and six DMI-S isolates representing different genetic backgrounds were selected for further real-time PCR analysis.

Relative expression of the *MfCYP51* and *MfABC1* genes. There were no statistical differences in mRNA levels between biological replicates for *MfCYP51* ($P = 0.624$) or *MfABC1* ($P = 0.565$); therefore, the datasets were merged (Table 3). The relative expression values of the *MfCYP51* gene in DMI-R and DMI-S isolates were significantly different ($P = 0.001$) and ranged from 5.2 to 11.2 and from 1.0 to 3.4, respectively. The addition of propiconazole to the mycelia of DMI-R and DMI-S

TABLE 4—Continued

Nucleotide at position ^a :															
759	793	954	1017	1092	1209	1230	1245	1272	1278	1401	1437	1470	1482	1503	1533
G	C	C	C	G	C	T	A	A	T	T	C	C	T	T	A
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	A	—	—	—	—	—	—	G	—	—	—	—	C	G	T
—	A	—	—	—	—	C	G	G	—	—	—	—	C	G	—
—	—	—	—	A	—	—	—	—	—	C	—	—	—	—	—
T	A	T	—	—	T	—	—	G	C	—	T	—	C	—	—
—	—	—	—	—	—	—	—	—	—	—	—	T	—	—	—
—	—	—	—	—	—	—	—	—	—	C	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	T	—	—	—	—	G	—	—	—	—	C	G	—

^a Dashes (—) indicate nucleotide sequences identical to the corresponding positions in the DMI-R isolates.

isolates did not affect expression levels of the *MfCYP51* gene ($P = 0.689$ and $P = 0.561$, respectively; Table 3). A slight but statistically significant difference of relative expression values of the *MeABC1* gene was observed between DMI-R and DMI-S isolates ($P = 0.041$); values ranged from 0.6 to 1.6 and from 1.0 to 2.8, respectively. The addition of propiconazole to mycelium of DMI-R isolates led to a statistically significant increase of *MeABC1* expression with average mean values increasing from 1.31 to 1.98 ($P = 0.004$). A similar increase in *MeABC1* mRNA levels was not observed in DMI-S isolates ($P = 0.32$; Table 3). All P values were determined by using the t test (SigmaStat for Windows version 3.00; Systat Software, Inc., San Jose, CA).

Determination of the relative *MfCYP51* copy numbers in DMI-R and DMI-S isolates. Real-time PCR was used to determine the relative copy number of the *MfCYP51* gene in genomic DNAs of DMI-R and DMI-S isolates. The mean

$2^{-\Delta\Delta C_T}$ values for DMI-R isolates Bmpc7, Bmpc10, Bmpc13, GADL193, GAAP5, and GAAP6 were 1.40, 1.31, 1.39, 1.31, 1.10, and 1.57, respectively. These values were statistically different ($P = 0.014$) from the $2^{-\Delta\Delta C_T}$ values of DMI-S isolates, which were 1.19, 1.10, 1.00, 1.11, 1.15, and 1.25 for isolates GAAP12, GADL3, SCDL72 (calibrator isolate), SC99A3, SC99A4, and Kac-18, respectively.

Cloning and sequence analysis of the region upstream the *MfCYP51* gene. Using nested PCR, a fragment of ~2,500 bp in length was amplified from the XmnI PCR library. This fragment contained 471 bp of the 5' region of the *MfCYP51* gene and 1,872 bp of the 5' upstream region, excluding the adaptor-specific primer sequence (AP2). Based on this sequence, the primers UpCYP-1F (located at position +71 of the 1,872-bp fragment) and UpCYP-1R (located at position +56 of the *MfCYP51* gene) were designed to amplify the majority of the upstream region and a small portion of the 5' region of the

FIG. 1. Phenogram constructed with binary MP-PCR data sets from DMI-R and DMI-S isolates of *M. fructicola*. The tree was constructed by using UPGMA according to the SAHN clustering procedure of NTSYS-pc 2.1.

FIG. 2. Simplistic model of the upstream region of the *MfCYP51* gene in *M. fructicola* isolates. P1, P2, and P3 indicate promoters 1, 2, and 3, respectively. A 65-bp repetitive element (Mona) was identified at bp -117 of the *MfCYP51* gene in DMI-R isolates.

MfCYP51 gene from all isolates. Sequence analysis revealed a 65-bp repetitive element at bp -117 of the *MfCYP51* translational start site in each of the DMI-R isolates but not in DMI-S isolates (Fig. 2). This repetitive element was named Mona. A BLAST search of the *MfCYP51* upstream region to nucleotide collection database (nr/nt) in GenBank plus EMBL plus DDBJ plus PDB showed no similarity to other sequences. The promoter prediction software identified three and two putative promoter sequences in DMI-R and DMI-S isolates, respectively (Fig. 2). Promoter 2 was located within Mona. The upstream sequences of DMI-R isolate Bmpc7 (identical with the other five DMI-R isolates), DMI-S isolates GAAP12, GADL3, SC99A4, Kac-18, SCDL72, and SC99A3 are available in GenBank under accession numbers EU035301, EU035302, EU035303, EU035304, EU035305, EU035306, and EU035307.

Abundance of Mona in *M. fructicola* field isolates. Real-time PCR was used to determine the relative copy number of Mona in genomic DNA of *M. fructicola* field isolates. For the six DMI-R isolates the mean $2^{-\Delta\Delta C_T}$ values (relative copy numbers to calibrator SCDL72) ranged from 1.3 to 33.9, and for the six DMI-S isolates the mean $2^{-\Delta\Delta C_T}$ values ranged from 1.0 to 85.6 (Table 5). There was no significant difference between the relative copy numbers of DMI-R and DMI-S isolates according to the Mann-Whitney rank sum test ($P = 0.485$; Table 5). Although we did not determine the absolute copy number, our results do indicate that some of our isolates possess at least 80 copies, assuming that the calibrator isolate contained at least one copy.

DISCUSSION

The 14 α -demethylase (*CYP51*) is a key enzyme of fungal sterol biosynthesis and represents the target enzyme for DMI fungicides. Resistance to DMI fungicides can be conferred in fungi by altering the protein structure at key positions, thereby reducing the binding affinity to the fungicide. In plant pathogenic fungi such as *Uncinula necator* and *Erysiphe graminis*, a single nucleotide mutation within the substrate recognition site of the *CYP51* gene, leading to the substitution of a phenylalanine residue for a tyrosine residue at position 136 in amino acid sequence, has been correlated with resistance (6, 7). Alterations in the sequence of the *CYP51* gene in clinical isolates of the yeast *Candida albicans* have been demonstrated to confer DMI fungicide resistance (9, 13, 16, 17, 26). A point mutation in the *CYP51* gene was also reported to contribute to

DMI fungicide resistance in clinical isolates of the ascomycete *A. fumigatus* (8). In the present study, all DMI-R and DMI-S isolates had identical predicted amino acid sequences in the *MfCYP51* protein-coding region. Therefore, the DMI resistance in *M. fructicola* field isolates was not due to changes in the amino acid sequence of the *MfCYP51* protein, which is in agreement with a study published previously on DMI fungicide-resistant *M. fructicola* field isolates from New York (21).

Increased expression of the *CYP51* gene is another potential mechanism of DMI fungicide resistance. Overexpression of the *CYP51* gene conferred resistance to DMI fungicide fluconazole in clinical isolates of *C. glabrata* (17). In the filamentous fungi *P. digitatum*, *Venturia inaequalis*, and *Blumeriella jaapii*, the overexpression of the *CYP51* gene has been linked to DMI fungicide resistance (10, 14, 30). In the present study, the expression of the *MfCYP51* gene in DMI-R isolates was 5- to 11-fold higher than that of DMI-S isolate Kac-18. In plant pathogenic fungi, similar levels of overexpression were reported. In *B. jaapii*, the expression of the *CYP51* gene in DMI-R isolates was 5- to 12-fold higher than in DMI-S isolates

TABLE 5. Relative copy number of Mona in *M. fructicola* field isolates

Isolate	Sensitivity to propiconazole ^a	Relative copy no. of Mona ^b	
		Mean	SD
Bmpc7	R	7.5	0.7
Bmpc10	R	6.0	1.7
Bmpc13	R	5.2	1.2
GADL193	R	1.3	0.3
GAAP5	R	1.4	0.7
GAAP6	R	33.9	1.3
GAAP12	S	2.1	0.7
GADL3	S	36.9	3.2
SCDL72	S	1.0	0.2
SC99A3	S	85.6	5.9
SC99A4	S	5.8	0.7
Kac-18	S	61.1	1.2

^a An isolate was considered resistant (R) when the relative mycelial growth was $\geq 20\%$ at the discriminatory dose of 0.3 μ g of propiconazole/ml; all other isolates were considered sensitive (S).

^b The C_T value of Mona was normalized with the *MfActin* gene C_T value (ΔC_T) and calibrated to the normalized C_T value of the isolate SCDL72 ($\Delta\Delta C_T$), which had the lowest Mona copy number. The relative copy number was calculated by the equation $2^{-\Delta\Delta C_T}$. Means are average values for three independent experiments.

(14). The *CYP51* gene in DMI-R strains of *V. inaequalis* containing a sequence inserted upstream the *CYP51* gene was up to 18-fold overexpressed compared to DMI-S strains (30). In *P. digitatum*, the *CYP51* gene was estimated to be 100-fold overexpressed in DMI-R strains compared to DMI-S strains (10). The latter, extremely high expression level was estimated by a less quantitative Northern hybridization analysis, whereas expression levels in the other three studies were determined by quantitative real-time PCR.

The driving factors for increased *CYP51* mRNA levels can vary. For example, in *C. glabrata*, the overexpression was based on an increase of *CYP51* gene copy numbers (17). In our study, the average MfCYP51 copy number in DMI-R isolates was greater than in DMI-S isolates (1.35 versus 1.13, respectively), but Southern hybridization analysis confirmed the presence of only one MfCYP51 gene copy in DMI-S isolate SCDL72 and in DMI-R isolate Bmpc7 (data not shown). Therefore, gene duplication cannot explain the increase of MfCYP51 mRNA. In plant pathogenic fungi, increased *CYP51* mRNA levels have been associated with large inserts in the upstream region of *CYP51* (10, 14, 30). In the present study we also found an insert (Mona) in the upstream region of the MfCYP51 gene in DMI-R isolates. However, Mona was smaller than any other insert associated with *CYP51* overexpression in plant pathogenic fungi, such as the 126-bp tandem repeat (four repeats) of a transcriptional enhancer in *P. digitatum* (10), a 553-bp insert in *V. inaequalis* (30), and >2,000-bp retrotransposons in *B. jaapii* (14). Interestingly, most of Mona was predicted to be a typical gene promoter sequence (promoter 2). Promoters 1 and 3 were present and identical in both DMI-R and DMI-S isolates; thus, they should not be responsible for the overexpression of the MfCYP51 gene. Except for Mona, we did not find other differences in the upstream regions that correlated with the resistance phenotype.

The fact that DMI-S isolate SCDL72 shares the same MfCYP51 sequence with the DMI-R isolates but does not contain Mona upstream the MfCYP51 gene strongly supports the linkage of Mona with the resistance phenotype. However, whether Mona is indeed driving the MfCYP51 gene overexpression could not be verified in the present study. Such analyses would have required promoter deletion studies that are difficult to conduct in a multinucleate fungus, such as *M. fructicola*. Agrobacterium-mediated transformation of *M. fructicola* has been achieved recently; however, the same study pointed out difficulties with obtaining homokaryotic mutants (12). Alternatively, heterologous expression of the MfCYP51 gene in yeast (28) with or without Mona could be conducted to confirm its role in MfCYP51 overexpression. The association between Mona and the MfCYP51 overexpression may also be confirmed in the future when more DMI-R isolates from other regions in GA or even from other states are discovered and analyzed.

The variability in relative copy numbers of Mona between isolates suggests that it is a mobile genetic element. Filamentous fungi harbor a large variety of mobile genetic elements, including introns, circular and linear plasmids, transposons, and mobile elements not associated with introns, plasmids, or transposons (23). Except for class I transposon SINEs (for short interspersed nuclear elements) and class II DNA transposon MITEs (for miniature inverted-repeat transposable elements), almost all of the mobile elements are able to encode

proteins involving transposition (23). Mona is too short to encode proteins; thus, it should be a SINE-like or MITE-like element. However, Mona does not have the typical features of a SINE either, i.e., the presence of an internal binding site for RNA polymerase III and adenine-rich 3' ends (23). Also, Mona lacks the terminal inverted repeats that are commonly described in class II transposons, including MITEs (4). The unique feature of Mona suggests that it may be a new type of repetitive genetic element.

It appears that if the DMI-R isolates arose recently as a consequence of fungicide selection, these isolates should be closely genetically related, possibly only differing by the presence of Mona upstream MfCYP51. In the present study, all DMI-R isolates revealed a MfCYP51 gene identical in nucleotide sequence. In addition, Mona was located at the same position upstream of the MfCYP51 gene, and no nucleotide variation was detected in Mona or in the surrounding upstream region. On the other hand, the MfCYP51 gene in DMI-S isolates did reveal ample nucleotide diversity. Still, DMI-R isolates were not genetically identical as determined by MP-PCR analysis. Furthermore, DMI-R isolates revealed various numbers of MfCYP51 copies. A possible explanation for these results is that the insertion event may have taken place some time ago, perhaps soon after the introduction of DMI fungicides in the 1970s and 1980s.

High expression of two *A. nidulans* genes (*atrA* and *atrB*) and a *P. digitatum* gene (*PMR1*), which encode ABC transporters, were linked to DMI fungicide resistance (5, 18). Likewise, Palani and Lalithakumari (20) reported energy-dependent efflux activity in penconazole-resistant laboratory mutants of *V. inaequalis*. The energy-dependent efflux transporter activity has been linked to epoxiconazole resistance in wheat tan spot, *Pyrenophora tritici-repentis*, and the application of the inhibitor of energy-dependent fungicide efflux transporters can shift the fungicide resistance isolates back to normal sensitivity levels (24). Interestingly, in the present study the constitutive expression of the MfABC1 gene was lower in DMI-R isolates than in DMI-S isolates. However, the mRNA levels of MfABC1 increased in DMI-R isolates after the mycelium was subjected to a nonlethal dose of propiconazole. These results indicate that the MfABC1 gene may be a minor genetic determinant for DMI resistance.

In conclusion, DMI resistance in *M. fructicola* isolates from Georgia is likely a result of increased synthesis of 14- α demethylase due to MfCYP51 gene overexpression. The resistance phenotype is also correlated with a novel 65-bp repetitive element (Mona) located near the translational start site. Mona will be a useful molecular marker for rapid detection of DMI-resistant isolates in field populations from Georgia and maybe elsewhere.

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