Overexpression of the *CYP51A1* Gene and Repeated Elements are Associated with Differential Sensitivity to DMI Fungicides in *Venturia inaequalis*

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ABSTRACT

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The involvement of overexpression of the *CYP51A1* gene in *Venturia inaequalis* was investigated for isolates exhibiting differential sensitivity to the triazole demethylation inhibitor (DMI) fungicides myclobutanil and difenoconazole. Relative expression (RE) of the *CYP51A1* gene was significantly greater ($P < 0.0001$) for isolates with resistance to both fungicides ($M_R D_R$ phenotype) or with resistance to difenoconazole only ($M_S D_R$ phenotype) compared with isolates that were resistant only to myclobutanil ($M_R D_S$ phenotype) or sensitive to both fungicides ($M_S D_S$ phenotype). An average of 9- and 13-fold increases in *CYP51A1* RE were

observed in isolates resistant to difenoconazole compared with isolates with $M_R D_S$ and $M_S D_S$ phenotypes, respectively. Linear regression analysis between isolate relative growth on myclobutanil-amended medium and $\log_{10}$ RE revealed that little to no variability in sensitivity to myclobutanil could be explained by *CYP51A1* overexpression ($R^2 = 0.078$). To investigate *CYP51A1* upstream anomalies associated with *CYP51A1* overexpression or resistance to difenoconazole, Illumina sequencing was conducted for three isolates with resistance to difenoconazole and one baseline isolate. A repeated element, “EL 3,1,2”, with the properties of a transcriptional enhancer was identified two to four times upstream of *CYP51A1* in difenoconazole-resistant isolates but was not found in isolates with the $M_R D_S$ phenotype. These results suggest that different mechanisms may govern resistance to different DMI fungicides in the triazole group.

Apple scab, caused by the fungal pathogen *Venturia inaequalis* (Cooke) G. Winter, is among the most prevalent and economically important diseases of apple in the northeastern United States (MacHardy 1996; MacHardy et al. 2001). In the United States, disease management relies on the application of multi- and single-site fungicides due to the paucity of apple cultivars that have both durable apple scab resistance and desired horticultural traits (Köller and Wilcox 2001; Lesniak et al. 2011; MacHardy 1996). Introduced in the 1980s, the sterol demethylation inhibitors (DMI) are a class of broad-spectrum, single-site fungicides that have consistently demonstrated high pre- and postinfection activity against a number of apple fungal pathogens, including *V. inaequalis* (Köller and Wilcox 1999; Szkolnik 1981; Villani et al. 2015), *Podosphaera leucotricha* (Yoder 2000), and *Gymnosporangium juniper-virginianae* (Yoder et al. 2014). Indeed, even 10 years after their introduction for use in commercial apple orchards in the United States, DMIs provided curative activity up to 96 h following infection by *V. inaequalis* (Beckerman et al. 2015).

The ability of DMI fungicides to arrest fungal growth during the infection process contributes to the success of this fungicide class as a potent curative fungicide. Specifically, DMI fungicides interrupt ergosterol biosynthesis by inhibiting demethylation at the 14- α carbon of lanosterol, a sterol intermediate of ergosterol, which is essential for hyphal growth (Köller and Scheinpflug 1987). This highly specific mode of action, in addition to their repetitive use as a postinfection fungicide, has gradually led to diminished efficacy and resistance in major apple production regions throughout the

United States and Canada (Braun and McRae 1992; Hildebrand et al. 1988; Jobin and Carisse 2007; Köller et al. 1997; Villani and Cox 2011; Villani et al. 2015).

Unfortunately, growers have been increasingly reporting instances of suspected or confirmed resistance to older DMI fungicide products, including Rally 40WSP/Nova 40W (myclobutanil) and Rubigan/Vintage SC (fenarimol). An extensive 10-year study on myclobutanil resistance in populations of *V. inaequalis* throughout the northeastern and mid-Atlantic United States concluded that 63% of the populations screened had practical resistance to the fungicide (Villani et al. 2015). During the same study, practical resistance to the more intrinsically active DMI fungicide, difenoconazole (Inspire), was not observed in any populations with confirmed resistance to myclobutanil (Villani et al. 2015). Although cross sensitivity within this class has historically been observed in other plant pathogens (Hsiang et al. 1997; Karaoglaniadis and Thanassouloupoulos 2003), cross-sensitivity between myclobutanil and difenoconazole was only apparent in DMI-sensitive populations and not in DMI-resistant populations of *V. inaequalis* from New York. This lack of cross-sensitivity between myclobutanil and difenoconazole in a population with practical resistance to myclobutanil suggests that different genetic mechanisms may be involved in resistance against the two fungicides (Hawkins et al. 2011; Villani et al. 2015).

The molecular basis of resistance to the DMI fungicides has been extensively studied in both human and plant fungal pathogens, including *V. inaequalis* (Cools et al. 2013; Hamamoto et al. 2000; Hulvey et al. 2012; Luo and Schnabel 2008; Lupetti et al. 2002; Ma et al. 2006; Schnabel and Jones 2001). The more common mechanisms of resistance include (i) missense mutations within the target *CYP51A1* gene (Asai et al. 1999; Becher and Wirsal 2012; Délye et al. 1997; Stammler et al. 2009) reducing the binding affinity of

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DMI fungicides, (ii) overexpression of energy-dependent ABC and MFS transporters encoding drug efflux pumps (Hayashi et al. 2002; Hulvey et al. 2012; Nakaune et al. 1998), and (iii) overexpression of the *CYP51A1* gene (Hamamoto et al. 2000; Ma et al. 2006; Schnabel and Jones 2001). Overexpression of the *CYP51A1* gene in phytopathogenic fungi has been attributed to insertions and repeated elements acting as transcriptional enhancers or to gene duplications increasing the copy number of the *CYP51A1* gene (Hawkins et al. 2011). In isolates of *V. inaequalis* from Michigan, overexpression of *CYP51A1* has been previously determined to be a mechanism of resistance to myclobutanil (Schnabel and Jones 2001). An insertion of 553 bp upstream of the *CYP51A1* gene was found to contain three predicted promoters and coincided with higher expression values in myclobutanil-resistant isolates. This insertion was not always present in myclobutanil-resistant or high-expressing isolates, and overexpression of *CYP51A1* was not always present in isolates resistant to myclobutanil, indicating that other mechanisms could be responsible for resistance to that fungicide.

The widespread adaptation of difenoconazole into apple scab management programs in conjunction with a lack of cross-sensitivity between myclobutanil and difenoconazole in isolates resistant to myclobutanil (Villani et al. 2015) prompted us to reexamine the role of *CYP51A1* in DMI fungicide resistance and determine whether different molecular mechanisms for resistance were present. Specifically, our objectives were to (i) evaluate the relative expression (RE) of the *V. inaequalis* *CYP51A1* gene and the presence of mutations within the coding sequence of the *CYP51A1* gene for isolates with varying sensitivity phenotypes to myclobutanil and difenoconazole and (ii) conduct traditional Sanger sequencing and Illumina sequencing with de novo assembly to identify upstream anomalies associated with *CYP51A1* overexpression or resistance to either DMI fungicide.

MATERIALS AND METHODS

Collection of *V. inaequalis* isolates. The contribution of the *CYP51A1* gene in resistance to the DMI fungicides myclobutanil or difenoconazole was evaluated for 50 isolates. Isolates were selected during the 2010 and 2011 seasons from four research and commercial apple orchards located in western New York, Maine, and West Virginia, and two western New York baseline orchards. According to records submitted by growers and research personnel, there were no fewer than 20 historical DMI fungicide applications in the research and commercial orchards surveyed for this study. Isolates collected from baseline orchards were never exposed to any single-site fungicide classes, including anilinopyrimidines, benzimidazoles, DMIs, guanidines, quinone outside inhibitors (QoI), or succinate dehydrogenase inhibitors.

All isolates selected for this study were obtained from sporulating apple scab lesions originating from infections on young, fully expanded leaves. Each single clonal conidial lesion isolate was phenotyped for sensitivity to the DMI fungicides myclobutanil and difenoconazole using microscopy-aided mycelial relative growth assays, as previously described and validated (Frederick et al. 2014; Köller et al. 2004; Villani et al. 2015). Briefly, individual sporulating clonal conidial lesions (Köller et al. 2004) were removed using a sterile cork borer (5 mm in diameter), placed in 1.2 ml of sterile distilled water, and shaken for 60 s to dislodge conidia from the lesion. The leaf discs were promptly removed and 100- μ l suspensions of 10^3 to 10^4 *V. inaequalis* conidia ml⁻¹ were evenly distributed on potato dextrose agar (PDA; Difco Laboratories) amended with streptomycin sulfate (50 μ g ml⁻¹), chloramphenicol (50 μ g ml⁻¹), and either analytical grade myclobutanil or difenoconazole (Sigma-Aldrich) dissolved in acetone (Sigma-Aldrich) at a discriminatory dose of 0.1 μ g ml⁻¹ (Villani et al. 2015) or no fungicide. Single-conidial lesion isolates were incubated at 22°C for 6 days, and mycelial growth of five randomly selected microcolonies originating from single spores was determined by measuring microcolony diameter or germ tube length (for spores with greatly inhibited

growth) using a SPOT Idea digital camera with the SPOT Imaging Basic software package (Diagnostic Instruments Inc.) attached to an Olympus SZX12 stereoscope (Olympus America Inc.). The in vitro sensitivity response for each single conidial isolate was expressed as mean percent relative growth (%RG), as previously described (Frederick et al. 2014; Smith et al. 1991; Villani et al. 2015).

Each clonal conidial lesion isolate was characterized as being sensitive to or having practical resistance to myclobutanil or difenoconazole based on previously validated thresholds for practical resistance (Villani et al. 2015). Isolates that had RG values greater than 60% on either myclobutanil- or difenoconazole-amended medium were characterized as resistant to the respective fungicide, whereas any isolate with RG values less than 60% on either DMI-amended medium was considered sensitive because labeled rates of Rally 40WSP or Inspire (difenoconazole) could still effectively control isolates with such phenotypes under field conditions (Villani et al. 2015).

Extraction of RNA. From each isolate, a single conidium was selected and grown on PDA in either the presence or absence of a DMI fungicide. To maintain phenotypes with varying sensitivities to DMI, isolates with practical resistance (RG > 60%) to myclobutanil, difenoconazole, or both DMI fungicides were maintained on PDA amended with a low concentration (0.01 μ g ml⁻¹) of myclobutanil, difenoconazole, or both fungicides, respectively. Alternatively, isolates sensitive to both DMI fungicides were maintained on PDA in the absence of either fungicide. Following 8 weeks of incubation at 22°C, 50 to 100 mg of mycelium was harvested from the agar surface and ground in liquid nitrogen using a sterile mortar and pestle. Total RNA was extracted from the 50 isolates using the Omega Bio-Tek E.Z.N.A. Plant RNA Kit (Omega Bio-Tek). DNA was removed with the TURBO DNA-free Kit (Ambion by Life Technologies). For all isolates, RNA was standardized to a concentration of 5 ng ml⁻¹ prior to expression analysis of the *CYP51A1* gene.

cdNA synthesis and reverse-transcription quantitative polymerase chain reaction. Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) was used to determine the RE of the *CYP51A1* gene for the 50 selected isolates. The iScript One-Step RT-PCR with SYBR Green Kit (Bio-Rad Laboratories Inc.) utilizing the SYBR Green I reporter dye was used for first-strand DNA synthesis and all subsequent RT-qPCR. To normalize the concentration of RNA, a 162-bp fragment of the *V. inaequalis* actin housekeeping gene (accession number AF269254) (Schnabel and Jones 2001) was amplified with primers ViActin-F and ViActin-R (Table 1). Primers ViCyp51-F and ViCyp51-R (Table 1) were designed to amplify a 161-bp region within the *CYP51A1* gene (accession number AF227916). Reactions were carried out in 25- μ l volumes and contained 12.5 μ l of 2 $\times$ SYBR Green RT-PCR mix (final concentration 1 $\times$), 300 nM each primer, 0.5 μ l of iScript RT for one-step RT-PCR, and 12.5 ng of RNA. Amplifications were performed in a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories Inc.) with the following parameters: 10 min at 50°C, 5 min at 95°C, followed by 40 cycles of 10 s at 95°C and 30 s at 57°C. For each isolate, three technical replicates were included and the experiment was repeated. A final melt curve analysis was also included to ensure proper amplification of a single amplicon. For three isolates (baseline, myclobutanil-resistant/difenoconazole-sensitive [M_RD_S], and myclobutanil-sensitive/difenoconazole-resistant [M_SD_R]), induced expression of the *CYP51A1* gene was evaluated by applying a mixture of myclobutanil and difenoconazole at their maximum labeled field rates of 333 g ha⁻¹ and 293 ml ha⁻¹, respectively, using an airbrush sprayer (Aztek 320A; Testor). The fungicides were applied for 5 s over two *V. inaequalis* colonies (approximately 40 mm in diameter) and RNA was extracted 24 h following application.

The comparative threshold cycle (C_T) method (Livak and Schmittgen 2001) was used to determine *CYP51A1* expression of test isolates relative to the expression of calibrator isolate 26A-22-11, the isolate with the greatest mean difference in C_T values between *CYP51A1* and the *actin* gene (Δ C_T) (Hulvey et al. 2012; Schnabel and Jones

2001) and, thus, the lowest RE of *CYP51A1*. Data were calibrated by determining the difference in ΔC_T values between the test isolate and the calibrator isolate ($\Delta\Delta C_T$), and RE of the *CYP51A1* gene for each isolate was calculated using the formula $2^{-\Delta\Delta C_T}$, as described previously (Livak and Schmittgen 2001; Schnabel and Jones 2001). A validation experiment was performed with dilutions of the calibrator isolate prior to utilizing the C_T method, to ensure that the efficiency of both *CYP51A1* amplification and *actin* amplification were approximately equal (slope = 0.01).

The effect of *CYP51A1* RE on DMI sensitivity phenotype (resistance to neither, both, or one DMI fungicide) was determined using the generalized linear mixed models with the GLIMMIX procedure of SAS (version 9.4; SAS Institute Inc.). Differences in RE of isolates exhibiting different sensitivity phenotypes was determined using the lsmeans statement of GLIMMIX at the 5% level of significance. Simple linear regression analysis was conducted to determine the relationship between isolate relative growth for each fungicide and mean RE ($\log_{10}$ transformed) (SigmaPlot version 11.0; Systat Software Inc.).

Extraction of genomic DNA. Prior to extraction of genomic DNA (gDNA), isolates were grown and maintained in a manner identical to that described above for RNA extraction. Following 8 weeks of incubation at 22°C, 50 to 100 mg of mycelium from each isolate was harvested from the growth medium and ground in liquid nitrogen using a sterile mortar and pestle. Extraction of gDNA was accomplished using the Omega Bio-Tek E.Z.N.A. Plant DNA Kit (Omega Bio-Tek) in accordance with the manufacturer's instructions.

PCR amplification of the *CYP51A1* gene in *V. inaequalis*. In order to determine whether mutations altering the coding sequence of the *CYP51A1* gene were present in isolates resistant to the DMI fungicide difenoconazole, the entire *CYP51A1* gene in *V. inaequalis* was sequenced for 15 isolates representing different difenoconazole sensitivity phenotypes. Primers were designed to amplify the *CYP51A1* gene based on the sequence of *V. inaequalis* isolate Ent23 (accession number AF227916) (Schnabel and Jones 2001). Using the primer sets SVcyp-38-F/SVcyp400-R, SVcyp300-

F/SVcyp1200-R, and SVcyp1080-F/SVcyp1720-R (Table 1), the 1,674-bp *CYP51A1* gene was amplified for three baseline isolates (3a-27-10, 3a-19-10, and 4a-45-10), three $M_S D_S$ isolates (26a-45-11, 1a-31-10, and 42a-30-10), three $M_R D_R$ isolates (38b-45-11, 40b-33-11, and 7a-27-10), an $M_S D_R$ isolate (40b-40-11, 42a-2-11, and 42a-10-11), and three $M_R D_S$ isolates (40b-28-11, 45b-33-11, and 7a-1-10). For each primer set, PCR was performed in 25- μ l reaction volumes and contained 1 $\times$ PCR buffer, 0.4 μ M each primer, 2.5 mM $MgCl_2$, 200 μ M each dNTP, 0.625 U of GoTaq Flexi DNA Polymerase (Promega Corp.) and 5 to 10 ng of gDNA. All amplifications were performed in a T100 Thermal Cycler (Bio-Rad Laboratories Inc.) with the following program: 2 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C; followed by a final extension of 10 min at 72°C.

PCR products were separated on a 2% agarose gel (Bio-Rad Laboratories Inc.) stained with ethidium bromide in 1 $\times$ Tris-acetate-EDTA buffer at 100 V for 1 h. Photographs of the gel were taken on a KODAK Gel Logic 200 Imaging System (Eastman Kodak Company). Using the QiaQuick PCR Purification Kit (Qiagen), PCR products were purified and sequenced with internal primers in both directions for each primer set (Table 1) using an Applied Biosystems Automated 3730xl DNA Analyzer at the Cornell University DNA sequencing facility in Ithaca, NY. For each isolate, sequences of *CYP51A1* were assembled with using CLC Main Workbench (version 6.8.2; CLCBio). After removing two introns previously characterized by Schnabel and Jones (2001), the resulting sequences were translated and aligned to identify any anomalies in amino acid sequence among the isolates.

Cloning of the *CYP51A1* upstream promoter region. To obtain the gDNA sequence upstream of the *V. inaequalis* *CYP51A1* coding region, 5' rapid amplification of genomic ends (RAGE) was carried out on baseline isolate 3a-27-10 in accordance with Liu and Baird (2001). Following the addition of a poly(C) tail with terminal transferase, PCR was performed using primer Vicyp22R (Table 1), which was designed to anneal to the 5' end of the *V. inaequalis* *CYP51A1* coding region, and a selective anchored

TABLE 1. Oligonucleotide primers used in this study and description of their amplification target

Primer	Sequence (5' to 3')	Amplification targets ^a
ViActin-F	GAT GGA GCA AAA GAA GTT CGT CA	qPCR primers for determining the expression level of the actin gene in <i>Venturia inaequalis</i>
ViActin-R	GAA TTG AGG GTA GCG TTT GGT ATA G	
ViCyp51-F	GAT GGA GCA AAA GAA GTT CGT CA	qPCR primers for determining the expression level of the <i>CYP51A1</i> gene in <i>V. inaequalis</i>
ViCyp51-R	GAA TTG AGG GTA GCG TTT GGT ATA G	
Vicyp22R	GCA AAG GAG AGA GGA GTC CCA T	Primers used during RAGE cloning to elucidate the far upstream/promoter region of the <i>CYP51A1</i> gene in <i>V. inaequalis</i>
Vicyp11R	AGG AGT CCC ATT GTT GGT GGT GAG	Amplification of the region -290 to +578 relative to the <i>CYP51A1</i> upstream sequence of <i>V. inaequalis</i> isolate 3a-27-10
AJ468F	ATC CCT GAA TTT GAG AGC ACT	Sequencing of the PCR product generated by primers AJ468F and SV395R
SV395R	ACA TAG GAT TTG AGA GCT TCG GTG GTG AGA C	Amplification of EL 3,1,2 in immediate <i>CYP51A1</i> upstream region
AJ414	TGC CAA GAA GAA TGA AGG TAA A	Amplification of the region -738 to -134 relative to the <i>CYP51A1</i> upstream sequence of <i>V. inaequalis</i> isolate 3a-27-10
Vi23F	GAA ACC TAG GTG CAA GAG CAA	Sequencing of the PCR product generated by primers Vi23F and Vi-134R
Vi-134R	TTA CCC TGG CCC TGG CTT TGG CTT T	Amplification of the region -38 to +400 relative to the first 5' adenosine of <i>CYP51A1</i>
Vi-185R	TGG CAT GGC CCA CCA ATC ACA TGC T	Amplification of the region +300 to +1,200 relative to the first 5' adenosine of <i>CYP51A1</i>
SVcyp-38-F	ATA CAG ACG AGC AAC ACC ACA CT	Amplification of the region +1,080 to +1,720 relative to the first 5' adenosine of <i>CYP51A1</i>
SVcyp400-R	TCC TCT GCG TTG ACA TGG CTC T	Sequencing of the PCR product generated by primers SVcyp-38-F and SVcyp400-R
SVcyp300-F	TTT ACC TTC ATT CTT CTT GGC A	Sequencing of the PCR product generated by primers SVcyp300-F and SVcyp1200-R
SVcyp1200-R	ATG CAT GCG AAG AGT TTC TTT G	Sequencing of the PCR product generated by primers SVcyp1080-F and SVcyp1720-R
SVcyp1080-F	GGA AGA ACT ATA CCA AGA ACA A	Amplification of EL 3,1,2 in immediate <i>CYP51A1</i> upstream region
SVcyp1720-R	TCC CAG GCT GTT CAC CAA ACG T	Amplification of EL 3,1,2 in Vi-134 ⁺⁵⁹ to 228 <i>CYP51A1</i> upstream region
seqSVcyp160-F	CAC TGG TTT CCC TTC TTC GGC A	
seqSVcyp260-R	TTG CAT TCT CAT TGT CGC ATT AC	
seqSVcyp620-F	ACC CTC AAT TCA AAG GCG AAA	
seqSVcyp720-R	AAC CTC CTT GCC TTG TAG TGA G	
seqSVcyp1340-F	AGA AGT GGG AGC CTC ACC GTT G	
seqSVcyp1440-R	GAG TCC ATA GCC GTA ATC CTC T	
EL3,1-for	TGA GCC GTG TCC GAG GTG AGT C	
Vi-185F	AGC ATG TGA TTG GTG GGC C	
7535hiup-R	GTC GGT GGT AGA CTC ACC ACC AT	

^a qPCR = quantitative polymerase chain reaction and RAGE = rapid amplification of genomic ends.

primer complimentary to the cytosine tail (Liu and Baird 2001). To improve specificity and improve DNA concentration, PCR using the product from the first reaction was conducted with universal amplification primer (Liu and Baird 2001) and Vcyp11R (Table 1). The purified product was cloned into PGEM-Teasy (Promega Corp.) according to the manufacturer's instructions, and 10 clones were subjected to Sanger sequencing, as described above.

Amplification and sequencing of the *CYP51A1* upstream promoter region for DMI-resistant isolates of *V. inaequalis*.

To determine putative upstream anomalies associated with resistance to difenoconazole or myclobutanil, PCR and Sanger sequencing were conducted for all 50 isolates of *V. inaequalis* with varying sensitivity to the two fungicides. Based on the upstream sequences obtained by RAGE and subsequent cloning, primer set Vi23F and Vi-134R (Table 1) was designed and used to amplify the region -738 to -135 bp upstream of the *V. inaequalis CYP51A1* gene (relative to baseline isolate 3a-27-10). PCR was performed in 25- μ l volumes as described above. Amplifications were performed in a T100 Thermal Cycler (Bio-Rad Laboratories Inc.) with the following program: 2 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 56°C, and 60 s at 68°C; followed by a final extension of 7 min at 68°C. PCR products were separated on a 2% agarose gel (Bio-Rad Laboratories Inc.) stained with ethidium bromide in 1 $\times$ Tris-acetate-EDTA buffer at 100 V for 1 h. Photographs of the gel were taken on a KODAK Gel Logic 200 Imaging System (Eastman Kodak Company). PCR products were purified and sequenced as described above using primer Vi-185R (Table 1).

To determine the presence of anomalies in the region immediately upstream of the *CYP51A1* gene not amplified by Vi23F and Vi-134R (i.e., -134 to the first 5' adenine relative to the start), PCR was performed using primers AJ468F (Schnabel and Jones 2001) and SV395R (Table 1). PCR was performed in 25- μ l volumes as described above. Amplifications were performed in a T100 Thermal Cycler (Bio-Rad Laboratories Inc.) using the following program: 2 min at 94°C; 35 cycles of 30 s at 94°C, 45 s at 53°C, and 2 min at 72°C; followed by a final extension of 7 min at 72°C. PCR products were separated on a 1% agarose gel (Bio-Rad Laboratories Inc.) stained with ethidium bromide in 1 $\times$ Tris-acetate-EDTA buffer at 100 V for 1 h. Photographs of the gel were taken on a KODAK Gel Logic 200 Imaging System (Eastman Kodak Company). PCR products were purified and sequenced as described above using primer AJ414 (Schnabel and Jones 2001) (Table 1).

Upstream sequence assembly and prediction of promoter elements. For isolates in which upstream fragments were successfully amplified for both sets of primers (Vi23F and Vi-134R; AJ468F and SV395R), assembly and alignments were made using CLC Main Workbench (version 6.8.2; CLCBio). For all isolates, the prediction of promoter elements within the upstream sequence of *CYP51A1* was carried out using the Berkeley Drosophila Genome Project's Neural Network Promoter Prediction software, with a standard cutoff score of 0.8.

Illumina sequencing, de novo assembly, and annotation of *CYP51A1* upstream sequences. Illumina sequencing was conducted to elucidate the upstream region of *CYP51A1* for three isolates in which no fragment was generated with primer pair AJ468F/SV395R. Illumina sequencing of isolates 40b-40-11 ($M_S D_R$), 41b-14-11 ($M_S D_R$), and 43b-40-11 ($M_R D_R$) was accomplished using the Illumina MiSeq v3 600bp kit. Additionally, a baseline isolate, 3a-27-10, in which the 738-bp upstream sequence of *CYP51A1* had already been assembled using PCR and Sanger sequencing, was included for comparison. gDNA from each of the four isolates was extracted using the Omega Bio-Tek E.Z.N.A. Plant DNA Kit, as previously described. Sample preparation, including library construction, was completed at the Cornell University Genomic Facility using the TruSeq DNA Library Prep Kit (Illumina). Digital PCR was performed at the Cornell University Genomic Facility on all samples to ensure optimum DNA loading for proper clustering on the Illumina sequencer. A single MiSeq run with 300-bp paired-

end reads was performed on the four pooled isolate samples expected to provide 15 GB of sequence data. After sequencing, low-quality Illumina MiSeq data were filtered and raw reads were assembled for each isolate using CLC Genomics Workbench with default settings (version 8.0.1; CLC Bio). Additional assemblies were also made on the combined read data from the three difenoconazole-resistant isolates to maximize coverage of the *CYP51A1* upstream region.

RESULTS

DMI fungicide sensitivity and expression of the *CYP51A1* gene. DMI fungicide sensitivity and expression of the *V. inaequalis CYP51A1* gene was evaluated for 50 isolates that were collected from six orchard populations in 2010 and 2011. The mean %RG of isolates on medium amended with a discriminatory concentration of myclobutanil and difenoconazole ranged from 0 to 131.2 and 0 to 138.3%, respectively. Mean RE of the *CYP51A1* gene using the comparative C_T method ranged from 1.00 to 179.21, with the calibrator isolate, 26A-22-11, and an isolate resistant to difenoconazole, 7a-48-10, having the lowest and highest RE, respectively (Table 2). Application of difenoconazole or myclobutanil to mature colonies of three *V. inaequalis* isolates 24 h prior to RNA extraction in an attempt to induce target gene expression did not affect the level of *CYP51A1* RE compared with copies of the isolates that did not receive the fungicide application prior to extraction (data not shown). Hence, only noninduced RE was determined for the remaining isolates. Isolates resistant to difenoconazole ($n = 23$) had a mean *CYP51A1* RE of 45.91 ± 8.3 , which was significantly higher ($P < 0.0001$) than for isolates sensitive to difenoconazole (mean RE = 4.19 ± 0.6 ; $n = 27$). For isolates resistant to myclobutanil ($n = 24$), mean *CYP51A1* RE was slightly greater than for isolates sensitive to myclobutanil ($n = 26$; mean 27.68 ± 5.9 compared with 19.08 ± 7.6) but these differences were not significant ($P = 0.275$).

To understand the extent of the contribution of *CYP51A1* expression in resistance to myclobutanil and difenoconazole, linear regression analysis of isolate mean %RG on difenoconazole- or myclobutanil-amended medium and $\log_{10}$ RE values was performed. The regression analysis revealed significant relationships between isolate %RG on either difenoconazole- or myclobutanil-amended medium and $\log_{10}$ RE ($P \leq 0.0001$ and $P = 0.0489$, respectively). However, although *CYP51A1* expression explained some of the variability in isolate sensitivity to difenoconazole ($R^2 = 0.630$) (Fig. 1A), little to none of the variability in isolate sensitivity to myclobutanil could be explained by *CYP51A1* RE ($R^2 = 0.078$) (Fig. 1B).

In order to gain further insight into the role of *CYP51A1* overexpression in difenoconazole resistance or myclobutanil resistance, isolates were further characterized into four groups based on their in vitro sensitivity to myclobutanil and difenoconazole. Of the 50 isolates evaluated in this study, 15 were resistant to myclobutanil and difenoconazole ($M_R D_R$), nine were resistant to myclobutanil but sensitive to difenoconazole ($M_R D_S$), eight were sensitive to myclobutanil but resistant to difenoconazole ($M_S D_R$), and 18 were sensitive to both fungicides ($M_S D_S$) (Table 2). For isolates resistant to difenoconazole ($M_R D_R$ and $M_S D_R$) RE was significantly greater ($P \leq 0.0001$) compared with isolates sensitive to myclobutanil. Conversely, when the difenoconazole sensitivity classification remained constant (i.e., $M_R D_S$ versus $M_S D_S$), no significant differences in RE of *CYP51A1* were observed between isolates resistant or sensitive to myclobutanil (Fig. 2).

PCR amplification of the *CYP51A1* gene in *V. inaequalis*. The 1,674-bp *CYP51A1* gene was sequenced for 15 isolates: 3 representative isolates from each of the four DMI-sensitivity classifications and 3 baseline isolates. The sequence of the *CYP51A1* gene for the 15 isolates had 100% identity to *V. inaequalis* isolate Ent54 (accession number AF262756). Following identification of two previously characterized introns (Schnabel and Jones

2001), translation revealed no missense mutations in the coding region of the gene (data not shown).

Cloning of the *CYP51A1* upstream promoter region. 5' RAGE was used to clone the upstream region of the *V. inaequalis* *CYP51A1* gene for baseline isolate 3a-27-10 (GenBank accession number KT694303). A 738-bp region was isolated upstream of the *CYP51A1* open reading frame and had 99% nucleotide identity to the upstream region of *V. inaequalis* isolate Ent23 (accession number AF227916) (Schnabel and Jones 2001). A novel 387-bp region of DNA was discovered in the region directly upstream of the

previously characterized upstream region of the *V. inaequalis* *CYP51A1* gene (Schnabel and Jones 2001). Given that none of the previously described upstream insertions (Schnabel and Jones 2001) were detected in the upstream region of the baseline isolate, all insertions or deletions in the promoter region of *CYP51A1* will be presented in the context of isolate 3a-27-10 unless otherwise noted.

Amplification of the region –738 to –135 bp upstream of the *CYP51A1* gene was accomplished using primer set Vi23F and Vi-134R and yielded fragments of 593, 604, 797, 828 or 1,103 bp.

TABLE 2. Demethylation inhibitor (DMI) sensitivity classification, *CYP51A1* relative expression (RE), size of insertions present in the region –738 to –135 bp upstream of *CYP51A1*, amplification success of the region –134 bp upstream of *CYP51A1*, and the frequency of element (EL) 3,1,2 in 50 isolates of *Venturia inaequalis* with differing sensitivity to the DMI fungicides myclobutanil and difenoconazole

Isolate	DMI sensitivity classification ^a	<i>CYP51A1</i> RE ^b	Insertion size (Vi23F/Vi-134R) ^c	Amplification with AJ468-F/SV395RC ^d	Frequency of EL 3,1,2 in <i>CYP51A1</i> promoter region
38b-45-11	M _R D _R	34.53	0	–	2
38b-24-11	M _R D _R	38.05	0	–	2
40a-35-11	M _R D _R	36.00	0	–	2
40a-45-11	M _R D _R	41.64	0	–	2
40b-25-11	M _R D _R	5.77	0	–	4
40b-33-11	M _R D _R	5.46	499	+	0
40b-7-11	M _R D _R	100.42	0	–	2
41a-37-11	M _R D _R	53.94	0	–	2
41b-37-11	M _R D _R	71.50	0	–	2
42a-1-11	M _R D _R	36.25	0	–	2
43b-40-11	M _R D _R	20.39	0	–	2
45b-24-11	M _R D _R	6.10	0	–	2
7a-15-10	M _R D _R	105.73	0	–	2
7a-27-10	M _R D _R	25.16	0	+	0
7a-7-10	M _R D _R	35.22	0	–	2
40b-40-11	M _S D _R	27.28	0	–	2
41b-14-11	M _S D _R	64.44	0	–	2
41b-36-11	M _S D _R	58.89	0	–	2
42a-10-11	M _S D _R	23.10	0	+	0
42a-2-11	M _S D _R	42.51	0	+	0
42a-40-11	M _S D _R	2.65	0	–	4
45b-4-11	M _S D _R	40.78	0	–	2
7a-48-11	M _S D _R	179.21	0	–	2
40b-28-11	M _R D _S	2.39	352	+	0
7a-1-10	M _R D _S	8.90	0	+	0
7a-4-10	M _R D _S	3.00	0	–	0
45b-33-11	M _S D _S	11.23	0	+	0
43a-20-11	M _S D _S	8.75	0	+	0
44a-23-11	M _S D _S	4.82	0	+	0
42a-30-11	M _S D _S	9.64	0	+	0
7a-6-10	M _S D _S	8.88	499	+	0
6a-21-10	M _R D _S	1.31	0	–	0
6a-44-10	M _R D _S	3.29	0	–	0
6a-29-10	M _R D _S	2.09	0	+	0
6a-37-10	M _R D _S	4.06	0	+	0
6a-23-10	M _S D _S	3.96	0	–	0
1a-32-10	M _R D _S	11.28	499	+	0
1a-22-10	M _S D _S	2.55	499	+	0
1a-31-10	M _S D _S	5.17	0	–	0
26a-15-11	M _S D _S	4.53	224	+	0
26a-22-11	M _S D _S	1.00	224	+	0
26a-27-11	M _S D _S	2.49	0	+	0
26a-45-11	M _S D _S	2.58	499	+	0
3a-18-10	M _S D _S	1.19	0	+	0
3a-19-10	M _S D _S	1.28	0	+	0
3a-27-10	M _S D _S	2.81	0	+	0
3a-41-10	M _S D _S	1.06	0	+	0
3a-50-10	M _S D _S	1.32	0	+	0
4a-37-10	M _S D _S	2.07	0	+	0
4a-45-10	M _S D _S	1.34	193	+	0

^a DMI sensitivity classification for isolates of *V. inaequalis* based on phenotypic response of the isolate on potato dextrose agar amended with myclobutanil or difenoconazole at 0.1 mg ml^{–1}. Isolates in which mean percent relative growth (%RG) was ≥60% were characterized as resistant to myclobutanil (M_R) or to difenoconazole (D_R). Isolates with mean %RG values <60% on myclobutanil- or difenoconazole-amended medium were characterized as sensitive (M_S or D_S, respectively).

^b RE of the *CYP51A1* gene is the relative amount of mRNA of the test isolate *CYP51A1* gene compared with the calibrator isolate 26a-22-11. The comparative threshold cycle method was used to determine *CYP51A1* expression of test isolates relative to the expression of calibrator isolate.

^c Size (bp) of insertions present in the region –738 to –135 bp upstream of the *V. inaequalis* *CYP51A1* gene amplified with the primer pair Vi23F and Vi-134R.

^d Successful (+) or unsuccessful (–) amplification of the region –134 bp upstream of *CYP51A1* using primer pair AJ468F and SV395R.

The anticipated 604-bp fragment was amplified in 40 of the 50 *V. inaequalis* isolates and was present across all DMI sensitivity classifications. For 9 of the 50 isolates, sequence analysis of the three larger fragments revealed individual insertions of 193, 224, or 499 bp, respectively. The 224- and 499-bp insertions upstream of *CYP51A* were identical to those reported by Schnabel and Jones (2001). The 193-bp insert was located in a single isolate –321 bp upstream of the *CYP51A1* gene (4a-45-11; GenBank accession number KT694304), while the 224- and 499-bp insertions were both located at position –323. In one isolate, 40b-28-11 (GenBank accession number KT694305), a 363-bp region located between positions –686 and –324 bp upstream of *CYP51A1* was deleted and replaced by a 352-bp sequence. This substituted region had 279 continuous nucleotides in common with the 499-bp insertion. The entire sequences of both the 193-bp insertion and the 224-bp insertion were embedded within the 499-bp insertion; however, they were disrupted by a 304-bp sequence and a 275-bp sequence, respectively, containing a predicted promoter elements (see below).

Using a cutoff score of 0.8, two promoter elements, designated promoter element 1 (5'-AGGCCCCGATTGATATATTACGCCGA GACGGCAATTCCCGTGGCAACCCGC-3'; score = 0.96) and promoter element 2 (5'-ATCGTAACCCATAATAAAGGCGTAGGA TTGGAAGTTTAATAAGTGAATC-3'; score = 0.93), were predicted within the 499- and 352-bp insertions. Promoter element 2 was the only promoter element predicted within the 193- and 224-bp insertions. These promoter elements were identical to those identified within the 225- and 500-bp insertions characterized previously (Schnabel and Jones 2001). The presence of either or both promoter elements in the region –738 to –135-bp upstream of *CYP51A1* did not affect expression of the *CYP51A1* gene. RE of isolates with anomalies in this region ranged from 2.55 to 11.28, whereas the RE of isolates that contained no insertions (604-bp fragment) in this upstream region ranged from 1.06 to 179.21. In addition, the 499-bp insertion, containing promoter elements 1 and 2, was detected in isolates that were resistant or sensitive to both DMI fungicides (Table 2).

Amplification of the region –134 bp upstream of the *CYP51A1* gene using primers AJ468F and SV395R produced an 868-bp fragment in 25 of 50 isolates and a 1,051-bp fragment for a single *V. inaequalis* isolate. Sequence analysis of PCR products revealed an insertion of 183 bp in a single isolate (1A-22-10) with an M_SD_S phenotype (GenBank accession number KT694306). The insertion was located –65 bp relative to the first 5' adenine of *CYP51A1* and contained a single promoter element (5'-GACGAACTTAT TTATAGAAGGTCTGTGCGTAGGTGGGCCGACGTTTAGC-3')

with a score of 0.94. Upstream sequences for the other 25 isolates with the 868-bp fragment were identical sequences and no insertions were identified.

For 24 of the 50 isolates, no amplification of the region –134 bp upstream of the *CYP51A1* gene was achieved using the AJ48F/SV395R primer set. Several other primer combinations located within 200 bp of the AJ48F/SV395R primer sites also failed to amplify (data not shown). RE was significantly greater ($P = 0.0005$) for isolates in which amplification of the immediate upstream region was not obtained (mean RE = 39.28 ± 8.3) compared with isolates in which amplification was achieved (mean RE = 7.48 ± 1.9). In addition, the majority (79%) of these isolates were characterized as resistant to difenoconazole. Within the four DMI sensitivity characterizations, no amplification of the region –134 bp upstream

Fig. 2. Mean *CYP51A1* relative expression (RE) according to demethylation inhibitor sensitivity phenotypes: myclobutanil-resistant (R)/difenoconazole-R (M_RD_R, $n = 15$), myclobutanil-sensitive (S)/difenoconazole-R (M_SD_R, $n = 8$), myclobutanil-R/difenoconazole-S (M_RD_S, $n = 9$), and myclobutanil-S/difenoconazole-S (M_SD_S, $n = 18$). Bars denoted by the same letter are not significantly different as denoted by the lsmeans statement of the GLIMMIX procedure in SAS v9.4 at the 5% level of significance.

Fig. 1. Log₁₀ relative expression (RE) values of *CYP51A1* plotted against mean percent relative growth of 50 isolates of *Venturia inaequalis* on **A**, difenoconazole-amended medium and **B**, myclobutanil-amended medium at a discriminatory concentration of 0.1 µg/ml. A linear distribution function [$y = (y_0 + ax)$] was fitted to the log₁₀ RE values of *CYP51A1* and mean percent relative growth values for each respective fungicide.

of *CYP51A1* was achieved for 87 and 75% of isolates with the $M_R D_R$ or $M_S D_R$ phenotype, respectively, compared with 33 and 11% for those with the $M_R D_S$ or $M_S D_S$ phenotypes, respectively.

Illumina sequencing and bioinformatics. Illumina MiSeq sequence reads for one baseline isolate and three isolates resistant to difenoconazole yielded an average of 9 million paired-end reads of Illumina sequence data. For each isolate, an average of 10,000 contigs of approximately 300 bp in length were assembled. To obtain maximum coverage of the *CYP51A1* upstream region, genomic reads were combined and assembled for all three difenoconazole-resistant isolates. The combined assembled genome of the three difenoconazole-resistant isolates revealed that the region –134 bp upstream of *CYP51A1* was split between two contigs. Alternatively, the region –738 bp upstream of *CYP51A1*, which had previously been sequenced for isolate 3a-27-10, was found on a single contig for the baseline isolate. Due to the number of repeated elements (see below) and presumed distance between the two upstream contigs (≥ 46 kbp), assembly of the two contigs upstream of *CYP51A1* could not be accomplished for the difenoconazole-resistant isolates.

In the combined genome assembly for difenoconazole-resistant isolates, a novel sequence of 474 bp (Fig. 3) was identified upstream of *CYP51A1* on the contig containing the *CYP51A1* gene (GenBank accession number KT694307). In this sequence, a unique 169-bp repeatable element, designated “EL 3,1,2”, was detected 379 bp upstream from the 5′ end of *CYP51A1* and again 59 bp downstream of the Vi-134 primer binding site (Vi-134⁺⁵⁹ to ⁺²²⁸) (Fig. 3). This element was not located anywhere in the genome of the baseline isolate but was repeated four times throughout the genome of isolate 40b-40-11 and twice throughout the genomes of isolates 41b-14-11 and 43b-40-11. A BLAST database search of EL 3,1,2 identified no similarity to any known sequences.

Three shorter repeated elements were identified within EL 3,1,2 and throughout the genome of the combined assembly for the three difenoconazole-resistant isolates. The first, designated “EL1”, was a 42-bp sequence that was observed twice upstream of *CYP51A1* in EL 3,1,2 in all three resistant isolates (Fig. 3). Throughout the assembled genome sequence data, EL1 was found once in the baseline isolate and as many as six times in the assembled genome of the difenoconazole-resistant isolates. The second element, “EL2”,

was a 67-bp sequence found three times upstream of *CYP51A1*: twice in EL 3,1,2 and once by itself (Fig. 3). Throughout the assembled genomes, EL2 was found once in the baseline isolate and up to 10 times in the assembled genome of the difenoconazole-resistant isolates. Element “EL3”, a 60-bp sequence, was identified four times upstream of *CYP51A1* in the assembled genome of the difenoconazole-resistant isolates (Fig. 3). Throughout the assembled genomes, it was found six times in the assembled genome of the difenoconazole-resistant isolates but not found in the baseline isolate.

Based on the combined assembled genome of the three *V. inaequalis* isolates, primers were designed to verify the correct assembly of the *CYP51A1* upstream region and also to detect the presence of EL 3,1,2 in isolates of *V. inaequalis* in which amplification of the region –134 bp upstream of *CYP51A1* was unsuccessful. Primer pair EL3,1-for/SV395R (Table 1) amplified a 907-bp fragment directly upstream of *CYP51A1* that contained EL 3,1,2 (Fig. 4). To detect the presence of EL 3,1,2 in the region downstream of the Vi-134 primer binding site (Vi-134⁺⁵⁹ to ⁺²²⁸), primers Vi-185F and 7535hiup-R (Table 1) amplified a 450-bp fragment for 17 of 23 isolates with difenoconazole resistance in which a single insertion of EL 3,1,2 was present (GenBank accession number KT694308). In two isolates, 42a-40-11 and 40b-25-11, a 957-bp fragment was produced (Fig. 4). Sequence analysis of these two isolates revealed that EL 3,1,2 was repeated in tandem four times (GenBank accession number KT694309); however, EL 3,1,2 was not present in the region directly upstream of *CYP51A1* for these two isolates. EL 3,1,2 was repeated a minimum of two times upstream of *CYP51A1* in 19 of 23 (83%) isolates resistant to difenoconazole and was not observed in the *CYP51A1* region for any isolates sensitive to difenoconazole (Table 2).

Determination of putative *CYP51A1* transcriptional enhancers.

No promoter elements with a score of 0.8 or greater were identified within EL 3,1,2. In addition, no promoter elements were detected within the 474-bp sequence (see above) characterized upstream of *CYP51A1* for isolates with EL 3,1,2. Interestingly, analysis of transcription factor (TF) binding sites within 474 bp upstream of *CYP51A1* revealed differences in TF binding sites relevant to *CYP51A1* expression or DMI fungicide resistance.

Fig. 3. Illustration of the upstream region of the *Venturia inaequalis* *CYP51A1* gene based on the combined assembly of difenoconazole-resistant isolates 40b-40-11, 41b-14-11, and 43b-40-11. A gray box labeled Start represents the first nucleotide in the coding region of *CYP51A1*, whereas gray boxes labeled Vi-185 and Vi-134 represent positions –185 and –134 bp, respectively, upstream from the Start position. Positions of repeated elements mapped downstream of the approximately 46-kb region between Vi-134 and the *CYP51A1* gene are relative to the start of the gene (i.e., Start^{–50} to ^{–84}), whereas those mapped prior to the approximately 46-kb region are relative to position –134 (i.e., Vi-134⁺⁵⁹ to ⁺²²⁸). An illustration of a baseline isolate is included to provide comparison of element insertion positions. Positions of elements and insertions are meant to demonstrate relative position and are not drawn to scale.

Within the 474 bp upstream of *CYP51A1* for isolate 40b-40-11, there were 15 binding sites for Stb5p, a TF activator for *ERG11* (*CYP51A1*) in yeast, compared with only 6 in the baseline isolate (3a-27-10). Additionally, isolate 40b-40-11 had fewer TF binding sites 474 bp upstream of *CYP51A1* for the ergosterol biosynthesis repressor Mot3p (Montañés et al. 2011) in comparison with the baseline isolate (3a-27-10), with 3 and 5 binding sites, respectively.

DISCUSSION

Sterol DMI fungicides target the 14 α -demethylase enzyme, an essential precursor to ergosterol biosynthesis encoded by the *CYP51A1* gene. Resistance to DMIs has been attributed to a number of mechanisms, including amino acid alterations in the target enzyme, overexpression of genes encoding drug efflux pumps, and overexpression of the *CYP51A1* gene. Although several studies have previously focused on elucidating the molecular mechanisms of resistance for a single DMI fungicide (Cools et al. 2013), in the present study, we found that several regulatory elements associated with resistance to difenoconazole were not present in isolates of *V. inaequalis* with resistance to myclobutanil. To our knowledge, this is the first report of a plant pathogen exhibiting differential resistance to two triazole DMI fungicides in a single isolate.

Overexpression of the *CYP51A1* gene has been implicated in DMI fungicide resistance for several phytopathogenic fungi, including *Penicillium digitatum* (Hamamoto et al. 2000; Sun et al. 2013), *Blumeriella jaapii* (Ma et al. 2006), *Monilinia fructicola* (Luo and Schnabel 2008), *Puccinia triticina* (Stammler et al. 2009), and *Mycosphaella graminicola* (Cools et al. 2010). In this study, RE of *CYP51A1* was evaluated for 50 isolates of *V. inaequalis* exhibiting varying levels of sensitivity to difenoconazole and myclobutanil. Isolates resistant to difenoconazole (those with the M_RD_R and M_SD_R phenotype) had a 9-fold increase in *CYP51A1* RE compared with isolates with an M_RD_S phenotype and a 13-fold increase in RE compared with isolates an M_SD_S phenotype. This level of expression was similar to other phytopathogenic fungi resistant to DMI fungicides, where expression values were observed to be more than fivefold greater when target gene overexpression was the primary mechanism of resistance (Hamamoto et al. 2000; Luo and Schnabel 2008; Ma et al. 2006).

CYP51A1 overexpression and subsequent DMI fungicide resistance can result from a number of factors, including duplication of the *CYP51* gene, insertions of DNA-containing promoter elements or transcriptional enhancers upstream of *CYP51*, and single nucleotide polymorphisms within the coding region of the gene. In the human pathogen *Candida glabrata* (Marichal et al. 1997) and the plant pathogen *Fusarium graminearum* (Liu et al. 2011), multiple copies of *CYP51* were found to be the driving force of target gene overexpression. In this study, genomic sequencing of a baseline isolate and of three difenoconazole-resistant isolates found no paralogous *CYP51* genes. However, a unique 169-bp repeatable element upstream of *CYP51A1* was identified. This element, EL 3,1,2, was repeated two to four times upstream of *CYP51A1* in 83% of difenoconazole-resistant isolates but not in isolates sensitive to difenoconazole.

Although EL 3,1,2 is highly associated with difenoconazole resistance and *CYP51A1* overexpression, the repeatable element was not found in the upstream region of *CYP51A1* for two isolates with M_SD_R phenotypes and for two isolates with M_RD_R phenotypes. The absence of EL 3,1,2 in these four isolates further illustrates the complexity of DMI fungicide resistance in *V. inaequalis* because resistance to DMIs not only may be governed by different mechanisms but also the molecular basis of resistance may differ between isolates as well. For example, in myclobutanil-resistant isolates from Michigan, the presence of a 553-bp insertion upstream of *CYP51A1* was linked to overexpression of the *V. inaequalis* *CYP51A1* gene (Schnabel and Jones 2001). However, this insertion was only present in 23% of myclobutanil-resistant isolates and in

isolates with a *CYP51A1* RE value of 5.0 or greater. Furthermore, we previously identified the 553-bp insertion in two DMI-sensitive isolates of *V. inaequalis* from Virginia (Cox et al. 2008). In the present study, the correlation of EL 3,1,2 and difenoconazole resistance was more prevalent in isolates with overexpression of *CYP51A1* (Schnabel and Jones 2001), being identified in the majority (83%) of isolates with difenoconazole resistance.

The presence of EL 3,1,2 solely in isolates with difenoconazole resistance strongly supports its association with resistance to difenoconazole and as a driving factor of *CYP51A1* overexpression. Similar relationships between repeated elements in the upstream region of *CYP51A1* and overexpression of the gene have previously been characterized. In *Penicillium digitatum*, a 126-bp element acting as a transcriptional enhancer was tandemly repeated five times upstream of *CYP51* in DMI-resistant strains and only once in DMI-sensitive strains. Although no promoter elements were predicted within the 126-bp tandem repeat, it was suggested that the presence of known fungal and vertebrate TF binding sites within the sequence might serve as a mechanism for *CYP51* transcriptional enhancement (Hamamoto et al. 2000).

Similar to the 126-bp tandemly repeated element found in *P. digitatum*, TF binding sites but no predicted promoter elements were found in EL 3,1,2. Although there were only 15 TF binding sites present, 8 had the capacity to bind the transcriptional enhancer zinc cluster protein TF Stb5p. Like many zinc cluster proteins (MacPherson et al. 2006; Rank et al. 1975; Saunders and Rank 1982; Wehrschütz-Sigl et al. 2004), Stb5p has been implicated as a positive regulator of multidrug resistance genes and also as a direct activator of *ERG11* (*CYP51A1*) (Laroche et al. 2006; MacPherson et al. 2006) in yeast. The introduction of eight Stb5p binding sites within each EL 3,1,2 insertion upstream of *CYP51A1* may have contributed to the observed higher *CYP51A1* expression values in difenoconazole-resistant *V. inaequalis* isolates. Due to the paucity of predicted promoter elements in the upstream region of *CYP51A1*, we also searched for TF binding sites 474 bp upstream of the first 5' adenine of *CYP51A1* for both baseline isolate 3a-27-10 and M_SD_R isolate 40b-40-11. Compared with the baseline isolate, which had 6 Stb5p binding sites within the queried region, 15 Stb5p binding sites

Fig. 4. Amplification of a 907-bp fragment containing EL 3,1,2 in the region directly upstream of *CYP51A1* (lanes 2 to 5 and 8, primers EL3,1-for/SV395R) or a 450- or 957-bp fragment containing EL 3,1,2 at downstream of position Vi-134⁺⁵⁹ to 228 (lanes 5 to 7 and 9, primers Vi-185F/7535hiup-R). EL 3,1,2 was repeated in tandem four times in isolates that produced the 957-bp fragment. Lane 1, Quick-Load 100-bp DNA Ladder (New England Bio-Labs); lanes 2 and 5, isolate 38b-24-11; lanes 3 and 6, isolate 40b-25-11; lanes 4 and 7, isolate 1a-31-10; lanes 8 and 9, nontemplate control.

were identified for the difenoconazole-resistant isolate, which included the 8 from El 3,1,2.

Two additional binding sites for the TF Mot3p were confirmed in the *CYP51A1* upstream region of the baseline isolate compared with the difenoconazole-resistant isolate. Unlike Stb5p, Mot3p represses the transcription of ergosterol biosynthesis genes (Montañés et al. 2011). The presence of these two transcription regulators, which appear to function antagonistically, raises questions regarding the importance of their ratio (Kim and Forger 2012) in *CYP51A1* expression and subsequent difenoconazole resistance. In baseline isolates, the ratio of Mot3p (repressor) to Stb5p (activator) was 5:7, compared with 3:15 in the difenoconazole-resistant isolates. Thus, it may not simply be the introduction of Stb5p binding sites into the immediate upstream region of *CYP51A1* but also the lower ratio of the TF repressing ergosterol biosynthesis.

The absence of EL 3,1,2 and the relatively low levels of *CYP51A1* RE in isolates of *V. inaequalis* with the M_RD_S phenotype suggests that the molecular basis of resistance may be different between myclobutanil and difenoconazole. Indeed, mean relative *CYP51A1* expression for isolates of *V. inaequalis* with resistance to myclobutanil only (M_RD_S) was not significantly different ($P > 0.05$) than isolates exhibiting the M_SD_S phenotype, indicating that the contribution of target gene overexpression in myclobutanil resistance may be minor. Regression analysis of isolate %RG on myclobutanil- or difenoconazole-amended medium and log₁₀ *CYP51A1* RE values further supported the observation that *CYP51A1* overexpression was not a primary genetic determinant of resistance to myclobutanil. Indeed, a similar relationship was observed for propiconazole-resistant isolates of *Sclerotinia homoeocarpa* and the *S. homoeocarpa* efflux transporter, *ShatrD*, which led to the conclusion that *ShatrD* overexpression and not *CYP51* overexpression was the driving force behind propiconazole resistance in that pathosystem (Hulvey et al. 2012). The low levels of *CYP51A1* expression for isolates with M_RD_S phenotypes in this study are in stark contrast to *CYP51A1* expression values for myclobutanil-resistant isolates of *V. inaequalis* from Michigan, which had an 18-fold increase in *CYP51A1* RE (Schnabel and Jones 2001). Although it is difficult to speculate regarding the reasons for this discrepancy between the two studies, it is not beyond the realm of possibility that the isolates screened in the 2001 study could have had a multiple DMI resistance phenotype that was mediated by overexpression of the target gene.

Resistance to DMI fungicides has also been conferred in fungi by the presence of point mutations leading to a change in the amino acid sequence of *CYP51A1* (Becher and Wirsal 2012; Délye et al. 1997; Gadoury et al. 2012; Wyand and Brown 2005). Depending on the location of an amino acid substitution, the binding affinity of DMI fungicides can be significantly reduced. Unlike the target genes of QoI fungicides, where amino acid substitutions are generally conserved across all fungal genera (Fernández-Ortuño et al. 2008), *CYP51* point mutations conferring DMI resistance are not frequently conserved in phytopathogenic fungi (Becher and Wirsal 2012; Cools et al. 2013). Upon verifying the predicted *CYP51A1* amino acid sequence (Schnabel and Jones 2001), we found no alterations within the coding sequence among any of the isolates or any of the mutations that have previously been associated with DMI resistance in other phytopathogens (Cools et al. 2013).

Overall, the results of this study have shown that the *CYP51A1* gene is overexpressed in isolates of *V. inaequalis* with practical resistance to difenoconazole (M_SD_R) or difenoconazole and myclobutanil (M_RD_R) but not in the majority of isolates with practical resistance to myclobutanil only. This finding suggests that resistance between myclobutanil and difenoconazole may develop independently and helps to explain the apparent lack of cross-sensitivity in several isolates with practical resistance to myclobutanil. The extent that overexpression of *CYP51A1* contributes to resistance of other DMI fungicides in *V. inaequalis* is still undetermined. Although we were unable to elucidate the mechanism of myclobutanil resistance, the involvement of overexpressed genes

encoding drug efflux transporters (Hayashi et al. 2002; Hulvey et al. 2012; Nakaune et al. 1998) has yet to be evaluated for *V. inaequalis*. Identification and expression analysis of undiscovered drug efflux pumps and the mechanisms promoting their overexpression not only might provide explanation for myclobutanil resistance in *V. inaequalis* but also could provide insight into multiple-fungicide resistance for this pathogen.

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