

The 14 α -Demethylase (*CYP51A1*) Gene is Overexpressed in *Venturia inaequalis* Strains Resistant to Myclobutanil

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ABSTRACT

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We identified the cytochrome P450 sterol 14 α -demethylase (*CYP51A1*) gene from *Venturia inaequalis* and optional insertions located upstream from *CYP51A1* and evaluated their potential role in conferring resistance to the sterol demethylation-inhibitor (DMI) fungicide myclobutanil. The *CYP51A1* gene was completely sequenced from one myclobutanil sensitive (S) and two myclobutanil-resistant (R) strains. No nucleotide variation was found when the three sequences were aligned. Allele-specific polymerase chain reaction (PCR) analysis indicated that a previously described single base pair mutation that correlated with resistance to DMI fungicides in strains of other filamentous fungi was absent in 19 S and 32 R strains of *V. inaequalis* from Michigan and elsewhere. The sequencing results and PCR analyses suggest that resistance in these

strains was not due to a mutation in the sterol demethylase target site for DMI fungicides. Expression of *CYP51A1* was determined for strains from an orchard that had never been sprayed with DMI fungicides (baseline orchard), and the data provided a reference for evaluating the expression of strains collected from a research orchard and from three commercial Michigan apple orchards with a long history of DMI use and a high frequency of R strains. Overexpression of *CYP51A1* was significantly higher in 9 of 11 R strains from the research orchard than in S strains from the baseline orchard. The high expression was correlated with the presence of a 553-bp insertion located upstream of *CYP51A1*. Overexpression of the *CYP51A1* gene was also detected in eight of eight, five of nine, and nine of nine R strains from three commercial orchards, but the insertion was not detected in the majority of these strains. The results suggest that overexpression of the target-site *CYP51A1* gene is an important mechanism of resistance in some field resistant strains of *V. inaequalis*, but other mechanisms of resistance also appear to exist.

Sterol demethylation inhibitors (DMI's) are broad-spectrum fungicides that are active against yeast, scab, mildew, and rust fungi. They are systemic compounds with highly protective and curative activity at low doses but also have selective action against nontarget organisms (7,23). However, because of their site-specific mode of action, the intensive use of DMI's has led to the development of resistance in a number of fungi of medical (16,18,44) and agricultural importance (14,37,41). Currently, in an effort to develop new drugs and antiresistance strategies, the molecular basis for DMI fungicide resistance in *Candida albicans*, an important opportunistic pathogen of humans, is being intensively studied. Fluconazole resistance in clinical isolates of *C. albicans* has been associated with a combination of several distinct mechanisms (12,39,44). The most common mechanisms include mutations in the target enzyme of azoles, a cytochrome P450 catalyzing the 14 α -demethylation of lanosterol (29,38,43). The gene encoding 14 α -demethylase has been named *P-450_{14dm}*, *Erg11*, and *CYP51A1*; the last designation will be used here as recommended by Nelson et al. (26). Energy-dependent drug efflux mechanisms have also been implicated in the acquired resistance of *Candida* spp. to azole antifungal agents (1,6,12,30,39,44). Phytopathologists have recently demonstrated that both mechanisms can confer resistance to DMI fungicides in filamentous fungi. Mutations within the 14 α -demethylase gene confer DMI resistance in laboratory mutants of *Ustilago maydis* and *Penicillium italicum* and in field strains of *Uncinula necator* and *Erysiphe graminis* (6,9,11,19). High expression of two *Aspergillus nidulans* genes (*AtrA* and *AtrB*) and a *Penicillium digitatum* gene (*PMR1*), which

encode drug efflux proteins of the ATP-binding cassette (ABC) transporter family, has been associated with DMI resistance (8,25). Other mechanisms include a defect in $\Delta 5,6$ desaturation or high levels of 14 α -demethylase caused by the overexpression of the *CYP51A1* gene (15,42). As yet, very few of these mechanisms of resistance have been characterized in resistant field strains of plant pathogens.

Field resistance to DMI fungicides in *Venturia inaequalis* arose in the mid-1980s and has increased significantly in Michigan and worldwide (4,21,24,33,35). This development of DMI-resistant field strains poses a real threat, because the selection of fungal strains with normal virulence and a relatively high degree of resistance can cause failure in disease control. To the best of our knowledge, no reports have been published on the molecular basis of DMI fungicide resistance in *V. inaequalis*. Such information could lead to improved spray recommendations for apple growers, quick methods for detecting DMI fungicide resistance in field populations, and improved design of antifungal compounds.

We identified and sequenced the complete 14 α -demethylase gene (*CYP51A1*) from *V. inaequalis*, encoding the target enzyme of DMI fungicides, as well as optional insertions located upstream from the gene and evaluated their potential for conferring resistance to DMI fungicides in *V. inaequalis* field strains.

MATERIALS AND METHODS

Strains of *V. inaequalis*, DNA isolation, and sensitivity to myclobutanil. The source of most *V. inaequalis* strains used in this study are listed in Table 1. Additional strains of *V. inaequalis* were isolated in 1997 from the Entomology Farm (Ent strains) and in 1999 from the Botany Farm (BF strains). Both farms are research orchards located in East Lansing, MI. Strains from commercial orchards in Michigan (ER, JM, and HM) were isolated in

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1999. The Botany Farm and orchards ER, JM, and HM had been exposed to at least four DMI sprays per year since the introduction of DMI fungicides in the early to mid-1980s, and all isolates were from lesions collected from trees treated with myclobutanil. The Entomology Farm orchard had never been sprayed with DMI fungicides. It was located 0.1 km east of the Botany Farm orchard.

Genomic DNA was isolated from strains of *V. inaequalis* as described previously (31). Nuclear DNA (nDNA) was purified and concentrated by centrifugation of genomic DNA in a CsCl density gradient containing the fluorescent dye bisbenzimidazole (13).

The reaction of each strain to myclobutanil was determined in mycelium growth assays on potato dextrose agar amended with myclobutanil at 0 and 0.3 µg/ml. Relative growth (RG) values were determined with at least two replicates per isolate. The RG value was defined as the diameter of a mycelial colony on media amended with 0.3 µg/ml of myclobutanil per colony diameter on unamended media × 100. Colony diameters were measured after 4 weeks of colony growth at 20°C. A strain was classified as sensitive (S) to myclobutanil if its RG was 30 or less or as resis-

tant (R) to myclobutanil if its RG was 70 or higher. Isolates with RG values between 31 and 69 were intermediate isolates and were not included in this study.

Creating polymerase chain reaction libraries of *V. inaequalis* DNA. Total DNA was digested separately with restriction enzymes *DraI*, *SacI*, and *SspI*. Subsequently, an adaptor (5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCCGCCCGGGCAGGT-3') and (5'-P-ACCTGCCC-NH₂-3') was ligated onto the ends of the digested DNA fragments (32). The resulting *DraI*, *SacI*, and *SspI* polymerase chain reaction (PCR) libraries were stored at -20°C.

PCR amplification of *CYP51A1* and its upstream flanking sequence. Internal fragments of *CYP51A1* were amplified with primer pairs AJ235 (5'-GARCCACCWGTGTGTTTCA-3') and AJ236 (5'-GTASTYYTCTTCGGCGTTGAC-3'), AJ240 (5'-TTCACTGGTTTCCCTTCTTC-3') and AJ241 (5'-KWKATTAKK-GKKACATAGGA-3') and AJ291 (5'-GTCTCACCACCGAAGCTCTCA-3') and AJ313 (5'-GCRAAYTKCTDCCRATRC-ABCAT-3') (Fig. 1). Reaction mixtures (50 µl) contained 1 µl

TABLE 1. Reaction of field strains of *Venturia inaequalis* from different geographical regions to myclobutanil and allele specific amplification-polymerase chain reaction (ASA-PCR) analysis of *CYP51A1* DNA

Origin (reference)	Amplification success with primer pair ^a		Strains	Reaction ^b
	AJ250-F, AJ244-R	AJ251-F, AJ244-R		
Michigan (this study)	+	0	Ent3, Ent4, Ent7, Ent15, Ent23, Ent24, Ent25, Ent26, Ent27, Ent28, Ent31, Ent50, Ent52, Ent56	S
Germany (34)	+	0	Winden10, Winden11, Winden12	S
France (17)	+	0	TUM2, UZ4	S
Michigan (32; this study)	+	0	F335, F336, F349, F411, F445, F446, F853, MSU33, MSU40, MSU37, MSU18, Ent2, Ent21, Ent38, Ent45, Ent54, BF1, BF5, BF6, BF8, BF18, BF19, BF20, BF26, BF29, BF35	R
Israel (17)	+	0	IL-21, IL-22	R
France (17)	+	0	TUFR1, TU86R2	R
Germany (34)	+	0	B1, B9	R

^a Two rounds of PCR were performed for each strain. First, a 404-bp fragment spanning codon 133 was PCR amplified from total DNA with primers AJ243 and AJ244. Then, using a 100-fold dilution of the first round product as template, wild-type primer AJ250 and mutant primer AJ251 were used with AJ244 to selectively amplify *CYP51A1* genes with nucleotides TAT and TTT at codon 133, respectively.

^b Sensitivity to 0.3 µg/ml of myclobutanil was determined in mycelium growth assays on potato dextrose agar. A strain was considered sensitive (S) to the fungicide if the relative growth (RG) was 30 or less and resistant (R) if RG was 70 or higher.

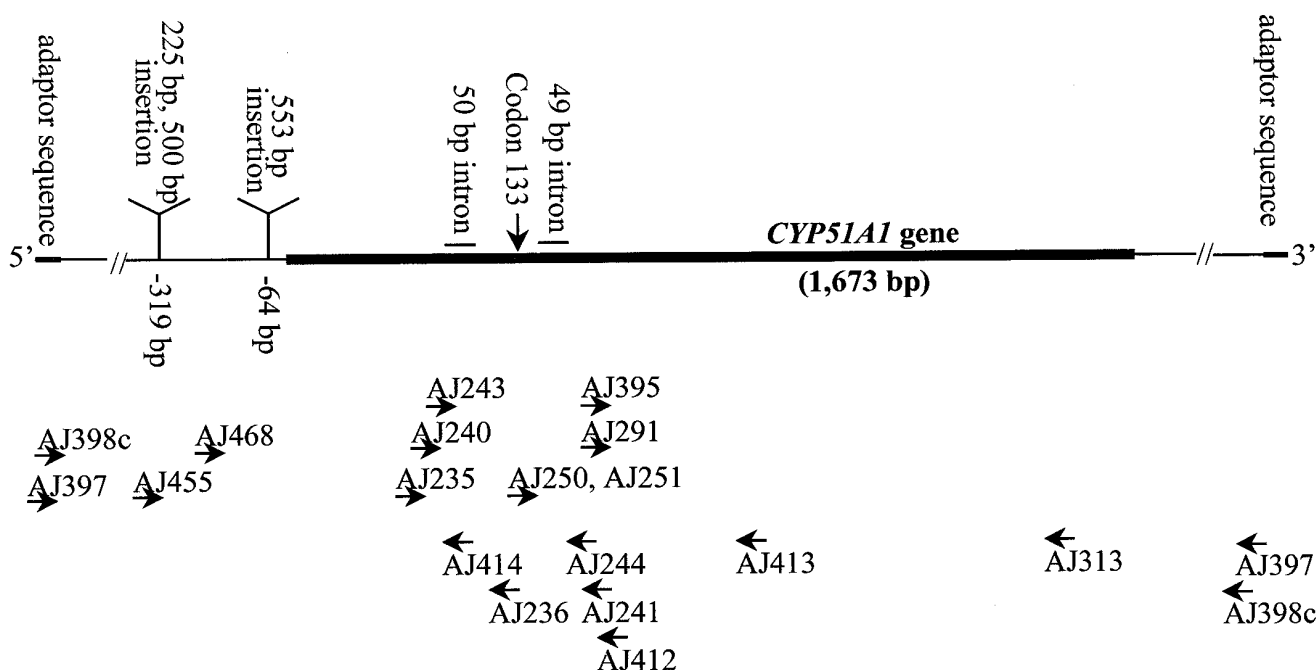


Fig. 1. Location for three insertions found upstream from the 5' end of the *CYP51A1* gene. Screening for the 55-bp insertion was performed by polymerase chain reaction (PCR) with primers AJ468 and AJ414 using total DNA. Also shown are the locations of primers used to amplify *CYP51A1* fragments and its 5' and 3' flanking sequences. Genomic DNA was digested with restriction enzymes *SacI* or *DraI* and an oligonucleotide adaptor sequence was ligated to the ends of the digestion products. Allele-specific primers AJ397 and AJ398c were used to PCR amplify the 5' and 3' ends of *CYP51A1*.

ATGGGACTCCTCTCTCCTTTGCTCGCCTNGTTACCGGGCAGCGACCGCAGTTGGTTATTTTACTCTTGCCCTCCTTCGG 80
 M G L L S P L L A X L P G S D R S W L F Y T L A S F G 27
 CTTACCGTTGCAATCGTCGCCCAACCTTGTCAAGCAACTCTTATTCTCAAACCCAAACGAACCTCCAGTAGTCTTCC 160
 F T V A I V A A N L V K Q L L F S N P N E P P V V F 53
 ACTGGTTTCCCTTCTTCGGCAACACGGTCGTCTACGGCATCGATCCTATCAAGTTTTCGCGGAGTGCAAGGAAAAGgta 240
 H W F P F F G N T V V Y G I D P I K F F A E C K E K 79
 atgcgacaatgagaatgcaagttgctggagctaacttgtctcccagCATGGCGATATCTTTACCTTATTCTTCTTGGC 320
 H G D I F T F I L L G 90
 AGGAAAACAACAGTCTACATTGGTACAAAGGGAAACGAATTCATTCTCAATGGCAAACAGAGCCATGTCAACGCAGAGGA 400
 R K T T V Y I G T K G N E F I L N G K Q S H V N A E E 117
 AATCTATAGCCCCCTGACGACGCCCGTCTTCGGCTCCGATGTTGTCT**A**TGATTGCCAAACTCGAAATTGATGGAGCAAA 480
 I Y S P L T T P V F G S D V V Y D C P N S K L M E Q 143
 AGAAGgtatgctgccaccattctttcgcaagagactcctgctgatatccacaagTTCGTCAAGTACGGTCTCACCACCGA 560
 K K F V K Y G L T T E 154
 AGCTCTCAAATCCTATGTCACCCTCATCCAACAAGAAGTGAAGACTATACCAAACGCTACCCTCAATTCAAAGGCGAAA 640
 A L K S Y V T L I Q Q E V E D Y T K R Y P Q F K G E 180
 AGGGCAGCTTCGATGTTTGGCGTTCATGGCCGAAATCACAATCTTCACTGCTTCCCGCTCACTACAAGGCAAGGAGGTT 720
 K G S F D V C A S M A E I T I F T A S R S L Q G K E V 207
 CGCGACAAGTTTGACGCCAGCTTTCAGACCTCTTCCACGATTTGGATATGGGCTTCTCCTATCAACTTCATGCTTCC 800
 R D K F D A S F A D L F H D L D M G F S P I N F M L P 234
 CTGGGCCCTCTCCACACAATCGTCGCCGAGATGCCGGAACAAAAGATGACGGAGACATATTTGGAAATTATCCAAT 880
 W A P L P H N R R R D A A N K K M T E T Y L E I I Q 260
 CGAGAAAAGCAGAGGGCGTCAAAAAGGATTCAGAGGACATGATTTGGAATTTGATGCAGTGTGTATACAAGAATGGCACT 960
 S R K A E G V K K D S E D M I W N L M Q C V Y K N G T 287
 CCCATCCCGGACAAAAGAAATCGCCACATGATGATCGCGCTGCTCATGGCCGGCCAGCACTCGTCCTCTAGCACCTCGTC 1040
 P I P D K E I A H M M I A L L M A G Q H S S S S T S S 314
 CTGGATTCTACTTCGACTAGCTACCAGACCTGATATCCAGGAAGAACTATACCAAGAACAATTCGGGTTTGGCGGCTG 1120
 W I L L R L A T R P D I Q E E L Y Q E Q I R V C G A 340
 ATCTTCCACCGTTGCAGTACGAAGATCTTGCTCGCATGCCTCTCCACAACCAGATTATCAAAGAAACTCTTCGCATGCAT 1200
 D L P P L Q Y E D L A R M P L H N Q I I K E T L R M H 367
 TCGCAATTCACAGCATCTTGGCTGCCGTCAAACAGCCTATGCCTGTGCAAGGAACCTTACACCATCCCCACCTCGCA 1280
 S P I H S I L R A V K Q P M P V E G T P Y T I P T S H 394
 TGTTCTCCTTGCTGCTCCCATCGCATCTGGAGGCTCGCCAATGTACTTTCCAGCTCCTGAGAAGTGGGAGCCTCACCGTT 1360
 V L L A A P I A S G G S P M Y F P A P E K W E P H R 420
 GGGACGAAGGATCAGGAGGAACCAACATCTCGGGCGCGAAAACGGTGGCGAAGAGAAAGAGGATTACGGCTATGGACTC 1440
 W D E G S G G T N I S G G E N G G E E K E D Y G Y G L 447
 ATCACAAGGGCGCCAGCTCGCGTACCTTCCGTTCCGGCGTGAAGACATAGGTGTATCGGCGAACAATTTGCATATAT 1520
 I T K G A S S P Y L P F G A G R H R C I G E Q F A Y M 474
 GCAGTTGAACACGGTTCTCGCGACGCAAGTTCGCGAATTCAGTTTCAAGTTTCAAGTTTGAAGGAAGGAGAGTTCCTCCCAAGACCG 1600
Q L N T V L A T Q V R E F K F S L R E G E S F P K T 500
 ACTTCTCTTCTATTTTCTGGACCTCTACGCCCGCGTGGTTGAACTGGGAACGTAGAGAGAAGTCTCATGA 1674
 D F S S L F S G P L R P A W L N W E R R E K S S . 525

Fig. 2. Nucleotide and deduced amino acid sequence of the *CYP51A1* gene from *Venturia inaequalis* (*CYP51A1*). The deduced amino acid sequence is indicated by the single-letter amino acid code below the nucleotides. Exons of *CYP51A1* are in upper case, introns are in lower case. The underlined amino acid sequences correspond to regions CR1 to CR6, known to be conserved among *CYP51A1* genes. Sequences for the *CYP51A1* genes from myclobutanil-resistant strains F445 and Ent54 and myclobutanil-sensitive strain Ent27 were identical. The possible mutation site at codon 133 (nucleotide 448) is indicated in bold.

(20 to 60 ng) of nDNA, 1.5 μ M each primer, 200 μ M each dNTP, 1.25 units of *Taq* (DNA polymerase) (Gibco BRL, Gaithersburg, MD), 20 mM Tris-HCl (pH 8.4), 50 mM KCl, and 1.5 mM MgCl₂. Mineral oil was added to prevent evaporation. Cycling parameters were 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 45°C for 1 min, and 72°C for 1 min. Reactions were performed in a minicycler (Model PTC-150-16, MJ Research Inc., Watertown, MA). All PCR products were analyzed by electrophoresis with 1% agarose gels in 0.5 \times Tris-Borate-EDTA buffer.

The 5' and 3' ends of *CYP51A1* and the 1.5-kb upstream region were amplified by a PCR method for walking in uncloned total DNA (32). Basically, nested sets of gene-specific and allele-specific primers were used to amplify the flanking sequences of *CYP51A1* from the PCR libraries. The 5' and 3' ends of the *CYP51A1* gene were successfully amplified using the *SacI* library and the *DraI* library, respectively, as a template. The gene-specific primers used to amplify the 5' end of *CYP51A1* were AJ413 (5'-AATCCTTTTGGACGCCCTCTGCTTTTCTCG-3') followed by AJ412 (5'-TTGCCTTGTAGTGAGCGGGAAGCAGTGAAGATTGTGATT-3'). The gene-specific primer used to amplify the 3' end of *CYP51A1* was AJ395 (5'-GTCTCTCACACCGAAGCTCTCAAATCCTATGT-3') for both rounds of PCR. Adaptor-specific primers AJ397 (5'-GGATCCTAATACGACTCACTATAGGGC-3') and AJ398c (5'-AATAGGGCTCGAGCGGC-3') were used for the first and second rounds of PCR, respectively (Fig. 1). Reactions were performed in a total volume of 50 μ l containing 2.5 μ l (50 to 100 ng) of each PCR library, 0.3 μ M each primer, 350 μ M each dNTP, 2.5 units of Expand long template DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN), and 1 \times Expand buffer 3. Mineral oil was added to prevent evaporation. Cycling parameters were 94°C for 1 min followed by 35 cycles (first PCR) and 23 cycles (second PCR) at 94°C for 30 s and 68°C for 6 min.

Upstream regions of the *CYP51A1* gene were amplified with forward primers AJ455 (5'-CGACGGCGATGTTGTTACGAGATGTA-3') and AJ468 (5'-ATCCCTGAATTTGAGAGCACT-3') and with reverse primer AJ414 (5'-CTGCCAAGAAGAATGAGGTAAA-3') as described above for the internal *CYP51A1* fragments at an annealing temperature of 50°C.

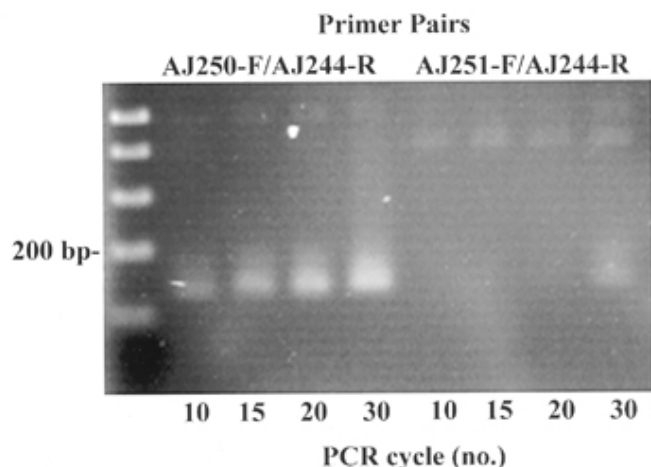


Fig. 3. The effect of the number of cycles on allele-specific amplification-polymerase chain reaction (ASA-PCR) of *CYP51A1* DNA from myclobutanil-sensitive *Venturia inaequalis* strain Ent50. ASA-PCR primers AJ250 (5'-CTCCGATGTTGTCTA-3') and AJ251 (5'-CTCCGATGTTGTCTT-3') were identical except for the base at the 3' end (underlined). Primer AJ250 detected the complementary T nucleotide in the second position of codon 133, whereas AJ251 would detect a possible mutation for demethylation-inhibitor resistance at this position. ASA-PCR was performed simultaneously with both primers with the product from PCR amplification, with primers AJ243 and AJ244 as a template. Products were analyzed by agarose gel electrophoresis after 10, 15, 20, and 30 PCR cycles.

PCR amplification of an *actin* gene fragment from *V. inaequalis*. An approximately 770-bp fragment was PCR amplified from genomic DNA by degenerate primers AJ665 (5'-AAYTGGGAYGAYATGGARAA-3') and AJ666 (5'-ATCCACATYTTGTTGRAANGT-3'). The reaction mixture was identical to the reaction mixture and amplification protocol used for PCR amplifying of the initial *CYP51A1* fragment.

Allele-specific amplification-PCR. Two rounds of PCR were performed for each strain. First, a 404-bp fragment spanning codon 133 was PCR amplified from total DNA with primers AJ243 (5'-GTTTCCCTTCTTCGGCAACAC-3') and AJ244 (5'-TGAGAGCTTCGGTGGTGAGAC-3'). Second, with a 100-fold dilution of the first round product as template, wild-type primer AJ250 (5'-CTCCGATGTTGTCTA-3') and mutant primer AJ251 (5'-CTCCGATGTTGTCTT-3') were used with AJ244 to selectively amplify *CYP51A1* genes with nucleotides TAT and TTT at codon 133, respectively (Fig. 1). PCR reactions and cycle parameters were identical to those used for amplifying internal fragments of *CYP51A1* (described previously), except the annealing temperature for both PCR reactions was 56°C and the number of cycles for allele-specific amplification (ASA)-PCR was 20.

Prediction of promoter sequences. Promoter sequences were predicted with Neural Network, a method that finds eukaryotic and prokaryotic promoters in a DNA sequence (28).

DNA sequencing. PCR products were purified with a PCR DNA purification system (Wizard PCR Preps DNA Purification System, Promega, Madison, WI) and sequenced at the Plant Research Laboratory Sequencing Facility, Michigan State University, East Lansing.

Isolation of RNA. The extraction procedures described below were performed at room temperature, and the centrifugations were conducted at 4°C for 10 min at 10,000 rpm. Single spore isolates of *V. inaequalis* not older than 3 weeks were ground with a tissue grinder, transferred into 250-ml flasks containing 50 ml of potato dextrose broth (PDB), and incubated for 3 to 4 weeks. Cultures from some R strains were also amended with 0.3 μ g/ml of myclobutanil (Nova 40W, Rohm and Haas Company, Philadelphia) 16 h

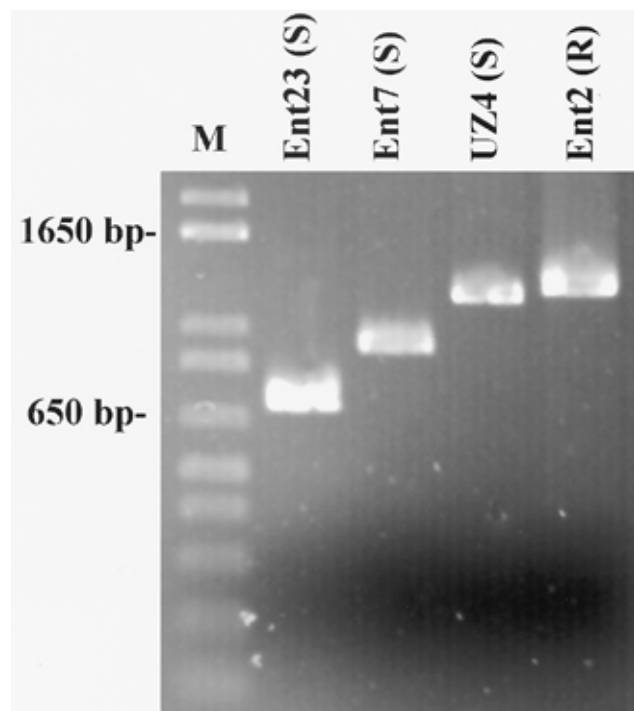


Fig. 4. Gel electrophoresis analysis of polymerase chain reaction products amplified with primers AJ455 and AJ414 from myclobutanil-sensitive (S) strains Ent23, Ent7, and UZ4 and from myclobutanil-resistant (R) strain Ent2. Lane M = 1-kb plus ladder.

before RNA extraction. Mycelium was washed once with sterile water and dried between paper towels. Approximately 100 mg of mycelium was pulverized in a mortar with liquid nitrogen and transferred into a mixture of 2 ml of lysis buffer (0.6 M NaCl, 10 mM EDTA, 100 mM Tris-HCL (pH 8.0), and 4% sodium dodecyl sulfate) and 2 ml of phenol (saturated with 0.1 M Tris-HCL at pH 8.0). The mixture was shaken vigorously for at least 20 min and centrifuged. The upper phase was extracted with phenol, mixed with 0.75 vol of 8 M LiCl, stored at 4°C overnight, vortexed, and centrifuged. The pellet was resuspended in 0.3 ml of double-distilled water and mixed with 0.03 ml of 3 M sodium acetate (pH 5.2) and 0.75 ml of ethanol. The solution was stored at -80°C for 30 min. After centrifugation, the pellet was washed once with 80% ethanol and resuspended in 50 to 100 µl of deionized formamide. The samples were stored at -20°C until further use.

cDNA synthesis. RNA in formamide was resuspended in water, DNase treated and reverse transcribed for real-time PCR analysis. Approximately 5 to 10 µg of RNA in formamide was added to 295 µl of diethylpyrocarbonate water, and 0.03 ml of 3 M sodium acetate (pH 5.2) and 0.75 ml of ethanol were added. The sample was chilled for 30 min at -80°C. The RNA was pelleted by centrifugation for 10 min at 13,000 rpm and subsequently washed with 70% ethanol. The pellet was air dried and resuspended in 5 µl of RNase-free water. DNase treatment was performed according to the manufacturer's protocol (RQ1 RNase-free DNase, Promega, Madison, WI). Reverse transcription was performed in a total of 20 µl using SUPERScript II (Gibco BRL) with 0.13 µl of 10× Hexanucleotide Mix (Boehringer GmbH, Mannheim, Germany) following the manufacturer's recommendations.

Quantification of CYP51A1 expression using real-time PCR. Real-time PCR is a fluorescence-based method of quantifying PCR products during the exponential phase of amplification. As target DNA is amplified, fluorescence increases as a result of the binding of the dsDNA to the DNA stain SYBR Green (Perkin-Elmer Applied Biosystems, Foster City, CA). Reactions were performed in an ABI sequence detection system (Prism 7700, Perkin-Elmer) following the manufacturer's recommendations. The point in time during cycling at which an amplification plot intersects with an arbitrary threshold limit (C_T) was detected for each sample. The comparative C_T method (separate tubes) was chosen to determine *CYP51A1* expression relative to the expression of the sample with the least copies of *CYP51A1* mRNA using *actin* expression for normalizing the data. The validation experiment indicated equal efficiency of the target amplification and the

reference amplification (slope = 0.07). For each strain, the ΔC_T value was determined by subtracting the average *actin* C_T from the average *CYP51A1* C_T value. To calibrate the data, the $\Delta\Delta C_T$ was calculated for each strain by subtracting the ΔC_T from the ΔC_T from strain Ent50, the strain with the highest ΔC_T value and thus the lowest amount of *CYP51A1* mRNA. The relative expression of *CYP51A1* ($CYP51A1_{REL}$) was calculated according to the formula: $2^{-\Delta\Delta C_T}$ with $\Delta\Delta C_T + s$ and $\Delta\Delta C_T - s$, where s = the standard deviation of the $\Delta\Delta C_T$ value.

All experiments were performed in a sequence detector using SYBR Green chemistry (SYBR Green RT-PCR kit, Perkin-Elmer Applied Biosystems). The assay targeted a 67-bp region of *CYP51A1* amplified with primers AJ753 (5'-GAAACTC-TTCGCATGCATTTCG-3') and AJ754 (5'-TCGACAGGCATA-GGCTGTTTG-3') and a 65-bp region of *actin* amplified with primers AJ755 (5'-CCGATCGTATGCAAAAGGAAA-3') and AJ756 (5'-GGGCAATGATTTTGACCTTCA-3'). Both primer pairs were designed with Primer Express primer design software from Applied Biosystems. The *actin* and *CYP51A1* gene fragments were amplified in the same run but in separate tubes. Reactions (30 µl) were performed containing 1× SYBR PCR buffer, 5 mM MgCl₂, 0.3 mM each dATP, dGTP, dCTP, 0.6 mM dUTP, 0.3 mM of each primer, AmpliTaq Gold DNA polymerase (0.025 units/µl), and 0.7 µl of 1:5 diluted cDNA. Both *actin* and *CYP51A1* cDNA were amplified in triplicate reactions following thermocycling program: one cycle of polymerase activation and template denaturation at 95°C for 10 min and 40 cycles at 95°C for 15 s and primer annealing and extension at 58°C for 1 min.

Nucleotide sequence accession numbers. The *CYP51A1* sequence data for *V. inaequalis* strains Ent27, Ent54, and F445 were deposited in GenBank, Accession Nos. AF227920, AF262756, and AF262757, respectively. The *CYP51A1* upstream sequences for strains Ent23, Ent2, Ent7, and UZ4 were deposited under Accession Nos. AF227916, AF227917, AF227918, and AF227919, respectively. The partial *actin* sequence was deposited under Accession No. AF269254.

RESULTS

Identification and characterization of CYP51A1. Alignment of the *Ucinula necator*, *Penicillium italicum*, and *Ustilago maydis* *CYP51A1* sequences (Accession Nos. U83840, Z49750, and Z48164, respectively) revealed several areas of high homology. Degenerate oligonucleotide primers for amplification of *CYP51A1* fragments were designed based on amino acid sequences in four

TABLE 2. Predicted promoters upstream from the *CYP51A1* gene from *Venturia inaequalis* and their location relative to the genes 5' terminal nucleotide. Shown are 51 bases spanning from -40 to +10, where +1 is the predicted transcription start site region^a

Strain	Promoter sequence	Score	Relative location
Ent23	CTTGGAGTAAAATACAGACGAGCAACACCACACTTCCTCACCACCAACA	0.59	-50
	-40 +1 +10		
Ent7	CTTGGAGTAAAATACAGACGAGCAACACCACACTTCCTCACCACCAACA	0.59	-50
	ATCGTAACCATAATAAAGGCGTAGGATTGGAAGTTAATAAGTGGAATCT	0.93	-440*
	-40 +1 +10		
UZ4	CTTGGAGTAAAATACAGACGAGCAACACCACACTTCCTCACCACCAACA	0.59	-50
	ATCGTAACCATAATAAAGGCGTAGGATTGGAAGTTAATAAGTGGAATCT	0.93	-439*
	AGGCCCGATTGATATATTACGCCGAGACGGCAATCCCGTGGCAACCCGC	0.96	-538*
	-40 +1 +10		
Ent2	CTTGGAGTAAAATACAGACGAGCAACACCACACTTCCTCACCACCAACA	0.59	-50
	TTGGGTAGTGTAATAAGAAAGTGGGTCAATGGCGGAGCCATTGATTAAG	0.64	-220*
	CCCCCGGCCCATTAATAATCATCGTTTCACCCAATCACAGTCACGGAA	0.76	-346*
	TCATACTACCCTGTACAACTCCTACACCCCGGCCCCCATTAATAATCA	0.59	-373*
	-40 +1 +10		

^a The cutoff value for the determination of scores was 0.5. The score indicates the likelihood of the nucleotide sequence being a promoter sequence. An asterisk indicates that the promoter is located within an insertion sequence.

conserved regions. A 350-bp fragment was amplified and sequenced with degenerate primers AJ235 and AJ236. Based on the sequence data, specific primer AJ240 was designed and combined with degenerate primer AJ241 to PCR amplify and sequence a 420-bp region of the gene. Specific primer AJ291 and degenerate primer AJ313 were used to amplify a 900-bp downstream flanking sequence (Fig. 1). Adaptor-ligation PCR with gene-specific and adaptor-specific primers allowed amplification of a 4-kb fragment containing the 5' end of *CYP51A1* and a 3.5-kb fragment containing the 3' end of *CYP51A1* from the *SacI* and *DraI* PCR libraries, respectively. The *CYP51A1* gene including introns was 1,674 bp in length and was predicted to have two introns of 50 and 49 bp located after nucleotides 236 and 485, respectively (Figs. 1 and 2). The predicted 5' GT and 3' AG splice sites of the introns were in agreement with typical fungal introns (36). The nucleotide sequence of *CYP51A1* was 48.6, 50.6, 52.0, and 40.8%, and the amino acid sequence was 61.6, 63.6, 59.8, and 51.3% identical to the *CYP51A1* genes from *Uncinula necator*, *E. graminis*, *Penicillium italicum*, and *Ustilago maydis*, respectively (GenBank Accession Nos. U83840, AF052515, Z49750, and Z48164, respectively).

Screening for a mutation at codon 133 in *CYP51A1*. Amino acid sequence alignment of *CYP51A1* from *Uncinula necator* and *V. inaequalis* revealed that codon 136 for tyrosine in *Uncinula necator* corresponded to codon 133 in *V. inaequalis* (Fig. 2). A 404-bp fragment containing codon 133 was PCR amplified from each strain with primers AJ243 and AJ244 and used as a template for allele-specific amplification (Fig. 1). DNA from 19 S and 32 R strains of *V. inaequalis* from Michigan, Israel, France, and Germany was examined by ASA-PCR for a single base pair change at codon 133. After 20 cycles, each isolate yielded the expected 150-bp fragment with primers AJ250 and AJ244. None yielded a product with primer pair AJ251 and AJ244 (Fig. 3, Table 1).

Polymorphism upstream from the 5' end of *CYP51A1*. PCR amplification with primers AJ455 and AJ414, using total DNA of 14 single-spore strains, yielded fragments of either 725, 949, 1,225, or 1,278 bp (Fig. 4). Sequence analysis revealed that the three larger PCR products contained insertions of 225, 500, and 553 bp for strains Ent7, UZ4, and Ent2, respectively (Fig. 1). The 225- and 500-bp insertions were located between nucleotides -319 and -320, and the 553-bp insertion was located between nucleotides -64 and -65 relative to the first 5' adenine (A) of the *CYP51A1* gene from strain Ent23. All alleles had a common predicted promoter sequence located at nucleotide -50, and 1, 2, or 3 predicted promoter sequences in the 225-, 500-, and 553-bp insertions, respectively (Table 2). S and R strains from five orchards in Michigan were screened for the 553-bp insertion using PCR with primers AJ468 and AJ414. The insertion was present in 9 of 11 R strains from the Botany Farm and in one R strain each from the Entomology Farm and orchard HM; none of the S strains contained the insertion (Table 3). The 225-bp and the 500-bp insertions were found in both R and S strains from East Lansing (data not shown).

Sensitivity to myclobutanil and *CYP51A1* gene expression in *V. inaequalis* strains from Michigan orchards. A total of 50, 47, 41, 37, and 48 isolates from the Entomology Farm, Botany Farm, and commercial orchards JM, HM, and ER, respectively, were tested for myclobutanil sensitivity (Fig. 5). The RG value for most isolates from the Entomology Farm were at the sensitive end of the distribution; however, a few R strains were also detected in this population. The RG values for the isolates in the Botany Farm research orchard and each of the three commercial orchards were clearly shifted to the resistance end of the distribution. The discriminatory dose of myclobutanil use in this study had little or no effect on the RG of the isolates from these orchards.

For normalizing the *CYP51A1* expression data, the *actin* gene from *V. inaequalis* was identified and partially sequenced. Degenerate primers were designed based on the *actin* sequences

TABLE 3. Relative expression of the *CYP51A1* gene, myclobutanil sensitivity, and presence or absence of the 553-bp insertion in myclobutanil sensitive (S) and resistant (R) strains of *Venturia inaequalis* from five orchards

Population strain ^a	Sensitivity ^b	553-bp insertion ^c	<i>CYP51A1</i> _{REL} ^d
Entomology Farm			
Ent2	R	+	5.0 (4.2 – 5.8)
Ent4	S	–	1.8 (1.1 – 2.6)
Ent7	S	–	3.5 (2.7 – 4.3)
Ent23	S	–	2.5 (1.6 – 3.4)
Ent24	S	–	2.6 (1.6 – 3.5)
Ent27	S	–	1.3 (0.6 – 2.0)
Ent28	S	–	2.4 (1.8 – 3.1)
Ent31	S	–	1.8 (0.9 – 2.7)
Ent50	S	–	1.00 (0.3 – 1.7)
Ent52	S	–	2.2 (1.6 – 2.9)
Botany Farm			
BF1	R	+	5.1 (4.1 – 6.0)
BF5	R	+	12.9 (12.0 – 13.8)
BF6	R	+	12.4 (11.5 – 13.2)
BF8	R	+	4.5 (3.6 – 5.4)
BF17	R	+	17.8 (17.0 – 18.6)
BF18	R	+	8.7 (7.9 – 9.5)
BF19	R	+	7.9 (7.0 – 8.9)
BF20	R	–	2.9 (2.1 – 3.7)
BF26	R	+	8.4 (7.5 – 9.2)
BF29	R	–	3.3 (2.4 – 4.2)
BF35	R	+	8.0 (7.1 – 8.9)
ER-orchard			
ER4	R	–	190.4 (189.7 – 191.2)
ER7	R	–	72.5 (71.6 – 73.4)
ER8	R	–	76.6 (75.8 – 77.4)
ER13	R	–	17.6 (16.7 – 18.4)
ER20	R	–	21.9 (21.1 – 22.8)
ER22	R	–	20.7 (19.8 – 21.5)
ER26	R	–	73.3 (72.4 – 74.2)
ER32	R	–	32.0 (31.2 – 32.8)
JM-orchard			
JM2	R	–	1.4 (0.5 – 2.3)
JM8	R	–	2.2 (1.3 – 3.2)
JM10	R	–	18.9 (18.1 – 19.8)
JM12	R	–	1.5 (0.7 – 2.3)
JM13	R	–	1.4 (0.4 – 2.3)
JM19	R	–	13.9 (13.0 – 14.8)
JM21	R	–	4.7 (3.8 – 5.7)
JM23	R	–	4.4 (3.5 – 5.2)
JM31	R	–	4.3 (3.5 – 5.2)
HM-orchard			
HM3	R	–	45.3 (44.4 – 46.1)
HM12	R	–	20.1 (19.4 – 20.9)
HM13	R	–	19.5 (19.0 – 20.0)
HM18	R	–	86.2 (85.3 – 87.1)
HM24	R	–	83.3 (82.5 – 84.1)
HM25	R	+	5.4 (4.5 – 6.2)
HM26	R	–	25.5 (24.5 – 26.4)
HM31	R	–	6.0 (5.2 – 6.8)
HM35	R	–	22.0 (21.0 – 22.8)

^a All strains were isolated from individual lesions. The Botany Farm and Entomology Farm were experimental farms, only 0.1 km apart. Orchards ER, JM, and HM were commercial orchards located in Michigan.

^b The reaction of each strain to myclobutanil was determined in mycelial growth assays on potato dextrose agar amended with myclobutanil at 0 and 0.3 µg/ml as described previously (31). A strain was classified as sensitive (S) to myclobutanil if its relative mycelium growth was inhibited by more than 70% or resistant (R) to myclobutanil if its growth was inhibited by less than 30% (Fig. 5).

^c The presence (+) or absence (–) of the 553-bp insert was determined by polymerase chain reaction amplification by primers AJ468 and AJ414.

^d *CYP51A1*_{REL} is the relative amount of *CYP51A1* mRNA as compared with the mRNA level from strain Ent50. This strain was chosen for comparison because it had the lowest amount of mRNA from all samples tested. The amount of target, normalized to an endogenous reference, and relative to a calibrator, is given by $2^{-\Delta\Delta CT}$. The range given of *CYP51A1*_{REL} relative to Ent50 is determined by evaluating the expression $2^{-\Delta\Delta CT}$ with $\Delta\Delta CT + s$ and $\Delta\Delta CT - s$, where s = the standard deviation of the $\Delta\Delta CT$ value.

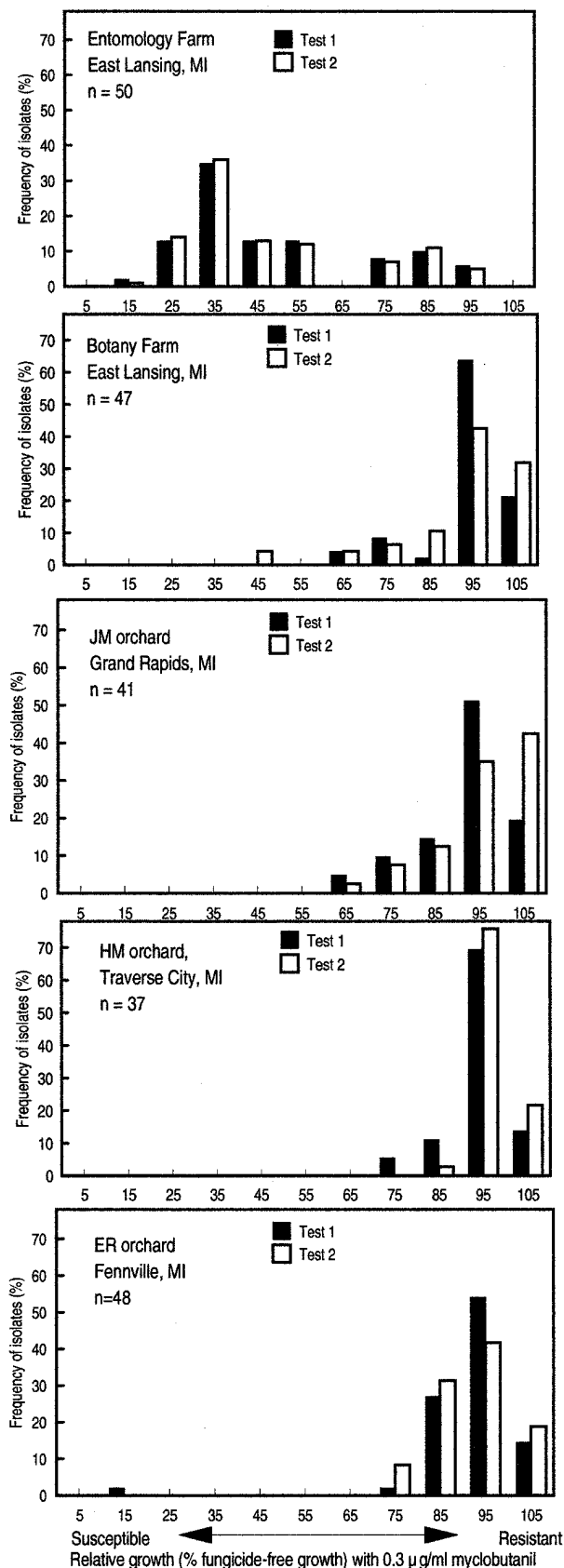


Fig. 5. Frequency distribution based on relative growth on myclobutanil-amended medium (0.3 $\mu\text{g/ml}$) of individual isolates of *Venturia inaequalis* from a baseline orchard (Entomology Farm), a research orchard for testing demethylation-inhibitor fungicides (Botany Farm), and three commercial Michigan apple orchards with poor control of scab. Relative growth equals the colony diameter on amended medium per colony diameter on unamended medium \times 100.

from *Homo sapiens*, *Neurospora crassa*, and *Phytophthora infestans* (GenBank Accession Nos. NM001614, U78026, and M59715). Sequence analysis of a 451-bp *actin* fragment revealed highest nucleotide similarities (85%) to the *actin* genes of *N. crassa*, *Colletotrichum gloeosporioides* (GenBank Accession No. AF112537), and *Ajellomyces capsulatus* (GenBank Accession No. U17498).

Expression of the *CYP51A1* gene was determined for nine S strains with RG values less than 30 from an orchard that had never been sprayed with DMI fungicides (baseline orchard Entomology Farm), and 37 R strains with RG values of 95 or higher collected from a research orchard (Botany Farm) and from three commercial Michigan apple orchards (ER, JM, and HM) with a long history of DMI use and a high frequency of R strains (Table 3, Fig. 5). Strain Ent50 was chosen to calibrate all data because among the strains tested it contained the least amount of *CYP51A1* mRNA. The $CYP51A1_{REL}$ values of R strains from the research orchard were significantly different from the values obtained from the baseline orchard ($P < 0.05$ according to LSD). Nine of eleven R strains had higher $CYP51A1_{REL}$ values than any of the S strains. The $CYP51A1_{REL}$ values from R strains from the commercial orchards HM and ER were significantly different from the values obtained from the baseline orchard ($P < 0.05$ according to the Tukey test). Overexpression of the *CYP51A1* gene was detected in eight of eight, five of nine, and nine of nine R strains from orchards ER, JM, and HM, respectively. The addition of 0.3 $\mu\text{g/ml}$ of myclobutanil to the PDB culture medium 24 h before RNA was extracted from the mycelium did not increase the level of *CYP51A1* expression (data not shown).

DISCUSSION

The putative amino acid sequence of the gene identified in this study closely resembles the cytochrome P450 sterol gene identified in other fungal species. It contains six regions known to be conserved and characteristic for *CYP51A1*s in mammals, yeast, and fungi (2). The sequence also contains two introns that are located exactly at the same position as the introns in *Uncinula necator* (10). This data strongly suggests that we identified the gene encoding cytochrome P450 sterol 14 α -demethylase in *V. inaequalis*.

The gene encodes the target enzyme for DMI fungicides, thus resistance could develop from changes in the structure of the *CYP51A1* gene thereby reducing the affinity of the target enzyme to these antifungal agents. Several single-site mutations within this gene were linked to DMI resistance in yeast. Most of the mutations found in fluconazole-resistant strains of the yeast fungus *Candida albicans* were located in the substrate recognition site; they reduced the affinity of the target enzyme for DMI fungicides (3,29). In filamentous fungi such as *Uncinula necator*, *Penicillium italicum*, and *E. graminis*, a single nucleotide mutation within the substrate recognition site, causing an amino acid change from phenylalanine to tyrosine, has been correlated with resistance (6,9,11). No mutations in the *CYP51A1* gene from *V. inaequalis* were found when two R and one S strains were completely sequenced. In addition, the point mutation reported to correlate with DMI resistance in filamentous fungi was not detected in a collection of strains from Michigan, Germany, Israel, and France. These results indicate that a resistance mechanism other than mutation of the *CYP51A1* gene is primarily the cause of DMI resistance in *V. inaequalis* strains from Michigan and elsewhere.

Increased expression of *CYP51A1* is another mechanism of DMI resistance. Recently, it was shown that overexpression of the *Saccharomyces cerevisiae CYP51A1* gene, under control of the regulatable promoter *GALI*, can result in azole resistance (22). In fluconazole-resistant clinical isolates of *C. albicans*, resistance was associated with less than a fivefold increase in the level of *CYP51A1* mRNA (1,30,42). Heterologous expression of the *Peni-*

Penicillium italicum CYP51A1 gene in *Aspergillus niger* revealed a twofold to fivefold increase of DMI resistance (39,40). The CYP51A1_{REL} values for R strains from the Botany Farm research orchard and commercial orchards ER and HM were significantly higher than the values for S strains from the baseline Entomology orchard. Many CYP51A1_{REL} values exceeded dramatically the less than fivefold increase in expression found in fluconazole-resistant *C. albicans* isolates (1,30,42). Heterologous expression of the *V. inaequalis* CYP51A1 gene from strain Ent2 in a PDR5::Tn5 *S. cerevisiae* strain revealed a fivefold increase of myclobutanil resistance (data not shown). These findings provide strong evidence that overexpression of the CYP51A1 gene is associated with DMI resistance in field strains of *V. inaequalis* collected from research and commercial orchards with practical resistance to DMI fungicides.

Overexpression of CYP51A1 in resistant strains theoretically could be a result of gene amplification, up-regulation of transcription, or a higher degree of mRNA stability all resulting in elevated CYP51A1 mRNA levels. Sequence analysis of the CYP51A1 5' flanking sequence revealed the presence of three insertions within 320 bp of the CYP51A1 5' end. Interestingly, the 553-bp insertion, which contained three predicted promoter sequences, was found in every R strain from the Botany Farm with CYP51A1_{REL} values ≥ 4.5 , but not in R strains with lower levels of CYP51A1 expression nor in S strains. Promoters located upstream or downstream from a gene often regulate gene expression by stimulating or inhibiting the initiation of transcription. The presence of a 126-bp repeat unit in the promoter region is responsible for CYP51A1 overexpression in DMI resistant *Penicillium digitatum* strains (15). Insertions located upstream and downstream of a gene promoter generate different patterns of gene expression (5). Therefore, the 553-bp insertion or its promoters may serve as a transcriptional enhancer of CYP51A1 expression, resulting in DMI resistance. Unfortunately, no S strains were detected among the isolates collected from the myclobutanil-sprayed trees in the Botany Farm orchard. Instead, we examined the S strains from the nearby Entomology Farm orchard, because short distance gene flow likely exists between adjacent *V. inaequalis* populations (34). Gene flow between the two orchards is supported by detection of a few R strains among a high frequency of S strains in the Entomology Farm orchard. Therefore, it is likely the R strains migrated into this orchard from the Botany Farm, the only other apple orchard in the surrounding area. However, other insertions or mechanisms seem to be responsible for the dramatic overexpression of CYP51A1 in many of the R strains from commercial orchards.

DMI-resistant field strains of *V. inaequalis* can be controlled to a certain degree by increased DMI fungicide application rates (20). However, even at higher rates the level of control may be commercially unacceptable, in particular where curative control is needed (21). The enhanced concentration of CYP51A1 mRNA in many R strains most likely results in higher levels of 14 α -demethylase in fungal cells. Elevated enzyme production may explain why increasing rates of DMI fungicides have been required to maintain control. The detection of R strains in the experimental and one commercial orchard without increased levels of enzyme production indicates that other mechanisms might also contribute to myclobutanil resistance in *V. inaequalis*. Recently, Palani and Lalithakumari (27) provided evidence that penconazole-resistant laboratory mutants of *V. inaequalis* possess an energy-dependent efflux mechanism. It remains to be investigated whether R strains without increased 14 α -demethylase production have increased levels of drug efflux activity. However, regardless of the mechanism for resistance at each orchard site, the detection of R strains at high frequency in a commercial orchard in 1995 (21) and in three additional commercial orchards in 1999 (this study), indicates that apple scab is becoming increasingly hard to control with DMI fungicides.

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