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Cloning and Sequence Analysis of the Eburicol 14 α -Demethylase Encoding Gene (*CYP51*) from the Japanese Pear Scab Fungus *Venturia nashicola*

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With 1 figure

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Abstract

The slow growth of many filamentous fungi *in vitro* prevents rapid isolation of large quantities of genomic DNA, thereby hampering molecular studies. Using a polymerase chain reaction (PCR)-based adapter ligation cloning technique that requires only nanogram quantities of genomic DNA we have successfully cloned the complete *CYP51* gene encoding the eburicol 14 α -demethylase, the target for DMI fungicides, from the Japanese pear scab fungus, *Venturia nashicola*. Analysis of predicted amino acid sequences of *CYP51*s from strains less sensitive to DMIs revealed no alterations when compared to the sensitive reference strain.

Introduction

Venturia nashicola is the causal agent of scab, a major disease of both Japanese and Chinese pears (Tanaka and Yamamoto, 1964). Since 1986 the DMI fungicides, superceding the now ineffective benzimidazole fungicides, have been the principal agents in controlling this disease. The rapid development of resistance to benzimidazole fungicides, from their introduction in 1971 to complete loss of efficacy in 1975 (Ishii, 1997), provoked concern over the possible development of resistance to DMI fungicides. Therefore, careful monitoring studies have been undertaken since the introduction of DMIs to detect possible shifts in sensitivity to these compounds (Tomita and Ishii, 1998). To date, no signs of practical resistance to DMI fungicides have been detected. However, a few strains of *V. nashicola* with significantly lower sensitivities to DMIs have been identified and this has prompted a greater interest in the possible molecular mechanisms involved.

Gene sequencing studies have proved a useful tool in the elucidation of the adaptive genetic mechanisms involved in the development of fungicide resistance.

Sequencing of genes (*CYP51*s) encoding the target for DMI fungicides, the eburicol 14 α -demethylase (Yoshida and Aoyama, 1987), a key enzyme in the ergosterol biosynthesis pathway (Aoyama et al., 1989), has led to the identification of a number of mutations that may confer DMI resistance by causing conformational changes that alter inhibitor binding, but do not effect the capacity for endogenous substrate binding. One such mutation, resulting in the substitution of phenylalanine for tyrosine in conserved region 2 (CR-2), has been identified in *CYP51* genes sequenced from DMI-resistant strains of *Candida albicans* (Sanglard et al., 1998), *Penicillium italicum* (De Waard, 1996) *Uncinular necator* (Delye et al., 1997b) and *Erysiphe graminis* f. sp. *hordei* (Delye et al., 1998).

The vast majority of fungal gene sequences have been obtained by screening genomic libraries. However, few genomic libraries for filamentous plant pathogenic fungi are currently available and their construction is a time-consuming, labour-intensive process requiring large amounts of genomic DNA. Polymerase chain reaction (PCR)-based cloning techniques have proved useful in the genetic analysis of slow growing fungi as only nanogram quantities of template DNA are required to rapidly amplify target genes.

This paper describes the use of degenerate primers to amplify a large fragment of the *CYP51* gene from the DMI-sensitive *V. nashicola* strain JS-18. The remainder of the *CYP51* gene was obtained using an adapter ligation PCR-based cloning kit (Siebert et al., 1995). Comparison of *CYP51* sequences of strains less sensitive to DMIs to that of the sensitive reference isolate JS-18, with particular emphasis on those regions in which mutations correlated with resistance have been found in *C. albicans*, *P. italicum*, *U. necator* and *E. graminis* f. sp. *hordei*, has also been undertaken.

Materials and Methods

Fungal strains

All strains were isolated from sporulating scab lesions on pear leaves collected from commercial orchards in Ibaraki and Saga prefectures, Japan. Strains with reduced sensitivity to DMIs, NA-9, OK-8 and NH-1, isolated in 1997, and ND-8, ND-1, SE-9 and ND-10, isolated in 1998, were kindly donated by the Plant Protection Office, Ibaraki, Japan. Strain 3-29-1 was isolated from Saga Prefecture in 1992. Sensitive strain JS-18 was isolated in 1979 from a non-DMI treated orchard in Ibaraki Prefecture.

Conidia collected from lesions were suspended in sterile distilled water. Drops of conidial suspensions were placed on water agar plates and incubated at 15°C for 35 days. Individual germinated conidia were cut out in agar blocks with a steel needle and transferred on potato-dextrose agar (PDA) slants amended with penicillin G potassium salt (50 mg/l), streptomycin sulphate (50 mg/l) and lactic acid (0.6 g/l). After incubation at 20°C, monoconidial cultures of *V. nashicola* strains were used in the fenarimol sensitivity tests.

Fungicide sensitivity assays

Each strain was cultured on PDA plates at 20°C for 60 days to supply inoculum. Mycelial discs, 4 mm in diameter, were cut from colony margins and transferred onto PDA plates containing 0, 0.01, 0.05, 0.1, 1, 5, 10, 50 and 100 mg AI per litre of a commercial 120 g/kg fenarimol WP. Three replicate plates were used per concentration. After 3 weeks incubation at 20°C in the dark, the diameter of each colony was measured and the EC₅₀ value for fenarimol calculated using a log-linear model (ZEN-NOH, Tokyo, Japan).

Amplification of a fragment of *CYP51* gene from *V. nashicola*

Genomic DNA was extracted from *V. nashicola* strain JS-18 following the protocols described by Wheeler et al. (1995). Degenerate primers (kindly supplied by Dr. J. Hargreaves, IACR-Long Ashton Research Station) L1a (5'-YTNACNACNCCNGTNTTCGG-3') and R1a (5'-GCRAAYTGYTCNCCDATRCA-3') correspond, as determined by sequence alignment between fungal *CYP51* genes, to conserved amino acid sequences L-T-T-P-V-F-G and C-I-G-E-Q-F-A, respectively. These were used to amplify a fragment of expected size (1105 bp) of the *CYP51* gene, using the Expand High Fidelity PCR System (Boehringer Mannheim, Mannheim, Germany) from 100 ng of genomic DNA extracted from DMI-sensitive *V. nashicola* strain JS-18. PCR reactions were carried out in a Biometra T3 thermocycler (Biotron GmbH, Göttingen, Germany). Conditions were as follows: an initial 40 cycles of 1-min denaturation at 94°C, 1.5-min annealing at 54°C and 2-min extension at 72°C, followed by a single cycle of 1-min denaturation at 94°C, 2-min annealing at 50°C and 4-min extension at 72°C. The amplified fragment was cloned into the pGem-Easy vector (Promega, Madison, WI, USA) and sequenced. The

sequence was analysed and a homology search performed using the NCBI BLAST server

Cloning of the complete *CYP51* gene from *V. nashicola*

The full-length *CYP51* gene was obtained using the Universal GenomeWalker kit (Clontech, Palo Alto, CA, USA). Five infrequently cutting restriction enzymes (*Eco* RV, *Dra* I, *Pvu* II, *Sca* I and *Stu* II) individually digested 2.5 µg of genomic DNA from strain JS-18 for 30 min. The resulting pool of DNA fragments from each digest were blunt-end ligated to the GenomeWalker adapter, creating five adapter-ligated GenomeWalker libraries. Six primers, specific to the previously obtained *CYP51* sequence (Gene-Specific Primers (GSPs)), were designed. Three primers for amplifications of *CYP51* fragments upstream of the known partial sequence (towards the 5'-end) and three for amplifications downstream of the known partial sequence (towards the 3'-end) of the *CYP51* gene (Fig. 1; Table 1).

All GenomeWalker reactions were carried out using the Biometra T3 Thermalcycler and the Extensor Long Template PCR System (Abgene, Epsom, UK). Optimal PCR conditions for amplifications using this system were as follows: an initial denaturation at 94°C for 2 min, followed by 10 cycles of 10-s denaturation at 94°C, 4.5 min of a combined annealing/extension step at 68°C, followed by 20 cycles of 10-s denaturation at 94°C, 4 min 20 s of combined annealing/extension at 68°C and a final prolonged extension step of 7 min at 68°C. Optimal conditions were maintained across all reactions with the exception of the JSR7 (annealing 64°C) and the JSF6 reaction (annealing 66°C). Primer concentrations for both GSPs and Adapter-specific Primers (APs) were 0.1 µM.

Two microlitres of DNA from each adapter ligation was used as template for two primary PCR reactions using the innermost GSPs (JSR2 for the upstream and JSF2 for the downstream reaction), with the outer adapter primer (AP1). Primers were removed from the resulting primary PCR mixture using a PCR product purification kit (Boehringer Mannheim, Indianapolis, IN, USA) and 2 µl used as template for the secondary reactions. The two GSPs, JSR7 (upstream reaction) and JSF6 (downstream reaction) used in the secondary PCR reactions, in combination, again with AP1, anneal to regions of the obtained *CYP51* sequence beyond the 5'-end of the primary GSPs, thus, 'walking' along the *CYP51* gene. The final PCR reaction, using the purified PCR mixture from the secondary PCR reaction as template, used GSPs JSR7N and JSF6N, which anneal to sequences directly adjacent to the 5'-end of JSR7 and JSF6, respectively. The adapter-specific primer in this reaction, AP2, partially anneals to the 3'-end of the AP1 sequence. Products obtained after this final nested reaction were visualized on an ethidium bromide stained agarose gel, cloned into pGem-Easy vector and sequenced (two clones for each amplified fragment).

1 ATGGGACTCCTCTCTGCTCTCCTCGCCCCCTTAGCGGGTAGCGACCGGGTGGCTATTCTACACCCCTCGCCTCGTTC
M G L L S A L L A P L A G S D R **G W L F Y T L A S F**
Putative transmembrane domain
GGTTTCACCGTCGCAATCGTTCGCAACGTCCTCAAGCAAGTCTACTCAAAAACCCCAATGAACCTCCCGTGGTC
G F T V A I V V A N V L K Q V L L K N P N E P V V
TTCCACTGGTTTCCCTTTTCGGCAACACGGTCTACGGCATCGACCCATCAAGTTCTTTGCTGAGTGCAAGAA
F 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
AAGgtaacgcgtccagctctagtagcaaggacacaagttgcctcgagcttacttgtctccagCATGGCGATATCTTTAC
K intron 1 H G D I F T
CR-1
CTTCATTCTCCTCGGCAGGAAAACAACAGTCTATATTGGGACAAAGGGTACGAGTTTCAATCTCAATGGAAAACAGAG
F I L L G R K T T V Y I G T K G Y E F I L N G K Q S
CCATGTCAACGAGGAAAATCTATAGCCCCCTGACGACGCCCTCTTCGGTCCGATGTTGTCTATGATGCCCCAAA
H V N A E E I Y S P **L T T P V F G S D V V Y D C P N**
CR-2
TTCAAAATTGATGGAGCAAAAAGgtataccggccttcattctttaacatgactcctactgatttttcacaagTTCG
S K L M E Q K K **intron 2** F V
TCAAGTACGGTCTCACCACGAAGCCCTCAAATCCTACGTCACCCCTATCCAAAGAGAAGTCGAAGATTATGCCAAC
K Y G L T T E A L K S Y V T L I Q R E V E D Y A K R
GCTACTCTCAATTCAAAGGCGAGAAGGGTAGTTTCGATGTTTTCGCTACCATGGCGAGATCACAATATCTCACTGCTT
Y S Q F K G E K G S F D V C A T M G E I T I F T A S
CCCCTTCAATACAAGCAAGGAGTTTCGCGACAAGTTGACGCCAGCTTCGACACCTCTCCACGATCTGGACATGG
R S L Q G K E V R D K F D A S F **A D L F H D L D M G**
CR-3
GCTTCTCTCCATCAACTTCATGCTTCCATGGCGCCTCTCCACACAATCGTCGCGAGACGCCCGAACAAGAAGA
F S P I N F M L P W A P L P H N R R R D A A N K K M
TGACGGAGACATACTTGAAAATTATAGATCGAGAAAAGTAGAGGCGCTAAGAAGACTCAGAGGACATGATTTGGA
T E T Y L E I I R S R K V E G A K K D S E D M I W N
ATCTGATGCAATGTGTATACAAGAATGGCACGCCATCCCGGACAACGAAATCGCCCATATGATGATCGCCTTCTCA
L M Q C V Y K N G T P I P D N **E I A H M M I A L L M**
CR-4
TGGCGGTCAACACTCGTCCCTAGCACCTCGTCCGGATGCTGTTTCGACTAGCTACCAGACCCGATATCCAAGAAG
A G Q H S S S S T S S W M L F R L A T R P D I Q E E
AACTTTACCAGGAACAAATCCGGTCTGCGGCGCTGATCTTCCCGGTTGAAAATACGACGACCTTGCACGATGCCTC
L Y Q E Q I R V C G A D L P P L K Y D D L A R M P L
TCCACAACCAGCAGATCATTAAGGAAACCTTCGCATGCATTCGCCGATTCACAGCATTTCGCGCGCGTCAAACAGC
H N Q Q I **I K E T L R M H S P I H S I L R A V K Q P**
CR-5
CTATGCCCATCGAAGAACTCCTTATACCAATCCACGTCGCATGTCCTCCTTGTCTGCCATCGCATCTGGAGGTT
M P I E G T P Y T I P T S H V L L A A P I A S G G S
GCCAATGTACTTCCCGGCCCTGAAAAGTGGGAGCCTCACCGTGGGACGAAGGATCCGGAGGAACCAACATTTCCGGT
P M Y F P A P E K W E P H R W D E G S G G T N I S G
GGCGATAATGGTGACGAAGAGAAGGAAGATTACGGCTATGGTCTGATCAGCAAGGGTCCAGTTCCCGTACCTTCCGT
G D N G D E E K E D Y G Y G L I T K G A S S **P Y L P F**
TTGGCGTGGAAGACATAGATGCATTTGGCGAACAATTTGCATATATGCAATTGAACACGGTGTCTGCGACGCAAGTCCG
G A G R H R C I G E Q F A Y M Q L N T V L A T Q V R
CR-6
CGAGTTCAAGTTCAAGTTAGGGAAGGAGAATCATCCCAAGACTGACTTCTCTTCCCTGTTTCTGGACCTCAACGC
E F K F S F R E G E S F P K T D F S S L F S G P Q R
CCGGCGTGGTTGAATTGGGAACGCAGAGAGAAGTTCATCATCATAG
P A W L N W E R R E K S S S *

Fig. 1 Translated nucleotide sequence of *Venturia nashicola* *CYP51* gene. Putative transmembrane domain and conserved regions (CR-*) shown in bold type. Tyr133 is underlined. GenomeWalker primers are underlined

Table 1
GenomeWalker primers

| Primer | Sequence (5'-3') | Direction | Position (nt) |
|--------|------------------------------|----------------------|---------------|
| JSR-2 | TCCGGGATGGGCGTGCCATTCTTGAT | Upstream | 952-979 |
| JSR-7 | ATGAAGGCCGGTATACCTTCTTTGCTC | Upstream | 482-510 |
| JSR-7N | CCGAAGACGGGCGTCGTCAGGGGGCTAT | Upstream | 413-441 |
| JSF-2 | ACAAATCCGGGTCTGCGGCGCTGATCTT | Downstream | 1105-1133 |
| JSF-6 | GGTGGCGATAATGGTGACGAAGAGAA | Downstream | 1403-1429 |
| JSF-6N | CCGTACCTTCCGTTTGGCGCTGGAAGA | Downstream | 1472-1499 |
| AP1 | GTAATACGACTCACTATAGGGC | GenomeWalker adapter | |
| AP2 | ACTATAGGGCACGCGTGGT | GenomeWalker adapter | |

The full-length *CYP51* gene was amplified using primers VN14DM.1 (5'-GGCCACCACCTCACCACCAAC-3') and VN14DM2 (5'-CTTTCACCTCTATCGCACTTCC-3') which anneal beyond the 3'-end of the inferred ATG start codon and beyond the 5'-end of the TAG stop codon, respectively. Primers were at final concentrations of 0.1 μ M. The annealing temperature was 62°C.

Sequence analysis of the *CYP51* gene from *V. nashicola* strains less sensitive to DMIs

Isolation of large amounts of pure genomic DNA from *V. nashicola* strains was impaired by the slow growth of the fungus *in vitro*. Therefore small amounts of crude genomic DNA were isolated by scraping mycelia directly from a culture on a PDA plate and freeze-drying the mycelia overnight. The freeze-dried mycelia were then crushed into a fine powder in a pestle and mortar with glass beads (212300 mesh) and the DNA extracted as described in Wheeler et al. (1995).

Primers VN14DM1 and VN133 (5'-CCGTTCTCC AAGCGCTGTTTC-3') were used to amplify a 761-bp fragment, encompassing the second conserved region (CR-2), of the *CYP51* gene from *V. nashicola* isolates NA-9, ND-10, ND-1, SE-9, ND-8, NH-1, OK-8 and 3-29-1 at an annealing temperature of 64°C and a primer concentration of 0.1 μ M. These fragments were cloned into the pGem-Easy vector and sequenced.

Results

Fungicide sensitivity assays

EC₅₀ values of fenarimol for strains shown in Table 2 were obtained in tests carried out in September 1999. All strains were originally considered less fenarimol-sensitive, as EC₅₀ values obtained from sensitivity tests performed directly after isolation were greater than 1 μ g/ml. Although, sensitivities to fenarimol increased for most strains with subsequent continuous sub-culturing and storage, all were still clearly different from the sensitive reference isolate JS-18.

Cloning of the *V. nashicola* *CYP51* gene

Amplifications with degenerate primers L1a and R1a produced a 1105-bp fragment, encoding a potential 352 amino acid polypeptide, interrupted by a putative 49-bp intron. The polypeptide displayed 58% identity and 71% similarity with the corresponding region of

the *CYP51* gene from *E. graminis* f. sp. *hordei* (Delye et al., 1998).

Amplifications towards the 5'-end of the *CYP51* gene, upstream of the known sequence produced a 200-bp fragment in the *Eco* *RV* library. Subsequent cloning and sequencing revealed this fragment to encode a 67 amino acid polypeptide homologous to the corresponding regions of CYP51s from related fungi. A 460-bp fragment from the *Dra* *I* library encoded, as shown by sequence homology, the remaining upstream 122 amino acids of the CYP51 interrupted by a putative 58-bp intron and extending beyond the ATG start codon. The sequencing of fragments from the remaining libraries was, therefore, deemed unnecessary. Amplifications towards the 3'-end, downstream of the known sequence produced an 1100-bp fragment in the *Eco* *RV* library. This fragment, encompassing the TAG stop codon, encoded the final 54 amino acids of the *V. nashicola* CYP51. Primers VN14DM.1 and VN14DM.2 annealing beyond the 3'-end of the start codon and the 5'-end of the stop codon, respectively, were used to amplify the full-length coding sequence of the *CYP51* gene. Sequencing of the full-length *CYP51* clone confirmed the previously inferred sequence (Fig. 1; GenBank accession: AJ314649).

Sequence analysis of the *CYP51* gene from *V. nashicola*

The predicted 526 amino acid CYP51, encoded by the 1578-bp *CYP51* gene coding sequence was aligned with representative CYP51 sequences other fungi. The strongest homology (94% identity and 96% similarity) was with *V. inaequalis* (Schnabel and Jones, 2000). Identities over 60% were seen with *Aspergillus fumigatus* (GenBank accession: AF338660), *Botryotinia fuckeliana* (GenBank accession: AF279912), *Monilinia fructicola* (GenBank accession: AF470621), *Tapesia acufiformis* (GenBank accession: AF208657), *T. yallundae* (Woods et al., 2001) and *E. graminis* f. sp. *hordei* (Delye et al., 1998). All *CYP51* gene sequences displayed greater than the 40% identity with that from *V. nashicola*, the homology required for two *CYP51* genes to belong to the same family.

The inferred CYP51 protein has a predicted molecular mass of 59.2 kDa. Six conserved domains typical of CYP51s, were identified upon alignment with fungal and mammalian CYP51s. The CR-2, CR-3 and CR-4 domains are believed to be involved in substrate recognition. The CR-6 domain, the cytochrome P450

| Strain | Year isolated | Prefecture | EC ₅₀ (μ g/ml) |
|-------------------|---------------|------------|--------------------------------|
| JS-18 (sensitive) | 1979 | Ibaraki | 0.040 |
| 3-29-1 | 1992 | Saga | 0.590 |
| NA-9 | 1997 | Ibaraki | 0.825 |
| OK-8 | 1997 | Ibaraki | 0.957 |
| NH-1 | 1997 | Ibaraki | 0.498 |
| ND-8 | 1998 | Ibaraki | 2.498 |
| ND-1 | 1998 | Ibaraki | 1.118 |
| SE-9 | 1998 | Ibaraki | 0.921 |
| ND-10 | 1998 | Ibaraki | 0.213 |

Table 2
Sensitivity of field strains of *Venturia nashicola* to fenarimol

haeme-binding site, contains the conserved cysteine residue that binds to the haeme iron atom at position 448. Hydrophobicity plotting (data not shown) indicated a putative transmembrane region in the N-terminal part of the sequence that may be involved in anchorage of the *V. nashicola* CYP51 to the outer endoplasmic reticulum membrane. As detected by similarity to the 5'-GTRNGT and 3'-YAG fungal intron consensus sequences, the *CYP51* gene from *V. nashicola* contains two introns. The first splices the highly conserved CR-1 region (K⁷⁹-H⁸⁰) and the second the CR-2 region (K¹⁴⁵-F¹⁴⁶).

Comparison of *CYP51* gene sequences from *V. nashicola* strains less sensitive to DMI fungicides

Sequence analysis of DMI-resistant strains of *P. italicum* (De Waard, 1996), *U. necator* (Delye et al., 1997b), *E. graminis* f. sp. *hordei* (Delye et al., 1998) and *C. albicans* (Sanglard et al., 1998) have correlated DMI-resistance with an alteration at position 133 (position based on predicted *V. nashicola* CYP51 sequence) in the CR-2 region of the *CYP51* gene (Tyr to Phe). Amplification and subsequent sequencing of the CR-2 region from *V. nashicola* strains shown to have lower sensitivities to DMIs (strains NA-9, ND-8, ND-1, SE-9, ND-10, NH-1, OK-8, and 3-29-1) revealed no sequence differences compared to the DMI-sensitive strain JS-18. Furthermore, analysis of the full length *CYP51* gene from the least fenarimol sensitive strain ND-8 (fenarimol EC₅₀ 2.498 µg/ml), showed no alterations in the predicted amino acid sequence when compared to strain JS-18.

Cross-resistance of *V. nashicola* strains to triazoles

All strains, isolated on the basis of fenarimol sensitivity, exhibit some degree of cross-resistance to triazole DMI fungicides (Table 3), with the exception of strains OK-8 and ND-1 to difenconazole, which tested sensitive.

Discussion

As with many filamentous fungi, no genomic library is currently available for *V. nashicola* and its slow growth *in vitro* impedes rapid isolation of large quantities of genomic DNA making construction of such a library a time-consuming process. Using degenerate primers, annealing to the highly conserved CR-2 and CR-6 regions, a large portion of the *CYP51* gene from *V. nashicola* was successfully amplified and sequenced.

A PCR-based adapter ligation technique (Siebert et al., 1995), the 'GenomeWalker kit', principally designed for the selective amplification of unknown sections of mammalian genes, was effectively adapted for fungal DNA, and used to rapidly obtain the remainder of the *CYP51* gene using only nanogram quantities of genomic DNA.

Comparison of CYP51 of *V. nashicola* with CYP51s from related fungi highlights six conserved domains (CR-regions) essential for enzymatic function. A mutation at Tyr¹³³ in the CR-2 region has been correlated with DMI-resistance in *E. graminis* f. sp. *hordei*, *U. necator*, *P. italicum* and *C. albicans*. Sequencing of the CR-2 region of all *V. nashicola* strains with reduced sensitivity to DMIs revealed no alterations when compared to the sensitive strain JS-18. Furthermore, sequencing of the full-length putative CYP51 protein from the least fenarimol sensitive strain ND-8, showed no changes in predicted amino acid sequence when compared to strain JS-18.

The absence of alterations in the *CYP51* genes from DMI-resistant isolates of *V. nashicola* suggests that an alternative mechanism is responsible for the altered sensitivity of these strains. Studies have associated four major mechanisms with DMI resistance: over-expression of the *CYP51* gene (Sanglard et al., 1995; Hamamoto et al., 2000; Schnabel and Jones, 2000), decreased intracellular accumulation of DMIs (De Waard and Van Nistelrooy, 1980), inactivation of the sterol $\Delta^{5(6)}$ desaturase (Watson et al., 1989; Joseph-Horne et al., 1995; Kelly et al., 1996) and mutations in the *CYP51* gene resulting in reduced affinity for DMIs (Sanglard et al., 1998; Marichal et al., 1999).

Inactivation of the sterol $\Delta^{5(6)}$ desaturase (Kelly et al., 1996) is an established mechanism of resistance in DMI-resistant clinical isolates of *C. albicans*. However, this mechanism has not been associated with DMI-resistance in field isolates of plant pathogens and would therefore be unlikely to be responsible to the resistant phenotype observed in the *V. nashicola* isolates in this study.

Up-regulation of a constitutively expressed ABC-transporter (*PMR1*) has been implicated as the primary determinant of DMI resistance in field isolates of *Penicillium digitatum* (Nakaune et al., 1998). This is the first report to suggest that an active toxicant efflux mechanism is responsible for DMI-resistance in field isolates of plant pathogens, although this mechanism

Table 3
Resistance factors (EC₅₀ resistant strain/EC₅₀ JS-18) of *Venturia nashicola* strains to a pyrimidine (fenarimol) and four triazole fungicides

| Strain | Resistance factor (RF) | | | | |
|--------|------------------------|---------------------------|-------------------------|-------------------------|--------------------------|
| | Fenarimol (pyrimidine) | Imibenconazole (triazole) | Myclobutanil (triazole) | Hexaconazole (triazole) | Difenconazole (triazole) |
| NA-9 | 46 | 195 | 30 | 29 | 31 |
| OK-8 | 12 | 9.5 | 18 | 3 | S |
| NH-1 | 10 | 2.7 | 14 | 12 | 4 |
| ND-8 | 62 | 0.5 | 2.8 | 57 | 5.8 |
| ND-1 | 10 | 195.5 | 22 | 7 | S |
| SE-9 | 25 | 354 | 72.8 | 31 | 9 |
| ND-10 | 16 | 10 | 9.5 | 5 | 9 |

is well established in clinical isolates of *C. albicans* (Sanglard et al., 1995) and laboratory isolates of *A. nidulans* (De Waard and Van Nistelrooy, 1980), *Botrytis cinerea* (Stehmann and De Waard, 1995) and *P. italicum* (De Waard and Van Nistelrooy, 1984). Furthermore, *PMRI* homologues were detected in several species of plant pathogenic fungi belonging to the classes *Ascomycetes*, *Basidiomycetes* and *Deuteromycetes*. *V. nashicola* strains are, therefore, likely to have a *PMRI* homologue, and, consequently, a potential ABC-transporter mediated DMI-resistance mechanism.

Over-expression of the *CYP51* gene has recently been correlated with resistance in strains of *V. inaequalis* isolated of the basis of myclobutanil sensitivity and *P. digitatum* strains resistant to the imidazole, triflumizole. In *V. inaequalis*, it was suggested a 533-bp insertion, containing three predicted promoter sequences in the 5'-flanking sequence could act as a transcriptional enhancer of *CYP51* expression, increasing intracellular target eburicol 14 α -demethylase levels, thereby conferring DMI-resistance (Schnabel and Jones, 2000). However, resistant strains without elevated *CYP51* levels were also identified, indicating the contribution of other mechanisms. The authors suggested that an energy-dependent efflux mechanism, implicated in penconazole resistance in *V. inaequalis* (Palani and Lalithakumari, 1999), might also be involved. A similar 126-bp insertion unit in the *P. digitatum CYP51* promoter, found tandemly repeated in resistant strains, is thought to be responsible for *CYP51* over-expression (Hamamoto et al., 2000). Additionally, in *P. digitatum* strains in which *CYP51* is over-expressed, disruption of the efflux pump encoding *PMRI* gene renders strains DMI sensitive. Suggesting that both mechanisms contribute to the final resistant phenotype (Nakaune et al., 1998).

The absence of substitutions in the predicted amino acid sequences of *CYP51*s from *V. nashicola* strains less sensitive to DMIs indicates that resistance in these strains is conferred either by enhanced toxicant efflux, mediated by the up-regulation of ABC-transporter homologues, or over-expression of the *CYP51* gene itself. The observed cross-resistance to triazoles (Table 3) supports this conclusion. Furthermore, in light of recent studies of DMI resistance in *V. inaequalis* and *P. digitatum*, and considering the substantial variation in resistance levels between strains, it may be a combination of these mechanisms that is responsible for the resistance phenotype in *V. nashicola*.

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