Involvement of *FgERG4* in ergosterol biosynthesis, vegetative differentiation and virulence in *Fusarium graminearum*

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SUMMARY

The ergosterol biosynthesis pathway is well understood in *Saccharomyces cerevisiae*, but currently little is known about the pathway in plant-pathogenic fungi. In this study, we characterized the *Fusarium graminearum* *FgERG4* gene encoding sterol C-24 reductase, which catalyses the conversion of ergosta-5,7,22,24-tetraenol to ergosterol in the final step of ergosterol biosynthesis. The *FgERG4* deletion mutant Δ FgErg4-2 failed to synthesize ergosterol. The mutant exhibited a significant decrease in mycelial growth and conidiation, and produced abnormal conidia. In addition, the mutant showed increased sensitivity to metal cations and to various cell stresses. Surprisingly, mycelia of Δ FgErg4-2 revealed increased resistance to cell wall-degrading enzymes. Fungicide sensitivity tests revealed that Δ FgErg4-2 showed increased resistance to various sterol biosynthesis inhibitors (SBIs), which is consistent with the over-expression of SBI target genes in the mutant. Δ FgErg4-2 was impaired dramatically in virulence, although it was able to successfully colonize flowering wheat head and tomato, which is in agreement with the observation that the mutant produces a significantly lower level of trichothecene mycotoxins than does the wild-type progenitor. All of these phenotypic defects of Δ FgErg4-2 were complemented by the reintroduction of a full-length *FgERG4* gene. In addition, *FgERG4* partially rescued the defect of ergosterol biosynthesis in the *Saccharomyces cerevisiae* *ERG4* deletion mutant. Taken together, the results of this study indicate that *FgERG4* plays a crucial role in ergosterol biosynthesis, vegetative differentiation and virulence in the filamentous fungus *F. graminearum*.

INTRODUCTION

Fusarium graminearum (teleomorph: *Gibberella zeae*) is an economically important plant pathogen, causing Fusarium head blight (FHB) on various cereal crops, including wheat, barley, oats, rye and maize (Goswami and Kistler, 2004; Starkey *et al.*, 2007). The disease can result in huge yield losses and cause health problems to humans and animals by contaminating grains with mycotoxins, such as deoxynivalenol (DON) and zearalenone

(Dublin *et al.*, 1997; McMullen *et al.*, 1997; Pestka and Smolinski, 2005). The serious damage caused by FHB highlights the need for the development of feasible disease control strategies, which may be derived from an understanding of fungal biology and the mechanisms of host–pathogen interaction.

Ergosterol is a predominant sterol constituent in the plasma membrane of fungi, and is a fungal-specific sterol (Georgopapadakou and Walsh, 1994; Lees *et al.*, 1999). Ergosterol is involved in a remarkable diversity of functions and processes, including the maintenance of cell membrane integrity and fluidity, regulation of membrane permeability and the activity of membrane-bound enzymes (Lees *et al.*, 1995; Parks *et al.*, 1995). In fungi, ergosterol is synthesized from acetyl-coenzyme A (acetyl-CoA) through a complex pathway. The biosynthetic steps of ergosterol have been well characterized in *Saccharomyces cerevisiae* (Baudry *et al.*, 2001; Fryberg *et al.*, 1973; Gachotte *et al.*, 2001; Henneberry and Sturley, 2005; Lees *et al.*, 1999; Mo and Bard, 2005; Mo *et al.*, 2003, 2004; Osumi *et al.*, 1978; Soustrea *et al.*, 2000). In *S. cerevisiae*, some enzymes (e.g. Erg6, Erg2, Erg3, Erg4) in the ergosterol biosynthesis pathway are dispensable, whereas others (e.g. Erg9, Erg1, Erg7, Erg11, Erg24) are essential for fungal viability (Lees *et al.*, 1995). Similarly, in the human pathogenic yeast *Candida albicans*, several ergosterol biosynthetic genes, including *ERG1*, *ERG18* and *ERG26*, are essential for fungal viability (Aaron *et al.*, 2001; Kennedy *et al.*, 2000; Pierson *et al.*, 2004). In addition, the inactivation of sterol $\Delta^{5,6}$ -desaturase (Erg3) attenuates the virulence of *C. albicans* in mice (Chau *et al.*, 2005).

To date, very little information is available on the ergosterol pathway in filamentous fungi. Thus, it would be interesting to investigate the functions of ergosterol biosynthesis genes in *F. graminearum*, which would be helpful in exploring novel targets for drug design. In previous studies, we have characterized *FgCYP51A*, *FgCYP51B* and *FgCYP51C*, *FgERG24A* and *FgERG24B*, and *FgERG2* genes in *F. graminearum* (Liu *et al.*, 2011a, b). In the current study, we further investigate the functions of the *ERG4* gene in this pathogen.

Sterol C-24 reductase, encoded by *ERG4*, catalyses the final step of the ergosterol biosynthesis pathway for the conversion of ergosta-5,7,22,24(28)-tetraenol to ergosterol. The *ERG4* mutant of *S. cerevisiae* is not lethal but fails to biosynthesize ergosterol. The mutant is hypersensitive to divalent cations and several drugs (including cycloheximide, miconazole, 4-nitroquinoline, fluconazole and sodium dodecylsulphate) (Zweyck *et al.*, 2000). Recent

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studies have shown that Erg4 is involved in cell polarity by interacting with the p21-activated kinase (PAK) Ste20, and is required for the polarized localization of Ste20 during *S. cerevisiae* mating (Tiedje *et al.*, 2007). More recently, Aguilar *et al.* (2010) have reported that *ERG4* is required for proper cell shape remodelling and cell fusion during yeast mating. Despite the fact that the function of *ERG4* in *S. cerevisiae* has been well documented, the roles of Erg4 in filamentous fungi have not been reported to date. Thus, the main objective of this study was to investigate the role of *FgERG4* in the physiology and virulence of *F. graminearum*. In the current study, we found that, in addition to playing a crucial role in ergosterol biosynthesis, *FgERG4* is also involved in the regulation of vegetative differentiation and virulence in *F. graminearum*.

RESULTS

Identification of *FgERG4* in *F. graminearum*

Using *S. cerevisiae* sterol C-24 reductase Erg4 as query, we identified the locus FGSG_10003.3 from the *F. graminearum* genome (http://www.broad.mit.edu/annotation/genome/fusarium_graminearum/Home.html). The *FgERG4* nucleotide sequence is 1902 bp in length and encodes 596 amino acids. The *FgERG4* gene is interrupted by two introns. The first intron is 59 bp in length and is located between the 36th and 94th nucleotides; the second intron is 52 bp in length and is located between 1839 and 1890 bp of the nucleotide sequence. Sequencing of the 1791-bp cDNA of *FgERG4* verified the existence of the two introns.

Phylogenetic analysis using the neighbour-joining method (Kumar *et al.*, 2008) showed that the amino acid sequence of FgErg4 is highly homologous to that from yeasts and other filamentous fungi. The deduced amino acid sequence of FgErg4 from *F. graminearum* shares 64.50% and 37.73% identity with NcErg4 of *Neurospora crassa* (NcErg4, XP_961419.2) and ScErg4 of *S. cerevisiae* (ScErg4, NP_011503.1), respectively (Fig. S1a, see Supporting Information). The alignment of the amino acid sequences of FgErg4 with those from *S. cerevisiae* and *C. albicans* showed that Erg4s from different fungi are conserved in their C-terminal parts, but highly variable in the N-terminal parts (Fig. S1b).

Disruption and complementation of *FgERG4* in *F. graminearum*

For a detailed functional analysis of *FgERG4*, we generated *FgERG4* deletion mutants using a double homologous recombination strategy. Using the *Agrobacterium tumefaciens*-mediated fungal transformation method, we identified eight *FgERG4* deletion mutants from 14 hygromycin-resistant transformants by polymerase chain reaction (PCR) analysis with primers A5 + A6 (Table S1, see Supporting Information). This primer pair amplified

1767- and 2220-bp fragments from the *FgERG4* deletion mutants and wild-type strain HN9-1, respectively. As shown in Fig. S2b (see Supporting Information), reverse transcription PCR with the primer pair ERG4-RT-F + ERG4-RT-R amplified an expected 276-bp fragment from the wild-type progenitor HN9-1 and complemented strain Δ FgErg4-9C, but not from the *FgERG4* deletion mutant Δ FgErg4-2 (Fig. S2b). PCR results were further verified by Southern blotting analysis. The genomic DNAs of HN9-1, Δ FgErg4-2 and Δ FgErg4-9C, digested by *Sall*, were blotted and hybridized with a digoxigenin (DIG)-labelled *FgERG4* downstream fragment (Fig. S2a). The wild-type strain had an expected hybridizing band of 6357 bp, whereas this band was replaced by a 3797-bp fragment in Δ FgErg4-2. In addition, the Southern hybridization pattern of Δ FgErg4-9C confirmed that a single copy of the full-length *FgERG4* gene was introduced into the genome of Δ FgErg4-2 (Fig. S2c).

Lack of ergosterol biosynthesis in the *FgERG4* deletion mutant

High-performance liquid chromatography (HPLC) analysis of ergosterol extracted from mycelia of HN9-1, Δ FgErg4-2 and Δ FgErg4-9C revealed that there is an ergosterol-specific absorption peak at the retention time of 15.5 min in wild-type HN9-1 and the complemented mutant Δ FgErg4-9C, but not in the *FgERG4* deletion mutant Δ FgErg4-2, suggesting that the mutant Δ FgErg4-2 fails to produce ergosterol, and *FgERG4* is necessary for the biosynthesis of ergosterol in *F. graminearum* (Fig. 1). Furthermore, the analysis of total sterols showed that the sterol intermediates in Δ FgErg4-2 were different from those in the parental strain HN9-1 and the complemented mutant Δ FgErg4-9C (Fig. S3, see Supporting Information), suggesting that sterol intermediates may be accumulated in the *FgERG4* deletion mutant.

Involvement of *FgERG4* in the regulation of hyphal growth and conidiation in *F. graminearum*

Disruption of *FgERG4* resulted in reduced mycelial growth on potato dextrose agar (PDA) medium (Fig. 2a). When grown in mung bean liquid (MBL) medium, Δ FgErg4-2 produced significantly less conidia than the wild-type progenitor and the complemented strain (Fig. 2b). Moreover, the conidia of Δ FgErg4-2 lacked the typical morphology and were shorter than those of the wild-type strain (Fig. 3a). Calcofluor white staining assays showed that 12.47% of the conidia of the mutant had a single septum, and 22.22%, 55.83%, 7.86% and 1.63% of the conidia had two, three, four and five septa, respectively. In contrast, most of the wild-type conidia had four or five septa (Figs 3b and 4a). We did not observe any wild-type conidia to have one or two septa (Fig. 4a). Although Δ FgErg4-2 produced conidia with abnormal morphology, the conidia of the mutant were able to germinate and to form normal

Fig. 1 Deletion of *FgERG4* resulted in a complete depletion of ergosterol. Comparative production of ergosterol in the wild-type progenitor HN9-1 (a), the deletion mutant Δ FgErg4-2 (b) and the complemented strain Δ FgErg4-9C (c) as determined by high-performance liquid chromatography (HPLC) assays. (d) Commercial standard of ergosterol was used as the control. The retention time for ergosterol is marked with arrows.

germ tubes (Fig. 3b). In addition, Δ FgErg4-2 had significantly longer hyphal cells when compared with the wild-type strain (Fig. 4b), indicating that hyphae of Δ FgErg4-2 also have less septa than those of the wild-type strain.

A previous study has shown that *S. cerevisiae* cells can take up exogenous sterols under anaerobic conditions, but not under aerobic conditions (Raychaudhuri and Prinz, 2006). Therefore, we were interested in determining the ability of Δ FgErg4-2 to take up exogenous sterols (ergosterol and cholesterol) under different conditions. As shown in Figs 5 and S4 (see Supporting Information), the defects of mycelial growth of Δ FgErg4-2 could not be restored when the mutant was grown on PDA medium supplemented with ergosterol or cholesterol, which suggested that *F. graminearum* might not be able to absorb ergosterol or chole-

Fig. 2 Reduced mycelial growth and conidiation in the *FgERG4* deletion mutant. (a) Comparison of mycelial growth rates among the wild-type progenitor HN9-1, the deletion mutant Δ FgErg4-2 and the complemented strain Δ FgErg4-9C on potato dextrose agar (PDA) medium. (b) Comparison of conidiation among HN9-1, Δ FgErg4-2 and Δ FgErg4-9C in mung bean liquid (MBL) medium. Bars denote standard errors from three repeated experiments.

sterol from the culture medium. To further confirm this hypothesis, we extracted ergosterol from mycelia of HN9-1, Δ FgErg4-2 and Δ FgErg4-9C after they had been incubated in the medium amended with ergosterol at the concentrations described in Fig. 5 for 3 days under either aerobic or anaerobic conditions. HPLC analyses showed that no ergosterol was detected from Δ FgErg4-2 (data not shown). These results further confirm that *F. graminearum* cells cannot take up exogenous sterols under either anaerobic or aerobic conditions.

Requirement for *FgERG4* for full virulence of *F. graminearum*

Pathogenicity assays indicated that disruption of *FgERG4* caused a significant reduction in the virulence of *F. graminearum*. As shown in Fig. 6a, 10 days after inoculation, the wild-type progenitor and the complemented strain caused typical scab symptoms in inoculated and nearby spikelets of flowering wheat heads. Under the same conditions, however, Δ FgErg4-2 caused scab symptoms only in the inoculated spikelet, but not in any nearby spikelets. As

Fig. 3 Deletion of *FgERG4* led to changes in conidial morphology. (a) Compared with the wild-type progenitor HN9-1 and the complemented strain ΔFgErg4-9C, the deletion mutant ΔFgErg4-2 produced shorter conidia in mung bean liquid (MBL) medium. (b) The conidia of ΔFgErg4-2 showed less septation (top), but germinated normally (bottom). Conidia were examined after incubation in 2% sucrose (wt/vol) for the times indicated in the figure, followed by staining with calcofluor white.

shown in Fig. 6b, 3 days after inoculation on nonhost tomato, the water-soaked rot lesion caused by ΔFgErg4-2 was significantly smaller than that caused by the wild-type progenitor or the complemented strain. These data suggest that *FgERG4* is essential for full virulence of *F. graminearum*.

Involvement of *FgERG4* in the regulation of DON biosynthesis

Previous studies have shown that DON is an important virulence factor in *F. graminearum* (Desjardins *et al.*, 1996; Proctor *et al.*, 1995; Seong *et al.*, 2009). As the scab symptoms caused by ΔFgErg4-2 on wheat heads were restricted to the inoculated spikelets, we were interested in determining DON production in the *FgERG4* deletion mutant. As shown in Fig. 7, the amounts of DON produced by the wild-type progenitor and the complemented strain were 18-fold higher than that produced by ΔFgErg4-2.

Sensitivity of the *FgERG4* deletion mutant to divalent and trivalent cations, osmotic and oxidative stresses, and sterol biosynthesis inhibitors (SBIs)

In *S. cerevisiae*, the *ERG4* deletion mutant is more sensitive than the wild-type progenitor to various divalent cations; thus, we also tested the sensitivity of ΔFgErg4-2 to divalent cations. As shown in

Fig. 8, ΔFgErg4-2 exhibited a significantly increased sensitivity to the divalent cations Cu^{2+} , Mg^{2+} and Ca^{2+} . In addition, the mutant showed increased sensitivity to the trivalent cations Fe^{3+} and Al^{3+} .

As the *FgERG4* deletion mutant was unable to produce ergosterol, which may result in the disturbance of membrane permeability and osmosis, we tested the sensitivity of the *FgERG4* deletion mutant to osmotic and oxidative stresses. As shown in Fig. 9, ΔFgErg4-2 revealed significantly increased sensitivity to osmotic stress mediated by NaCl and D-sorbitol, and to oxidative stress generated by H_2O_2 and paraquat.

As the *FgERG4* deletion mutant was unable to biosynthesize ergosterol, we were interested in determining the sensitivity of the mutant to ergosterol biosynthesis inhibitors. As shown in Fig. 10, ΔFgErg4-2 exhibited increased tolerance to SBIs, including triadimefon and tebuconazole (targeting sterol 14 α -demethylase), the amine fungicides tridemorph, fenpropidin and spiroxamine (targeting sterol C-14 reductase or sterol $\Delta^7,8$ -isomerase) and the polyene fungicides nystatin and amphotericin B (binding to ergosterol). In contrast, the mutant did not show any change in the sensitivity to the dicarboximide fungicide iprodione. These results are further supported by the observation that the expression of several SBI target genes (*FgCYP51A*, *FgCYP51B*, *FgCYP51C* and *FgERG24A*, *FgERG24B*) was up-regulated in the mutant. Real-time polymerase chain reaction (RT-PCR) assays indicated that the expression levels of all 10 *ERG* genes tested were increased dramatically in ΔFgErg4-2 (Table 1).

Fig. 4 Deletion of *FgERG4* led to changes in conidial and hyphal cell length. (a) Comparison of the percentage of conidia with different numbers of septa among HN9-1, ΔFgErg4-2 and ΔFgErg4-9C. (b) Comparisons of hyphal cell length among HN9-1, ΔFgErg4-2 and ΔFgErg4-9C.

Resistance of the *FgERG4* deletion mutant to cell wall-degrading enzymes

In *S. cerevisiae*, *ERG4* has been shown to be involved in cell wall assembly, and the deletion of *ERG4* resulted in hypersensitivity to the cell wall-degrading enzyme zymolyase (Tiedje *et al.*, 2007). To determine the possible role of *FgERG4* in cell wall integrity in *F. graminearum*, we examined the sensitivity of the *FgERG4* deletion mutant to cell wall-degrading enzymes. As shown in Fig. 11, after treatment with cellulase, lysozyme and snailase at 30 °C for 3 h, ΔFgErg4-2 produced fewer protoplasts than the wild-type progenitor and the complemented strain, indicating that the *FgERG4* mutant shows increased resistance to cell wall-degrading enzymes.

FgErg4 partially complements the defect of ergosterol biosynthesis in an *S. cerevisiae* *ERG4* deletion mutant

In *S. cerevisiae*, ergosterol biosynthesis was blocked in the *ERG4* deletion mutant. In order to further determine the functions of FgErg4, we tested whether or not FgErg4 could complement the yeast *ERG4* mutant. An expression vector pYES2 containing the full-length *FgERG4* cDNA was transformed into the *ERG4* mutant

BY4741Δ*ERG4*. As a control, the mutant was also transformed with the pYES2 vector. After transformation and PCR identification, ergosterol was extracted and detected by HPLC analysis. As shown in Fig. 12a,c, an ergosterol-specific absorption peak at the retention time of 15.5 min was observed in the wild-type BY4741 + pYES2 and the complemented strain BY4741Δ*ERG4* + pYES2-*FgERG4*, but not in the deletion mutant BY4741Δ*ERG4* + pYES2 (Fig. 12b). In addition, a compound with an absorption peak with a retention time of 11 min was detected in BY4741Δ*ERG4* + pYES2, but not in the wild-type strain BY4741 + pYES2, suggesting that this compound could be the accumulated sterol precursor (Fig. 12b). Interestingly, the absorption peak at the retention time of 11 min was also observed in the complemented strain BY4741Δ*ERG4* + pYES2-*FgERG4*, indicating that *F. graminearum* FgErg4 partially complements the *S. cerevisiae* *ERG4* mutant in ergosterol biosynthesis.

DISCUSSION

Ergosterol is the major sterol component in the fungal plasma membrane (Georgopapadakou and Walsh, 1994; Lees *et al.*, 1999) and is responsible for structural membrane features, such as fluidity and permeability (Lees *et al.*, 1995; Parks *et al.*, 1995). The important biochemical characters of ergosterol make the enzymes involved in its biosynthesis pathway attractive inhibition sites for antifungal agents. In *S. cerevisiae*, the deletion of *ERG4* is not lethal, but completely blocks ergosterol biosynthesis (Lees *et al.*, 1995). In contrast, over-expression of the *ERG4* gene can promote ergosterol production (He *et al.*, 2003). In this study, we found that *FgERG4* deletion mutants were impaired in ergosterol production, indicating that *FgERG4* is also essential for ergosterol biosynthesis in *F. graminearum*. Furthermore, *F. graminearum* FgErg4 partially complements the ergosterol biosynthesis defect of a *S. cerevisiae* *ERG4* mutant, indicating that FgErg4 is a homologue of ScErg4.

Ergosterol biosynthesis is an oxygen-dependent process as several enzymes of the postsqualene ergosterol biosynthetic pathway require molecular oxygen (Rosenfeld and Beauvoit, 2003). When grown under aerobic conditions, *S. cerevisiae* is able to synthesize sterols, and yeast cells can only take up exogenous sterols under anaerobic conditions (Andreasen and Stier, 1953; Raychaudhuri and Prinz, 2006). Unlike *S. cerevisiae*, the filamentous fungi *Aspergillus fumigatus* (Xiong *et al.*, 2005), *Aspergillus niger* (Chowdhury *et al.*, 1998; Nemec and Jernejc, 2002) and *Chrysosporium keratinophilum* (al Musallam and Radwan, 1990) are able to import sterols under aerobic conditions. In this study, we observed that the *FgERG4* mutant of *F. graminearum* cannot absorb exogenous ergosterol and cholesterol under either aerobic or anaerobic conditions. These results indicate that different fungi show significantly different ability to take up exogenous ergosterol.

Fig. 5 Exogenous application of ergosterol could not restore normal mycelial growth of Δ FgErg4-2. The wild-type progenitor HN9-1, the deletion mutant Δ FgErg4-2 and the complemented strain Δ FgErg4-9C were cultured on potato dextrose agar (PDA) medium supplemented with ergosterol at the concentrations indicated in the figure under aerobic (a) and anaerobic (b) conditions.

Fig. 6 *FgERG4* is important for full virulence of *Fusarium graminearum*. (a) Flowering wheat heads were point inoculated with a conidial suspension of the wild-type progenitor HN9-1, the deletion mutant Δ FgErg4-2 and the complemented mutant Δ FgErg4-9C. Infected wheat heads were photographed 10 days after inoculation. (b) Tomatoes were inoculated with a conidial suspension of each strain and infected fruits were photographed 3 days after inoculation.

As a main component of the plasma membrane, ergosterol plays an important role in the maintenance of membrane heterogeneity and the prevention of water penetration, as well as the maintenance of the rigidity of the plasma membrane interfacial region (Abe *et al.*, 2009). As a result of a lack of ergosterol and/or accumulation of its precursor, the *FgERG4* deletion mutant displayed several defects in plasma membrane function, such as increased sensitivity to metal cations and osmotic stresses (Figs 8 and 9). Tolerance to metal cations is often conferred by membrane ATPases, which serve as pumps to protect cells from toxic compounds (Hampsey, 1997). Ergosterol depletion has been found to

inactivate vacuolar ATPase function, impairing vacuolar acidification (Zhang *et al.*, 2010). In addition, as the mutant showed an increased osmo-sensitivity, paraquat and H_2O_2 may penetrate the plasma membrane of the mutant more easily than that of the wild-type, resulting in increased sensitivity of the mutant to these stress agents. In *S. cerevisiae* and *C. albicans*, the mutants with impaired ergosterol synthesis and a low content of ergosterol exhibited enhanced synthesis of chitin and abnormal distribution of chitin in their cell walls (Chiew *et al.*, 1982; Marisco *et al.*, 2011; Schmidt *et al.*, 1999). In this study, we found that the cell wall of Δ FgErg4-2 was more resistant to the mixture of cellulase, lys-

Fig. 7 Reduced deoxynivalenol (DON) production in the deletion mutant $\Delta FgErg4-2$. The amounts of DON (mg/mg of fungal DNA) produced by the wild-type progenitor HN9-1, the deletion mutant $\Delta FgErg4-2$ and the complemented strain $\Delta FgErg4-9C$ in infected wheat kernels. Bars denote standard errors from three repeated experiments.

Fig. 8 Comparison of the sensitivity to metal cations among the wild-type progenitor HN9-1, the deletion mutant $\Delta FgErg4-2$ and the complemented strain $\Delta FgErg4-9C$ on minimal medium amended with each metal cation at the concentration indicated in the figure. Bars denote standard errors from three repeated experiments.

ozyme and snailase than was that of the wild-type strain. These results suggest that a lack of ergosterol may also enhance chitin biosynthesis. Although $\Delta FgErg4-2$ exhibited increased resistance to cell wall-degrading enzymes, it is surprising that it showed less septation in the conidia and hyphae of *F. graminearum*. These results indicate that the lack of ergosterol and/or *FgErg4* may affect cell wall integrity in *F. graminearum*.

In addition to the involvement in ergosterol biosynthesis, *Erg4* also plays important roles in other biological processes. In *S. cerevisiae*, *Erg4* plays an important role in cell polarity by interacting with *Ste20*. The deletion of either *ERG4* or *STE20* results in various polarity defects (Tiedje *et al.*, 2007). In this study, deletion of *FgERG4* did not result in recognizable polarity defects in hyphal growth and conidial germination in *F. graminearum*. Furthermore, deletion of *FgSTE20* did not show recognizable phenotypic changes (Wang *et al.*, 2011; X. Liu and Z. Ma, unpublished data).

Fig. 9 Comparison of the sensitivity to cell stress among the wild-type progenitor HN9-1, the deletion mutant $\Delta FgErg4-2$ and the complemented strain $\Delta FgErg4-9C$ on potato dextrose agar (PDA) medium amended with various cell stress agents. Bars denote standard errors from three repeated experiments.

Fig. 10 Comparison of the sensitivity to different fungicides among the wild-type progenitor HN9-1, the deletion mutant $\Delta FgErg4-2$ and the complemented mutant $\Delta FgErg4-9C$ on potato dextrose agar (PDA) medium amended with various fungicides. Bars denote standard errors from three repeated experiments.

These results indicate that the mechanisms for the regulation of cell polarity in yeast are different from those in filamentous fungi. In this study, we observed, for the first time, that the *FgERG4* deletion mutant forms smaller and shorter conidia with less septation. The mutant was also significantly reduced in conidiation, indicating that *FgErg4* is involved in the regulation of conidial development and maturation processes in *F. graminearum*.

In *S. cerevisiae*, the *ERG4* mutant cells are hypersensitive to certain drugs, including cycloheximide, terbinafine, miconazole and fluconazole (Zweyick *et al.*, 2000). These effects can be explained in part by the lack of ergosterol in the plasma membrane of the *ERG4* mutant, which may facilitate the import of these drugs into the mutant cells. In addition, as some membrane-bound transporters may require ergosterol for optimum activity, lack of ergosterol could result in a less efficient efflux of these

Accession number (gene)	Putative enzyme encoded by the gene	Fold change value*
FGSG_04092 (<i>FgCYP51A</i>)	Sterol 14 α -demethylase	311.9 $\pm$ 11.4
FGSG_01000 (<i>FgCYP51B</i>)	Sterol 14 α -demethylase	29.17 $\pm$ 7.423
FGSG_11024 (<i>FgCYP51C</i>)	Sterol 14 α -demethylase	5.384 $\pm$ 0.303
FGSG_07315 (<i>FgERG2</i>)	C-8 sterol isomerase	11.93 $\pm$ 2.909
FGSG_05950 (<i>FgERG7</i>)	Lanosterol cyclase	14.58 $\pm$ 4.569
FGSG_09381 (<i>FgERG9</i>)	Squalene synthetase	3.032 $\pm$ 0.045
FGSG_02783 (<i>FgERG6A</i>)	Sterol 24-C-methyltransferase	3.056 $\pm$ 0.064
FGSG_05740 (<i>FgERG6B</i>)	Sterol 24-C-methyltransferase	4.732 $\pm$ 2.593
FGSG_06606 (<i>FgERG24A</i>)	Sterol C-24 reductase	40.52 $\pm$ 3.104
FGSG_02346 (<i>FgERG24B</i>)	Sterol C-24 reductase	378.5 $\pm$ 31.69

*Fold-change value represents the fold expression in Δ FgErg4-2 relative to that in the wild-type HN9-1 detected by quantitative real-time polymerase chain reaction. Results are means $\pm$ standard deviation of three repeated experiments.

Fig. 11 Deletion of *FgERG4* resulted in increased resistance of *Fusarium graminearum* to cell wall-degrading enzymes. Fresh hyphae of the wild-type progenitor HN9-1, the deletion mutant Δ FgErg4-2 and the complemented strain Δ FgErg4-9C were digested with a mixture of cellulase, lysozyme and snailase for 3 h at 30 °C. Circular protoplasts are marked with arrows.

Table 1 Relative expression level of 10 *ERG* genes in the *FgERG4* deletion mutant Δ FgErg4-2.

Fig. 12 *FgERG4* partially complements the *Saccharomyces cerevisiae* *ERG4* mutant. Comparison of ergosterol production in the wild-type strain BY4741 (a) and the *ERG4* deletion mutant BY4741 Δ ERG4 (b) transformed with empty pYES2 vector, and the complemented strain BY4741 Δ ERG4 + pYES2-FgERG4 (c) determined by high-performance liquid chromatography (HPLC) assays. A commercial standard of ergosterol was used as control (d). The retention time for ergosterol is marked with arrows.

drugs mediated by these transporters (Catty *et al.*, 1997). Surprisingly, in contrast with *S. cerevisiae*, we found that the *FgERG4* deletion mutant of *F. graminearum* showed enhanced resistance to various ergosterol biosynthesis inhibitors. This finding was further supported by the over-expression of SBI target genes (including sterol demethylation inhibitor fungicide targets *FgCYP51A*, *FgCYP51B* and *FgCYP51C*, and amine fungicide targets *FgERG24A* and *FgERG24B*) (Table 1) and certain sterol intermediates in *FgERG4* deletion mutants. These results suggest that a feedback regulatory mechanism exists in *F. graminearum*. Disruption of *FgERG4* blocked ergosterol biosynthesis, which resulted in the over-expression of *EGR* genes upstream of *FgERG4* in the pathway, subsequently leading to the accumulation of certain sterol intermediates (Fig. S3) and to increased sensitivity of the mutant to SBIs.

Plant infection results demonstrated that the *FgERG4* deletion mutant Δ FgErg4-2 could successfully colonize wheat head and tomato, but lost its aggressiveness in plant tissues (Fig. 6a,b). These data suggest that *FgERG4* plays an important role in the virulence of *F. graminearum*. The markedly reduced virulence of the mutant appeared to be caused by defects in multiple regulatory functions. First, the mycelia of the *FgERG4* mutant grew significantly more slowly than those of the wild-type progenitor. Second, it is well known that reactive oxygen species (ROS) play an important role in pathogen–host interactions. Under pathogen attack, plants use the oxidative burst as an early defence reaction. The *FgERG4* mutant showed increased sensitivity to H_2O_2 , which may be related to the virulence impairment of the *FgERG4* mutant in plant tissues. Third, DON is an end product of the trichothecene biosynthetic pathway; it has been identified as an important virulence factor and plays a significant role in the spread of FHB within a spike (Desjardins *et al.*, 1996; Proctor *et al.*, 1995; Seong *et al.*, 2009). Δ FgErg4-2 showed a significant reduction in DON production, which is consistent with the observation that Δ FgErg4-2 can cause scab symptoms in the inoculated spikelet, but cannot spread within the spike.

The results of this study showed that a number of properties of the *S. cerevisiae* *ERG4* mutants were not shared by the *F. graminearum* *FgERG4* deletion mutants, although *ERG4* genes are essential for ergosterol biosynthesis in both fungal species. Thus, future experimentation is necessary to address the different roles of *ERG4* genes in yeast and filamentous fungi.

EXPERIMENTAL PROCEDURES

Fungal strains and growth conditions

Fusarium graminearum strain HN9-1, used as the parental wild-type, was collected from Henan Province, China. The wild-type and its derivative mutants were routinely cultured at 25 °C on PDA (200 g potato, 20 g dextrose, 20 g agar and 1 L water) to assess mycelial growth and colony

characteristics, and in MBL (40 g mung beans boiled in 1 L water for 20 min, and filtered through cheesecloth) (Bai and Shaner, 1996) for the examination of conidiation. For the mycelial growth rate assay, 5-mm mycelial plugs cut from the edge of a 3-day-old colony of each strain were placed on PDA plates and incubated at 25 °C. Colony diameter was measured every 12 h. Each experiment was carried out with three replicates. For the conidiation assay, six 5-mm 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 of MBL. The flasks were incubated at 25 °C for 4 days in a shaker (180 rpm). The amount of conidia in the broth was counted for each sample using a haemocytometer. There were three replicates for each strain. The conidial septum was examined using the calcofluor white (Sigma, St. Louis, MO, USA) staining method, as described previously (Harris, 2005). In addition, to examine the ability of the *FgERG4* mutant to absorb exogenous ergosterol and cholesterol, 5-mm mycelial plugs of the *F. graminearum* strains were inoculated on PDA plates amended with technological ergosterol or cholesterol (Shangon Co., Shanghai, China) at different concentrations as indicated in Figs 5 and S4, and incubated under aerobic and anaerobic conditions for 3 days at 25 °C. All experiments were repeated three times independently.

Generation of *FgERG4* deletion mutants

FgERG4 deletion vector pCA-erg4-Del was constructed by inserting two flanking sequences of *FgERG4* into the left and right sides of *HPH* (hygromycin resistance gene) in the pBS-HPH1 vector (Liu *et al.*, 2007). Briefly, using the primer pair A1 + A2 (Table S1), a 990-bp upstream flanking sequence fragment of *FgERG4* was amplified from HN9-1 genomic DNA and inserted into the *Xho*I-*Sal*I sites of the pBS-HPH1 vector to generate the plasmid pBS-erg4-U. Subsequently, a 910-bp downstream flanking sequence fragment of *FgERG4* amplified from HN9-1 genomic DNA using the primers A3 + A4 (Table S1) was inserted into the *Hind*III-*Bam*HI sites of the pBS-erg4-U vector to generate the plasmid pBS-erg4-UD. Finally, the 3403-bp fragment containing the *FgERG4*-upstream-*HPH*-*FgERG4*-downstream cassette was excised by digestion of the plasmid pBS-erg4-UD with *Xho*I and *Bam*HI, and ligated into the *Xho*I-*Bam*HI sites of pCAMBIA 1300 (Cambia, Canberra, Australia). The gene deletion vector pCA-erg4-Del was then transformed into *A. tumefaciens* strain C58C1. *Agrobacterium tumefaciens*-mediated fungal transformation was performed as described previously (Mullins *et al.*, 2001). For the selective growth of transformants, PDA medium supplemented with hygromycin (100 mg/L) was used.

Complementation of the *FgERG4* gene deletion mutant

In order to confirm that the phenotypic changes of the *FgERG4* deletion mutant Δ FgErg4-2 were caused by the disruption of the gene, Δ FgErg4-2 was complemented with the full-length *FgERG4*. The complement plasmid pCA-FgERG4-COM was constructed using the backbone of pCAMBIA1300-NEO. The full-length *FgERG4* gene, including a 988-bp upstream region and a 720-bp terminator region, was amplified from genomic DNA of HN9-1 using the primers ERG4-com-F + ERG4-com-R (Table S1), and cloned into the *Bam*HI and *Pst*I sites of pCAMBIA1300-NEO to generate the complement plasmid pCA-FgERG4-COM. Before the

plasmid pCA-FgERG4-COM was transformed into *A. tumefaciens* strain C58C1, the *FgERG4* gene in this plasmid was sequenced to ensure flawlessness of the sequence. Transformation of Δ FgErg4-2 with the full-length *FgERG4* gene was conducted as described above, except that G418 (100 mg/L) was used as a selection agent.

Determination of ergosterol biosynthesis

To extract ergosterol, a 100- μ L spore suspension (10^5 spores/mL) of each strain was inoculated in 100 mL yeast glucose (YG) medium (5 g yeast extract, 15 g glucose, 1 L), and incubated at 25 °C for 2 days. Mycelia were harvested by filtration and washed three times with sterile water. Total ergosterol was extracted using a previously published protocol (Davey *et al.*, 2009). The ergosterol content was quantified using a Prominence SPD-20A HPLC system (Shimadzu Corporation, Kyoto, Japan). Ergosterol was separated at 25 °C on a Hypersil BDS C18 (250 nm $\times$ 4.6 nm, 5 μ m) analytical column using 100% methanol (chromatography pure) as mobile phase. The detection wavelength was 282 nm, and the identification of ergosterol was based on the retention time and co-chromatography of a commercial standard of ergosterol (Sigma). The experiment was repeated twice independently.

In addition, to extract total sterols, 100 μ L of the spore suspension (10^5 conidia/mL) of each strain were 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 the dried mycelia were ground into a powder. The ground mycelia (0.1 g) were added to 3 mL of 25% alcoholic potassium hydroxide solution (25 g of KOH and 36 mL of sterile distilled water, brought to 100 mL with 100% ethanol) and mixed by vortex for 1 min. After incubation at 80 °C for 1 h, the mixture was allowed to cool at room temperature. Sterols were then extracted by the addition of a mixture of 1 mL of sterilized water and 3 mL of *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, a 4-mL sterol extract was diluted five-fold in 100% ethanol and determined using a SpectraMax® Plus 384 spectrophotometer (Molecular Devices Inc., Sunnyvale, CA, USA) with the spectral range 190–450 nm.

Yeast strains and complementation assays

The yeast strain BY4741 (wild-type) and an *ERG4* deletion mutant BY4741 Δ ERG4 were ordered from EUROSCARF (EUROpean Saccharomyces Cerevisiae ARchive for Functional Analysis, Frankfurt, Germany). The full-length *FgERG4* cDNA was amplified using the primer pair YES2-erg4-F + YES2-erg4-R. The PCR product was digested with *Bam*HI and *Eco*RI, cloned into the pYES2 vector (Invitrogen, Carlsbad, CA, USA) and transformed into the yeast mutant BY4741 Δ ERG4. Yeast transformants were selected on synthetic medium lacking Uracil (Clontech, Palo Alto, CA, USA). In addition, the wild-type strain BY4741 and the BY4741 Δ ERG4 mutant transformed with the pYES2 vector were used as controls. For the complementation assays, the yeast transformants were grown on SG medium (0.67% yeast nitrogen base without amino acids, 2% galactose and 1% raffinose) for 2 days and the cell culture was harvested by filtration and washed three times with sterile water. Ergosterol was extracted and detected using the protocol described above.

Plant infection assays

Conidia harvested from 4-day-old cultures of *F. graminearum* strains in MBL were resuspended in 0.01% (vol/vol) Tween 20 and adjusted to 10^5 conidia/mL. Pathogenicity tests on flowering wheat heads were performed as described previously (Wu *et al.*, 2005). Briefly, a 10- μ L aliquot of a conidial suspension was injected into a floret in the middle spikelet of a wheat head of susceptible cultivar Jimai22 at early anthesis. There were 10 replicates for each strain. After inoculation, the plants were kept at 25 °C and 100% humidity for 2 days, and then maintained in a growth chamber. Infected spikelets in each inoculated wheat head were recorded 10 days after inoculation. The experiment was repeated three times. To examine the ability to colonize tomato, a 10- μ L aliquot of a conidial suspension was injected into the wounded tomato after surface sterilization. There were five replicates for each strain. Inoculated tomatoes were incubated at 25 °C and 100% humidity with 12 h of daylight, and were photographed 3 days after inoculation. The experiment was repeated three times.

Determination of the sensitivity of the *FgERG4* mutant to fungicides and to metal cations and cell stresses

Mycelial growth tests were performed on PDA or minimal medium (MM) plates supplemented with the following chemicals: NaCl, D-sorbitol, H₂O₂ and paraquat (Shangon Co.), SBI fungicides triadimefon, tebuconazole, tridemorph, fenpropidin and spiroxamine, polyene fungicides nystatin and amphotericin B, the dicarboximide fungicide iprodione or metal ions (CuCl₂, MgCl₂, CaCl₂, FeCl₃, AlCl₃) at the concentrations indicated in the figures. The percentage of inhibition of mycelial radial growth (PIMG) was calculated using the formula, $PIMG = [(C - N)/(C - 5)] \times 100$, where *C* is the colony diameter of the nontreated mycelium and *N* is the colony diameter of the fungicide-treated mycelium. The experiments were repeated twice.

Expression analysis of *ERG* genes

To extract RNA, mycelia of each strain were inoculated into PDB medium, and cultured for 2 days at 25 °C in a shaker. Total RNA was extracted from the mycelia of each strain using TaKaRa RNAiso Reagent (TaKaRa Biotechnology Co., Dalian, China), and 10 mg of each RNA sample was used for reverse transcription with the oligo(dT)₁₈ primer employing a RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas Life Sciences, Burlington, Canada). The expression of 10 *ERG* genes was determined by quantitative RT-PCR with the primer pairs listed in Table S1. The quantitative RT-PCR amplifications were performed in a DNA Engine Opticon 4 System (MJ Research, Waltham, MA, USA) 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 Laboratories, Hercules, CA, USA), 1 μ L reverse transcription product and 0.5 μ L of each primer (Table S1). There were two replicates for each sample. The quantitative RT-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 had been completed, melting curves were obtained to identify the

PCR products. For each sample, PCR amplifications with the primer pair F-actin-F + F-actin-R (Table S1) for the quantification of expression of the actin gene were performed as a reference. The experiment was repeated three times. The expression of 10 *ERG* genes in the wild-type strain and the deletion mutant $\Delta FgErg4-2$ was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Resistance of the *FgERG4* deletion mutant to cell wall-degrading enzymes

For each strain, 1 mL of spore suspension (10^5 spores/mL) was inoculated in 100 mL YEPD (1% yeast extract, 2% peptone, 2% glucose). After incubation at 25 °C overnight, fresh hyphae of each strain were harvested, and treated with cellulase, lysozyme and snailase (2% each) (Kaiyang Co., Shanghai, China) for 3 h in 0.7 M NaCl at 30 °C. The resulting protoplast of each strain was examined under a microscope.

Determination of DON production

Diseased wheat kernels were harvested from inoculated spikelets 20 days after inoculation and used for the assay of DON production employing a previously described protocol (Bluhm *et al.*, 2007; Mirocha *et al.*, 1998). The extracts were purified with a PuriTox^{SR} DON column TC-T200 (Trilogy Analytical Laboratory, Washington, MO, USA), and the amount of DON in each sample was determined using a Prominence SPD-20A HPLC system (Shimadzu Corporation). In addition, the amount of *F. graminearum* DNA in each sample was determined using a quantitative RT-PCR method (Jiang *et al.*, 2011). The experiment was repeated three times, and the data were analysed using the analysis of variance (ANOVA) procedure of SAS (SAS version 8.0; SAS Institute, Cary, NC, USA).

Standard molecular methods

Fungal genomic DNA was extracted using a previously published protocol (McDonald and Martinez, 1990). Plasmid DNA was isolated using a plasmid miniprep purification kit (BioDev Co., Beijing, China). Southern blot hybridization analysis of the *FgERG4* gene in each strain of *F. graminearum* was performed using the probes indicated in Fig. S2a. Probe was labelled with DIG using a high prime DNA labelling and detection starter kit II according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany).

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REFERENCES

Aaron, K.E., Pierson, C.A., Lees, N.D. and Bard, M. (2001) The *Candida albicans* *ERG26* gene encoding the C-3 sterol dehydrogenase (C-4 decarboxylase) is essential for growth. *FEMS Yeast Res.* **1**, 93–101.

- Abe, F., Usui, K. and Hiraki, T. (2009) Fluconazole modulates membrane rigidity, heterogeneity, and water penetration into the plasma membrane in *Saccharomyces cerevisiae*. *Biochemistry*, **48**, 8494–8504.
- Aguilar, P.S., Heiman, M.G., Walther, T.C., Engel, A., Schwudke, D., Gushwa, N., Kurzchalia, T. and Walter, P. (2010) Structure of sterol aliphatic chains affects yeast cell shape and cell fusion during mating. *Proc. Natl. Acad. Sci. USA*, **107**, 4170–4175.
- Andreasen, A.A. and Stier, T.J. (1953) Anaerobic nutrition of *Saccharomyces cerevisiae*. I. Ergosterol requirement for growth in a defined medium. *J. Cell. Physiol.* **41**, 23–36.
- Bai, G.H. and Shaner, G. (1996) Variation in *Fusarium graminearum* and cultivar resistance to wheat scab. *Plant Dis.* **80**, 975–979.
- Baudry, K., Swain, E., Rahier, A., Germann, M., Batta, A., Rondet, S., Mandalai, S., Henry, K., Tint, G.S., Edlind, T., Kurtzi, M. and Nickels, J. (2001) The effect of the *erg26-1* mutation on the regulation of lipid metabolism in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **276**, 12 702–12 711.
- Bluhm, B.H., Zhao, X., Flaherty, J.E., Xu, J.R. and Dunkle, L.D. (2007) RAS2 regulates growth and pathogenesis in *Fusarium graminearum*. *Mol. Plant-Microbe Interact.* **20**, 627–636.
- Catty, P., de Kerchove d'Exaerde, A. and Goffeau, A. (1997) The complete inventory of the yeast *Saccharomyces cerevisiae* P-type transport ATPases. *FEBS Lett.* **409**, 325–332.
- Chau, A.S., Gurnani, M., Hawkinson, R., Laverdiere, M., Cacciapuoti, A. and McNicholas, P.M. (2005) Inactivation of sterol $\Delta^{5,6}$ -desaturase attenuates virulence in *Candida albicans*. *Antimicrob. Agents Chemother.* **49**, 3646–3651.
- Chiew, Y.Y., Sullivan, P.A. and Shepherd, M.G. (1982) The effects of ergosterol and alcohols on germ-tube formation and chitin synthase in *Candida albicans*. *Can. J. Biochem.* **1**, 15–20.
- Chowdhury, B., Das, S.K. and Bose, S.K. (1998) Use of resistant mutants to characterize the target of mycobacillin in *Aspergillus niger* membranes. *Microbiology*, **144**, 1123–1130.
- Davey, L., Nybakken, L., Kausrud, H. and Ohlson, M. (2009) Fungal biomass associated with the phyllosphere of bryophytes and vascular plants. *Mycol. Res.* **113**, 1254–1260.
- Desjardins, A.E., Proctor, R.H., Bai, G.H., McCormick, S.P., Shaner, G. and Hohn, T.M. (1996) Reduced virulence of trichothecene-nonproducing mutants of *Gibberella zeae* in wheat field tests. *Mol. Plant-Microbe Interact.* **9**, 775–781.
- Dublin, Y.Y., Glichrist, L., Reeves, J. and McNab, A. (eds) (1997) *Fusarium Head Scab: global status and future prospects*. In: *Proceedings of a Workshop held at CIMMYT-Centro Internacional de Mejoramiento de Maiz y Trigo, International Centre for the Improvement of Maize and Wheat*, El Batán, Mexico, pp. 13–17. El Batán: CIMMYT.
- Fryberg, M., Oehlschlager, A.C. and Unrau, A.M. (1973) Biosynthesis of ergosterol in yeast. Evidence for multiple pathways. *J. Am. Chem. Soc.* **95**, 5747–5757.
- Gachotte, D., Eckstein, J., Barbuch, R., Hughes, T., Roberts, C. and Bard, M. (2001) A novel gene conserved from yeast to humans is involved in sterol biosynthesis. *J. Lipid Res.* **42**, 150–154.
- Georgopapadakou, N.H. and Walsh, T. (1994) Human mycoses: drugs and targets for emerging pathogens. *Science*, **264**, 371–373.
- Goswami, R.S. and Kistler, H.C. (2004) Heading for disaster: *Fusarium graminearum* on cereal crops. *Mol. Plant Pathol.* **5**, 515–525.
- Hampsey, M. (1997) A review of phenotypes in *Saccharomyces cerevisiae*. *Yeast*, **13**, 1099–1133.
- Harris, S.D. (2005) Morphogenesis in germinating *Fusarium graminearum* macroconidia. *Mycologia*, **97**, 880–887.
- He, X.P., Zhang, B. and Tan, H. (2003) Overexpression of a sterol C-24(28) reductase increases ergosterol production in *Saccharomyces cerevisiae*. *Biotechnol. Lett.* **25**, 773–778.
- Henneberry, A.L. and Sturley, S.L. (2005) Sterol homeostasis in the budding yeast, *Saccharomyces cerevisiae*. *Semin. Cell Dev. Biol.* **16**, 155–161.
- Jiang, J., Yun, Y., Yang, Q., Shim, W.B., Wang, Z. and Ma, Z. (2011) A type 2C protein phosphatase FgPtc3 is involved in cell wall integrity, lipid metabolism, and virulence in *Fusarium graminearum*. *PLoS ONE*, **6**, e25311.
- Kennedy, M.A., Johnson, T.A., Lees, N.D., Barbuch, R., Eckstein, J.A. and Bard, M. (2000) Cloning and sequencing of the *Candida albicans* C-4 sterol methyl oxidase gene (*ERG25*) and expression of an *ERG25* conditional lethal mutation in *Saccharomyces cerevisiae*. *Lipids*, **35**, 257–262.
- Kumar, S., Dudley, J., Nei, M. and Tamura, K. (2008) MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief. Bioinform.* **9**, 299–306.

- Lees, N.D., Skaggs, B., Kirsch, D.R. and Bard, M. (1995) Cloning of the late genes in the ergosterol biosynthetic pathway of *Saccharomyces cerevisiae*. *Lipids*, **30**, 221–226.
- Lees, N.D., Bard, M. and Kirsch, D.R. (1999) Biochemistry and molecular biology of sterol synthesis in *Saccharomyces cerevisiae*. *Crit. Rev. Biochem. Mol. Biol.* **34**, 33–47.
- Liu, X., Yu, F., Schnabel, G., Wu, J., Wang, Z. and Ma, Z. (2011a) Paralogous *cyp51* genes in *Fusarium graminearum* mediate differential sensitivity to sterol demethylation inhibitors. *Fungal Genet. Biol.* **48**, 113–123.
- Liu, X., Fu, J., Yun, Y., Yin, Y. and Ma, Z. (2011b) A sterol C-14 reductase encoded by *FgERG24B* is responsible for the intrinsic resistance of *Fusarium graminearum* to amine fungicides. *Microbiology*, **157**, 1665–1675.
- Liu, X.H., Lu, J.P., Zhang, L., Dong, B., Min, H. and Lin, F.C. (2007) Involvement of a *Magnaporthe grisea* serine/threonine kinase gene, *MgATG1*, in appressorium turgor and pathogenesis. *Eukaryot. Cell*, **6**, 977–1005.
- Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods*, **25**, 402–408.
- Marisco, G., Saito, S.T., Ganda, I.S., Brendel, M. and Pungartnik, C. (2011) Low ergosterol content in yeast *adh1* mutant enhances chitin maldistribution and sensitivity to paraquat-induced oxidative stress. *Yeast*, **28**, 363–373.
- McDonald, B.A. and Martinez, J.P. (1990) Restriction fragment length polymorphisms in *Septoria tritici* occur at high frequency. *Curr. Genet.* **17**, 133–138.
- McMullen, M., Jones, R. and Gallenberg, D. (1997) Scab of wheat and barley: a reemerging disease of devastating impact. *Plant Dis.* **81**, 1340–1348.
- Mirocha, C.J., Kolaczowski, E., Xie, W.P., Yu, H. and Jelen, H. (1998) Analysis of deoxynivalenol and its derivatives (batch and single kernel) using gas chromatography/mass spectrometry. *J. Agric. Food Chem.* **46**, 1414–1418.
- Mo, C.Q. and Bard, M. (2005) Erg28p is a key protein in the yeast sterol biosynthetic enzyme complex. *J. Lipid Res.* **46**, 1991–1998.
- Mo, C.Q., Millab, P., Athenstaedt, K., Ott, R., Ballianob, G., Daum, G. and Bard, M. (2003) In yeast sterol biosynthesis the 3-keto reductase protein (Erg27p) is required for oxidosqualene cyclase (Erg7p) activity. *Biochim. Biophys. Acta*, **1633**, 68–74.
- Mo, C.Q., Valachovic, M. and Bard, M. (2004) The *ERG28*-encoded protein, Erg28p, interacts with both the sterol C-4 demethylation enzyme complex as well as the late biosynthetic protein, the C-24 sterol methyltransferase (Erg6p). *Biochim. Biophys. Acta*, **1686**, 30–36.
- Mullins, E.D., Chen, X., Romaine, P., Raina, R., Geiser, D.M. and Kang, S. (2001) *Agrobacterium*-mediated transformation of *Fusarium oxysporum*: an efficient tool for insertional mutagenesis and gene transfer. *Phytopathology*, **91**, 173–180.
- al Musallam, A.A. and Radwan, S.S. (1990) Wool-colonizing micro-organisms capable of utilizing wool-lipids and fatty acids as sole sources of carbon and energy. *J. Appl. Bacteriol.* **69**, 806–813.
- Nemec, T. and Jernejc, K. (2002) Influence of Tween 80 on lipid metabolism of an *Aspergillus niger* strain. *Appl. Biochem. Biotechnol.* **101**, 229–238.
- Osumi, T., Taketani, S., Katsuki, H., Kuhara, T. and Matsumoto, I. (1978) Ergosterol biosynthesis in yeast. Pathways in the late stages and their variation under various conditions. *J. Biochem.* **83**, 681–691.
- Parks, L.W., Smith, S.J. and Crowley, J.H. (1995) Biochemical and physiological effects of sterol alterations in yeast. *Lipids*, **30**, 227–230.
- Pestka, J.J. and Smolinski, A.T. (2005) Deoxynivalenol: toxicology and potential effects on humans. *J. Toxicol. Environ. Health B: Crit. Rev.* **8**, 39–69.
- Pierson, C.A., Jia, N., Mo, C., Lees, N.D., Sturm, A.M., Eckstein, J., Barbuch, R. and Bard, M. (2004) Isolation, characterization, and regulation of the *Candida albicans* *ERG27* gene encoding the sterol 3-keto reductase. *J. Med. Mycol.* **42**, 461–473.
- Proctor, R.H., Hohn, T.M. and McCormick, S.P. (1995) Reduced virulence of *Gibberella zeae* caused by disruption of a trichothecene toxin biosynthetic gene. *Mol. Plant-Microbe Interact.* **8**, 593–601.
- Raychaudhuri, S. and Prinz, W.A. (2006) Uptake and trafficking of exogenous sterols in *Saccharomyces cerevisiae*. *Biochem. Soc. Trans.* **34**, 360–362.
- Rosenfeld, E. and Beauvoit, B. (2003) Role of the non-respiratory pathways in the utilization of molecular oxygen by *Saccharomyces cerevisiae*. *Yeast*, **20**, 1115–1144.
- Schmidt, C.L., Grey, M., Schmidt, M., Brendel, M. and Henriques, J.A.P. (1999) Allelism of *Saccharomyces cerevisiae* genes *PSO6*, involved in survival after 3-CPs + UVA-induced damage, and *ERG3*, encoding the enzyme sterol C-5 desaturase. *Yeast*, **14**, 1503–1510.
- Seong, K.Y., Pasquali, M., Zhou, X.Y., Song, J., Hilburn, K., McCormick, S., Dong, Y., Xu, J.R. and Kistler, H.C. (2009) Global gene regulation by *Fusarium* transcription factors *Tri6* and *Tri10* reveals adaptations to toxin biosynthesis. *Mol. Microbiol.* **72**, 354–367.
- Soustrea, I., Dupuyb, P.H., Silveb, S., Karsta, F. and Loisonb, G. (2000) Sterol metabolism and *ERG2* gene regulation in the yeast *Saccharomyces cerevisiae*. *FEBS Lett.* **470**, 102–106.
- Starkey, D.E., Ward, T.J., Aoki, T., Gale, L.R., Kistler, H.C., Geiser, D.M., Suga, H., Tóth, B., Varga, J. and O'Donnell, K. (2007) Global molecular surveillance reveals novel *Fusarium* head blight species and trichothecene toxin diversity. *Fungal Genet. Biol.* **44**, 1191–1204.
- Tiedje, C., Holland, D.G., Just, U. and Höfken, T. (2007) Proteins involved in sterol synthesis interact with Ste20 and regulate cell polarity. *J. Cell Sci.* **120**, 3613–3624.
- Wang, C.F., Zhang, S.J., Hou, R., Zhao, Z.T., Zheng, Q., Xu, Q.J., Zheng, D.W., Wang, G.H., Liu, H.Q., Gao, X.L., Ma, J.W., Kistler, H.C., Kang, Z.S. and Xu, J.R. (2011) Functional analysis of the kinome of the wheat scab fungus *Fusarium graminearum*. *PLoS Pathog.* **7**, e1002460.
- Wu, A.B., Li, H.P., Zhao, C.S. and Liao, Y.C. (2005) Comparative pathogenicity of *Fusarium graminearum* isolates from China revealed by wheat coleoptile and floret inoculations. *Mycopathologia*, **160**, 75–83.
- Xiong, Q., Hassan, S.A., Wilson, W.K., Han, X.Y., May, G.S., Tarrand, J.J. and Matsuda, S.P. (2005) Cholesterol import by *Aspergillus fumigatus* and its influence on antifungal potency of sterol biosynthesis inhibitors. *Antimicrob. Agents Chemother.* **49**, 518–524.
- Zhang, Y.Q., Gamarra, S., Garcia-Effron, G., Park, S., Perlin, D.S. and Rao, R. (2010) Requirement for ergosterol in V-ATPase function underlies antifungal activity of azole drugs. *PLoS Pathog.* **6**, e1000939.
- Zweytick, D., Hrastnik, C., Kohlwein, S.D. and Daum, G. (2000) Biochemical characterization and subcellular localization of the sterol C-24(28) reductase, erg4p, from the yeast *Saccharomyces cerevisiae*. *FEBS Lett.* **470**, 83–87.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 *FgERG4* gene of *Fusarium graminearum* encodes a sterol C-24 reductase (Erg4), which is homologous to those in yeasts and other filamentous fungi. (a) Phylogenetic tree generated using the neighbour-joining method with Mega 4.1 software on the basis of the deduced amino acid sequences of FgErg4 from *F. graminearum* strain HN9-1 (boxed) and those from *Aspergillus fumigatus* (AfErg4A, GenBank accession no. XP_750461.2; AfErg4B, XP_002376232.1), *Aspergillus nidulans* (AnErg4A, XP_001392043.1; AnErg4B, XP_662788.1), *Botrytis cinerea* (BcErg4, XP_001552616.1), *Candida albicans* (CaErg4, XP_717756.1), *Candida dubliniensis* (CdErg4, XP_002419017.1), *Colletotrichum higginsianum* (ChErg4, CCF43629.1), *Fusarium oxysporum* (FoErg4, FOXG_05355T0), *Fusarium verticillioides* (FvErg4, FVEG_02775T0), *Glomerella graminicola* (GgErg4, EFQ32368.1), *Magnaporthe oryzae* (MoErg4, XP_365763.1), *Neurospora crassa* (NcErg4, XP_961419.2), *Neurospora tetrasperma* (NtErg4, EGO60747.1), *Paracoccidioides brasiliensis* (PbErg4, EEH21282.1), *Penicillium marneffei* (PmErg4, XP_002149230.1), *Saccharomyces cerevisiae* (ScErg4, NP_011503.1), *Schizosaccharomyces pombe* (SpErg4, CAA45113.1), *Sclerotinia sclerotiorum* (SsErg4, XP_001593427.1), *Trichoderma atroviride* (TaErg4, EHK45190.1), *Ustilago maydis* (UcErg4, XP_757645.1) and *Verticillium dahliae* (VdErg4, EGY13563.1). The bootstrap values are indicated on the phylogenetic tree. (b) The alignment of the amino

acid sequences of FgErg4 with those from *S. cerevisiae* and *C. albicans*.

Fig. S2 Generation and identification of *FgERG4* mutant. (a) Gene replacement strategy for *FgERG4* gene. The hygromycin resistance cassette (*HPH*) is denoted by the large grey arrow. Primer binding sites are indicated by arrows (see Table S1 for the primer sequences). (b) Reverse transcription polymerase chain reaction analysis of *FgERG4* expression in the parental strain HN9-1, the deletion mutant Δ FgErg4-2 and the complemented mutant Δ FgErg4-9C using cDNA as template. NC is a negative control without template cDNA in the PCR amplification. (c) Southern blot hybridization analysis of HN9-1, Δ FgErg4-2 and Δ FgErg4-9C using a 910-bp *FgERG4* downstream fragment as a probe. The genomic DNA preparation of each strain was digested with *Sa*I.

Fig. S3 Spectrophotometric analysis of sterol intermediates in the parental strain HN9-1, the *FgERG4* gene deletion mutant (Δ FgErg4-2) and the complemented mutant Δ FgErg4-9C. OD, optical density.

Fig. S4 Exogenous application of cholesterol was unable to restore the mycelial growth defects of Δ FgErg4-2. The wild-type progenitor HN9-1, the deletion mutant Δ FgErg4-2 and the complemented strain Δ FgErg4-9C were cultured on potato dextrose agar (PDA) medium supplemented with cholesterol at the concentrations indicated in the figure under aerobic (a) or anaerobic (b) conditions.

Table S1 Oligonucleotide primers used in this study and their relevant characteristics.