



A sterol 14 α -demethylase is required for conidiation, virulence and for mediating sensitivity to sterol demethylation inhibitors by the rice blast fungus *Magnaporthe oryzae*

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ABSTRACT

The *Magnaporthe oryzae* genome contains two homologous *CYP51* genes, *MoCYP51A* and *MoCYP51B*, that putatively encode sterol 14 α -demethylase enzymes. Targeted gene deletion mutants of *MoCYP51A* were morphologically indistinguishable from the isogenic wild type *M. oryzae* strain Guy11 in vegetative culture, but were impaired in both conidiation and virulence. Deletion of *MoCYP51B* did not result in any obvious phenotypic changes compared with Guy11. The *Amocyp51A* mutants were also highly sensitive to sterol demethylation inhibitor (DMI) fungicides, while *Amocyp51B* mutants were unchanged in their sensitivity to these fungicides. Expression of both *MoCYP51A* and *MoCYP51B* was significantly induced by exposure to DMI fungicides. Analysis of intracellular localization of *MoCyp51A* showed that *MoCyp51A* was mainly localized to the cytoplasm of hyphae and conidia. Taken together, our results indicate that *MoCYP51A* is required for efficient conidiogenesis, full virulence and for mediating DMI sensitivity by the rice blast fungus.

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1. Introduction

In fungi, ergosterol regulates cell membrane fluidity and permeability and is essential for cell survival (Rodriguez et al., 1985; Lees et al., 1995; Parks et al., 1995). Sterol 14 α -demethylase (Cyp51) is a key enzyme in the ergosterol biosynthesis pathway. Ergosterol depletion, coupled with the accumulation of methylated sterol precursors, has been proposed to affect both membrane integrity and the function of membrane-bound proteins, resulting in inhibition of fungal growth (Lees et al., 1995; Ji et al., 2000; Ruge et al., 2005). Cyp51 is therefore an important drug target for control of fungal infections. Demethylase inhibitor fungicides (DMIs) inhibit the ergosterol biosynthesis by inhibiting the demethylation of precursor sterols at position 14, in a reaction catalyzed by Cyp51. DMI fungicides have been widely used in medicine and agriculture and constitute some of the most successful fungicides. However, resistance to DMIs has now been reported in many fungal species (Hildebrand et al., 1988; Delye et al., 1998; Ma et al., 2006; Leroux et al., 2007; Gao et al., 2009; Sombardier et al., 2010), and the molecular mechanism leading to DMI resistance has been investigated in several plant pathogenic species, including *Blumeriella jaapii* (Ma et al., 2006; Proffer et al., 2006), *Blumeria graminis* (Delye

et al., 1998; Wyand and Brown, 2005), *Monilinia fructicola* (Luo and Schnabel, 2008), *Mycosphaerella graminicola* (Leroux et al., 2007; Brunner et al., 2008; Bean et al., 2009), *Mycosphaerella fijiensis* (Canales-Gutierrez et al., 2009), *Penicillium digitatum* (Nakaune et al., 1998; Hamamoto et al., 2001; Zhao et al., 2007), *Tapesia yallundae* (Wood et al., 2001), *Venturia inaequalis* (Schnabel and Jones, 2001), *Fusarium asiaticum* and *Fusarium graminearum* (Yin et al., 2009). Currently, the DMI fungicides, such as propiconazole, are widely used for the control of rice blast disease (Skamnioti and Gurr, 2009). The interaction between sterol 14 α -demethylase from *Magnaporthe oryzae* and azoles has also been explored (Yang et al., 2009), but the genes encoding sterol 14 α -demethylase have yet to be functionally characterized.

In several yeasts, including *Saccharomyces cerevisiae*, *Candida albicans* and *Cryptococcus neoformans*, the Cyp51-encoding gene, *ERG11*, has been shown to be required for aerobic viability in yeasts (Bard et al., 1993; Geber et al., 1995; Sanglard et al., 2003; Revankar et al., 2004). Some filamentous fungi, however, contain multiple *CYP51* genes, such as *F. graminearum* (3), *Aspergillus fumigatus* (2), *Aspergillus nidulans* (2), *Aspergillus oryzae* (3) (Ferreira et al., 2005; Lepesheva and Waterman, 2007). In *A. fumigatus*, for instance, there are two distinct but related Cyp51 proteins encoded by *CYP51A* and *CYP51B* (Mellado et al., 2001). Targeted gene disruption mutants of *CYP51A* have been shown to be morphologically indistinguishable from the wild type strain, and retained the ability

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to cause pulmonary disease in neutropenic mice, but mutants were, however, more susceptible to azole fungicides, such as ketoconazole and fluconazole (Mellado et al., 2005). However, the biological functions of *CYP51* genes in most fungi, including important plant pathogenic species such as the rice blast fungus *M. oryzae*, have not been investigated. The characterization of these genes may contribute to a better understanding of the mechanism of action of azoles and the potential for fungicide resistance to emerge.

In this report, we present the functional characterization of two homologous *CYP51* genes (*MoCYP51A* and *MoCYP51B*) in *M. oryzae*. Rice blast disease continues to be an important constraint on world wide rice production causing losses of between 10% and 30% of the annual rice harvest (Ou, 1985; Talbot, 2003; Skamnioti and Gurr, 2009). We reasoned that understanding the likely target of one of the most widely used fungicides would provide new insight into the capacity for resistance to emerge and would also allow the biological role of ergosterol biosynthesis to be explored during the infection cycle of *M. oryzae*. The rice blast fungus has proved to be highly amenable to molecular genetic manipulation, allowing the principal signaling pathways that regulate plant infection to be identified (Tucker and Talbot, 2001; Talbot, 2003; Wang et al., 2005; Wilson and Talbot, 2009). We used targeted gene deletion to generate null mutants of *MoCYP51A* or *MoCYP51B* which were morphologically indistinguishable from an isogenic wild type strain of the fungus. We show that Δ *mocyp51A* mutants are, however, impaired in their ability to produce asexual spores and to cause rice blast disease and show high sensitivity to sterol DMI fungicides. Moreover, expression of both *MoCYP51A* and *MoCYP51B* is significantly induced by exposure to exogenous DMI fungicides. These data indicate that efficient ergosterol biosynthesis is required for conidiation, virulence and for mediating sensitivity to triazoles in *M. oryzae*.

2. Materials and methods

2.1. Strains, culture conditions and DNA analysis

All mutants described in the present study were generated from the *M. oryzae* (Couch and Kohn, 2002) wild type strain Guy11, as shown in Table 1. Standard growth and storage procedures for fungal strains were performed, as described previously (Talbot et al., 1993). *Escherichia coli* strain DH-5 α was used for routine bacterial transformations and maintenance of all plasmids in this study. Southern blot analysis was performed by the digoxigenin (DIG) high prime DNA labeling and detection starter Kit I (Roche, Mannheim, Germany). General procedures for nucleic acid analysis followed standard protocols (Sambrook et al., 1989).

2.2. Construction of vectors and fungal transformation

For construction of the *MoCYP51A* gene replacement vector, a 1.4 kb *HPH* gene cassette, which encodes hygromycin phosphotrans-

ferase under control of the *A. nidulans* TrpC promoter (Carroll et al., 1994), was amplified with primers HPH-AF and HPH-AR (Table 2) using pCB1003 as a template and cloned into pGEM-T easy vectors (Promega, Madison, WI, USA) to produce pGEM-AHPH. Flanking sequences of about 1.3 kb at each border of the *MoCYP51A* gene locus were amplified using primer pairs of 1F/1R and 2F/2R (Table 2) and cloned sequentially into pGEM-T vectors to generate pGEM-AL and pGEM-AR, respectively. The pGEM-AL construct was digested with *Xba*I and *Sac*I and the released fragment inserted into the corresponding site of pGEM-AHPH to produce pGEM-ALH. The pGEM-ALH vector was digested with *Kpn*I and *Sac*I before being inserted into pGEM-AR to generate pCYP-AKO. For construction of the *MoCYP51B* gene replacement vector, a 1.4 kb *HPH* gene cassette was amplified with primers HPH-BF and HPH-BR (Table 2) using pCB1003 as a template to produce pGEM-BHPH. Flanking sequences of 1.4 kb were amplified from either side of the *MoCYP51B* gene using primer pairs of 4F/4R and 5F/5R (Table 2) and cloned sequentially into pGEM-T easy to generate pGEM-BL and pGEM-BR, respectively. The pGEM-BR construct was then digested with *Bam*HI and *Ap*I and the released fragment was inserted into the corresponding site of pGEM-BHPH to produce pGEM-BHR. The pGEM-BHR was digested with *Xba*I and *Sal*I and the released fragment was inserted into pGEM-BL to generate pCYP-BKO. The resulting construct vectors, pCYP-AKO and pCYP-BKO, were linearized and transformed into *M. oryzae* Guy11 to generate null mutants, as described previously (Talbot et al., 1993).

The *MoCYP51A* C-terminal GFP tagging vector, pCYP51A-GFP, was constructed by amplification of a 3.1 kb fragment including the 1.6 kb *CYP51A* gene-coding sequence and 1.5 kb of upstream promoter region using primers HB-AF and HB-AR (Table 2). The 1.5 kb green fluorescent protein (GFP)-encoding gene (*sGFP*) carrying the *A. nidulans* trpC terminator (Chiu et al., 1996) was amplified using primers GFP-AF and GFP-AR (Table 2). The 3.1 kb fragment was cloned into pGEM-T easy to generate pGM-CYPA. The 1.5 kb GFP allele was cloned to pGEM-T easy vector and digested with *Kpn*I and *Spe*I to release the GFP allele, which was inserted into

Table 2
Primers used in this study.

Name	Sequence (5' → 3') ^a
1F	TTGCCAGTCTGTAAATGCCT
1R	ATTCTAGACAGCATCGGGTAAGGAGAC
2F	CCGGTACCATGACAAAACGAAAAGCAT
2R	GTTCTCAATCTTCGGCCAGT
HPH-AF	CGTCTAGATATTGAAGGAGCATTTTGG
HPH-AR	TTGGTACCTTCGAAATCAGCTCTTGT
3F	GCCTTCTCTCCCATCTG
3R	TAGGCCGAGAGAACATAGAA
4F	AAGACAGAACCAGCAACCT
4R	ACTCTAGATGTAAGGGGGGAATCGACAA
5F	ACGGATCCTACATAGAGTTATAGAGCT
5R	CGAAGACAAGAAGACGCCT
HPH-BF	CGTCTAGATATTGAAGGAGCATTTTGG
HPH-BR	GGATCCTTCTCGAATCAGCTCTTGT
6F	GATGCCTTCTACCAGCTG
6R	TTAGCGCCTCTCCAGTAAA
HB-AF	AAGCTTGTGTTTACTATGAATATGT
HB-AR	GGTACCCGAGCTTTCGGGCTCGCTC
GFP-AF	CGGGGTACCATGGTGAAGGAGGCGAGGA
GFP-AR	GGACTAGTGTGGAGATGTGGAGTGGGCGCTT
RT-AF	ACTCGTCCATCCATCCATC
RT-AR	CCGTAGCCGTAATCGACAAT
RT-BF	GTTGTCCCTTCGTCAAGCTTC
RT-BR	GTAAGATGTTGCCGTACTTC
Tub-F	ACCCTCGCAACGGAAGG
Tub-R	GACGAAGTGGATGCTACGC
OE-F	CCCAAGCTTATGGCTTCTTCTTCCCAT
4432-F	ATGGGCCTTCTACAGGACAC

^a Introduced restriction sites are underlined. *Xba*I = TCTAGA, *Kpn*I = GGTACC, *Bam*HI = GGATCC, *Hind*III = AAGCTT, *Spe*I = ACTAGT, *Xho*I = CTCGAG.

Table 1
Strains of *M. oryzae* used in this study.

Strain	Brief description	Reference
Guy11	Wild type, MAT1-2	Leung et al. (1988)
A51	Δ <i>mocyp51A</i> mutant of Guy11	This study
A50	Ectopic transformant of Guy11 with integration of pCYPA-KO	This study
A53	Ectopic transformant of Guy11 with integration of pCYPA-KO	This study
B102	Δ <i>mocyp51B</i> mutant of Guy11	This study
B108	Δ <i>mocyp51B</i> mutant of Guy11	This study
B112	Ectopic transformant of Guy11 with integration of pCYPB-KO	This study
HBA-1	A51 transformed with pCYP51A-GFP	This study
HBA-2	A51 transformed with pCYP51A-GFP	This study

the corresponding site of pGM-CYPA to create pGM-CYPA-GFP. The pGM-CYPA-GFP vector was then checked by DNA sequencing to confirm correct orientation of the GFP insertion and that the gene was in-frame. Finally, pGM-CYPA-GFP was digested with *Hind*III and *Spe*I to release the *CYP51A*:GFP gene fusion, which was cloned into pCB1532, which contains the *ILV1* allele conferring resistance to sulfonyleurea (Sweigard et al., 1997), to give pCYP51A-GFP. The resulting plasmid pCYP51A-GFP was used to transform into *Δmocy51A* mutant A51. Fungal transformation was performed, as described previously (Talbot et al., 1993). Transformants were selected carrying a single insertion and complementation of the *Δmocy51A* mutant phenotypes was verified. GFP fluorescence was observed using a Leica TCS SP5 inverted confocal laser scanning microscope (Leica, Wetzlar, Germany).

2.3. Fungal development assays – fungal growth, sporulation, appressorium formation and genetic crosses

Vegetative growth of *M. oryzae* was assessed by measurement of colony diameter on plate cultures grown on complete medium (CM) at 25 °C for 10 days (Talbot et al., 1993). Conidial development was assessed by harvesting conidia from the surface of 10-day-old plate cultures and determining the concentration of the resulting conidial suspension using a haemocytometer. Appressorium development was evaluated by allowing conidia (at a concentration of 5×10^4 conidia ml⁻¹) to germinate on hydrophobic GelBond films incubated in a humid environment at 25 °C. Each test was repeated three times. Fertility assays were carried out by pairing Guy11 (*MAT1-2*), *Δmocy51A* and *Δmocy51B* mutants with standard tester strain TH3 (*MAT1-1*) on oatmeal agar (OMA) plates (Talbot et al., 1993). The plate cultures were incubated at 25 °C with a 12-h photo-phase until the colonies contacted each other, and then placed under continuous white fluorescent light at 18 °C for 3–4 weeks. The junctions between the individuals were examined for the capacity to form perithecia. The mating test was repeated three times.

2.4. Pathogenicity assay

Plant infection assays were carried out using a seedling spray-inoculation method described previously (Talbot et al., 1993; Wang et al., 2007). Conidia harvested from 10-day-old CM cultures were diluted in 0.2% gelatin to 5×10^4 or 1×10^4 conidia ml⁻¹ for plant infections using 14-day-old rice cv. CO-39. Conidial suspensions were spray-inoculated using an artist's airbrush onto plant seedlings. Rice seedlings were incubated in plastic bags for 24 h to maintain high humidity and then transferred to controlled environment chambers at 25 °C and 90% relative humidity with illumination. Rice seedlings were incubated for 5 days for disease symptom development. Pathogenicity tests were repeated three times.

2.5. Quantitative RT-PCR

Total RNA was extracted from mycelium of *M. oryzae* Guy11, A51 and B102 (Table 1) using SV Total RNA Isolation System (Z3100, Promega) according to the manufacturer's instructions. Total RNA was utilized for synthesis of first strand cDNA using the PrimeScript™ 1st Strand cDNA Synthesis Kit (D6110A, TaKaRa, Tokyo). The resulting cDNA was used as a template for quantitative reverse transcription-PCR (qRT-PCR). qRT-PCR was performed with a SYBR Green Real-time PCR Master Mix Kit (QPK-201, TOYOBO, Osaka, Japan) using an iCycler iQ™ Multicolor Real-Time PCR Detection System (Bio-Rad, Munich, Germany). All qRT-PCR reactions were conducted in triplicate for each cDNA sample and were repeated three times. The primer pairs of RT-AF/RT-AR and

RT-BF/RT-BR (Table 2) were used to determine the relative expression of *MoCYP51A* and *MoCYP51B*, respectively. *M. oryzae* beta-tubulin gene (MGG_00604.6) amplified with the primer pairs of Tub-F/Tub-R was used as an endogenous control. The abundance of the gene transcripts was calculated relative to this control using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

For qRT-PCR assessment of the expression of *MoCYP51A* and *MoCYP51B* after being treated with DMIs, the wild type strain Guy11 was grown in CM liquid medium (150 ml per flask) for 2 days. Before mycelium was harvested for RNA extraction, each flask was treated with 1.09 μg ml⁻¹ propiconazole, 0.31 μg ml⁻¹ tebuconazole and 0.08 μg ml⁻¹ prochloraz for 6 h, respectively. The selected concentrations were EC₅₀ values (the effective concentration that results in 50% mycelial growth inhibition) for each fungicide (Table 3). Addition of 200 μl acetone was used as a control.

2.6. Sensitivity of *M. oryzae* strains to DMI and other fungicides

Sensitivity of *M. oryzae* strains to DMI and other group of fungicides was tested using a mycelial inhibition method. A 5-mm mycelial plug was taken from the edge of a 7-day-old colony and placed at the centre of a CM agar plate containing 2.5 μg ml⁻¹ triadimefon, 0.75 μg ml⁻¹ prochloraz, 0.5 μg ml⁻¹ tebuconazole, 1.0 μg ml⁻¹ propiconazole, 1.0 μg ml⁻¹ diniconazole, 1.0 μg ml⁻¹ difenoconazole, 1.0 μg ml⁻¹ flutriafol, 0.2 μg ml⁻¹ carbendazim, or 0.5 μg ml⁻¹ iprodione. Three replicates for each treatment were carried out. The results were examined and photographs taken after incubation at 25 °C for 12 days. The experiments were repeated three times.

For EC₅₀ assays, a 5-mm mycelial plug was taken from the edge of a 7-day-old colony and placed at the centre of a CM plate amended with the single compound tebuconazole at 0, 0.1, 0.3, 0.5, 0.6, 0.75, 1, and 5 μg ml⁻¹, propiconazole at 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 μg ml⁻¹ or prochloraz at 0, 0.01, 0.05, 0.1, 0.3, 0.5, 1, and 3 μg ml⁻¹. Three replicates for each concentration were used for each strain. After incubation at 25 °C for 10 days, colony diameters were measured. EC₅₀ values were calculated with the Data Processing System (DPS) computer program (Hangzhou Reifeng Information Technology Ltd., Hangzhou, China). The experiment was performed twice.

3. Results

3.1. Phylogenetic analysis of the two putative genes encoding sterol 14 α -demethylase in *M. oryzae*

Two putative genes encoding sterol 14 α -demethylase were identified from the *M. oryzae* genome (Dean et al., 2005) by similarity to known *CYP51* genes (Mellado et al., 2001; Ferreira et al., 2005) and named *MoCYP51A* and *MoCYP51B*, respectively. To confirm the position and size of the introns of the two genes, reverse transcription-PCR (RT-PCR) was carried out using the primer pairs of OE-F/HB-AR (Table 2) for *MoCYP51A* and 4432-F/6R (Table 2) for

Table 3
Sensitivity of *Magnaporthe* strains to tebuconazole, propiconazole and prochloraz.

Strain	EC ₅₀ (μg ml ⁻¹) ^a		
	Tebuconazole	Propiconazole	Prochloraz
Guy11	0.309 ± 0.054	1.092 ± 0.002	0.078 ± 0.005
A51 (<i>Δmocy51A</i>)	0.014 ± 0.002	0.001 ± 0.001	0.034 ± 0.004
B102 (<i>Δmocy51B</i>)	0.301 ± 0.009	1.003 ± 0.005	0.083 ± 0.011

^a EC₅₀ = effective concentration that results in 50% mycelial growth inhibition on CM. Data are means (± standard error) of two experiments.

MoCYP51B, and the cDNA sequences and genome sequences were compared. These confirmed that *MoCYP51A* has an open reading frame of 1548 bp without introns, and putatively encodes a 515 aa protein, *MoCYP51B* has an open reading frame of 1710 bp interrupted by a single intron of 129 bp and putatively encodes a 526 aa protein. The two genes, *MoCYP51A* and *MoCYP51B*, are 100% identical to the sequences of ID numbers MGG_04628.6 and MGG_04432.6 (Broad Institute) predicted by automated annotation of the *M. oryzae* genome sequence, respectively.

An amino acid alignment analysis showed that the predicted *M. oryzae* MoCyp51A protein showed 49.6% amino acid identity with MoCyp51B. The alignment results of MoCyp51A and MoCyp51B, compared to several fungal Cyp51 proteins showed that MoCyp51A was 64.4%, 60.8%, 61.8% and 57.2% amino acid identical to Cyp51A proteins from *Gibberella zeae* (Gene Bank: ACL93391), *A. fumigatus* (ACF17706), *Aspergillus flavus* (XP_002375123) and *Aspergillus clavatus* (XP_001271579), respectively, and that MoCyp51B was 71.6%, 59.8%, 49.25% and 53.7% amino acid identical to Cyp51B proteins from *G. zeae* (ACL93392), *A. fumigatus* (AAK73660), *A. flavus* (XP_002379130) and *A. clavatus* (XP_001273214), respectively. Comparisons of MoCyp51A and MoCyp51B proteins with the Cyp51 proteins from other fungal species showed that the percentage identity at the amino acid level was high enough to consider MoCyp51A and MoCyp51B to be members of the fungal Cyp51 family. Phylogenetic analysis of the putative Cyp51 genes from several fungal species is shown in Supplementary Fig. 1, indicating that MoCyp51A is most closely related to the *G. zeae* Cyp51A, and MoCyp51B is most closely related to the *Neurospora crassa* Cyp51 (XP_964049) and the *G. zeae* Cyp51B. Both MoCyp51A and MoCyp51B are genetically distant to the putative sterol 14 α -demethylase genes from *S. cerevisiae* (AAA34546) and *Ustilago maydis* (CAA88176).

3.2. Targeted gene deletion of *MoCYP51A* and *MoCYP51B*

To characterize *MoCYP51A* and *MoCYP51B*, we performed a targeted gene deletion of both genes using the gene replacement vectors pCYP-A-KO and pCYPB-KO (Fig. 1A and B), respectively. The gene replacement events of *MoCYP51A* were analyzed by PCR amplification with primers 3F and 3R (Table 2) from transformants and also by Southern blot analysis. The resulting $\Delta moCyp51A$ null mutant, A51 (Table 1), was selected based on the absence of hybridization to the deleted fragment probe (a gene fragment amplified with primers 3F and 3R using genomic DNA from Guy11 as a template), coupled with the presence of a single copy of the *HPH* gene cassette (Fig. 1C). Two of the transformants resulting from ectopically integrated pCYP-A-KO, A50 and A53, were used as control strains in subsequent analysis (Fig. 1C). Similarly, the gene replacement of *MoCYP51B* was analyzed by PCR amplification with primers of the replaced *MoCYP51B* sequence 6F and 6R (Table 2) from transformants. The resulting $\Delta moCyp51B$ null mutants, B102 and B108 (Table 1), were selected based on the absence of hybridization to the deleted fragment probe using the presence of a single copy of the *HPH* gene cassette (Fig. 1D). One of the transformants resulting from ectopically integrated pCYPB-KO, B112, was subsequently used as a control strain (Fig. 1D).

3.3. *MoCYP51A* is required for conidiation and virulence in *M. oryzae*

Targeted gene deletion mutants of either *MoCYP51A* or *MoCYP51B* were morphologically indistinguishable from the wild type strain Guy11 on CM medium (Fig. 2A) and still formed normal melanized appressoria on hydrophobic surfaces (Supplementary Fig. 2A), indicating that *MoCYP51A* or *MoCYP51B* are not necessary

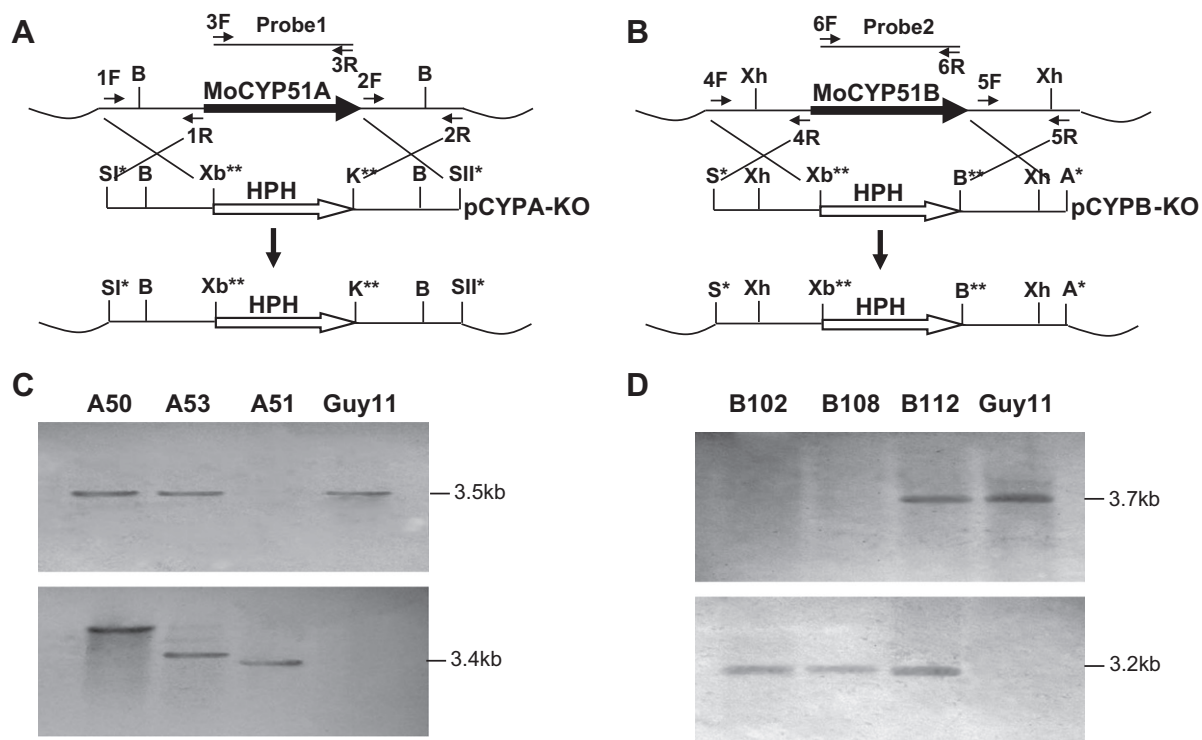


Fig. 1. Targeted gene deletion of *MoCYP51A* and *MoCYP51B*. A and B. Construction of *MoCYP51A* and *MoCYP51B* gene replacement vectors. The pCYP-A-KO and pCYP-B-KO vectors were linearized and transformed into *Magnaporthe oryzae* Guy11, respectively. B = BamHI; SI = SacI; Xb = XbaI; K = KpnI; SII = SacII; Xh = XhoI; S = SalI; A = ApaI. * represents restriction sites from pGEM-T vector. ** represents introduced restriction sites. C. $\Delta moCyp51A$ mutants confirmed by Southern blot analysis. Genomic DNAs from the wild type strain Guy11 and three transformants were digested with BamHI and probed with a 1.5 kb fragment (top) amplified with the primers 3F and 4R (Table 2) and a 1.4 kb *HPH* cassette (bottom), respectively. Lanes left to right: A50, ectopic transformant; A51, $\Delta moCyp51A$ mutant; A53, ectopic transformant; Guy11, the wild type strain. D. $\Delta moCyp51B$ mutants confirmed by Southern blot analysis. Genomic DNAs from Guy11 and three transformants were digested with XhoI and probed with a 1.7 kb fragment (top) amplified with the primers 6F and 6R (Table 2) and a 1.4 kb *HPH* cassette (bottom), respectively. Lane left to right: B102, $\Delta moCyp51B$ mutant; B108, $\Delta moCyp51B$ mutant; B112, ectopic transformant; Guy11, the wild type strain.

for vegetative growth and appressorium formation. In order to determine the role of *MoCYP51A* or *MoCYP51B* in sexual reproduction, the wild type Guy11 (*MAT1-2*), Δ *mocyp51A* mutant A51 and Δ *mocyp51B* mutant B102 were crossed with a standard tester TH3 (*MAT1-1*) isolate of *M. oryzae* to allow perithecia production. After 3 weeks of incubation, we observed numerous perithecia at the junctions between mated individuals (Supplementary Fig. 2B), indicating that *MoCYP51A* and *MoCYP51B* are also not essential for sexual reproduction in *M. oryzae*.

Interestingly, however, deletion of *MoCYP51A* caused defects in conidiation and virulence (Fig. 2B and C). The Δ *mocyp51A* mutant A51 displayed significantly reduced conidiation ($P < 0.01$), producing $2.05 \pm 3.11 \times 10^5$ conidia per plate after a 10-day incubation on CM at 25 °C, compared with the Δ *mocyp51B* mutant B102 which produced $2.83 \pm 1.26 \times 10^5$ and Guy11 which produced $3.30 \pm 4.69 \times 10^5$ per plate (Fig. 2B). To assess the ability to cause rice blast disease, conidia of the Δ *mocyp51A* mutant A51 and Δ *mocyp51B* mutant B102 at a concentration 5×10^4 conidia ml⁻¹ were used to inoculate leaves of the susceptible rice cv CO-39, respectively. The Δ *mocyp51B* mutant B102 was fully pathogenic on rice leaves, while the Δ *mocyp51A* mutant A51 was impaired in virulence on rice (Fig. 2C). Consistently, a quantitative analysis using conidial suspensions at a concentration 1×10^4 conidia ml⁻¹ to spray rice plants showed that the number of disease lesions was reduced significantly

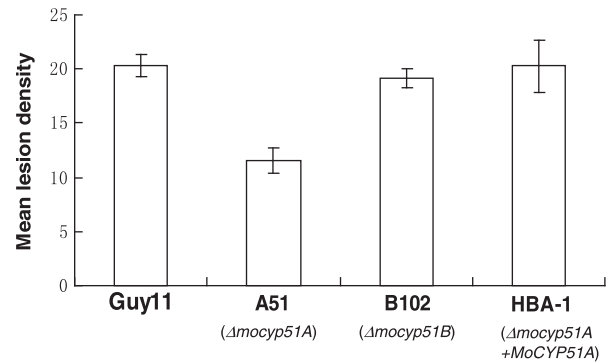


Fig. 3. Quantitative analysis of rice infection assays. Leaves of 14-day-old rice seedlings (cv. CO-39) were sprayed with conidia of Guy11, Δ *mocyp51A* mutant A51, Δ *mocyp51B* mutant B102 and complementation strain HBA-1 at a concentration 1×10^4 conidia ml⁻¹, respectively. Mean lesion density values recorded from 5 cm sections from 15 of the most infected leaves. Error bars represent the standard deviation.

on leaves inoculated with A51 compared with those infected with Guy11 and B102 ($P < 0.01$) (Fig. 3). However, the lesions caused by the Δ *mocyp51A* mutant were spreading blast lesions, suggesting that the fungus was able to grow within plant tissue following

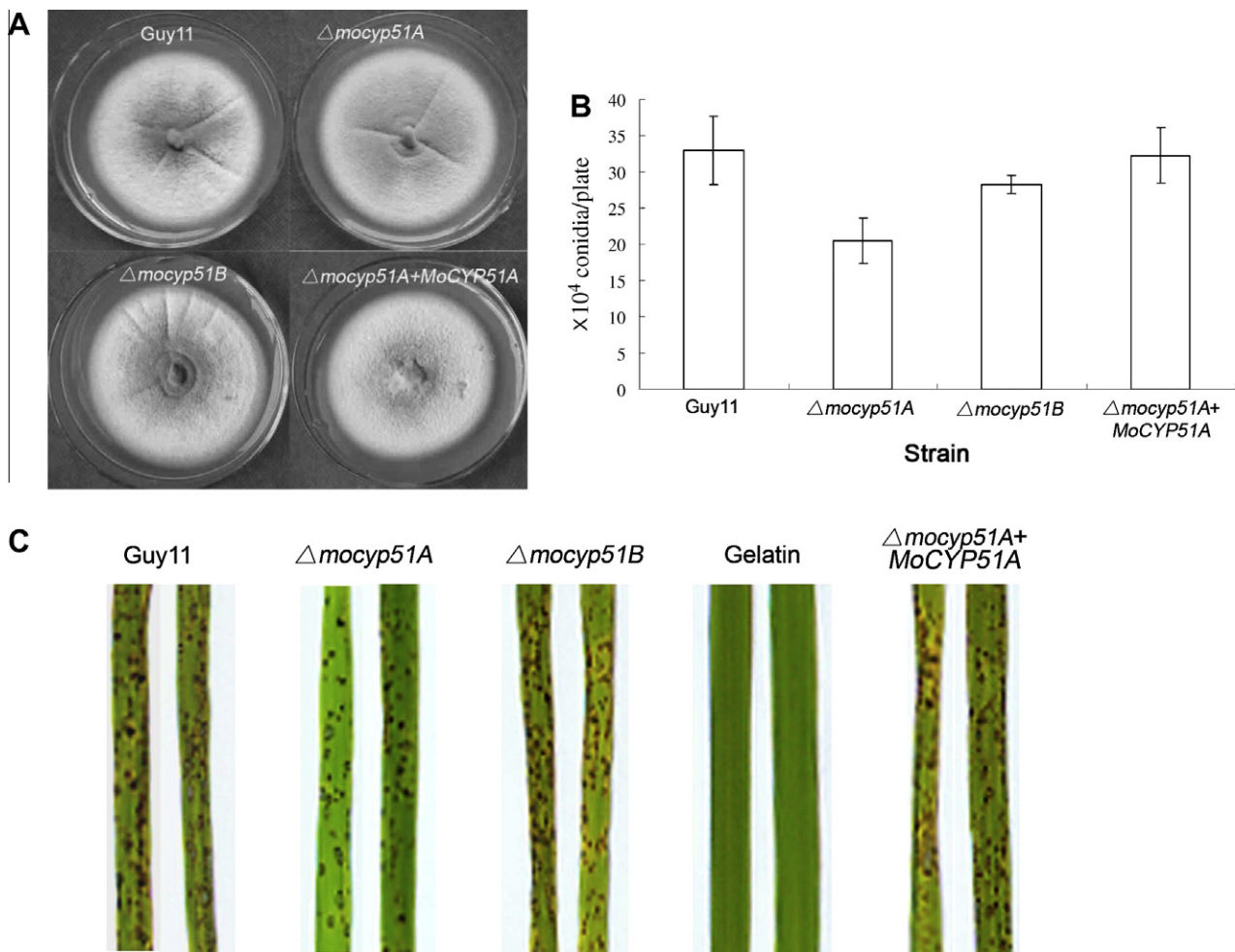


Fig. 2. Phenotypic analysis of Δ *mocyp51A* and Δ *mocyp51B* mutants. A. Colonies of the wild type strain Guy11, Δ *mocyp51A* mutant A51, Δ *mocyp51B* mutant B102 and complementation transformant HBA-1 (Δ *mocyp51A* + *MoCYP51A*). Photographs were taken after incubation on CM at 25 °C for 10 days. B. Bar chart showing the conidial development of different *Magnaporthe* strains. Conidiation was measured by counting the number of conidia harvested from a 10-day-old CM plate. Each bar represents the mean of three independent replications of the experiment with three plates per experiment, and the error bars represent the standard deviations. C. Leaves of 14-day-old rice seedlings (cv. CO-39) were sprayed with conidia of Guy11, Δ *mocyp51A* mutant A51, Δ *mocyp51B* mutant B102 and complementation strain HBA-1 at a concentration 5×10^4 conidia ml⁻¹, respectively. Solution of 0.2% gelatin was used as the control. Photographs were taken at 5 days after inoculation.

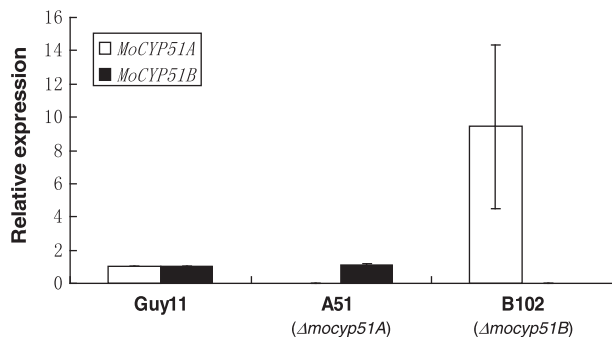


Fig. 4. Relative expression of *MoCYP51A* in $\Delta mocyp51B$ and *MoCYP51B* in $\Delta mocyp51A$. Measurements of gene transcripts obtained by quantitative RT-PCR analysis were normalized to β -tubulin and expressed as relative values, with 1 corresponding to Guy11. Error bars represent the standard deviation. Expression of *MoCYP51A* was significantly induced in $\Delta mocyp51B$ mutant B102, but expression of *MoCYP51B* was unchanged in $\Delta mocyp51A$ mutant A51.

infection, but that the frequency of plant infection was reduced. These results suggest that *M. oryzae MoCYP51A* is required for efficient conidiation and full virulence.

3.4. Expression of *MoCYP51A* is induced by deletion of *MoCYP51B*

To determine the relative expression of *MoCYP51B* and *MoCYP51A* in the $\Delta mocyp51A$ mutant A51 and $\Delta mocyp51B$ mutant B102, we performed qRT-PCR using the primer pairs of RT-AF/RT-AR and RT-BF/RT-BR (Table 2). As expected, the expression of *MoCYP51A* and *MoCYP51B* was not detected in the A51 mutant

and B102 mutant, respectively, due to the targeted gene deletion (Fig. 4). The expression of *MoCYP51A* was, however, significantly up-regulated (~9-fold) in $\Delta mocyp51B$ mutant B102, while the expression of *MoCYP51B* in $\Delta mocyp51A$ mutant A51 was similar to that observed in the wild type strain Guy11 (Fig. 4). The results suggest that expression of *MoCYP51A* is induced by the absence of *MoCYP51B* expression in *M. oryzae*, but not vice versa.

3.5. Deletion of *MoCYP51A* increases sensitivity of *M. oryzae* to DMI fungicides

Seven DMI fungicides were used to test the sensitivity of *M. oryzae* strains. The $\Delta mocyp51A$ mutant A51 was very sensitive to $1.0 \mu\text{g ml}^{-1}$ propiconazole, $0.75 \mu\text{g ml}^{-1}$ prochloraz, $2.5 \mu\text{g ml}^{-1}$ triadimefon or $0.5 \mu\text{g ml}^{-1}$ tebuconazole. By contrast, the $\Delta mocyp51B$ mutant B102 did not show hyper-sensitivity to these DMI fungicides, as shown in Fig. 5A. Similar levels of sensitivity of $\Delta mocyp51A$ or $\Delta mocyp51B$ were observed to other DMI fungicides, such as diniconazole, difenconazole or flutriafol (data not shown). EC_{50} values were calculated for each DMI fungicides as shown in Table 3. The EC_{50} values of tebuconazole, propiconazole and prochloraz to $\Delta mocyp51A$ were significantly lower than those to Guy11 and $\Delta mocyp51B$, respectively. In addition, sensitivity of $\Delta mocyp51A$ and $\Delta mocyp51B$ to carbendazim and iprodione was similar to that of Guy11 (data not shown), suggesting that deletion of *MoCYP51A* increases sensitivity of *M. oryzae* only to DMIs, but not to other groups of fungicides. These results indicate that deletion of *MoCYP51A*, but not *MoCYP51B*, significantly increased sensitivity to DMI fungicides in *M. oryzae*.

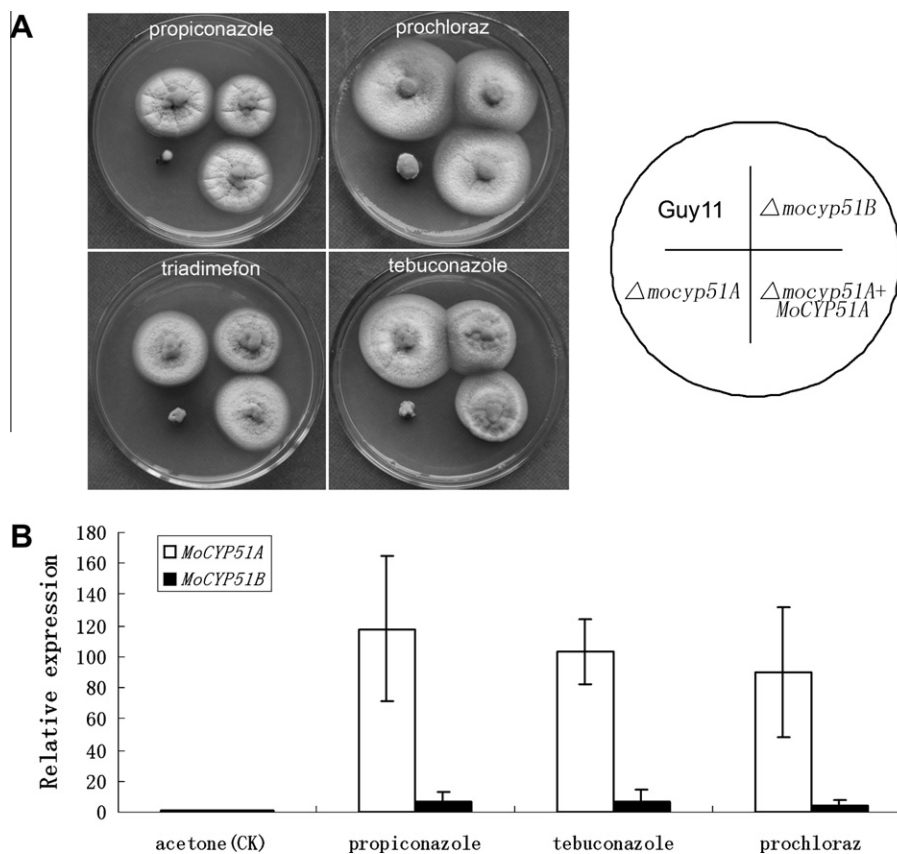


Fig. 5. Deletion of *MoCYP51A* increases sensitivity to DMIs and expression of *MoCYP51A* and *MoCYP51B* is induced by DMIs. A. Deletion of *MoCYP51A* increases sensitivity to DMIs. CM plates contained $1.0 \mu\text{g ml}^{-1}$ propiconazole, $0.75 \mu\text{g ml}^{-1}$ prochloraz, $2.5 \mu\text{g ml}^{-1}$ triadimefon, $0.5 \mu\text{g ml}^{-1}$ tebuconazole were used to detect sensitivity of *M. oryzae* to DMI fungicides. Photographs were taken after incubation at 25°C for 12 days. B. Expression of *MoCYP51A* and *MoCYP51B* is induced by DMIs. Measurements of gene transcripts obtained by quantitative RT-PCR analysis were normalized to β -tubulin and expressed as relative values, with 1 corresponding to Guy11. Error bars represent the standard deviation. DMI fungicides include $1.09 \mu\text{g ml}^{-1}$ propiconazole, $0.31 \mu\text{g ml}^{-1}$ tebuconazole and $0.08 \mu\text{g ml}^{-1}$ prochloraz.

In order to determine whether expression of the *CYP51* genes of *M. oryzae* was induced by exposure to DMI fungicides, we carried out qRT-PCR experiments to examine the expression of *MoCYP51A* and *MoCYP51B* after incubation in the presence of DMI fungicides, including propiconazole, tebuconazole and prochloraz. The results showed that the expression of both genes was significantly induced by these DMIs ($P < 0.01$), and that *MoCYP51A* was more highly expressed (117, 102 and 90-fold up-regulated in response to propiconazole, tebuconazole and prochloraz, respectively) than *MoCYP51B* (6, 7 and 4-fold upregulation) as shown in Fig. 5B.

3.6. *MoCYP51A* is expressed at low levels during vegetative growth, conidiation and appressorium formation

In order to investigate the temporal and spatial pattern of *MoCYP51A* expression in *M. oryzae*, a 1.5 kb promoter fragment upstream of the gene and the entire *MoCYP51A* protein-coding sequence was fused in-frame to *GFP*, and introduced into $\Delta moCyp51A$ mutant A51. Transformants carrying a single integration of the plasmid were selected by DNA gel blot analysis. An independent single pCYP51A-GFP insertion event was observed in two transformants, HBA-1 and HBA-2, respectively (see Table 1). HBA-1 was selected for further analysis by confocal laser scanning microscopy. Very faint GFP fluorescence was observed in the cytoplasm of hyphae, while relatively brighter GFP fluorescence was observed in the cytoplasm of conidia (Fig. 6A). Similarly, we also observed very faint GFP fluorescence in cytoplasm of germ tubes and appressoria during appressorium development (Fig. 6A). These results indicate that *MoCYP51A* is expressed at low levels during vegetative growth, conidiation and appressorium formation, and that *MoCyp51A* may mainly localize to cytoplasm.

Further experiments showed that bright GFP fluorescence was observed both in hyphae and conidia of HBA-1 harvested from CM cultures by adding exogenous $1.0 \mu\text{g ml}^{-1}$ propiconazole (Fig. 6B), indicating the expression of *MoCYP51A-GFP* was induced by the supplement of the fungicide. This was consistent with the result analyzed by qRT-PCR as shown in Fig. 5B. In this investigation, GFP distributed unevenly in cytoplasm of young mycelia and conidia (data not shown), while large brightly fluorescing globular structures was observed in mature mycelia and conidia as shown in Fig. 6B. Similar GFP fluorescent patterns were observed from the other strain HBA-2 by the treatment of propiconazole (data not shown). To verify the vacuolar localization of *MoCyp51A* under the induction with the DMI fungicide, we performed neutral red (NR) staining. The dye is used for plants or fungi as an acidotropic stain accumulating in vacuoles (Weber et al., 2001; Dubrovsky et al., 2006). The merged image of GFP and NR staining showed that *MoCyp51A-GFP* mainly localizes to the vacuoles of hyphae (Fig. 7). These results are consistent with movement of the *MoCyp51A* protein move into vacuoles once its concentration increases within cells, perhaps associated with greater turnover of the protein.

3.7. Re-introduction of *MoCYP51A* into the $\Delta moCyp51A$ mutants restores all phenotypes

To ensure that the phenotypic differences observed in $\Delta moCyp51A$ mutants were associated with the gene replacement event, we carried out phenotypic analysis of the complementation transformants HBA-1 and HBA-2 (Table 1). The GFP-expressing transformants exhibited full virulence to rice by spray-inoculation assays (Figs. 2C and 3). The phenotypes of $\Delta moCyp51A$ mutant A51, including conidiation and sensitivity to DMIs, were fully complemented by re-introduction of the gene (Figs. 2B and 5A). Taken together, we conclude that *MoCYP51A* is required for efficient

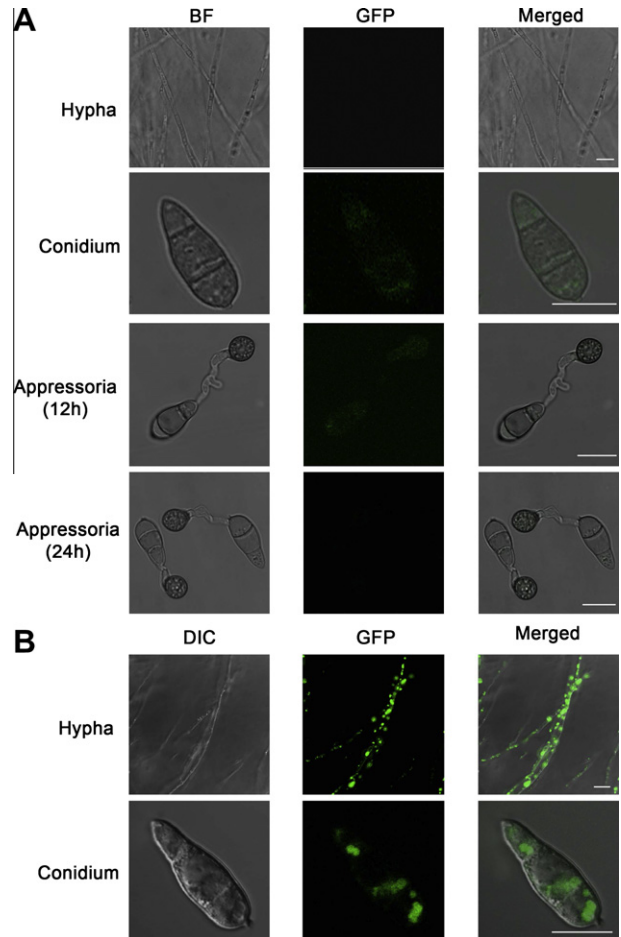


Fig. 6. Temporal and spatial expression of *M. oryzae MoCYP51A*. Transformant HBA-1 was used for the GFP observation by a confocal laser scanning microscope. Bar = 10 μm . A. Mycelia and conidia of HBA-1 were harvested from 10-day-old cultures on CM plates. Very faint GFP fluorescence was in the cytoplasm of hyphae, conidia and appressoria. BF = bright field. B. Mycelia and conidia of HBA-1 were harvested from 10-day-old cultures on CM plates containing $1.0 \mu\text{g ml}^{-1}$ propiconazole. Bright GFP fluorescence was unevenly distributed in the cells of hyphae and conidia. DIC = differential interference contrast.

conidiation, full virulence and for mediating sensitivity to DMI fungicides in *M. oryzae*.

4. Discussion

Ergosterol is a ubiquitous component of the plasma membrane of fungi, where it plays an important structural role to regulate membrane fluidity and permeability and indirectly to modulate the activity and distribution of integral membrane proteins, including enzymes, ion channels and components of signal transduction pathways (Lees et al., 1995; Lepesheva and Waterman, 2007). Sterol 14 α -demethylase (Cyp51) is the key enzyme in ergosterol biosynthesis. In fungi, only a small sub-set of *CYP51* genes have been functionally characterized to date. In *A. fumigatus*, targeted gene disruption mutants of *CYP51A* retained the ability to cause pulmonary disease in neutropenic mice (Mellado et al., 2005). In this report, two homologous *CYP51* genes (*MoCYP51A* and *MoCYP51B*) encoding sterol 14 α -demethylase were identified and functionally analyzed in the rice blast fungus *M. oryzae*. Deletion of *MoCYP51A* did not cause defects in vegetative growth and appressorium formation (Fig. 2A; Supplementary Fig. 2A), but did lead to a reduction in conidiation and impaired virulence on rice (Fig. 2B and C). By contrast, no observable phenotypes were

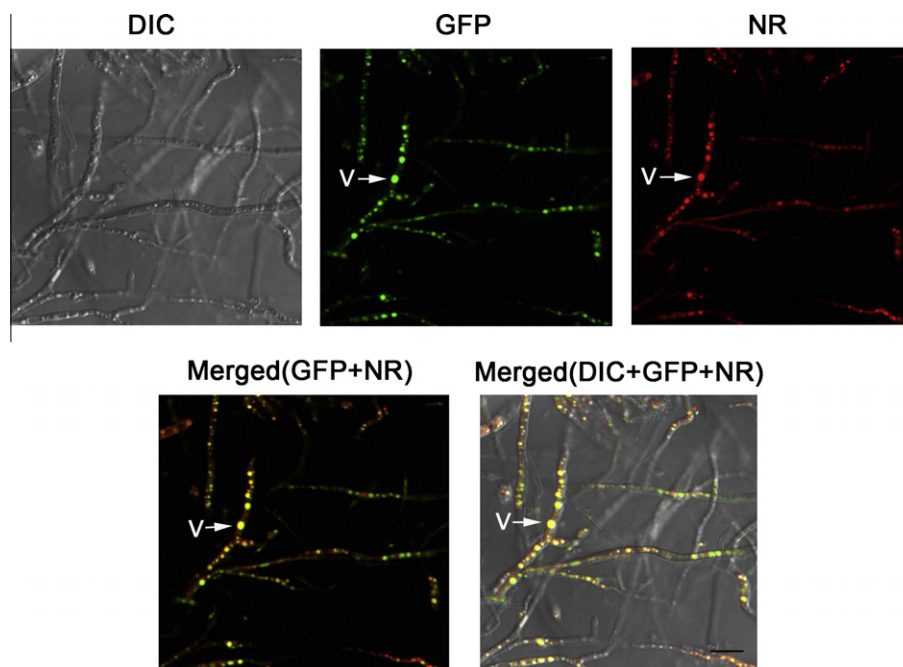


Fig. 7. MoCyp51A-GFP mainly localizes in vacuoles under the induction of propiconazole. Mycelia of HBA-1 were harvested from 10-day-old cultures on CM plates containing $1.0 \mu\text{g ml}^{-1}$ propiconazole and stained with neutral red (NR), an acidotropic stain accumulating in vacuoles. The merged image of GFP and NR staining showed that MoCyp51A-GFP mainly localizes to the vacuoles of hyphae. DIC = differential interference contrast. V = vacuole. Bar = $10 \mu\text{m}$.

detected following targeted gene deletion of *MoCYP51B*. The fact that we were unable to obtain $\Delta\text{mocyp51A } \Delta\text{mocyp51B}$ double mutants from more than 500 transformants (data not shown) suggests that loss of sterol 14α -demethylase activity may be lethal in *M. oryzae*. It also implies that the two Cyp51-encoding genes have partially overlapping functions, but that neither gene is essential on its own for cellular viability. Interestingly, MoCyp51A plays a far more significant role during plant infection, consistent with stage-specific functions and developmental regulation of each enzyme.

Recently, it has been reported that *A. fumigatus CYP51B* expression levels were not significantly induced by the disruption of *CYP51A* (Mellado et al., 2005). However, the expression of *A. fumigatus CYP51A* in *CYP51B* gene deletion mutants has not been investigated. In *F. graminearum*, the causal agent of *Fusarium* head blight (FHB) disease of wheat and barley, there are three homologous *CYP51* genes (*CYP51A*, *B*, and *C*) encoding sterol 14α -demethylase, and the expression of *CYP51A* and *CYP51C* was increased significantly in the deletion mutants of *CYP51B* (Liu and Ma, unpublished data). Consistent with these studies, we found that deletion of *MoCYP51B* led to a significant increase in expression of *MoCYP51A* in *M. oryzae*, but, conversely, the expression of *MoCYP51B* was not changed in $\Delta\text{mocyp51A}$ mutant (Fig. 4). Deletion of *MoCYP51B* may therefore be partially compensated by the induction of expression of *MoCYP51A* in *M. oryzae*, perhaps explaining why there was no distinct mutant phenotype associated with its loss of function. To explore whether the phenotypes of $\Delta\text{mocyp51A}$ mutants could be complemented by over-expression of *MoCYP51B*, we developed strains in which *MoCYP51B* was over-expressed in the $\Delta\text{mocyp51A}$ mutant A51 and $\Delta\text{mocyp51B}$ mutant B102, respectively (Supplementary Fig. 3). Quantitative RT-PCR analysis showed high level *MoCYP51B* expression in these strains (Supplementary Fig. 3A). Surprisingly, the hyper-sensitivity to DMIs shown by $\Delta\text{mocyp51A}$ mutants could not be complemented by over-expression of *MoCYP51B* (Supplementary Fig. 3B). Conversely, over-expression of *MoCYP51B* in $\Delta\text{mocyp51B}$ mutant did increase resistance to DMIs (Supplementary Fig. 3B). The detailed regulatory mechanisms con-

trolling the expression of the two *CYP51* genes in *M. oryzae* remain unknown, but clearly there must be a mechanism by which *MoCYP51A* is induced in expression by detecting a fall in Cyp51 activity in the cell due to loss of the *MoCYP51B* gene. Moreover, it is clear that *MoCYP51B* is regulated in a distinct manner that is not sensitive to the levels of Cyp51 activity.

Deletion mutants of *MoCYP51A* in *M. oryzae* increased sensitivity to all DMI fungicides tested, while the deletion mutants of *MoCYP51B* did not change sensitivity to DMIs. Very recently, it has been found that different DMI fungicides can target different Cyp51 proteins in *F. graminearum*, resulting in the development of a new potential mixture of triadimefon and tebuconazole with a synergistic, additive, effect on inhibition of the fungal growth (Liu and Ma, unpublished data). It will therefore be valuable to screen for compounds, to which $\Delta\text{mocyp51B}$ mutants show increased sensitivity. This may prove illuminating in developing new fungicide mixtures to control rice blast disease more durably in the field.

In mammalian species, the *CYP51* gene is ubiquitously expressed with the highest levels observed in the testis, ovary, adrenal gland, prostate, liver, kidney and lung (Seliskar and Rozman, 2007). The highest level of *CYP51* expression is, for instance, observed in testis (germ cells) of different mammals, including human, pig and rat (Seliskar and Rozman, 2007). Generally, Cyp51 resides in the endoplasmic reticulum (ER), but during male germ cell development, the enzyme is also detected in the Golgi, on the acrosome and acrosomal membranes during all phases of acrosome development, indicating that intracellular transport of Cyp51 occurs from the ER to the Golgi and then to the acrosome (Cotman et al., 2004; Seliskar and Rozman, 2007). In *S. cerevisiae*, indirect immunofluorescence microscopy revealed that Cyp51 is localized exclusively to ER (Homma et al., 2000). However, localization of Cyp51 proteins has not previously been investigated in filamentous fungi. In this study, MoCyp51A was fused to GFP at its C-terminus and the resulting fusion protein expressed in *M. oryzae* during vegetative growth and plant infection-related morphogenesis. MoCyp51A was mainly localized to the cytoplasm in mycelium

and conidia (Fig. 6A). However, owing to low levels of expression of MoCYP51A under normal conditions, it is still not clear whether MoCyp51A also localizes to membranes or cellular organelles such as the ER. Expression of MoCYP51A was, however, highly induced by exposure to DMI fungicides, such as propiconazole (Figs. 5B and 6B). Under these conditions MoCyp51A-GFP mainly localized to vacuoles of mycelia and conidia, although GFP was also observed in the cytoplasm (Figs. 6B and 7). Similar MoCyp51A-GFP localization patterns were, however, also observed when the native promoter of MoCYP51A was replaced by high level constitutive *A. nidulans* TrpC promoter (data not shown). It is therefore possible that the localization of MoCyp51A-GFP to vacuoles is related to its high level expression and that the protein moves into vacuoles for degradation once the concentration of MoCyp51A accumulates within cells.

In summary, MoCYP51A encodes one of two likely sterol 14 α -demethylases in *M. oryzae* and plays an important function during plant infection and sporulation by the fungus.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2010.09.005.

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