A transcription factor FgSte12 is required for pathogenicity in *Fusarium graminearum*

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SUMMARY

A conserved mitogen-activated protein kinase (MAPK) cascade homologous to the yeast Fus3/Kss1 mating/filamentation pathway is involved in the regulation of vegetative development and pathogenicity in *Fusarium graminearum*. However, little is known about the downstream transcription factors of this pathway. In *Saccharomyces cerevisiae*, the homeodomain protein Ste12 is a key transcription factor activated by Fus3/Kss1. In this study, we characterized a Ste12 orthologue FgSte12 in *F. graminearum*. The *FgSTE12* deletion mutant (Δ FgSte12) was impaired in virulence and in the secretion of cellulase and protease, although it did not show recognizable phenotype changes in hyphal growth, conidiation or deoxynivalenol (DON) biosynthesis. In addition, Δ FgSte12 and the *FgGPMK1* (a *FUS3/KSS1*-related MAPK gene) mutant shared several phenotypic traits. Furthermore, we found that FgGpmk1 controls the nuclear localization of FgSte12. Yeast two-hybrid and affinity capture assays indicated that FgSte12 interacts with the FgSte11–Ste7–Gpmk1 complex. Taken together, these results indicate that FgSte12 is a downstream target of FgSte11–Ste7–Gpmk1 and plays an important role in pathogenicity in *F. graminearum*.

Keywords: *Fusarium graminearum*, FgGpmk1, pathogenicity, transcription factor FgSte12.

INTRODUCTION

Fusarium graminearum (teleomorph *Gibberella zeae*) causes Fusarium head blight (FHB), which is a devastating disease of cereal crops worldwide. Infection of cereal crops with *F. graminearum* may lead to huge yield losses in severe epidemic years (Goswami and Kistler, 2004). More importantly, trichothecene mycotoxins, including deoxynivalenol (DON), acetyldeoxynivalenol (3-ADON or 15-ADON), nivalenol (NIV) and acetylnivalenol (4-ANIV), produced by *F. graminearum* in infested grains, pose a serious threat to human and animal health (McMullen *et al.*, 1997; Pestka and Smolinski, 2005). Despite the serious damage caused by FHB, efficient strategies for the management of FHB are not available to date (Steiner *et al.*, 2009). Understanding the fundamental

biology of this fungal pathogen can provide the basis for sustainable, long-term disease management.

In several plant-pathogenic fungi, the mitogen-activated protein kinase (MAPK), homologous to the *Saccharomyces cerevisiae* mating/filamentation MAPKs Fus3/Kss1, has been found to be involved in pathogenicity (Qi and Elion, 2005). The essential role of this MAPK (named Pmk1) during infection was first reported in the rice blast fungus *Magnaporthe grisea*. Fungal mutants lacking the *PMK1* gene were defective in appressorium formation and were unable to infect host plants. Subsequently, Fus3/Kss1 orthologues were also found to be essential for pathogenicity in other plant pathogens, including *Colletotrichum lagenarium* and *Botrytis cinerea* (Park *et al.*, 2006; Takano *et al.*, 2000; Zhao *et al.*, 2005, 2007; Zheng *et al.*, 2000). In *F. graminearum*, FgGpmk1 (the orthologue of Fus3/Kss1) is involved in vegetative differentiation and virulence (Jenczmionka *et al.*, 2003; Jenczmionka and Schafer, 2005). FgGpmk1 is known to control a variety of virulence-related functions (Zhao *et al.*, 2007); however, questions remain unanswered on how this MAPK regulates the pathogenicity of *F. graminearum*.

The transcription factor Ste12, which functions downstream of the MAPKs Fus3/Kss1, binds to pheromone and filamentation response elements in the promoters of target genes involved in invasive growth in *S. cerevisiae* (Madhani *et al.*, 1997). Ste12 orthologues have been identified and characterized in several plant-pathogenic fungal species. In *M. oryzae*, the appressoria formed by *MST12* mutants have normal appressorium turgor, but fail to develop penetration pegs (Park *et al.*, 2002, 2004). The orthologue of Ste12 in *C. lagenarium* is Cst1. Unlike Mst12 of *M. oryzae*, Cst1 is essential for penetration and infectious growth, but dispensable for appressorium formation (Tsuji *et al.*, 2003). Similarly, disruption of the Ste12 orthologue in *Cryphonectria parasitica* results in dramatically reduced virulence, suggesting an essential role of CpSte12 during the infection process (Wong Sak Hoi *et al.*, 2007).

A whole-genome search revealed that *F. graminearum* contains an orthologue of Ste12 (hereafter named FgSte12). Based on the previous studies, we hypothesized that FgSte12 might play an important role in the pathogenicity of *F. graminearum*. To address this hypothesis, the main objective of this study was to analyse the biological, genetic and biochemical functions of FgSte12. Our study revealed that the *FgSTE12* deletion mutants did not show recognizable phenotype changes in vegetative growth,

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conidiation or DON biosynthesis, but were impaired in pathogenicity on host plants. FgSte12 may function downstream of the FgGpmk1 in *F. graminearum*.

RESULTS

In silico analysis of FgSte12 in *F. graminearum*

The Ste12 orthologue of *F. graminearum* was retrieved by BLASTP search of the *Fusarium* genome database (http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html) with the *S. cerevisiae* Ste12 protein as a query. FgSte12 was predicted to encode a 695-amino-acid protein, which shares 60% similarity with the *S. cerevisiae* Ste12. In addition, protein domain analysis by SMART (<http://smart.embl-heidelberg.de/>) revealed that FgSte12 contains a STE domain and two C₂H₂ zinc finger domains (Fig. 1a), which are highly homologous to those of other Ste12 orthologues (Fig. 1a–c).

Deletion and complementation of FgSTE12 in *F. graminearum*

For a detailed functional analysis of FgSte12, we generated gene deletion mutants using a homologous recombination strategy

(Fig. 2a). Among 50 hygromycin-resistant transformants, 24 *FgSTE12* deletion mutants were identified by PCR analysis with the primer pair A5 + A6 (Table S1, see Supporting Information). The primer pair amplified 1865-bp and 1548-bp fragments from the deletion mutants and the wild-type progenitor HN9-1, respectively. Reverse transcription-polymerase chain reaction (RT-PCR) with the primer pair A7 + A8 amplified an expected 407-bp fragment from the wild-type progenitor HN9-1 and complemented strain ΔFgSte12-C, but not from the deletion mutant ΔFgSte12 (Fig. 2b). When probed with a 668-bp DNA fragment of *FgSTE12*, ΔFgSte12 had an anticipated 4076-bp band, but lacked a 2446-bp band, which was present in the wild-type progenitor (Fig. 2c). The Southern hybridization pattern confirmed that ΔFgSte12 is a null mutant resulting from the homologous recombination events at the *FgSTE12* locus. The complemented strain ΔFgSte12-C contains a single copy of the wild-type *FgSTE12*, which was inserted into the genome of ΔFgSte12 (Fig. 2c).

FgSte12 is dispensable for hyphal growth, conidiation and conidial germination

The *FgSTE12* mutant (ΔFgSte12) did not show obvious phenotypic changes when compared with the wild-type progenitor in

Fig. 1 Sequence alignment of Ste12 orthologues from *Fusarium graminearum* and other fungi. (a) Each Ste12 orthologue contains two functional protein domains, STE and C₂H₂ domains, which were identified by SMART (<http://smart.embl-heidelberg.de/>). (b) Alignments of amino acid sequences of STE domain of the Ste12 orthologue of *F. graminearum* with those of *Saccharomyces cerevisiae*, *Magnaporthe grisea*, *Candida albicans* and *Neurospora crassa*. The Boxshade program was used to highlight identical (black shading) or similar (grey shading) amino acids. (c) Alignments of amino acid sequences of two C₂H₂ domains of FgSte12 with those of *M. grisea* and *N. crassa*. The Boxshade program was used to highlight identical (black shading) or similar (grey shading) amino acids.

Fig. 2 Generation and identification of the *FgSTE12* deletion mutant and complemented strain. (a) Gene deletion strategy for *FgSTE12*. The hygromycin resistance cassette (*HPH*) is denoted by the large black arrow. Primer binding sites are indicated by arrows (see Table S1 for the primer sequences). A 668-bp downstream fragment of *FgSTE12* was used as a probe in Southern blot hybridization analysis. (b) Reverse transcription-polymerase chain reaction (RT-PCR) analysis for the transcription of *FgSTE12* in the wild-type HN9-1, the *FgSTE12* deletion mutant (ΔFgSte12) and the complemented strain (ΔFgSte12-C). NC is a negative control without cDNA template. (c) Southern blot hybridization analysis of *FgSTE12* locus in HN9-1, ΔFgSte12 and ΔFgSte12-C. The genomic DNA preparation of each strain was digested with *Xho*I.

Fig. 3 Effects of *FgSTE12* and *FgGPMK1* deletion on colony morphology. The wild-type HN9-1, ΔFgSte12, ΔFgSte12-C and *FgGPMK1* deletion mutant (ΔFgGpmk1) were grown on potato dextrose agar (PDA) and minimal medium (MM) at 25 °C for 3 days.

hyphal growth and colony morphology on potato dextrose agar (PDA) or minimal medium (MM) (Fig. 3). In addition, to determine the effects of *FgSTE12* deletion on conidiation, 10 mycelial plugs of each strain were inoculated in mung bean liquid (MBL) medium. After 4 days of incubation in a shaker, we found that ΔFgSte12 did not exhibit any defects in conidiation (Fig. S1a, see Supporting Information). Furthermore, the conidia of ΔFgSte12 germinated normally in 2% (w/v) sucrose solution (Fig. S1b).

FgSte12 is required for pathogenicity of *F. graminearum*

Pathogenicity of ΔFgSte12 was evaluated by point inoculation of conidial suspensions on flowering wheat heads. Fifteen days after inoculation, scab symptoms failed to develop, even in the spikelet inoculated with conidia of ΔFgSte12. Under the same conditions, however, the wild-type HN9-1 and the complemented strain caused serious scab symptoms on wheat heads (Fig. 4a). In

Fig. 4 Virulence of *FgSTE12* and *FgGPMK1* deletion mutants. (a) Wheat heads were point inoculated with conidial suspensions of the wild-type HN9-1, $\Delta FgSte12$, $\Delta FgSte12-C$ and $\Delta FgGpmk1$. Infected wheat heads were photographed 15 days after inoculation. The negative control (NC) was only injected with water. (b) Each strain was inoculated on wheat-head medium and incubated at 25 °C for 3 days. (c) Tomatoes were inoculated with a conidial suspension of each strain and infected fruits were photographed 3 days after inoculation. The negative control (NC) was only injected with water (without fungal conidia).

addition, each strain was incubated on wheat-head medium. As revealed in Fig. 4b, the mutant showed no detectable vegetative defects when compared with the wild-type HN9-1, indicating that, although a growth medium made with wheat heads provides the necessary nutrients for growth, the mutant is still unable to cause disease on wheat heads.

DON is an important virulence factor in *F. graminearum* (Proctor *et al.*, 1995; Seong *et al.*, 2009; Starkey *et al.*, 2007). As $\Delta FgSte12$ is impaired in the infection of flowering wheat heads, we were also interested in analysing DON production for the mutant. After incubation on wheat kernels for 20 days, $\Delta FgSte12$ produced similar amounts of DON as the wild-type HN9-1 (Fig. S2, see Supporting Information), suggesting that *FgSte12* is not involved in the regulation of DON biosynthesis in *F. graminearum*.

To examine the ability of $\Delta FgSte12$ to colonize tomato, a 10- μ L aliquot of conidial suspension of each strain was injected into wounded tomato after surface sterilization. As shown in Fig. 4c, the disease lesion caused by $\Delta FgSte12$ on wounded tomato was dramatically smaller than that caused by the wild-type HN9-1. To

test whether the reduction in virulence is associated with a penetration defect of the mutant, we performed a cellophane penetration assay. As shown in Fig. 5a, $\Delta FgSte12$ was unable to, but the wild-type and complemented strains were able to, penetrate cellophane sheet. Furthermore, the addition of $NaNO_3$ or NH_4NO_3 had no positive effects on the penetration of $\Delta FgSte12$ into the cellophane sheet (Fig. S3, see Supporting Information). In addition, electron scanning microscopy examination showed that the hyphae of the wild-type progenitor formed penetration structures on wheat spikelets, but such penetration structures were not observed for $\Delta FgSte12$ (Fig. 5b). These results suggest that *FgSte12* is required for penetration and extension during infection of *F. graminearum* on host plants.

***FgSte12* regulates sexual development**

As a homothallic fungus, sexual reproduction plays a significant role in the infection cycle of *F. graminearum* (Min *et al.*, 2012; Trail, 2009). In order to characterize the function of *FgSte12* in sexual

Fig. 5 Penetration of *FgSTE12* and *FgGPMK1* deletion mutants into cellophane membrane and wheat glumes. (a) Fungal colonies were grown for 2 days at 25 °C on top of cellophane membranes placed on minimal medium (Before). The cellophane membranes with the fungal colonies were removed, and the plates were incubated for an additional day to examine the presence of mycelial growth on the plate, indicating penetration of the cellophane (After). (b) Infection assay on dissected wheat glumes. Wheat head spikelets were inoculated with conidia of each strain. Samples were taken 8 days after inoculation, and examined with a scanning electron microscope. Infection structures (FS, foot structure; LA, lobate appressorium indicated by the arrows) were observed in the wild-type (HN9-1), but not in the mutant Δ FgSte12. Bar, 5 μ m.

development, we determined the ability of the mutant to form perithecia on carrot agar. Consistent with the finding from a previous report (Jenczmionka *et al.*, 2003), we found that the *FgGPMK1* mutant was sexually sterile. The *FgSTE12* deletion mutant produced significantly ($P < 0.05$) less perithecia than the wild-type HN9-1 (Fig. 6).

FgSte12 regulates the secretion of cell wall-degrading enzymes

Cell wall-degrading enzymes secreted by *F. graminearum* have been found to be important pathogenicity factors. A previous

study has indicated that FgGpmk1 regulates the activities of extracellular endoglucanase, and xylanolytic and proteolytic enzymes (Jenczmionka and Schafer, 2005). Furthermore, this MAPK was also responsible for the induction of secreted lipolytic activities (Jenczmionka and Schafer, 2005). As Δ FgSte12 was unable to penetrate a cellophane sheet, we were also interested in analysing the secreted proteolytic and endoglucanase activity for the mutant. As shown in Fig. 7a,b, Δ FgSte12 exhibited reduced proteolytic and cellulase activities, which could partially explain the reduced virulence of the mutant on the host plant. However, Δ FgSte12 did not show a detectable alteration in lipolytic

Fig. 6 Effects of the deletion of *FgSTE12* and *FgGPMK1* on sexual development of *Fusarium graminearum*. The wild-type HN9-1, Δ*FgSte12* and Δ*FgGpmk1* were incubated on carrot agar for 2 weeks to induce the formation of perithecia. Line bars denote standard errors of three repeated experiments. Values on the open bars followed by the same letter are not significantly different according to Fisher's least significant difference (LSD) test at $P = 0.05$.

activity compared with the wild-type HN9-1 (Fig. S4, see Supporting Information).

In *S. cerevisiae*, the *cis*-acting sequence of TGAACA was recognized by Ste12 (Yuan and Fields, 1991). We also identified four cellulase genes which contain the sequence of TGAACA in their promoters. Quantitative real-time PCR assays showed that the expression levels of these four genes were decreased significantly in Δ*FgSte12* (Fig. 8). In contrast, the transcription levels were similar to the wild-type for four other cellulase genes without the sequence of TGAACA in their promoters (Fig. S5, see Supporting Information); this suggests that *FgSte12* might normally bind to the *cis*-acting sequence in *F. graminearum*.

Identification of *FgSte12*-interacting proteins

To identify *FgSte12*-interacting proteins, we conducted an affinity capture experiment. Briefly, *FgSte12* was tagged with green fluorescent protein (GFP), and proteins co-purified with *FgSte12*-GFP were analysed by mass spectrometry. Interestingly, *FgSte7* (encoded by the *FGSG_09897* gene) was co-purified with *FgSte12*-GFP (Table 1). BLASTP analysis revealed that *FgSte7* is homologous to *S. cerevisiae* ScSte7, which functions as a kinase upstream of *FgGpmk1*. In *F. graminearum*, the interaction of *FgGpmk1* with *FgSte7*, but not with *FgSte12*, was demonstrated by yeast two-hybrid assay (Fig. 9).

To further investigate the relationship between *FgGpmk1* and *FgSte12*, we generated *FgGPMK1* mutants. Phenotypic analyses showed that, similar to *FgSTE12* mutants, the *FgGPMK1* mutant exhibited defects in pathogenicity, penetration of cellophane sheet, secretion of protease and cellulose, and was unable to form perithecia (Figs 4–7). In addition, the transcription level of *FgSTE12* was increased three-fold in the *FgGPMK1* mutant

Fig. 7 Comparison of the activities of cell wall-degrading enzymes secreted by the wild-type HN9-1, Δ*FgSte12* and Δ*FgGpmk1*. (a) Cellulase activity was measured spectrophotometrically using carboxymethylcellulose as substrate. One unit of enzymatic activity is defined as 1 nmol/min reducing sugars released from the substrate. Line bars denote the standard errors of three repeated experiments. (b) Proteolytic activity. One unit of enzymatic activity is defined as 1 nmol/min aromatic amino acids released from the substrate. Line bars denote standard errors of three repeated experiments.

(Fig. S6a, see Supporting Information), indicating that *FgGpmk1* positively regulates the transcription of *FgSTE12*. Western blotting assays showed that the phosphorylation of *FgGpmk1* in Δ*FgSte12* did not change dramatically when compared with that in the wild-type progenitor (Fig. S7, see Supporting Information). To further understand how *FgGpmk1* regulates *FgSte12*, we analysed the subcellular localization of *FgSte12* in Δ*FgGpmk1*. Briefly, the entire *FgSte12* protein coding sequence was fused in-frame to GFP under the strong constitutive promoter RP27, and introduced into Δ*FgGpmk1* and Δ*FgSte12*, respectively. As shown in Fig. 10, *FgSte12*-GFP localized to the nucleus in mycelia in the Δ*FgSte12* background strain. However, *FgSte12*-GFP localized to both the nucleus and cytoplasm in the Δ*FgGpmk1* background strain, indicating that *FgGpmk1* regulates the subcellular localization of *FgSte12*. Taken together, these results indicate that *FgSte12* may function downstream of *FgGpmk1* in *F. graminearum*.

Fig. 8 Effects of *FgSTE12* deletion on the transcription of cellulase genes. The relative transcription level of each cellulase gene in Δ FgSte12 is the relative amount of mRNA of each gene in the wild type HN9-1. These four genes contain the *cis*-element TGAAACA in their promoters. Line bars in each column denote standard errors of three repeated experiments. Values on the grey bars followed by the same letter are not significantly different according to Fisher's least significant difference (LSD) test at $P = 0.05$.

Table 1 Identification of FgSte12 interacting proteins by affinity capture assays.

Proteins	Putative encoding protein
FGSG_09897	Ste7
FGSG_10042	Signal transduction histidine kinase
FGSG_07397	Predicted unusual protein kinase
FGSG_04382	Serine/threonine protein kinase nrc-2
FGSG_06957	Serine/threonine protein kinase Cla4
FGSG_09903	Serine/threonine protein kinase carbon catabolite derepressing protein kinase
FGSG_05912	Mevalonate kinase
FGSG_11938	ATP phosphoribosyltransferase
FGSG_10198	Aconitase A
FGSG_09908	Regulatory subunit of cAMP-dependent protein kinases
FGSG_09892	Uncharacterized conserved protein, contains RING Zn-finger
FGSG_09616	Cullin, a subunit of E3 ubiquitin ligase
FGSG_09572	5,10-Methylenetetrahydrofolate reductase
FGSG_09555	Electron transfer flavoprotein, β subunit
FGSG_09020	Cation transport ATPase
FGSG_08529	Phosphoserine phosphatase
FGSG_05683	Predicted flavoprotein involved in K^+ transport
FGSG_05447	Rho5
FGSG_05293	Predicted divalent heavy-metal cation transporter
FGSG_05132	Predicted phosphatase
FGSG_04948	Uncharacterized conserved protein, contains RING Zn-finger
FGSG_04555	Uncharacterized conserved protein
FGSG_03412	Amino acid transporters
FGSG_02570	Translation elongation factors (GTPases)
FGSG_02460	Cation transport ATPase
FGSG_01947	Sulphite oxidase and related enzymes
FGSG_01866	Predicted GTPases (dynamin-related)
FGSG_01329	Adenylylsulphate kinase and related kinases
FGSG_01006	Sulphate permease and related transporters
FGSG_00852	Dienosine tetraphosphatase and related serine/threonine protein phosphatases

DISCUSSION

The Ste12 and Ste12-like proteins are transcription factors found exclusively in the fungal kingdom. Ste12 was originally reported to be involved in the regulation of mating and invasive growth as a target of the Fus3 and Kss1 MAPK cascade in *S. cerevisiae* (Errede

and Ammerer, 1989; Gavrias *et al.*, 1996; Madhani and Fink, 1997). In this study, we identified a Ste12 orthologue from *F. graminearum*. Similar to other Ste12 orthologues, FgSte12 contains a characteristic STE homeodomain in the N-terminal region of the protein (Fig. 1a). Although the fungal STE homeodomain is highly divergent, a sequence of KQKVFVFSVA in this domain is

Fig. 9 Yeast two-hybrid analyses of interactions between FgSte7, FgGpmk1 and FgSte12. Serial decimal dilutions of yeast cells (cells/mL) transferred with the bait and prey constructs indicated in the figure were assayed for growth on SD-Leu-Trp-His [synthetic medium lacking leucine (Leu), tryptophan (Trp) and histidine (His)] plates. The transformant bearing the pair of plasmids pGBKT7-53 and pGADT7 served as a positive control. The transformants bearing the pairs of plasmids pGBKT7-Lam and pGADT7, pGBKT7 and pGADT7-FgGpmk1, pGBKT7-FgSte7 and pGADT7, and pGBKT7-FgSte12 and pGADT7 were used as negative controls.

Fig. 10 Subcellular localization of FgSte12 in *Fusarium graminearum*. The entire FgSte12 protein coding sequence was fused to green fluorescent protein (GFP) with the constitutive promoter RP27, and introduced into Δ FgGpmk1 and Δ FgSte12. FgSte12-GFP was localized in the nucleus in mycelia of the Δ FgSte12 background strain (left panel), and into both cytoplasm and nucleus in the Δ FgGpmk1 background strain (right panel). Bar, 5 μ m. DIC, differential interference contrast. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

considerably conserved (Yuan and Fields, 1991). In *S. cerevisiae*, a pheromone response element (PRE) containing a sequence of TGAAACA is able to interact with the Ste12 protein (Yuan and Fields, 1991). The STE domain has also been found to be required for DNA binding in *Cryptococcus neoformans* (Chang *et al.*, 2004). A recent study has shown that each base pair from the *cis*-element sequence of TGAAACA recognized by Ste12 is important for binding, except for the last A, in *Colletotrichum lindemuthianum* (Wong Sak Hoi *et al.*, 2007). In *F. graminearum*, we found that four cellulase genes (FGSG_10922, FGSG_04678, FGSG_03795 and FGSG_11487) contain this *cis*-element in their promoter regions. Quantitative real-time PCR assays showed that the transcription levels of these genes were decreased dramatically, but the transcription levels of other cellulase genes without the *cis*-element in their promoters did not change significantly, in Δ FgSte12. These results indicate that FgSte12 may regulate the transcription of target genes by binding to this *cis*-element in *F. graminearum*.

Ste12 proteins have been shown to be required for infection in several pathogenic fungi, including *Alternaria brassicicola*, *M. grisea*, *Fusarium oxysporum*, *C. lagenarium* and *C. lindemuthianum* (Asuncion Garcia-Sanchez *et al.*, 2010; Park *et al.*, 2002; Schamber *et al.*, 2010). In *M. grisea*, the *MST12* mutant showed a defect in microtubule reorganization during penetration peg formation, which possibly leads to penetration defects (Park *et al.*, 2004). In *C. lindemuthianum*, the *STE12* mutant showed decreased pectinase activity (Wong Sak Hoi *et al.*, 2007). However, Ste12 is not required for the activation of pectinase genes in *F. oxysporum* (Asuncion Garcia-Sanchez *et al.*, 2010). In contrast with pectinase activity, the activities of extracellular amylase and cellulase were positively regulated by Ste12 in *F. oxysporum* (Asuncion Garcia-Sanchez *et al.*, 2010). In our study, we observed that FgSte12 is required for *F. graminearum* penetration of the cellophane sheet and host plant tissue. The defects in penetration may partially result from the low levels of secretion of the cell wall-degrading enzymes in Δ FgSte12. In *A. brassicicola*, the *STE12* mutant failed to penetrate host plants to cause disease symptoms. The mutants produced multiple hyphae at several locations of immature conidia and were unable to form appressoria (Cho *et al.*, 2009). These results indicate that, although Ste12 proteins are required for infection in various plant-pathogenic fungi, the mechanisms for the involvement of Ste12 in pathogenicity vary dramatically in different fungal species.

To test whether FgSte12 functions downstream of the MAPK, FgGpmk1, we also generated *FgGPMK1* mutants and compared the phenotypes of Δ FgSte12 and Δ FgGpmk1 systematically. Δ FgSte12 and Δ FgGpmk1 share several phenotypic traits, including defects of penetration into cellophane, induction of cell wall-degrading enzymes, sexual development and pathogenicity. In addition, a high transcript level of *FgSTE12* was detected in the *FgGPMK1* mutant. Furthermore, FgGpmk1 regulates the nuclear

localization of FgSte12. These results indicate that FgSte12 may be associated with the FgGpmk1 pathway. In the yeast two-hybrid assay, we did not observe the direct interaction between FgSte12 and the MAPK FgGpmk1 (Fig. 9). However, we found that FgSte7 was co-purified with FgSte12 in the affinity capture assay (Table 1). As reported recently, FgGpmk1 and FgSte11–FgSte7 form a complex (Wang *et al.*, 2011). In our yeast two-hybrid assays, we observed the physical interaction between FgSte7 and FgGpmk1 (Fig. 9). Thus, we propose that FgSte12 interacts with the FgSte11–FgSte7–FgGpmk1 complex in *F. graminearum*. Furthermore, Western blotting assays showed that FgGpmk1 phosphorylation was not detectable in the Fgste11 and Fgste7 mutants (Ramamoorthy *et al.*, 2007), indicating that FgSte11 and FgSte7 function upstream of FgGpmk1. However, phosphorylation of FgGpmk1 was not affected by the deletion of *FgSTE12* (Fig. S7), indicating that FgSte12 may be a downstream target of the FgSte11–Ste7–Gpmk1 complex.

Although Δ FgSte12 and Δ FgGpmk1 shared several characteristic phenotypes, we also identified some defects of Δ FgGpmk1 that were not observed for Δ FgSte12. The *FgGPMK1* mutant exhibited decreased hyphal growth and conidiation. A delay in conidial germination was observed in Δ FgGpmk1 (Jenczmionka *et al.*, 2003). By contrast, deletion of *FgSTE12* had no detectable effects on hyphal growth, conidiation or conidial germination (Fig. 3, Fig. S1a,b), indicating that, although FgSte12 may be one of the outputs of FgGpmk1, other downstream transcription factor(s) might be involved in the regulation of vegetation differentiation in *F. graminearum*.

In *S. cerevisiae*, the phosphorylation of Ste12 by either Fus3 or Kss1 was established as a key step for the induction of the activity of Ste12 (Wong Sak Hoi and Dumas, 2010). Phosphorylated Ste12, dissociated from its regulatory proteins (Dig1 and Dig2), is input into the nucleus and activates its target genes (Bardwell *et al.*, 1998; Cook *et al.*, 1996). In the current study, we found that deletion of FgGpmk1 only had a partial effect on the nuclear localization of FgSte12 (Fig. 10), indicating that other unknown upstream component(s) may also act on FgSte12 (Fig. 11). In our affinity capture assays, we found that several kinases (including FGSG_10042, FGSG_07397, FGSG_06957 and FGSG_04382) interact with FgSte12 (Table 1). These kinases might also play important roles in the phosphorylation of the FgSte12 protein. Thus, further characterization of these FgSte12-interacting proteins will be helpful for a full understanding of the functions of Ste12 in filamentous fungi.

EXPERIMENTAL PROCEDURES

Fungal strains, growth assays and sporulation tests

Fusarium graminearum strain HN9-1 was used as the wild-type progenitor for the construction of various gene deletion mutants. The wild-type strain,

Fig. 11 Proposed functions of FgSte12 in *Fusarium graminearum*. FgSte12 regulates penetration, sexual development and activities of lytic enzymes downstream of the FgSte11–Ste7–Gpmk1 complex, whereas the remaining FgGpmk1-controlled functions may be mediated by other unidentified factors. In addition, other unknown kinase(s) may also regulate FgSte12.

resultant mutants and complemented strains were cultured at 25 °C on PDA (200 g potato, 20 g dextrose, 20 g agar and 1 L water), MM (10 mM K_2HPO_4 , 10 mM KH_2PO_4 , 4 mM $(NH_4)_2SO_4$, 2.5 mM NaCl, 2 mM $MgSO_4$, 0.45 mM $CaCl_2$, 9 mM $FeSO_4$, 10 mM glucose and 1 L water, pH 6.9) or wheat-head medium (200 g ground fresh wheat heads and 20 g agar in 1 L double-distilled H_2O) for mycelial growth tests.

For conidiation assays, 10 mycelial plugs (5 mm in diameter) of each strain, taken from the periphery of a 3-day-old colony, were inoculated in a 50-mL flask containing 20 mL of MBL (10 g mung beans boiled in 1 L water for 20 min and filtered through cheesecloth) (Mirocha *et al.*, 1989). The flasks were incubated at 25 °C for 4 days in a shaker (180 rpm). The number of conidia was determined for each strain using a haemocytometer. The experiment was repeated three times independently.

Constructed gene deletion and complemented strains

The gene deletion vectors and transformation of *F. graminearum* were carried out using the protocols described previously (Jiang *et al.*, 2011a). The primers used to amplify the flanking sequences for each gene are listed in Table S1. Putative gene deletion mutants were identified by PCR assays with the relevant primers (Table S1), and were further analysed by the Southern blotting assay (Fig. 2c). All of the mutants generated in this study were preserved in 15% glycerol at –80 °C.

Microscopic examinations of hyphal and conidial morphology

The hyphal morphology of each mutant was examined with a Leica TCS SP5 imaging system (Wetzlar, Hesse-Darmstadt, Germany) using fresh mycelia harvested from a 3-day-old colony of each strain growing on PDA plates. For the observation of GFP signals (GFP fused with FgSte12), fresh mycelia were examined with a Zeiss LSM780 confocal microscope (Gottingen, Niedersachsen, Germany). To observe nuclei, fresh mycelia were washed with sterilized water and stained with 10 µg/mL of 4',6'-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO, USA).

Assays for sexual development

Aerial hyphae of a 3-day-old culture of each strain on carrot agar were pressed down with 300 µL of sterilized 0.1% Tween 60. Perithecia formation was examined after 1 week of incubation at 25 °C, as described previously (Jenczmionka *et al.*, 2003). The experiment was repeated three times.

Pathogenicity assays

The pathogenicity of each strain on flowering wheat heads was assessed as described previously (Jiang *et al.*, 2011a). Briefly, each strain was cultured in MBL for 5 days. The resultant conidia were collected by filtration through three layers of cheese cloth and subsequently resuspended in sterilized water to a concentration of 10^6 conidia/mL. A 10-µL aliquot of conidial suspension was injected into a floret in the central section spikelet of a single flowering wheat head of susceptible cultivar Zimai22. The control heads were inoculated with 10 µL of sterilized water. There were 20 replicates for each strain. After inoculation, the plants were kept under 100% humidity at 22 ± 2 °C for 2 days, and then maintained in a glass-house. Fifteen days after inoculation, the infected spikelets in each inoculated wheat head were recorded. The experiment was repeated four times independently. To analyse the virulence defect of the mutants in detail, infected wheat spikelets were sampled 8 days after inoculation. Hyphal structures of each strain growing in the spikelet were examined with a Hitachi TM-1000 tabletop microscope (Hitachi, Tokyo, Japan).

To examine the ability to colonize tomato, a 10-µL aliquot of a conidial suspension was injected into 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.

To analyse the virulence defect of the mutants in detail, the penetration behaviour of each strain was examined on cellophane membranes, as described previously (Lopez-Berges *et al.*, 2010). Briefly, each strain was grown on MM covered with a cellophane membrane. After 2 days of incubation, the cellophane membrane with the colony was removed from each plate. After the plates had been incubated for an additional day, mycelial growth on each plate was examined. The presence of mycelial growth on the plate indicated penetration of the cellophane membrane. The experiment was repeated three times.

Determination of DON production

A 50-g aliquot of healthy wheat kernels was sterilized and inoculated with five mycelial plugs of each strain. A negative control was prepared with wheat kernels inoculated with five agar plugs. After incubation at 25 °C for 20 days, DON was extracted using a previously described protocol (Mirocha *et al.*, 1998). The DON extracts were purified with a PuriToxSR DON column TC-T200 (Trilogy Analytical Laboratory, Washington, MO, USA), and the amount of DON (per milligram of fungal DNA) in each sample was determined using a Waters 1525 high-performance liquid chromatography (HPLC) system (Shimadzu Corporation, Kyoto, Japan) (Jiang *et al.*, 2011b). The amount of *F. graminearum* DNA in each sample

was determined using a quantitative real-time PCR method (Yin *et al.*, 2009). The experiment was repeated three times, and the data were analysed using analysis of variance (SAS version 8.0; SAS Institute, Cary, NC, USA).

RNA extraction and quantitative real-time PCR

To extract total RNA, mycelia of each strain were inoculated in potato dextrose broth (PDB) and cultured for 2 days at 25 °C in the dark. Mycelia were harvested by filtration over two layers of Miracloth and washed with sterilized water. Harvested mycelia were then lyophilized and ground in liquid nitrogen. Total RNA was extracted from mycelia of each sample using the TaKaRa RNAiso Reagent (TaKaRa Biotechnology Co., Dalian, China), and 10 mg of each RNA sample was used for reverse transcription with a RevertAid H Minus First Strand cDNA Synthesis Kit employing the oligo(dT)₁₈ primer (Fermentas Life Sciences, Burlington, ON, Canada). The expression levels of *FgSTE12* and cellulase genes were determined by quantitative real-time PCR with the primers listed in Table S1. For each sample, PCR amplification with the primer pair actin-F + actin-R (Table S1) for the quantification of the expression of the actin gene was performed as a reference. The experiment was repeated three times independently.

Cell wall-degrading enzyme activity assays

Total proteolytic activity was assayed by measuring the release of aromatic amino acids from the substrate using a previously described protocol (Jenczmionka and Schafer, 2005). The activity of cellulase was assayed by measuring the reducing sugars set free from the substrates using a previously published protocol (Jenczmionka and Schafer, 2005). For the determination of extracellular lipolytic activity, fresh mycelia of each strain were incubated in 50 mL of water supplemented with 2% (v/v) wheat germ oil (a lipase inducer) for different times, as indicated in the legend to Fig. S4. Lipolytic activity was determined using a previously published protocol (Voigt *et al.*, 2005). The experiment was repeated three times, and data were analysed using analysis of variance (SAS version 8.0; SAS Institute).

Western blotting assay

Six mycelial plugs were inoculated into 150 mL PDB and incubated at 25 °C in a shaker (200 rpm) for 36 h. Mycelia were then harvested, washed with deionized water and ground in liquid nitrogen. Approximately 200 mg of finely ground mycelia were resuspended in 1 mL of extraction buffer [50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 2 mM phenylmethylsulphonylfluoride (PMSF)] and 10 µL of protease inhibitor cocktail (Sangon, Shanghai, China). After homogenization with a vortex shaker, the lysate was centrifuged at 14 000 *g* in a microcentrifuge for 20 min at 4 °C. The resulting proteins were separated on 10% denaturing polyacrylamide gel (sodium dodecylsulphate-polyacrylamide gel electrophoresis, SDS-PAGE) and transferred to Immobilon-P transfer membrane (Millipore, Billerica, MA, USA) with a Bio-Rad electroblotting apparatus (Hercules, CA, USA). The total and phosphorylated FgGpmk1 were detected with a PhosphoPlus p44/42 MAPK antibody kit (Cell Signaling Technology Inc., Danvers, MA, USA). The total FgSte12-GFP was detected

with anti-GFP antibody (Abcam, Cambridge, MA, USA). The input protein samples were also detected with anti-histone H3 antibody (Abcam) as a reference. Incubation with a secondary antibody and chemiluminescent detection were performed as described previously (Yang *et al.*, 2012). The experiment was conducted three times independently.

Yeast two-hybrid assays

To construct plasmids for yeast two-hybrid analyses, the coding sequence of each tested gene was amplified from cDNA of HN9-1 with the primer pairs indicated in Table S1. The cDNA fragment was inserted into the yeast GAL4 binding domain vector pGBKT7 and GAL4 activation domain vector pGADT7 (Clontech, Mountain View, CA, USA). The pairs of yeast two-hybrid plasmids were co-transformed into *S. cerevisiae* strain AH109 following the LiAc/SS-DNA/PEG transformation protocol (Schieftl and Gietz, 1989). In addition, a pair of plasmids, pGBKT7-53 and pGADT7, served as a positive control. The following pairs of plasmids, pGBKT7-Lam and pGADT7, pGBKT7 and pGADT7-FgGpmk1, pGADT7 and pGBKT7-FgSte7, pGADT7 and pGBKT7-FgSte12, were used as negative controls. Transformants were grown at 30 °C for 3 days on synthetic medium lacking leucine (Leu) and tryptophan (Trp), and were then transferred to medium lacking histidine (His), Leu and Trp, and containing 5 mM 3-aminotriazole (3-AT), to assess binding activity. The experiment was repeated twice independently.

Affinity capture assays

FgSte12 was tagged with GFP and transferred to the *FgSTE12* deletion mutant. The resultant transformant was used for protein extraction as described above. After centrifugation at 14 000 *g* in a microcentrifuge for 20 min at 4 °C, supernatant (800 µL) was transferred into a sterilized tube. About 50 µL of anti-GFP agarose (Abmart, Shanghai, China) was added to capture FgSte12-GFP-interacting proteins, following the manufacturer's instructions. After incubation at 4 °C overnight, the agarose was washed three times with 500 µL of TBS (20 mM Tris-HCl, 500 mM NaCl, pH 7.5). Proteins binding to the beads were boiled with 60 µL of TBS supplemented with 10 µL of 10% SDS. After centrifugation at 14 000 *g* in a microcentrifuge for 5 min at 4 °C, the supernatant was digested with trypsin as described previously (Tao *et al.*, 2005; Zhou *et al.*, 2007). Tryptic peptides were analysed by mass spectrometry by BGI Co. (Shenzhen, China), as described previously (Ding *et al.*, 2010).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Effect of *FgSTE12* deletion on conidiation and conidial germination of *Fusarium graminearum*. (a) Conidia were counted after incubation of the wild-type HN9-1, the mutant Δ FgSte12 and the complemented strain Δ FgSte12-C in mung bean broth for 4 days in a shaker. Line bars in each column denote the standard errors of three experiments. (b) Conidia of HN9-1, Δ FgSte12 and Δ Fg12-C were suspended in 2% (w/v) sucrose solution. After incubation for 6 h, conidial germination of 150 conidia was examined. Line bars in each column denote standard errors of three repeated experiments. Values on the black bars followed by the same letter are not significantly different at $P = 0.05$.

Fig. S2 Effect of *FgSTE12* deletion on deoxynivalenol (DON) production in *Fusarium graminearum*. The amount of DON (per milligram of fungal ergosterol) produced by each strain in infected wheat kernels was determined after 20 days of inoculation. Line bars in each column denote the standard errors of three replicated experiments. Values on the black bars followed by the same letter are not significantly different at $P = 0.05$.

Fig. S3 Penetration of *FgSTE12* and *FgGPMK1* deletion mutants into cellophane membrane (CM) on minimal medium (MM) supplied with different nitrogen sources. Fungal colonies were grown for 2 days at 25 °C on top of CM placed on MM containing 50 mM NaNO₃ or NH₄NO₃ (Before). CMs with the fungal colonies were then removed, and the plates were incubated for an additional day to examine the presence of mycelial growth on the plate, indicating the penetration of the CM (After). All the strains were unable to penetrate CM on MM supplied with 50 mM HN₄NO₃. The wild-type HN9-1 and complemented strain Δ FgSte12-C, but not the mutants Δ FgSte12 and Δ FgGpmk1, were able to penetrate CM on MM amended with 50 mM NaNO₃.

Fig. S4 Extracellular lipolytic activity of the wild-type HN9-1 and the mutants Δ FgSte12 and Δ FgGpmk1. One unit of lipolytic activity was defined as 1 nmol/min *p*-nitrophenol released from the substrate. Bars denote the standard errors of three repeated experiments.

Fig. S5 Effects of *FgSTE12* deletion on the expression of cellulase genes. Transcription levels of cellulase genes in the wild-type HN9-1 and Δ FgSte12 were determined by quantitative real-time PCR. The relative transcription level of each cellulase gene in Δ FgSte12 is the relative amount of mRNA of each gene in the wild type HN9-1. These four genes do not contain the *cis*-element TGAAACA in their promoters. PCR amplifications with the primer pair actin-F + actin-R for the quantification of expression of the actin gene were performed as a reference. Line bars in each column denote the standard errors of three repeated experiments. Values on the open bars followed by the same letter are not significantly different at $P = 0.05$.

Fig. S6 *FgSTE12* was up-regulated in the *FgGPMK1* deletion mutant. (a) The relative transcription level of FgSTE12 in Δ FgGpmk1 is the relative amount of mRNA of FgSTE12 in the wild type HN9-1. The line bar in the column denotes the standard errors of three repeated experiments. Values on the grey bars followed by the same letter are not significantly different at $P = 0.05$. (b) The protein levels of FgSte12 in wild-type and Δ FgGpmk1. The entire FgSte12 protein coding sequence was fused to green fluorescent protein (GFP) under the native promoter, and introduced into Δ FgGpmk1 and Δ FgSte12. The anti-GFP antibody was used to detect the protein levels of FgSte12-GFP.

Fig. S7 The deletion of *FgSTE12* did not affect FgGpmk1 phosphorylation dramatically. The phosphor-p44/42 mitogen-activated protein kinase (MAPK) antibody and p44/42 MAPK antibodies were used to detect phosphorylated FgGpmk1 and total FgGpmk1, respectively.

Table S1 Oligonucleotide primers used in this study.