A sterol C-14 reductase encoded by *FgERG24B* is responsible for the intrinsic resistance of *Fusarium graminearum* to amine fungicides

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Fusarium graminearum, the causal agent of wheat head blight, shows intrinsic resistance to amine fungicides. It is commonly accepted that the amines target sterol C-14 reductase and sterol Δ^8 – Δ^7 isomerase of ergosterol biosynthesis, encoded by the genes *ERG24* and *ERG2*, respectively. Analysis of the genome sequence of *F. graminearum* revealed that the fungus contains two paralogous *FgERG24* genes (*FgERG24A* and *FgERG24B*), which are homologous to the *ERG24* of *Saccharomyces cerevisiae*. In this study, we disrupted *FgERG24A* and *FgERG24B* in *F. graminearum*. Compared to the wild-type strain HN9-1, *FgERG24A* and *FgERG24B* deletion mutants did not show recognizable phenotypic changes in mycelial growth on potato dextrose agar or in virulence on wheat heads. HPLC analysis showed that the amount of ergosterol in *FgERG24A* or *FgERG24B* deletion mutants was not significantly different from that in the wild-type strain. These results indicate that neither of the two genes is essential for growth, pathogenicity or ergosterol biosynthesis in *F. graminearum*. *FgERG24B* deletion mutants exhibited significantly increased sensitivity to amine fungicides, including tridemorph, fenpropidin and spiroxamine, but not to non-amine fungicides. In contrast, *FgERG24A* deletion mutants did not show changed sensitivity to any amine tested. The resistance of the *FgERG24B* deletion mutant to amines was restored by genetic complementation of the mutant with wild-type *FgERG24B*. These results indicate that *FgERG24B* controls the intrinsic resistance of *F. graminearum* to amines. The finding of this study provides new insights into amine resistance in filamentous fungi.

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INTRODUCTION

The filamentous ascomycete *Fusarium graminearum* Schwabe [teleomorph *Gibberella zeae* (Schweinitz) Petch] is the primary causal agent of Fusarium head blight (FHB), which is a devastating disease of wheat and other cereal crops (Goswami & Kistler, 2004; Trail, 2009; Xu & Nicholson, 2009). This disease can cause huge economic losses worldwide in epidemic years (Starkey *et al.*, 2007). Besides the yield losses, infested cereals often are contaminated with deoxynivalenol (DON) and other mycotoxins which are harmful to human and animals (Walter *et al.*, 2010).

Since most wheat cultivars planted in the world are susceptible to *Fusarium* spp. (Snijders, 2004; Yin *et al.*, 2009), the primary method for management of FHB is by fungicide application (Blandino *et al.*, 2006; Lechoczki-Krsjak *et al.*, 2008). At present, sterol biosynthesis

inhibitors (SBIs) are the major fungicides used to control FHB worldwide. The SBIs target different enzymes involved in ergosterol biosynthesis, which is necessary for fungal development and survival (Alcazar-Fuoli *et al.*, 2008). To date, according to statistics by the FRAC (Fungicide Resistance Action Committee), more than 44 SBIs have been developed successfully and have made great contributions to world agricultural production. Based on the target enzymes, SBIs can be divided into four groups: demethylation inhibitors (DMIs), amines (formerly called morpholines), hydroxylanilides and squalene-epoxidase inhibitors. The former three groups have been used extensively for control of plant diseases.

Amine fungicides, including morpholines, piperidines and spiroketalamines, are very effective against *Blumeria graminis* (powdery mildew) and *Puccinia* spp. (cereal rust) on cereals (Baloch & Mercer, 1987; Mercer, 1991; Zocco *et al.*, 2008).

Biochemical studies on the mode of action of amine fungicides have shown that they inhibit ergosterol biosynthesis at the step subsequent to C-14 demethylation

Abbreviations: DMI, demethylation inhibitor; FHB, Fusarium head blight; SBI, sterol biosynthesis inhibitor.

Two supplementary figures are available with the online version of this paper.

and/or the step of Δ^8 – Δ^7 isomerization in several fungal species (Baloch *et al.*, 1984; Baloch & Mercer, 1987; Debieu *et al.*, 1992, 1998, 2000; Lorenz & Parks, 1991; Schneegurt & Henry, 1992; Ziogas *et al.*, 1991). Inhibition of the sterol C-14 reductase and sterol Δ^8 – Δ^7 isomerase leads to deficiency of ergosterol, but accumulation of fecosterol or Δ^8 -sterols, which are toxic to fungal cells (Lorenz & Parks, 1991; Marcireau *et al.*, 1990; Steel *et al.*, 1989).

Sterol C-14 reductase, encoded by *ERG24*, catalyses the demethylation at C-14 to yield 4,4-dimethyl zymosterol, which is essential for sterol biosynthesis (Lees *et al.*, 1999). This enzyme has been shown to be conserved among eukaryotes (Benveniste 1986; Taton *et al.*, 1989). In *Candida albicans*, the *ERG24* gene is involved in virulence and sensitivity to amines (Jia *et al.*, 2002). However, the phenotypic effect of *ERG24* in *Saccharomyces cerevisiae* depends on genetic background, type of medium, and CaCl_2 concentration (Shah Alam Bhuiyan *et al.*, 2007; Crowley *et al.*, 1998; Lorenz & Parks, 1992; Marcireau *et al.*, 1992). In *Arabidopsis*, *ERG24* is required for cell division and expansion, and is involved in proper organization of the embryo (Schrick *et al.*, 2000). In filamentous fungi, however, information on function of the *ERG24* gene is limited. Most fungal species contain only a single *ERG24* gene; however, genomic studies revealed the existence of two different but closely related *ERG24* genes in the genomes of several *Aspergillus* species and *F. graminearum* (Da Silva Ferreira *et al.*, 2005), but the functions of these paralogous *ERG24* genes remain undiscovered.

Fusarium species are pathogens of many important agricultural crops, and are often strong mycotoxin producers. Among the genus *Fusarium*, some species are very sensitive to fenpropimorph and fenpropidin, whereas others are tolerant (Debieu *et al.*, 1992, 2000). In a preliminary study, we found that all *F. graminearum* isolates collected from different locations in China appeared to be intrinsically resistant to amines. Thus, to gain basic information about the molecular mechanism of *F. graminearum* resistance to amines, the objective of this study was to investigate the functions of *FgERG24* genes in *F. graminearum* using a gene-disruption strategy.

METHODS

Strains and culture medium. *Fusarium graminearum* strain HN9-1, used as a wild-type parent, was collected from Henan province, China. The wild-type strain and transformants were grown on potato dextrose agar (PDA) (200 g potato, 20 g glucose, 20 g agar, and 1 l water) for mycelial growth tests, and in mung bean liquid medium (MBL; 40 g mung beans boiled in 1 l water for 20 min, and then filtered through cheesecloth) (Bai & Shaner, 1996) for sporulation tests.

Expression analysis and intron verification of *FgERG24* genes in *F. graminearum*. Through a BLASTP search at the *Fusarium* Comparative Database site (available at http://www.broadinstitute.org/annotation/genome/fusarium_graminearum/MultiHome.html) using the amino acid sequence of *S. cerevisiae* sterol C-14 reductase Erg24 (Lorenz & Parks, 1992) as a query, we identified two Erg24-homologous

sequences in *F. graminearum*, FGSG_06606.3 and FGSG_02346.3; these were named *FgERG24A* and *FgERG24B*, respectively.

Transcripts of the two *FgERG24* genes in *F. graminearum* were detected using genomic DNA and cDNA as templates with primer pairs A-spanintron-F + A-spanintron-R and B-spanintron-F + B-spanintron-R (Table 1), respectively. Each primer pair spans an intron in *FgERG24A* or *FgERG24B*. RNA was extracted from mycelia of wild-type HN9-1 using TaKaRa RNAiso Reagent (TaKaRa Biotech Co.), and used for reverse transcription with the primer oligo(dT)₁₈ using a RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas Life Sciences). PCR amplifications were conducted using the following parameters: initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 53 °C for 45 s, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. PCR products were separated on 1% agarose gels in Tris/acetate (TAE) buffer and photographed after staining with ethidium bromide.

To validate the existence of introns in *FgERG24* genes, PCR amplifications with primer pairs A-all-F + A-all-R and B-all-F + B-all-R (Table 1) were performed for *FgERG24A* and *FgERG24B* using genomic DNA and cDNA as templates, respectively. The resultant PCR product was purified, cloned and sequenced.

Construction of *FgERG24* gene deletion mutants. *FgERG24A* deletion vector pCA-erg24A-Del was constructed by inserting two flanking sequences of *FgERG24A* gene into the left and right sides of *HPH* (hygromycin-resistance gene) in the pBS-HPH1 vector (Liu *et al.*, 2007). Briefly, by using primer pair A1 + A2 (Table 1), a 761 bp upstream flanking sequence fragment of *FgERG24A* was amplified from HN9-1 genomic DNA, and was inserted into the *Xho*I–*Sal*I sites of the pBS-HPH1 vector to generate plasmid pBS-erg24A-up. Subsequently, a 1019 bp downstream flanking sequence fragment of *FgERG24A* amplified from HN9-1 genomic DNA using the primers A3 + A4 (Table 1) was inserted into *Hind*III–*Sac*I sites of the pBS-erg24A-up vector to generate plasmid pBS-erg24A-UD. Finally, the 3283 bp fragment containing the erg24A-upstream-HPH-erg24A-downstream cassette was obtained by digestion of plasmid pBS-erg24A-UD with *Xho*I and *Sac*I, and ligated into the *Xho*I–*Sac*I sites of pCAMBIA 1300 (CAMBIA, Canberra, Australia). Similar to pCA-erg24A-Del, we constructed the *FgERG24B* deletion vector pCA-erg24B-Del (see Figs 3a and 4a). The gene deletion vectors pCA-erg24A-Del and pCA-erg24B-Del were separately transformed into *Agrobacterium tumefaciens* strain C₅₈C₁.

The *A. tumefaciens*-mediated fungal transformation (ATMT) was performed as described by Mullins *et al.* (2001). PDA medium supplemented with hygromycin (100 µg ml^{−1}) was used to select transformants. After single-spore isolation, transformants were kept at 4 °C for subsequent experiments.

Genetic complementation of *FgERG24B* deletion mutant. To confirm that the phenotypic changes of the *FgERG24B* deletion mutant were due to disruption of the gene, the mutant was complemented with the wild-type *FgERG24B*. A 3022 bp sequence including the full-length *FgERG24B* gene with 1000 bp upstream and 469 bp terminator region was amplified using primer pair B-com-F + B-com-R (Table 1) from genomic DNA of wild-type strain HN9-1. This fragment was cloned into the *Xba*I–*Sal*I sites of pCAMBIA1300 to generate the complement plasmid pCA-erg24B-COM. Transformation of Δ erg24B-2 with the full-length *FgERG24B* gene was conducted as described above except that spiroxamine (10 µg ml^{−1}) was used as a selection agent.

Mycelial growth, sporulation, and pathogenicity assays. Mycelial growth assays for wild-type and gene deletion strains were performed on PDA plates; the colony diameter of each strain was measured every 24 h for 5 days. For sporulation assays, six 5 mm

Table 1. Oligonucleotide primers used in this study and their relevant characteristics

Primer	Sequence (5'–3')	Relevant characteristics
A1	ATCTCGAGTCTTGTGAAACCAACGGGT	PCR primers for amplification of the upstream fragment of <i>FgERG24A</i>
A2	ATGTCGACAAGACGCGGTAGAGGATTAAA	
A3	ATAAGCTTACCAACCCGAACGACCCCTA	
A4	ATGAGCTCTGTCTTGTCTCTCCCCATTCC	PCR primers for amplification of the downstream fragment of <i>FgERG24A</i>
B1	ATCTCGAGACCATTCTAGGCTTGGACTA	
B2	ATGTCGACTAGCGGCATTGGGTTGAGT	
B3	ATAAGCTTTTCATTTGGGACCCATCG	PCR primers for amplification of the downstream fragment of <i>FgERG24B</i>
B4	ATGAGCTCGAATCGGAGGCATTTGATTG	
A5	ACTATGGCCGTCAAGCCAAA	
A6	ACTTGCGGTGACACTTTTCGT	PCR primers for the identification of <i>FgERG24A</i> disruption mutants
B5	AAACTGTTCCGTCGATCTGA	
B6	TGTCAAGAGCTTTGTTCCGC	
A-all-F	ATGGCCGTCAAGCCAAAACAG	PCR primers for amplification of full cDNA sequence of <i>FgERG24A</i>
A-all-R	TTAGTAAAGCCGGGAACGA	
B-all-F	ATGAAGGCTCCCGACTCTCCTC	
B-all-R	TTAGTAAACACCAGGCCAAA	PCR primers for amplification of full cDNA sequence of <i>FgERG24B</i>
B-com-F	TCTAGACGTGGAAGAGACGAGAATGCT	
B-com-R	GTCGACGAATCGGAGGCATTTGATTG	
A-spanintron-F	GTCAAGCCAAAACAGCCTCCT	PCR primers for the detection of <i>FgERG24A</i> transcription
A-spanintron-R	ATCGGTATTTGAGTCTACCGC	
B-spanintron-F	TCTCCTCGCTATGAATTTGG	
B-spanintron-R	CGCAGTTTTGTACCAAGAACA	PCR primers for the detection of <i>FgERG24B</i> transcription
A-RT-F	TTTACTCTTGCTGTCTCGCC	
A-RT-R	ACCGATGAGAGGGATCTCAAT	
B-RT-F	CCTCTCTGTCTATCCCG	Quantitative real-time PCR primers for analysis of <i>FgERG24A</i> expression
B-RT-R	CCTGCAGGAAGAATAACGT	
F-actin-F	ATCCACGTCAACCACTTTCAA	
F-actin-R	TGCTTGAGATCCACATTTG	Quantitative real-time PCR primers for analysis of <i>FgERG24B</i> expression
		PCR primers for amplification of the reference actin gene in quantitative real-time PCR assays

mycelial plugs of each strain taken from the edge of a 3-day-old colony were inoculated in a 50 ml flask containing 10 ml MBL medium. The flasks were incubated at 25 °C for 7 days in a shaker (180 r.p.m.). The number of conidia in the broth was counted using a haemocytometer. The experiment was repeated three times. Pathogenicity tests were performed by the single-floret injection method as described by Wu *et al.* (2005). Briefly, 10 µl of conidial suspension (10^5 spores ml⁻¹) was injected into a single floret in the central section spikelet of single flowering wheat heads of susceptible cultivar Jimai22 at early anthesis. Ten spikes were used for each strain. Infected spikelets in each inoculated wheat head were recorded 14 days after inoculation. The experiment was repeated three times.

Fungicide sensitivity test. For this test, the amine fungicides tridemorph, fenpropidin and spiroxamine, the DMI fungicides triadimefon and tebuconazole, and the dicarboximide fungicide iprodione, were added to PDA, after sterilization, at the concentrations described in Results. These fungicides were kindly provided by the Institute of Zhejiang Chemical Industry or by the Institute for the Control of Agrochemicals, Ministry of Agriculture (ICAMA), China. To gain a precise sensitivity level of each strain to amine fungicides, the fungicide concentration that resulted in 50 % mycelial growth inhibition (EC₅₀) was also determined. Briefly, a mycelial plug (5 mm in diameter) was taken from the periphery of a 3-day-old colony and placed on the centre of a PDA plate containing tridemorph, fenpropidin or

Fig. 1. Phylogenetic tree generated by using the neighbour-joining method with MEGA 4.1 software on the basis of deduced amino acid sequences of FgErg24A and FgErg24B from *F. graminearum* strain HN9-1 (boxed), and those from *Arabidopsis thaliana* (AtErg24, GenBank accession no. NP_566975.1), *Aspergillus fumigatus* (AfErg24A, EDP55659.1; AfErg24B, EDP55908.1), *Aspergillus nidulans* (AnErg24, CBF74719.1), *Botrytis cinerea* (BcErg24, XP_001544681.1), *Candida albicans* (CaErg24, EEQ47440.1), *Candida glabrata* (CgErg24, XP_447380.1), *Fusarium oxysporum* (FoErg24A, FOXG_02348T0; FoErg24B, FOXG_10757T0), *Fusarium verticillioides* (FvErg24A, FVEG_05520T0; FvErg24B, FVEG_09413T0), *Homo sapiens* (HsErg24, NP_003264.2), *Magnaporthe oryzae* (MoErg24, XP_362903.2), *Mus musculus* (MmErg24, NP_082730.2), *Neurospora crassa* (NcErg24, XP_956111.1), *Saccharomyces cerevisiae* (ScErg24, NP_014119.1), *Schizosaccharomyces pombe* (SpErg24, SPBC16G5.18), *Sclerotinia sclerotiorum* (SsErg24, XP_001591653.1), *Verticillium alboatrum* (VaErg24, VDBG_02555) and *Verticillium dahliae* (VdErg24, VDAG_09749). The scale bar indicates genetic difference.

spiroxamine at 0, 0.001, 0.005, 0.025, 0.125, 0.625, 1, 5, 10, 20 or 40 $\mu\text{g ml}^{-1}$. Three replicates for each concentration were used for each strain. After incubation at 25 °C for 3 days, colony diameter on each plate was measured, with the original mycelial plug diameter (5 mm) subtracted from each measurement. EC_{50} values were calculated with the Data Processing System (DPS) computer program (Hangzhou Reifeng Information Technology). The experiment was repeated twice.

Ergosterol analysis. To extract ergosterol, 100 μl of a spore suspension (10^5 spores ml^{-1}) of each strain was inoculated in

Fig. 2. PCR amplifications of *FgERG24A* and *FgERG24B* fragments using genomic DNA and cDNA extracted from mycelium of the parental strain HN9-1 as template.

100 ml yeast glucose medium (YG: yeast 5 g, glucose 15 g, 1 l). After incubation at 25 °C for 2 days, mycelia were harvested by filtration and washed three times with sterilized water. The resulting mycelia were incubated in 100 ml sterilized water amended with tridemorph, fenpropidin or spiroxamine at 10, 14 and 17 $\mu\text{g ml}^{-1}$ (EC_{50} values of the parental strain to these fungicides), respectively, at 25 °C for 3 days. Then the mycelia were harvested, and dried at 60 °C for 3 h. Mycelia incubated in sterilized water were used as the control. Ergosterol was extracted from dried mycelia using a previously published protocol (Davey *et al.*, 2009). The experiment was performed twice. Ergosterol content was quantified using a Prominence SPD-20A HPLC system (Shimadzu). Briefly, ergosterol was separated at 25 °C on a Hypersil BDS C18 250 nm $\times$ 4.6 nm, 5 μm analytical column using 100% methanol (chromatography pure) as mobile phase. The detection wavelength for ergosterol was 282 nm, and the identification of ergosterol was based on retention time and co-chromatography of a commercial ergosterol standard (Sigma).

Additionally, to extract total sterols from each strain, 100 μl of a spore suspension (10^5 conidia ml^{-1}) was incubated in 100 ml YG at 25 °C for 2 days. Mycelia were harvested and washed three times with sterile water. The resulting mycelia were dried at 60 °C for 3 h, and dried mycelia were ground into powder. The ground mycelia (0.1 g) were added into 3 ml of 25% alcoholic potassium hydroxide solution (25 g KOH and 36 ml sterile distilled water, brought to 100 ml with 100% ethanol) and mixed by vortexing for 1 min. After incubation at 80 °C for 1 h, the mixture was allowed to cool down at room temperature. Sterols were then extracted by addition of 1 ml sterilized water and 3 ml n-heptane, followed by vigorous vortexing for 3 min. Subsequently, the heptane layer was transferred to a clean borosilicate glass tube and stored at -20 °C for up to 24 h. Finally, for each sample, 4 ml sterol extract was diluted fivefold in 100% ethanol and the total sterol content determined using a SpectraMax Plus 384 spectrophotometer (Molecular Devices) with a spectral range from 190 to 450 nm.

Analysis of *FgERG24* gene expression. To extract total RNA, mycelia of the parental strain HN9-1 and deletion mutants were inoculated into potato dextrose broth medium, and cultured for 2 days at 25 °C in a shaker. Before mycelia were harvested for RNA extraction, each culture was treated for 6 h with the amine fungicide tridemorph, fenpropidin or spiroxamine, at 10, 14 and 17 $\mu\text{g ml}^{-1}$, respectively. A culture without fungicide treatment was used as the

Fig. 3. Generation and identification of a *FgERG24A* gene deletion mutant. (a) Gene replacement strategy for the *FgERG24A* gene. The hygromycin-resistance cassette (HPH) is denoted by the large black arrow. Primer-binding sites are indicated by arrows (see Table 1 for the primer sequences). (b) Reverse transcription PCR analysis of *FgERG24A* expression in the parental strain (HN9-1) and gene disruption mutant Δ erg24A-1 using cDNA as template. NCK is a negative control without template cDNA in the PCR amplification. (c) Southern blot hybridization analysis of HN9-1 and Δ erg24A-1 using a 761 bp *FgERG24A* fragment as a probe. A genomic DNA preparation of each strain was digested with *EcoRI*.

control. Total RNA was extracted from mycelia of each sample using the TaKaRa RNAiso Reagent, and 10 μ g of each RNA sample was used for reverse transcription with the oligo(dT)₁₈ primer using a RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas). Expression of *FgERG24A* and *FgERG24B* was determined by quantitative real-time PCR (RT-PCR) with primer pair A-RT-F + A-RT-R and B-RT-F + B-RT-R, respectively. The RT-PCR amplifications were performed in a DNA Engine Opticons 4 System (MJ Research) using SYBR Green I fluorescent dye detection. Amplifications were conducted in a 20 μ l volume containing 10 μ l iQ SYBR Green Supermix (Bio-Rad), 1 μ l reverse transcription product and 0.5 μ l of each primer (Table 1). There were two replicates for each sample. The real-time PCR amplifications were performed with the following parameters: an initial preheat at 95 °C

for 2 min, followed by 35 cycles at 95 °C for 15 s, 53 °C for 20 s, 72 °C for 20 s, and 75 °C for 3 s in order to quantify the fluorescence at a temperature above the denaturation of primer-dimers. Once the amplifications were completed, melting curves were obtained to identify PCR products. For each sample, PCR amplifications with primer pair F-actin-F + F-actin-R (Table 1) for the quantification of expression of the actin gene were performed as a reference. The experiment was repeated three times. The expressions of *FgERG24A* and *-B* in the wild-type strain and deletion mutants Δ erg24A-1 and Δ erg24B-2 were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001).

Standard molecular methods. Fungal genomic DNA was extracted using a previously published protocol (McDonald & Martinez, 1990).

Fig. 4. Generation and identification of a *FgERG24B* gene deletion mutant. (a) Gene replacement strategy for the *FgERG24B* gene. Primer-binding sites are indicated by arrows (see Table 1 for the primer sequences). (b) Reverse transcription PCR analysis of *FgERG24B* expression in the parental strain (HN9-1), gene disruption mutant Δ erg24B-2, and *FgERG24B* complemented strain Δ erg24B-com11 using cDNA as template. NCK is a negative control without template cDNA in the PCR amplification. (c) Southern blot hybridization analysis of HN9-1, Δ erg24B-2 and Δ erg24B-com11 using a 607 bp *FgERG24B* fragment as a probe. A genomic DNA preparation of each strain was digested with *EcoRI*.

Plasmid DNA was isolated using a plasmid miniprep purification kit (BioDev Co.). Southern blot hybridization analysis of the *FgERG24* gene in each strain of *F. graminearum* was performed using probes as indicated in Figs 3(a) and 4(a). Each probe was labelled with digoxigenin (DIG) using a High Prime DNA labelling and detection starter kit II according to the manufacturer's instructions (Roche Diagnostics).

RESULTS

Expression and structure of *FgERG24A* and *-B*

The nucleotide sequences of *FgERG24A* and *FgERG24B* were 1565 and 1553 bp in length, and encode 485 and 481 amino acids, respectively. *FgERG24A* has two predicted introns. One is 57 bp in length, located between nucleotides 63 and 119. The other is 50 bp in length, located from 425 to 474 bp of the nucleotide sequence. The deduced 481 amino acid sequence of the *FgERG24B* product is derived from one open reading frame split by two introns. Sequencing of the 1458 and 1446 bp cDNA of *FgERG24A* and *FgERG24B*, respectively, verified the existence of these introns.

Phylogeny construction was performed by MEGA 4.1 (<http://www.megasoftware.net/>) using the neighbour-joining method (Kumar *et al.*, 2008). The sequences of the *FgERG24* genes are homologous to those from yeasts, other filamentous fungi, mammals and plants. As shown in Fig. 1, the deduced amino acid sequence of FgErg24A from the wild-type strain HN9-1 showed 66.12% identity to NcErg24 of *Neurospora crassa* (NcErg24, XP_956111.1). The FgErg24B from HN9-1 showed 69.23% similarity to the MoErg24 of *Magnaporthe oryzae* (XP_362903.2).

Reverse transcription PCR generated the expected fragments from the cDNA of the wild-type strain and confirmed that both *FgERG24* genes are expressed in *F. graminearum* (Fig. 2). Additionally, real-time quantitative PCR assays showed that the expression level of *FgERG24A* was not significantly different from that of *FgERG24B* in mycelia or conidia of *F. graminearum* (data not shown).

Disruption and complementation of *FgERG24* genes

For a detailed functional analysis of the two *FgERG24* genes, targeted disruption mutants were generated using a gene-replacement approach. Using the *A. tumefaciens*-mediated fungal transformation method, 17 *FgERG24A* deletion mutants were identified from 24 hygromycin-resistant transformants by PCR analysis using primers A5 + A6 (Table 1). This primer pair amplified a 2208 and a 1503 bp fragment from the *FgERG24A* deletion mutant and the wild-type strain, respectively. As shown in Fig. 3(b), reverse transcription PCR with the primer pair A-RT-F + A-RT-R amplified the expected 273 bp fragment from the wild-type strain, but not from the *FgERG24A* deletion mutant Δ erg24A-1 (Fig. 3b). The PCR results were further

confirmed by Southern blot analysis. The total genomic DNA of wild-type HN9-1 and deletion mutant Δ erg24A-1 digested by *EcoRI* was blotted and hybridized with a DIG-labelled *FgERG24A* fragment (Fig. 3a). HN9-1 yielded a predicted band of 3547 bp, while in the deletion mutant Δ erg24A-1, this band was replaced by a 2934 bp fragment as expected (Fig. 3c). Since deletion of *FgERG24A* did not result in a changed phenotype, we did not reintroduce the full-length *FgERG24A* gene into the deletion mutant.

For the *FgERG24B* gene, we isolated nine gene deletion mutants from 12 hygromycin-resistant transformants by PCR analysis using primers B5 + B6 (Table 1). Reverse transcription PCR with the primer pair B-RT-F + B-RT-R was unable to amplify any product from the *FgERG24B* deletion mutant Δ erg24B-2, but could amplify the expected 244 bp fragment from the parental strain HN9-1 and from the complementary strain Δ erg24B-com11 (Fig. 4b). The total genomic DNA of HN9-1, Δ erg24B-2 and Δ erg24B-com11 digested by *EcoRI* was blotted and hybridized with a DIG-labelled *FgERG24B* fragment (Fig. 4a). In Southern blot analysis, the wild-type showed the predicted hybridizing band of 3874 bp, while in the Δ erg24B-2 mutant, this fragment was replaced by a 1779 bp fragment as expected. Additionally, the Southern hybridization pattern of Δ erg24B-com11 confirmed that a single copy of *FgERG24B* gene was reintroduced into the genome of Δ erg24B-2 (Fig. 4c).

FgERG24 genes are not involved in fungal growth, sporulation and pathogenicity

FgERG24A or *-B* disruption mutants were morphologically indistinguishable from the parent strain on PDA plates. No morphological changes were observed between the wild-type strain and the gene deletion mutants Δ erg24A-1 and Δ erg24B-2 when they were grown on PDA containing NaCl, KCl or H₂O₂ to generate osmotic and oxidative stresses (data not shown). Analyses of sporulation in MBL and virulence on wheat heads showed that deletion of either *FgERG24A* or *FgERG24B* did not cause recognizable changes in conidia formation or pathogenicity of *F. graminearum*.

FgERG24B controls the intrinsic resistance of *F. graminearum* to amines

Fungicide sensitivity tests showed that compared to the wild-type strain HN9-1, the *FgERG24A* deletion mutant did not show changed sensitivity to the amine fungicides tridemorph, fenpropidin and spiroxamine. Surprisingly, the *FgERG24B* deletion mutant showed significantly increased sensitivity to all three amines tested (Fig. 5, Table 2). As shown in Fig. 5, the wild-type HN9-1, *FgERG24A* deletion mutant Δ erg24A-1 and complementary strain Δ erg24B-com11 could grow on PDA plates containing tridemorph, fenpropidin or spiroxamine at 5 μ g ml⁻¹, but growth of the *FgERG24B* deletion mutant Δ erg24B-2 was

Fig. 5. Comparison of sensitivity to amine and non-amine fungicides among the parent isolate HN9-1, *FgERG24A* gene deletion mutant Δ erg24A-1, *FgERG24B* gene deletion mutant Δ erg24B-2, and *FgERG24B* gene complemented transformant Δ erg24B-com11 on PDA containing each fungicide at the concentrations indicated in the figure. CK, control (no fungicide).

totally inhibited by these fungicides. Consistent with Δ erg24B-2, eight other *FgERG24B* deletion mutants were unable to grow in the presence of 5 µg ml⁻¹ tridemorph, fenpropidin or spiroxamine (data not shown).

Sensitivity tests with non-amine fungicides showed that as compared to the wild-type HN9-1, *FgERG24A* and *-B* deletion mutants did not exhibit changed sensitivity to the DMI fungicides triadimefon and tebuconazole, or the dicarboximide fungicide iprodione (Fig. 5).

Quantitative real-time PCR analysis showed that expression levels of *FgERG24A* and *FgERG24B* in the Δ erg24A-1

and Δ erg24B-2 mutants were not significantly different from those in the wild-type strain either treated or untreated with amine fungicides (Table 3). Additionally, determination of ergosterol content by HPLC assay showed that the contents of ergosterol in *FgERG24A* or *-B* disruption mutants were not significantly different from that in the wild-type strain ($P > 0.05$) (Table 4). Furthermore, analysis of total sterols showed that the sterol intermediates in the *FgERG24A* and *-B* disruption mutants were not significantly different from these in the parental strain (see Supplementary Fig. S1, available with the online version of this paper).

Table 2. Sensitivity of wild-type strain HN9-1, deletion mutants Δ erg24A-1 and Δ erg24B-2, and the *FgERG24B*-complemented strain Δ erg24B-com11 to amine fungicides

Results are means $\pm$ SD of three repeated experiments. There were no significant differences among the three experiments ($P > 0.05$).

Strain	EC ₅₀ (µg ml ⁻¹) of		
	Tridemorph	Fenpropidin	Spiroxamine
HN9-1	10.10 $\pm$ 1.235	14.06 $\pm$ 2.431	17.39 $\pm$ 3.427
Δ erg24A-1	11.28 $\pm$ 0.724	13.11 $\pm$ 1.883	18.15 $\pm$ 2.945
Δ erg24B-2	0.270 $\pm$ 0.037	0.047 $\pm$ 0.013	0.068 $\pm$ 0.012
Δ erg24B-com11	9.773 $\pm$ 1.685	15.56 $\pm$ 2.766	16.47 $\pm$ 3.445

Table 3. Relative expression of *FgERG24A* and *-B* in the *FgERG24A* and *FgERG24B* deletion mutants treated or untreated with amine fungicides

The mutants were treated with tridemorph, fenpropidin or spiroxamine at 10, 14 and 17 $\mu\text{g ml}^{-1}$, respectively, for 6 h. The relative expression of *FgERG24* genes is the amount of mRNA of each gene relative to the wild-type parent HN9-1 without fungicide treatment. Results are means $\pm$ SD of three repeated experiments; there were no significant differences among the three experiments ($P>0.05$). –, Not determined.

Fungicide	Relative expression of	
	<i>FgERG24B</i> in $\Delta\text{erg24A-1}$	<i>FgERG24A</i> in $\Delta\text{erg24B-2}$
Tridemorph	–	1.174 ± 0.204
Fenpropidin	–	1.886 ± 0.059
Spiroxamine	–	1.690 ± 0.214
Untreated control	–	1.580 ± 0.178
Tridemorph	0.806 ± 0.063	–
Fenpropidin	0.748 ± 0.172	–
Spiroxamine	2.137 ± 0.325	–

DISCUSSION

Ergosterol is the major sterol component in fungal membranes and is involved in a variety of cellular functions, including cell fluidity, cell integrity and membrane-bound enzyme function (Gooday, 1995). The important biochemical characteristics of ergosterol make the enzymes involved in its biosynthesis pathway attractive inhibition sites for antifungal agents. The sterol C-14 reductase encoded by the *ERG24* gene was proposed to be the target of amine fungicides (Baloch & Mercer, 1987). To date, *ERG24* genes from several yeasts have been characterized (Crowley *et al.*, 1998; Lorenz & Parks, 1992; Marcireau *et al.*, 1992; Shah Alam Bhuiyan *et al.*, 2007), but information on functions of *ERG24* in filamentous fungi is very limited. Genomic studies revealed that most fungal species contain only a single *ERG24* gene; however, *F. graminearum* contains two paralogous *ERG24* genes (Da Silva Ferreira *et al.*, 2005). The predicted amino acid sequences FgErg24A and FgErg24B revealed that they are two different but closely related proteins. The percentages of identity between sterol C-14 reductases from *F.*

graminearum and these from other fungi are high enough to consider both FgErg24A and *-B* to be members of the sterol C-14 reductase family (Fig. 1).

In this study, reverse transcription PCR showed that both paralogous *FgERG24* genes are transcribed in *F. graminearum* (Fig. 2). The targeted disruption mutants of *FgERG24A* and *-B* are morphologically indistinguishable from their parent strain on PDA. Pathogenicity tests revealed that virulence of the deletion mutants did not change significantly as compared to the parental strain on wheat heads. Moreover, sterol assays revealed that disruption of *FgERG24A* or *-B* did not cause the arrest of sterol biosynthesis in *F. graminearum*. These results strongly indicated that the functions of the paralogous *FgERG24* genes in *F. graminearum* are interchangeable, and neither of the two genes is essential for fungal growth, pathogenicity and ergosterol biosynthesis. However, after many attempts, we were unable to obtain a double deletion mutant of *FgERG24A* and *-B*, indicating that disruption of both genes may be lethal in *F. graminearum* and the presence of at least one *FgERG24* gene is essential for growth.

Table 4. Ergosterol contents of the wild-type and mutants treated or not with amine fungicides

The amounts of ergosterol were determined in wild-type strain HN9-1, *FgERG24A* gene deletion mutant $\Delta\text{erg24A-1}$, *FgERG24B* deletion mutant $\Delta\text{erg24B-2}$ and the *FgERG24B* complemented strain $\Delta\text{erg24B-com11}$, untreated or treated with tridemorph, fenpropidin or spiroxamine at 10, 14 and 17 $\mu\text{g ml}^{-1}$, respectively, for 3 days in sterilized water. See Methods for details. The results are means $\pm$ SD of two repeated experiments; there were no significant differences between the two experiments ($P>0.05$).

Strain	Ergosterol content [$\text{mg (g dry mycelium)}^{-1}$]			
	Non-treated	Treated with tridemorph	Treated with fenpropidin	Treated with spiroxamine
HN9-1	3.951 ± 0.024	1.606 ± 0.023	1.621 ± 0.023	1.793 ± 0.041
$\Delta\text{erg24A-1}$	3.127 ± 0.166	1.738 ± 0.057	1.194 ± 0.034	1.925 ± 0.056
$\Delta\text{erg24B-2}$	3.285 ± 0.039	1.779 ± 0.014	1.897 ± 0.048	1.174 ± 0.068
$\Delta\text{erg24B-com11}$	4.113 ± 0.046	1.962 ± 0.058	2.051 ± 0.062	1.529 ± 0.063

The *F. graminearum* genome has fewer high-identity duplicated sequences than other related fungi (Cuomo *et al.*, 2007). In a previous study, we characterized three paralogous *cyp51* genes encoding sterol 14 α -demethylase in *F. graminearum*, and found that these genes are not essential for fungal growth but they participate in mediating sensitivity of *F. graminearum* to different DMIs (Liu *et al.*, 2011). Additionally, *F. graminearum* contains two paralogous β -tubulin genes (*FgTUB1* and -2). Functional analysis showed that neither of these genes is necessary for fungal growth; however, both of them are involved in mediating sensitivity of *F. graminearum* to benzimidazole fungicides (Chen *et al.*, 2007; Liu *et al.*, 2010). The reasons for the existence of paralogous genes in the genome of a single species and their precise functions remain unclear. It has been pointed out that paralogous genes in one species might have evolved through a process of duplication of an ancestral gene followed by functional and structural specialization (Da Silva Ferreira *et al.*, 2005; Mellado *et al.*, 2005). Existence of duplicated genes in one species could be a good strategy for adaptation to various environmental stresses, including fungicide treatment as shown in this and previous studies (Chen *et al.*, 2007; Liu *et al.*, 2010, 2011).

Although *FgERG24A* or -*B* deletion mutants did not show obvious changes in morphological characters without the stress of fungicide treatment, we did observe that the disruption of *FgERG24B* caused increased sensitivity of *F. graminearum* to amines. These results indicate that *FgERG24B* is responsible for the intrinsic resistance of *F. graminearum* to amines; amine fungicides may bind more tightly to the FgErg24A protein than to FgErg24B. This finding provides a new insight into the resistance mechanisms of plant pathogens to amines. Tridemorph, fenpropidin and spiroxamine are representatives of the morpholine, piperidine and spiroketalamine subgroups, respectively. It is interesting that tridemorph was more effective than the other two amines against wild-type *F. graminearum*, but when *FgERG24B* was deleted, the mutants were more sensitive to fenpropidin and spiroxamine than to tridemorph (Table 2). These results suggest that fenpropidin and spiroxamine are more effective than tridemorph against the FgErg24A protein in *F. graminearum*.

In this study, we found that *FgERG24A* deletion mutants did not show changed sensitivity to amines. Obviously, such mutants can be used as a screen for identification of new antifungal compounds that specifically target the FgErg24B protein. If a chemical is effective against a *FgERG24A* deletion mutant, a mixture of this compound with an amine fungicide will significantly enhance control efficacy for plant diseases caused by *Fusarium* species.

The sterol Δ^8 - Δ^7 isomerase encoded by *FgERG2* has been shown as a second target of amines in several fungal species (Baloch *et al.*, 1984; Baloch & Mercer, 1987; Debieu *et al.*,

1992, 1998, 2000; Lorenz & Parks, 1991; Schneegurt & Henry, 1992; Ziogas *et al.*, 1991). Searching the *F. graminearum* genome database showed that the fungus has a single *FgERG2* gene (Da Silva Ferreira *et al.*, 2005). The predicted amino acid sequence of FgErg2 shows a high level of identity to sterol Δ^8 - Δ^7 isomerase proteins in other filamentous fungi and yeasts (Supplementary Fig. S2). To investigate functions of *FgERG2* in *F. graminearum*, we also generated *FgERG2* deletion mutants. Similar to *FgERG24* deletion mutants, *FgERG2* deletion mutants were morphologically indistinguishable from the parental strain. Surprisingly, the *FgERG2* deletion mutants did not show changed sensitivity to amines. HPLC assays revealed that disruption of *FgERG2* did not lead to depletion of ergosterol in *F. graminearum*. These results indicate the protein encoded by *FgERG2* may not be necessary for ergosterol biosynthesis in *F. graminearum*.

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