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Genome-wide functional characterization of putative peroxidases in the head blight fungus *Fusarium graminearum*

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SUMMARY

Reactive oxygen species (ROS) are associated with various developmental processes and host–pathogen interactions in pathogenic fungi. Peroxidases are a group of ROS-detoxifying enzymes that are involved in the oxidative stress response and in a variety of physiological processes. In this study, we performed a genome-wide functional characterization of putative peroxidase genes in *Fusarium graminearum*, a head blight pathogen of cereal crops. We identified 31 putative peroxidase genes and generated deletion mutants for these genes. Twenty-six of the deletion mutants showed developmental phenotypes indistinguishable from that of the wild-type, and five deletion mutants exhibited phenotypic changes in at least one phenotypic category. Four deletion mutants, *fca6*, *fca7*, *fpx1* and *fpx15*, showed increased sensitivity to extracellular H_2O_2. Deletion mutants of *FCA7* also exhibited reduced virulence and increased trichothecene production compared with those of the wild-type strain, suggesting that Fca7 may play an important role in the host–pathogen interaction in *F. graminearum*. To identify the transcription factors (TFs) regulating *FCA6*, *FCA7*, *FPX1* and *FPX15* in response to oxidative stress, we screened an *F. graminearum* TF mutant library for growth in the presence of H_2O_2 and found that multiple TFs co-regulated the expression of *FCA7* under oxidative stress conditions. These results demonstrate that a complex network of transcriptional regulators of antioxidant genes is involved in oxidative stress responses in this fungus. Moreover, our study provides insights into the roles of peroxidases in developmental processes and host–pathogen interactions in plant-pathogenic fungi.

Keywords: *Fusarium graminearum*, oxidative stress response, peroxidase.

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INTRODUCTION

Fusarium graminearum, one of the most economically important plant pathogens, causes Fusarium head blight (FHB) in wheat, barley and rice, as well as ear rot in maize (Leslie and Summerell, 2006). Epidemics of FHB cause serious yield losses in major cereal crops worldwide (Goswami and Kistler, 2004; Windels, 2000). In addition to yield losses, this fungus is responsible for the contamination of grains with mycotoxins, such as trichothecenes and zearalenone, which are harmful to humans and to livestock (Desjardins and Proctor, 2007). In particular, trichothecenes are potent inhibitors of protein synthesis (Arunachalam and Doohan, 2013) and are well-studied virulence factors in *F. graminearum* (Proctor *et al.*, 1995).

Reactive oxygen species (ROS), such as superoxides ($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2), are generated as byproducts of aerobic respiration and metabolic pathways that primarily occur in mitochondria, peroxisomes and chloroplasts (Heller and Tudzynski, 2011). On the one hand, because excessive amounts of ROS can damage cellular components by oxidizing membrane lipids, cellular proteins and nucleic acids, living organisms possess efficient ROS-degrading mechanisms (Camhi *et al.*, 1995). On the other hand, some ROS, particularly H_2O_2, act as secondary messengers in important signal transduction pathways (Apel and Hirt, 2004). Therefore, the delicate balance between ROS generation and scavenging is expected to be tightly regulated by a complex antioxidant defence mechanism comprising both enzymatic and non-enzymatic components.

ROS also play an important role in plant–pathogen interactions. During plant infections, phytopathogenic fungi are often exposed to oxidative stress conditions caused by the oxidative burst, a rapid and transient accumulation of ROS (Mehdy, 1994; Wojtaszek, 1997). The oxidative burst is an immediate and non-specific plant defence response triggered by pathogen attack. The excessive accumulation of ROS induces other plant defence responses, such as the cross-linking of cell walls and programmed cell death of plant cells at infection sites, and can also directly kill microbial pathogens (Lamb and Dixon, 1997; Levine *et al.*, 1994; Torres *et al.*, 2006). Therefore, plant-pathogenic fungi have evolved effective ROS-scavenging mechanisms to detoxify plant-derived ROS and to successfully colonize host plants.

Peroxidases are major H_2O_2-decomposing enzymes that catalyse the oxidation of various organic and inorganic compounds using H_2O_2 or organic hydroperoxides as electron acceptors (Heller and Tudzynski, 2011). Peroxidases are involved in the oxidative stress response as antioxidant enzymes, and many studies have attempted to define their roles in ROS detoxification during the initial plant infection process (Garre *et al.*, 1998; Mir *et al.*, 2015; Robbertse *et al.*, 2003; Skamnioti *et al.*, 2007). Moreover, peroxidases, such as catalase, catalase-peroxidase and peroxiredoxin, are required as redox controllers in various physiological processes (Fourquet *et al.*, 2008; König *et al.*, 2012), demonstrating that they are also closely involved in a variety of biological processes.

Several important signal mediators, such as transcription factors (TFs), which orchestrate oxidative stress responses, have been identified in fungi. Yap1 and Skn7 are the best-characterized TFs; they play crucial roles in the oxidative stress response by regulating the expression of genes encoding antioxidant enzymes in *Saccharomyces cerevisiae* (Lee *et al.*, 1999). Several studies have revealed that Yap1 and Skn7 have a conserved function in the oxidative stress response in various plant-pathogenic fungi, including *Ustilago maydis*, *Magnaporthe oryzae*, *Alternaria alternata*, *Cochliobolus heterostrophus* and *Botrytis cinerea* (Chen *et al.*, 2012; Guo *et al.*, 2011; Lev *et al.*, 2005; Lin *et al.*, 2009; Molina and Kahmann, 2007; Shalaby *et al.*, 2014; Temme and Tudzynski, 2009). In *F. graminearum*, several known and novel TFs involved in the oxidative stress response have been characterized (Jiang *et al.*, 2015; Lysøe *et al.*, 2011; Montibus *et al.*, 2013; Wang *et al.*, 2011). These TFs have been reported to mediate the oxidative stress response by regulating the expression of genes encoding putative antioxidant enzymes (peroxidases). However, whether or not these putative antioxidant genes are indeed important for the oxidative stress response in *F. graminearum* remains unknown.

Recent studies have revealed that oxidative stress is also related to secondary metabolite biosynthesis in fungi (Hong *et al.*, 2013a; Montibus *et al.*, 2015). In *Aspergillus* species, aflatoxin biosynthesis is triggered in response to oxidative stress (Hong *et al.*, 2013a; Reverberi *et al.*, 2012). Accordingly an exogenous treatment with H_2O_2 leads to enhanced trichothecene production and induces the expression of trichothecene biosynthetic genes in *F. graminearum* (Ponts *et al.*, 2006, 2007). Several studies have suggested that secondary metabolites may act in concert with antioxidant enzymes to protect cells against oxidative stress (Hong *et al.*, 2013a,b).

In this study, we functionally characterized 31 putative peroxidase genes through an extensive phenome analysis of *F. graminearum*. The aims of this study were as follows: (i) to characterize the function of putative peroxidases in various developmental processes, including vegetative growth, conidiation, sexual development, virulence, mycotoxin production and the oxidative stress response; and (ii) to identify TFs that regulate the expression of the genes encoding these putative peroxidases in response to oxidative stress. To our knowledge, this is the first study to investigate the function of genome-wide putative peroxidase genes in the development and infection processes of phytopathogenic fungi. Our results provide insights into the roles of peroxidases in intracellular processes and host–pathogen interactions.

RESULTS

Identification of putative peroxidase genes in *F. graminearum*

We used the previously constructed fungal peroxidase database (http://peroxidase.riceblast.snu.ac.kr) to identify all of the putative peroxidase genes in *F. graminearum* (Choi *et al.*, 2014). The *F. graminearum* genome contains 23 haem peroxidases and eight non-haem peroxidases, including five previously reported putative monofunctional catalase genes (*FCA1*, *FCA2*, *FCA3*, *FCA4* and *FCA5*), two putative bifunctional catalase-peroxidase genes (*FCA6* and *FCA7*) (Lee *et al.*, 2014) and three NADPH oxidase genes (*NOXA*, *NOXB* and *NOXC*) (Takemoto *et al.*, 2007; Wang *et al.*, 2014) (Table 1). We designated the remaining 21 peroxidase genes as *FPX1* to *FPX21* (*F. graminearum* peroxidase) for convenience. To investigate the phylogenetic relationships between the peroxidases of *F. graminearum*, we constructed a phylogenetic tree based on the predicted amino acid sequences of these peroxidases (Fig. 1A,B). Because there was no evolutionary relationship between haem (Fig. 1A) and non-haem (Fig. 1B) peroxidases, a separate phylogenetic tree was constructed for each group. Peroxidases belonging to the same family generally clustered together, indicating the genetic similarity of their protein sequences. The relationships between peroxidase families were relatively weak, as indicated by the low bootstrap values.

To assess the genetic requirements for peroxidases in fungal developmental processes, we analysed transcript profiles during sexual and asexual development of the fungus (Fig. 1C) and during plant infection (Fig. 1D). RNA-sequencing (RNA-seq) and microarray results were obtained from previous studies (Harris *et al.*, 2016; Sikhakolli *et al.*, 2012; Son *et al.*, 2013, 2016), reanalysed and visualized using ClustVis (Metsalu and Vilo, 2015). Expression profiles during plant infection were additionally normalized to that of *β-tubulin* (FGSG_09530). Approximately two-thirds of the studied genes (*FPX5–FPX16* in Fig. 1C) were up-regulated during the initial and/or late stages of sexual reproduction, but most of the genes were down-regulated during conidiation. The other 10 genes (*FPX15–FPX19* in Fig. 1C) were up-regulated during asexual reproduction, but showed maintained or reduced expression after sexual induction, with the exception of *FPX9*, *NOXA* and *NOXB*. During the early infection of wheat,

Table 1 Putative peroxidases in *Fusarium graminearum*.

Types of peroxidase			Locus ID	Gene name	Reference
Haem peroxidase	Catalase superfamily	Catalase	FGSG_06554	*FCA1*	Lee *et al.* (2014)
			FGSG_06733	*FCA2*	Lee *et al.* (2014)
			FGSG_16526	*FCA3*	Lee *et al.* (2014)
			FGSG_02881	*FCA4*	Lee *et al.* (2014)
			FGSG_06596	*FCA5*	Lee *et al.* (2014)
	Class I peroxidase	Catalase-peroxidase	FGSG_02974	*FCA6*	Lee *et al.* (2014)
			FGSG_12369	*FCA7*	Lee *et al.* (2014)
		Cytochrome *c* peroxidase	FGSG_01245	*FPX1*	This study
			FGSG_10606	*FPX2*	This study
		Hybrid ascorbate-cytochrome *c* peroxidase	FGSG_04434	*FPX3*	This study
	Class II peroxidase	Other class II peroxidase	FGSG_16013	*FPX4*	This study
	Haloperoxidase superfamily	Haloperoxidase	FGSG_03708	*FPX5*	This study
			FGSG_17448	*FPX6*	This study
			FGSG_02341	*FPX7*	This study
			FGSG_08911	*FPX8*	This study
			FGSG_03436	*FPX9*	This study
	Peroxidase-cyclooxygenase superfamily	Prostaglandin H synthase (cyclooxygenase)	FGSG_17094	*FPX10*	This study
		Linoleate diol synthase (PGHS-like)	FGSG_02668	*FPX11*	This study
			FGSG_10960	*FPX12*	This study
			FGSG_11146	*FPX13*	This study
	NADPH oxidase superfamily	NoxA	FGSG_00739	*NOXA*	Wang *et al.* (2014)
		NoxB	FGSG_10807	*NOXB*	Wang *et al.* (2014)
		NoxC	FGSG_11195	*NOXC*	This study
Non-haem peroxidase	Peroxiredoxin superfamily	1-cysteine peroxiredoxin	FGSG_07536	*FPX14*	This study
		Typical 2-cysteine peroxiredoxin	FGSG_03180	*FPX15*	This study
		Atypical 2-cysteine peroxiredoxin (type Q, BCP)	FGSG_10296	*FPX16*	This study
		Atypical 2-cysteine peroxiredoxin (type II, type V)	FGSG_08677	*FPX17*	This study
			FGSG_00353	*FPX18*	This study
	Alkylhydroperoxidase superfamily	Carboxymuconolactone decarboxylase	FGSG_01796	*FPX19*	This study
		(no peroxidase activity)	FGSG_10039	*FPX20*	This study
	Glutathione peroxidase	Fungal–bacterial glutathione peroxidase	FGSG_06150	*FPX21*	This study

approximately one-half of the studied genes (*FPX15–FPX4* and *FCA5–FPX7* in Fig. 1D) were up-regulated at the initial stage of infection (1 and 2 days after inoculation), whereas the other one-half of the genes were up-regulated during the later days of infection (4 days after inoculation). These results suggest that peroxidase-mediated molecular processes are closely related to fungal development and infection in *F. graminearum*.

Targeted deletion of putative peroxidase genes in *F. graminearum*

To investigate the functions of the putative peroxidase genes, we performed targeted gene deletion by homologous recombination. Each peroxidase gene of the *F. graminearum* wild-type strain Z-3639 was replaced with the geneticin resistance gene (*GEN*) to create individual gene deletion mutants (Table S1, see Supporting Information). Successful disruption of 24 peroxidase genes was confirmed by Southern blot hybridization using a 5′ or 3′ flanking region as a probe (Fig. S1, see Supporting Information). The seven deletion mutants of the catalase and catalase-peroxidase genes, *fca1–fca7*, were derived in a previous study (Lee *et al.*, 2014).

We analysed 31 peroxidase deletion mutants for defects in various developmental processes, including vegetative growth, sexual and asexual development, trichothecene production, virulence and the oxidative stress response. Overall, we found that five peroxidase mutants were defective in at least one phenotypic category compared with the wild-type (Table 2). None of the peroxidase deletion mutants exhibited defects in vegetative growth or conidiation (Table S2, see Supporting Information). When cultured on complete medium (CM) and minimal medium (MM), there was no significant difference in radial growth or colony morphology between the wild-type and the peroxidase deletion mutant strains. Moreover, all of the 31 peroxidase deletion mutants were normal with regard to conidial production (Table S2). With respect to sexual development, only the *noxA* deletion mutants showed defects in perithecium production (Fig. 2A). The wild-type and other peroxidase deletion mutants produced normal perithecia 7 days after sexual induction.

NADPH oxidase genes in *F. graminearum*

The functions of two NADPH oxidase genes, *NOXA* and *NOXB*, in sexual development and pathogenicity have been characterized

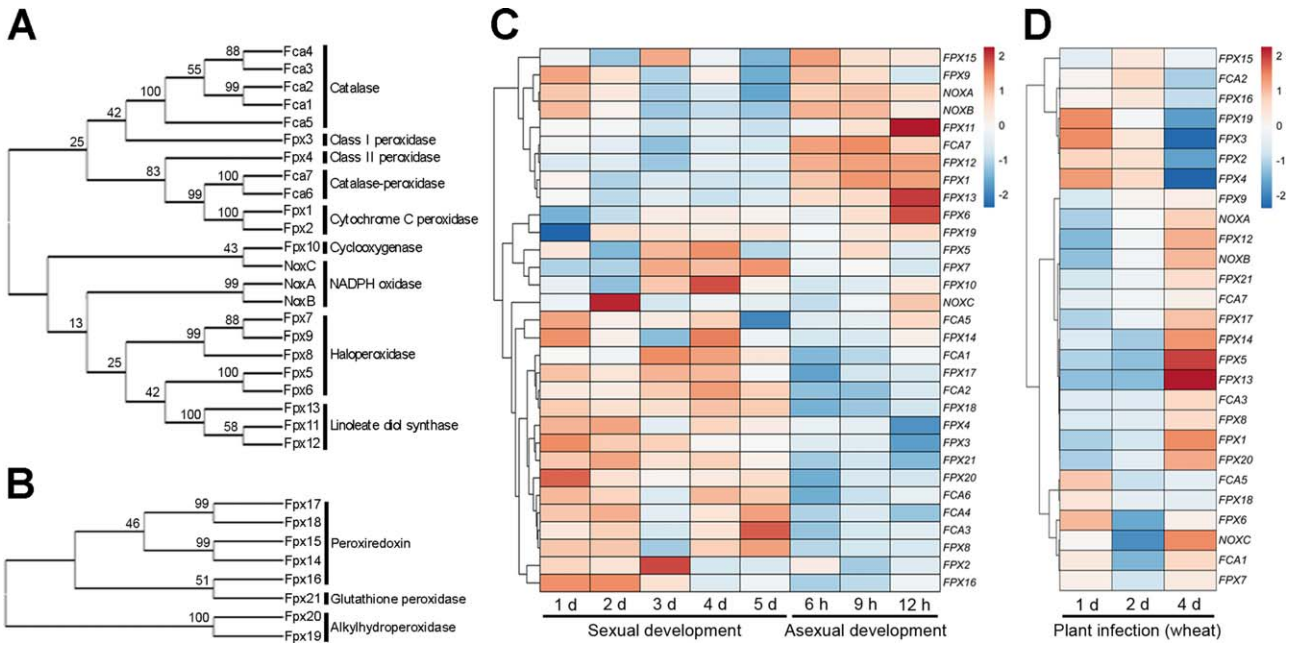

Fig. 1 Phylogenetic and transcriptional analysis of peroxidase genes. Phylogenetic trees based on the amino acid sequences of 23 putative haem peroxidases (A) and eight putative non-haem peroxidases (B) in *Fusarium graminearum*. The phylogenetic trees were constructed using the MEGA program (version 6.06) by the neighbour-joining method with 2000 bootstrap replicates (Tamura *et al.*, 2013). The numbers at the nodes represent the bootstrap percentages. (C, D) Heatmap visualization of the peroxidase gene transcriptional profiles during asexual and sexual development (C) and plant infection (D). The heatmap depicts peroxidase gene transcript abundances during various asexual and sexual developmental stages based on $\log_2$-based relative transcript abundances compared with the 0-day (sexual development), 3-h (asexual development) and mock-inoculated (plant infection) samples. Red and blue represent higher and lower expression, respectively. The rows represent transcriptional units. The expression data were obtained from previous studies (Harris *et al.*, 2016; Sikhakolli *et al.*, 2012; Son *et al.*, 2013, 2016) and visualized using CLUSTVIS (Metsalu and Vilo, 2015).

previously (Wang *et al.*, 2014; Zhang *et al.*, 2016). Here, we identified and characterized a novel NADPH oxidase gene, *NOXC*. Disruption of *NOXA* caused significant defects in sexual development, whereas deletion mutants of *NOXB* and *NOXC* showed no defects in perithecium production or maturation (Fig. 2A). Perithecia with cirrhi were observed in *noxB* and *noxC* mutants 10 days after sexual induction. In assays of fungal infection of wheat heads, the *noxA* deletion mutants showed significantly reduced virulence ($P < 0.01$, *t*-test), whereas the deletion mutants of *noxB* and *noxC* caused typical head blight symptoms (Fig. 2B).

Peroxidases involved in the oxidative stress response

To investigate the sensitivity of the 31 peroxidase deletion mutants to oxidative stress, all of the knockout mutants were cultured in CM supplemented with 10 mM H_2O_2. Only four deletion mutants (*fca6*, *fca7*, *fpx1* and *fpx15*) exhibited significantly altered sensitivity to oxidative stress mediated by H_2O_2 compared with that of the wild-type strain (Fig. 3A). Of these four deletion mutants, *fca7* and *fpx15* were much more susceptible to H_2O_2 than were the other mutants.

To determine whether the deletion of these four peroxidase genes leads to decreased peroxidase enzyme activity, we measured the peroxidase activity of the deletion mutants under oxidative conditions (Fig. 3B). The total peroxidase activities of the *fca6*, *fca7* and *fpx1* deletion mutants were significantly reduced compared with that of the wild-type strain. Under our experimental conditions, the peroxidase activity of the *fpx15* deletion mutant was similar to that of the wild-type strain. These results suggest that the oxidative stress sensitivities of the *fca6*, *fca7* and *fpx1* deletion mutants are mainly caused by decreased peroxidase enzyme activity.

To investigate the H_2O_2 degradation capabilities of Fca6, Fca7, Fpx1 and Fpx15, we measured the concentration of H_2O_2 in mycelia after short-term treatment of the cultures with H_2O_2 (Fig. 3C). We found that the H_2O_2 concentration in mycelia of the *fca7* strain was significantly increased compared with that of the wild-type. The concentration of H_2O_2 in the mycelia of the other deletion mutants was slightly higher than or similar to that of the wild-type.

Peroxidases required for virulence and mycotoxin production

To determine whether the deletion of peroxidase genes affects the virulence of *F. graminearum*, we tested the pathogenicity of

Table 2 Summary of the phenotypes of the *Fusarium graminearum* peroxidase mutants.

Developmental processes	Mutants showing defects
Vegetative growth*	None (same as wild-type)
Conidiation†	None (same as wild-type)
Sexual development‡	*noxA*
Oxidative stress response§	*fca6*, *fca7*, *fpx1*, *fpx15*
Pathogenicity¶	*noxA*, *fca7*
Trichothecene production**	*fca7*

*Radial growth was measured after 5 days of incubation on complete medium (CM) and minimal medium (MM).

†The conidia were counted after 3 days of incubation in carboxymethyl cellulose (CMC) medium.

‡The formation of perithecia was observed 10 days after sexual induction on carrot agar.

§The sensitivity to oxidants (H_2O_2) was tested.

¶The disease index was measured 21 days after inoculation.

**Trichothecene production in minimal medium containing 5 mM agmatine (MMA) was analysed.

the peroxidase deletion mutants on flowering wheat heads. We found that the *fca6*, *fpx1* and *fpx15* deletion mutants exhibited normal virulence, despite being highly susceptible to H_2O_2. However, only the *fca7* deletion mutant showed reduced (but not abolished) virulence compared with that of the wild-type strain (Fig. 3D).

We also analysed total trichothecene production (deoxynivalenol and 15-acetyl-deoxynivalenol) by the peroxidase deletion mutants. Interestingly, the accumulation of trichothecenes by the *fca7* deletion mutant was significantly enhanced compared with that of the wild-type. The other peroxidase deletion mutants, including *fca6*, *fpx1* and *fpx15*, produced amounts of trichothecenes similar to that of the wild-type strain (Fig. 3E). The quantitative real-time polymerase chain reaction (qRT-PCR) results demonstrated that the expression of the trichothecene biosynthetic genes *TRI5* and *TRI6* was highly induced in the *fca7* deletion mutant compared with the wild-type strain (Fig. 3F). These results indicate that Fca7 has non-redundant and crucial functions in virulence and trichothecene biosynthesis in *F. graminearum*. However, both the wild-type and the FCA7 overexpression (FCA7oe) strains produced similar levels of trichothecenes, perhaps because the

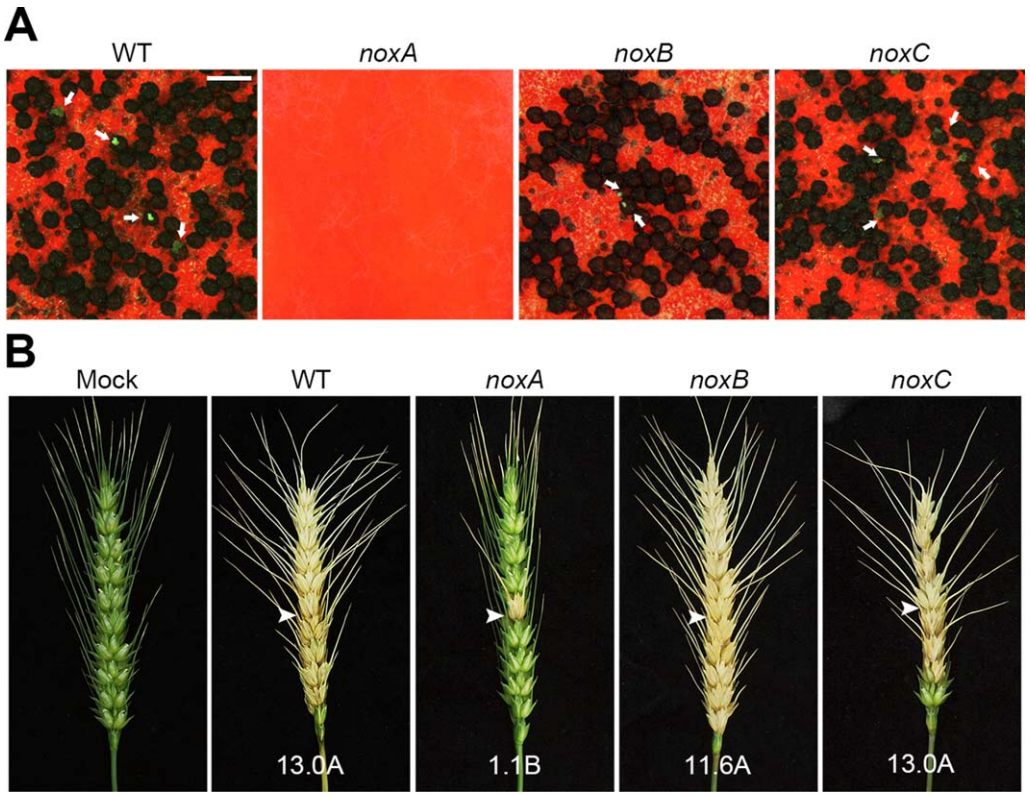

Fig. 2 Sexual development and virulence of NADPH oxidase deletion mutants. (A) Perithecium formation by the NADPH oxidase deletion mutants. Cirrhi (indicated by white arrows) were observed in the wild-type (WT) and in the *noxB* and *noxC* deletion mutants 10 days after sexual induction. Scale bar, 500 μm. (B) Virulence on wheat heads. The centre spikelet of each wheat head was injected with 10 μL of a conidial suspension, and photographs were taken at 21 days after inoculation. Arrowheads indicate the inoculated spikelets. 'Mock' indicates wheat heads that were mock inoculated with 0.01% Tween 20.

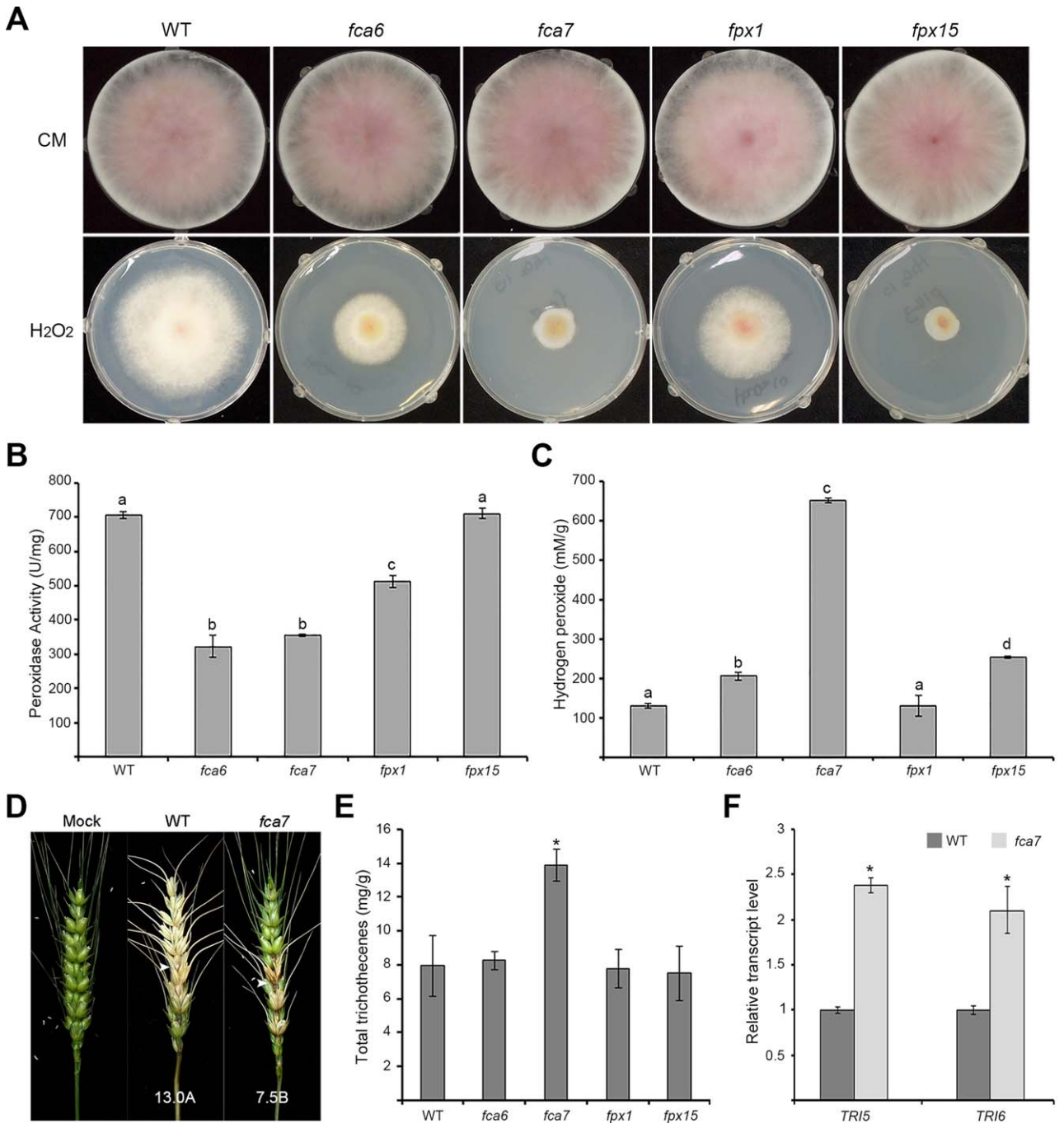

Fig. 3 Peroxidases involved in the oxidative stress responses in *Fusarium graminearum*. (A) Oxidative stress sensitivity of *F. graminearum* strains. The mycelial growth of four peroxidase deletion mutants was evaluated on complete medium (CM) with and without supplementation with 10 mM H_2O_2. Photographs were taken 5 days after inoculation. (B) Peroxidase enzyme activities of the *F. graminearum* strains. (C) Hydrogen peroxide detection in mycelia of the *F. graminearum* strains. (D) Virulence on wheat heads. The centre spikelet of each wheat head was injected with 10 μL of a conidial suspension, and photographs were taken 21 days after inoculation. Arrowheads indicate the inoculated spikelets. 'Mock' indicates wheat heads that were mock inoculated with 0.01% Tween 20. (E) Total trichothecene production by the *F. graminearum* strains. Each strain was grown in minimal medium containing 5 mM agmatine (MMA) for 7 days. Trichothecenes were analysed by gas chromatography-mass spectrometry (GC-MS) and quantified based on the biomass of each strain. (F) Transcript levels of *TRI5* and *TRI6* in the wild-type (WT) and *fca7* deletion mutant strains. The transcript levels were analysed by quantitative real-time polymerase chain reaction (qRT-PCR) 4 days after inoculation in MMA. Asterisk indicates significant difference: $P < 0.01$, Tukey's test.

basal expression of *FCA7* was sufficient to negatively regulate the production of trichothecenes under our experimental conditions (Fig. S2, see Supporting Information).

Identification of TFs involved in the oxidative stress response

To dissect the regulatory mechanisms of the major peroxidases, we first attempted to identify TFs involved in the oxidative stress response in *F. graminearum* among 657 TF mutants (Son *et al.*, 2011b). We first screened the TF mutants using various oxidative stress-inducing agents (10 mM H_2O_2, 0.1 mM menadione and 1 mM diamide). From the TF mutants showing altered sensitivity to oxidative stress-inducing agents, we preferentially selected eight TF mutants that were highly sensitive to H_2O_2 for further study (Fig. 4A and Table 3). Of these, three TFs (FgAp1, FgSkn7 and Zif1) have been functionally characterized previously in *F. graminearum* (Jiang *et al.*, 2015; Montibus *et al.*, 2013; Wang *et al.*, 2011). FGSG_05171 encodes a homologue of *Neurospora crassa* Cys-3 and *Aspergillus nidulans* MetR involved in the regulation of the sulfur regulatory circuit (Fu *et al.*, 1989; Natorff *et al.*, 2003), and FGSG_01100 encodes a homologue of FoxO1 required for the oxidative stress response in mammalian cell systems (Furukawa-Hibi *et al.*, 2005). The three putative TFs containing a $Zn(II)_2Cys_6$ DNA-binding domain (FGSG_08924 and FGSG_01293) and a C_2H_2 zinc finger domain (FGSG_01298) have not been functionally characterized in other fungi (Table 3).

Expression of four peroxidase genes in TF deletion mutants

We hypothesized that the TFs involved in the oxidative stress response regulate the expression of the four peroxidase genes that are required for the oxidative stress response. We performed qRT-PCR to measure the transcript levels of *FCA6*, *FCA7*, *FPX1* and *FPX15* in the wild-type strain and in the eight selected TF deletion mutants under normal and oxidative stress conditions (Fig. 4B). The transcript levels of *FCA6*, *FCA7*, *FPX1* and *FPX15* were markedly up-regulated in response to H_2O_2 in the wild-type strain. In the TF deletion mutants, the expression levels of *FCA6*, *FPX1* and *FPX15* were increased by H_2O_2 treatment (similar to the wild-type), whereas the transcript levels of *FCA7* showed a different pattern. In seven TF deletion mutants (*Fgap1*, *Fgskn7*, *gzbzip007*, *gzzc086*, *gzzc236*, *gzhome001* and *gzc2h010*), the expression of *FCA7* was not highly induced or was even reduced by H_2O_2 treatment (Fig. 4B). In the *zif1* mutants, the expression of *FCA7* was relatively highly expressed following H_2O_2 treatment compared with the expression in the other deletion mutants, indicating that *FCA7* expression is a key factor in the oxidative stress response in TF mutants.

Genetic relationship among TFs and *FCA7*

To investigate the genetic relationships between *FCA7* and the eight TFs, we generated TF mutants with *FCA7* overexpression by outcrosses (Table S1). Of the seven TF deletion mutants (*Fgap1*, *Fgskn7*, *gzbzip007*, *gzzc086*, *gzzc236*, *gzhome001* and *gzc2h010*) that showed unchanged or reduced expression of *FCA7* (Fig. 4B), the oxidative stress sensitivity of six (*Fgap1*, *Fgskn7*, *gzzc086*, *gzzc236*, *gzhome001* and *gzc2h010*) was restored to some degree by the overexpression of *FCA7* (Fig. 5). Of these, the *gzzc236* mutants have been reported previously to show reduced virulence (Son *et al.*, 2011b). Although the oxidative stress sensitivity of these mutants was restored by the overexpression of *FCA7*, virulence was not recovered (Fig. S4, see Supporting Information). Because the *gzbzip007* deletion mutants did not grow at all on CM supplemented with 10 mM H_2O_2, we assayed the sensitivities of the *gzbzip007* mutants to relatively low oxidative stress conditions (medium containing 3 mM H_2O_2). The vegetative growth of *gzbzip007* under mild oxidative stress conditions was not restored by the overexpression of *FCA7* (Fig. S3, see Supporting Information). The oxidative stress sensitivity of the *zif1* mutant was slightly restored by *FCA7* overexpression, consistent with the qRT-PCR results. Taken together, these results demonstrate that the altered sensitivities of the TF mutants to oxidative stress are primarily caused by the repression of *FCA7*.

We also outcrossed the TF deletion mutants to a heterothallic *fca7* deletion strain, *mat2 fca7*. If these TFs are involved in a different antioxidant system, the double mutation would be expected to produce synergistic defects in the oxidative stress response. We found that two TF deletion mutants (*zif1* and *gzzc086*) showed markedly increased sensitivity to oxidative stress when combined with the deletion of *FCA7* (Fig. 5). Based on the phenotypes of the *gzzc086* mutants carrying *FCA7* deletion or overexpression, we suspected that *GzZC086* has a regulatory function for *FCA7* expression and that it is involved in multiple antioxidant pathways. Double deletion mutants of *fca7* and TFs (*Fgap1*, *Fgskn7*, *gzzc236*, *gzhome001* and *gzc2h010*) did not show synergistic effects on oxidative stress sensitivity compared with the corresponding single-gene deletion mutants.

Genetic regulatory network of the TFs involved in oxidative stress responses

We further examined the transcript levels of the TF genes in the wild-type strain under normal and oxidative stress conditions using qRT-PCR (Fig. 6A). The expression of five TF genes (*ZIF1*, *FgAP1*, *FgSKN7*, *GzbZIP007* and *GzZC086*) was significantly up-regulated in response to H_2O_2, whereas the expression of the other three TF genes (*GzZC236*, *GzHOME001* and *GzC2H010*) was not induced under our experimental conditions. To investigate the genetic regulatory network with which TF genes are associated, we examined the expression of the five TF genes showing

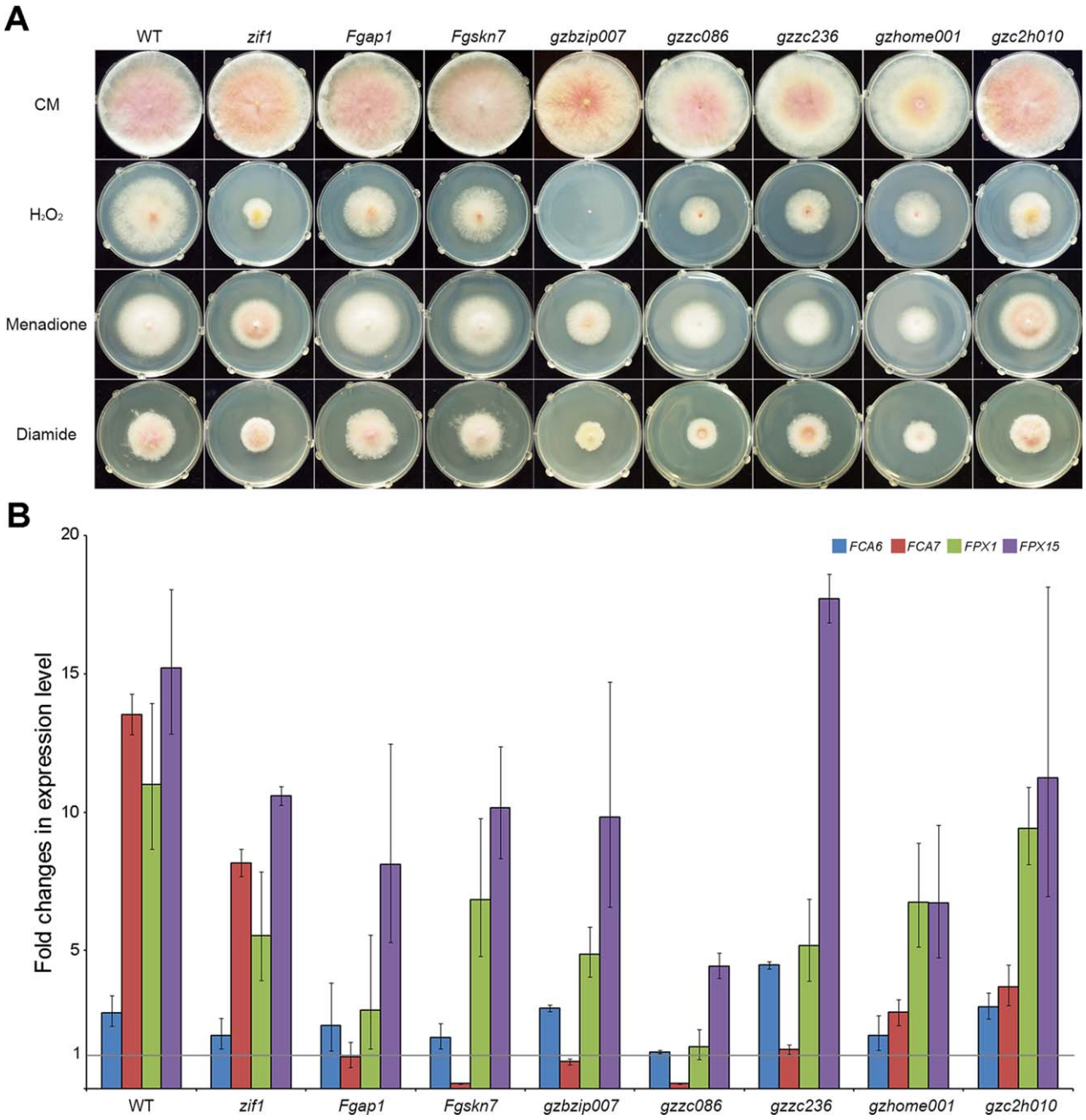

Fig. 4 Characterization of transcription factors (TFs) involved in the oxidative stress response. (A) Oxidative stress sensitivity of eight TF mutants. The mycelial growth of the TF deletion mutants was evaluated on complete medium (CM) and on CM supplemented with 10 mM H_2O_2, 0.1 mM menadione or 1 mM diamide. Photographs were taken 5 days after inoculation. (B) Transcript abundances of peroxidase genes in the TF mutants. The transcript levels of *FCA6, FCA7, FPX1* and *FPX15* were analysed by quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was isolated from wild-type (WT) and TF deletion mutant strains grown for 30 min in CM only or in CM supplemented with 5 mM H_2O_2.

increased expression under oxidative stress conditions in eight TF deletion mutants. We compared the fold change in expression of these five TF genes after H_2O_2 treatment in the eight TF deletion mutants with the fold change in the wild-type strain (Fig. 6B). H_2O_2-mediated induction of *ZIF1*, *FgAP1* and *FgSKN7* was nearly abolished in the *gzzc086* mutants, and *GzZC086* was not induced in *gzhome001*. We also found that the expression of *ZIF1* and *FgSKN7* did not increase in response to H_2O_2 in the *gzc2h010* and *gzzc236* mutants, respectively. The expression of *GzbZIP007* was not reduced in any of the TF deletion mutants, indicating that

Table 3 Putative transcription factors involved in the oxidative stress response.

Locus ID	Gene name	Description of the gene product	Species	Homologue	Reference
FGSG_01555	*ZIF1*	Related to bZIP transcription factor	*Fusarium graminearum*	*ZIF1*	Wang *et al.* (2011)
FGSG_08800	*FgAP1*	Related to AP1-like transcription factor	*F. graminearum*	*FgAP1*	Montibus *et al.* (2013)
FGSG_06359	*FgSKN7*	Related to SKN7	*F. graminearum*	*FgSKN7*	Jiang *et al.* (2015)
FGSG_05171	*GzbZIP007*	Related to regulatory protein cys-3	*Neurospora crassa*	*CYS-3*	Fu *et al.* (1989)
FGSG_08924	*GzZC086*	Conserved hypothetical protein	N/A	N/A	Son *et al.* (2011b)
FGSG_01293	*GzZC236*	Related to $Zn(II)_2Cys_6$ transcriptional activator	N/A	N/A	Son *et al.* (2011b)
FGSG_01100	*GzHOME001*	Related to LIM homeobox protein	Human	*FoxO1*	Furukawa-Hibi *et al.* (2005)
FGSG_01298	*GzC2H010*	Conserved hypothetical protein	N/A	N/A	Son *et al.* (2011b)

GzbZIP007 is involved in a completely independent regulatory system for oxidative stress responses. Furthermore, it appeared that feedback regulatory mechanisms among the TFs, *ZIF1–GzZC086*, *ZIF1–Gz236* and *FgAP1–GzZC236*, modulated the genetic regulatory networks. Based on our data, we propose a simplified genetic regulatory network that illustrates how TF genes are involved in the oxidative stress response in *F. graminearum* (Fig. 6C).

In conclusion, we found that, although the expression of *FCA7* was not the only component of the oxidative stress response, Fca7 was the major antioxidant enzyme produced. We also concluded that at least six TFs (*FgAP1*, *FgSKN7*, *GzZC086*, *GzZC236*, *GzHOME001* and *GzC2H010*) regulated the expression of *FCA7* under oxidative stress conditions, demonstrating the existence of a complex network of transcriptional activators of antioxidant genes.

DISCUSSION

Because plant-pathogenic fungi encounter both internal and external oxidative stresses during development and during the process of plant infection, they have evolved effective ROS-detoxifying mechanisms (Heller and Tudzynski, 2011). In particular, the rapid generation of H_2O_2 is one of the earliest plant defence responses to occur following the perception of signals associated with the presence of pathogens (Lamb and Dixon, 1997; Levine *et al.*, 1994), and the roles of H_2O_2-scavenging enzymes, such as catalases and catalase-peroxidases, during plant invasion have been investigated in various phytopathogenic fungi (Garre *et al.*, 1998; Robbertse *et al.*, 2003; Schouten *et al.*, 2002; Skamnioti *et al.*, 2007; Tanabe *et al.*, 2011). H_2O_2 also plays an important role as a signalling molecule in various developmental processes of fungi, such as cell differentiation and sexual development (Gessler *et al.*, 2007; Hansberg *et al.*, 1993; Lara-Ortíz *et al.*, 2003). Thus, cellular ROS levels should be extremely fine-tuned by peroxidases during fungal development. In this study, we investigated the roles of putative peroxidases in *F. graminearum*. We identified 31 genes encoding putative peroxidases in *F. graminearum* and characterized their functions, not only in the oxidative stress response, but also in various developmental processes.

The *F. graminearum* genome contains 23 peroxidases belonging to the 11 haem peroxidase families and eight peroxidases belonging to the six non-haem peroxidase families. In yeast, only two haem peroxidase families and five non-haem peroxidase families have been found (Choi *et al.*, 2014). A comparison of the predicted peroxidase genes in plant-pathogenic fungi, including *F. graminearum*, *M. oryzae*, *C. heterostrophus* and yeast, revealed that the genomes of plant-pathogenic fungi generally contain numerous haem peroxidase genes (Choi *et al.*, 2014). Several studies have reported that certain haem peroxidase genes, such as haloperoxidase genes and catalase-peroxidase genes, are mainly found in the genomes of phytopathogens (Gasselhuber *et al.*, 2015; Zámocký and Obinger, 2010; Zámocký *et al.*, 2012). These results imply a potential role of haem peroxidases in pathogenicity.

Phenome analysis of 31 peroxidase deletion mutants in *F. graminearum* revealed that only five peroxidase deletion mutants (*fca6*, *fca7*, *fpx1*, *fpx15* and *noxA*) were involved in the various developmental processes examined. Other peroxidases may be able to functionally compensate for the loss of a single peroxidase, indicating overlapping and redundant functions of these peroxidases. The clustered peroxidase gene expression profiles observed during reproductive processes also support the overlapping functions of peroxidases in *F. graminearum* (Fig. 1C). Likewise, the deletion of multiple catalase genes in *Cryptococcus neoformans* did not cause visible phenotypic changes, because of the presence of a robust and redundant antioxidant defence system in this species (Giles *et al.*, 2006). Moreover, in other filamentous fungi, such as *B. cinerea* and *M. oryzae*, deletion of the genes required for the oxidative stress response did not cause phenotypic changes in the fungal growth rate or conidia production (Huang *et al.*, 2011; Temme and Tudzynski, 2009).

With respect to sexual development, only *noxA* deletion mutants showed defective perithecia production. Previous studies have demonstrated that NADPH oxidase-dependent ROS signalling is important for cellular differentiation and development in fungi (Heller and Tudzynski, 2011; Takemoto *et al.*, 2007). In this study, we identified three NADPH oxidase homologues in *F. graminearum*: NoxA, NoxB and NoxC. The function of NoxC has been poorly studied in filamentous fungi, whereas the

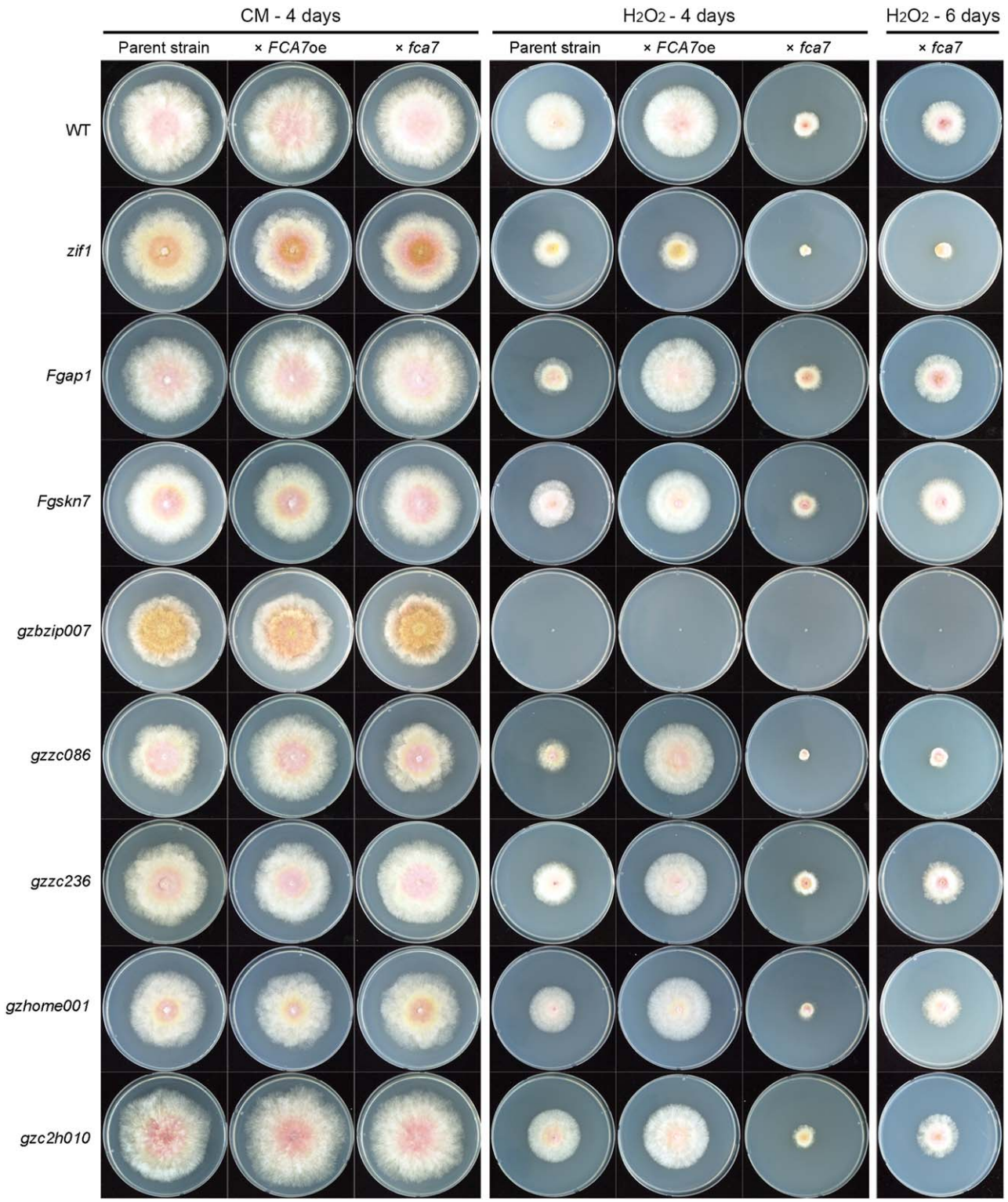

Fig. 5 Oxidative stress sensitivity of eight transcription factor (TF) mutants carrying an FCA7 deletion or FCA7 overexpression (*FCA7*oe). The mycelial growth of TF mutant strains on complete medium (CM) supplemented with 10 mM H_2O_2 is shown. Photographs were taken 4 and 6 days after inoculation. WT, wild-type.

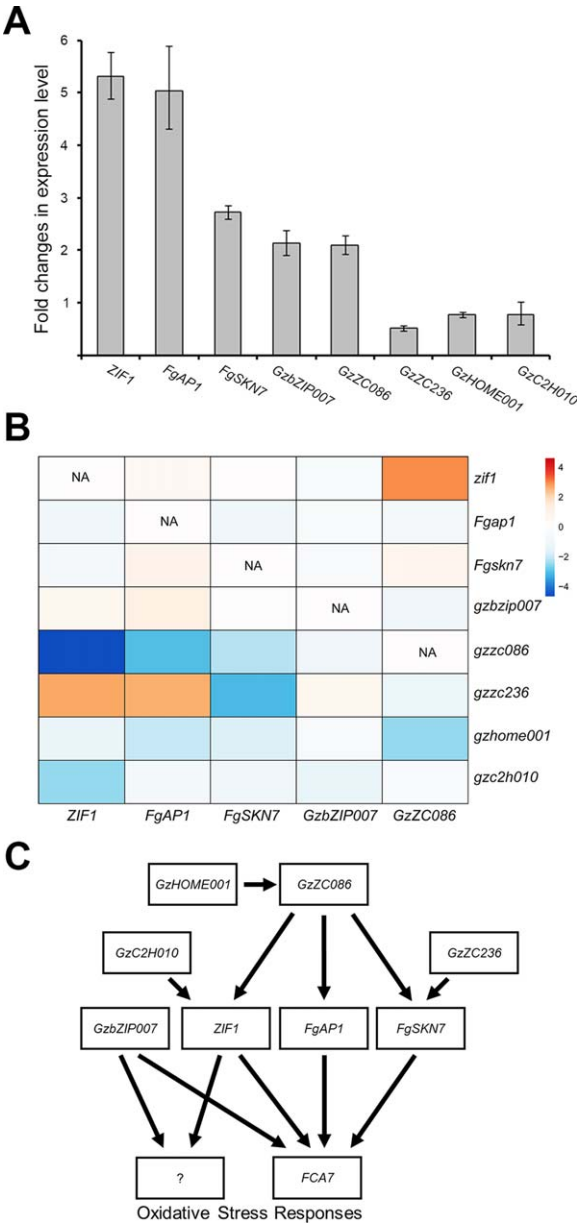

Fig. 6 Genetic network of transcription factor (TF) genes involved in the oxidative stress response in *Fusarium graminearum*. (A) Fold change values in the expression of TF genes after H_2O_2 treatment in the wild-type strain. The transcript levels of the TF genes were analysed by quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was isolated from the wild-type strain grown for 30 min in complete medium (CM) only or in CM supplemented with 5 mM H_2O_2. (B) Heatmap of selected TF genes that were up-regulated in the wild-type strain in response to H_2O_2. The log base ratio of the fold change in gene expression in each deletion mutant compared with that in the wild-type strain was converted to a heatmap using ClustVis (Metsalu and Vilo, 2015). (C) Proposed genetic network of TFs and FCA7 in the oxidative stress response. ?, unknown antioxidant components.

functions of NoxA and NoxB in various cellular differentiation processes, including sexual development and pathogenicity, have been well characterized (Cano-Domínguez *et al.*, 2008; Egan *et al.*, 2007; Giesbert *et al.*, 2008; Lara-Ortíz *et al.*, 2003; Scott and Eaton, 2008; Segmüller *et al.*, 2008; Takemoto *et al.*, 2007; Wang *et al.*, 2014; Zhang *et al.*, 2016). A recent study has reported that NoxC exists in only seven Ascomycota and that most of these seven species are phytopathogenic fungi, suggesting that NoxC may have a specialized function in pathogenicity (Takemoto *et al.*, 2007). Moreover, *NOXC* showed distinct and unique expression patterns during fungal reproduction compared with those of *NOXA* and *NOXB* (Fig. 1C). However, deletion of *NOXC* did not affect the pathogenicity of *F. graminearum*. Thus, the function of *NOXC* remains unclear.

Four peroxidase genes, *FCA6*, *FCA7*, *FPX1* and *FPX15*, were closely involved in the oxidative stress response, but deletion of *FPX15* did not affect peroxidase activity in *F. graminearum* (Fig. 3B). *FPX15* is predicted to encode a non-haem peroxidase belonging to the typical 2-cysteine peroxiredoxin family. Peroxiredoxins possess both peroxidase and molecular chaperone activities (Jang *et al.*, 2004), and the peroxidase-to-chaperone switch is triggered by oxidative stress (Jang *et al.*, 2004; Kim *et al.*, 2009). Based on previous and current results, we conclude that the increased sensitivity of *fpx15* to oxidative stress is not directly related to its peroxidase activity, but to its chaperone activity.

Although the total peroxidase enzyme activity of *fca6* was decreased to a level similar to that of *fca7*, the concentration of accumulated H_2O_2 in the mycelia of *fca7* was much higher than that in those of *fca6*. *FCA7* homologues have been predicted to encode an extracellular catalase-peroxidase (Zámocký *et al.*, 2009), and Fca7 contains a potential signal sequence for secretion predicted by SignalP 4.0 (Petersen *et al.*, 2011). These results suggest that Fca7 may play a predominant role in the detoxification of external H_2O_2 in *F. graminearum*. Consistent with the fact that plant-derived ROS scavenging by extracellular peroxidases is critical for successful infection, only *fca7* mutants were reduced in virulence compared with that of the wild-type strain.

Proteomic analysis of wheat spikelets during infection by *F. graminearum* revealed that proteins related to the oxidative burst pathway are induced at the early infection stage (Zhou *et al.*, 2005, 2006). In this study, we discovered that, although deletion mutants of *FCA6*, *FCA7*, *FPX1* and *FPX15* showed increased sensitivity to oxidative stress mediated by H_2O_2, only *fca7* deletion mutants exhibited reduced virulence compared with that of the wild-type strain. In a number of phytopathogenic fungi, deletion of a gene that is essential for survival in the presence of high concentrations of H_2O_2 does not lessen virulence; this may be a result of the presence of alternative antioxidant systems (Robbertse *et al.*, 2003; Temme and Tudzynski, 2009). Taken together, we conclude that Fca7 may function as a major H_2O_2-

scavenging enzyme and may play an irreplaceable role during the infection process in *F. graminearum*.

Recent studies have reported that oxidative stress and secondary metabolism are tightly linked in filamentous fungi, and have suggested that secondary metabolites play a protective role in the adaptation of plants to stress conditions (Hong *et al.*, 2013a; Montibus *et al.*, 2015). In *F. graminearum*, trichothecenes have been reported to be virulence factors for the fungal infection of wheat heads (Desjardins *et al.*, 1996; Maier *et al.*, 2006), and trichothecene biosynthesis has been shown to be triggered by exogenous H_2O_2 treatment associated with the increased expression of *TRI* genes (Ponts *et al.*, 2006, 2007). In this study, we found that the *fca7* deletion mutant produced more trichothecenes than the wild-type strain, and that Fca7 is the major H_2O_2-scavenging enzyme expressed in *F. graminearum* under oxidative stress conditions (Fig. 3C,E). Based on these findings, we conclude that Fca7-mediated modulation of H_2O_2 may be one of the major determinants of trichothecene production in *F. graminearum*.

Eight TFs involved in the oxidative stress response were identified in *F. graminearum*. Previously, homologues of Yap1 and Skn7 were identified and functionally characterized in *F. graminearum* (Jiang *et al.*, 2015; Montibus *et al.*, 2013). Yap1 and Skn7 are central TFs that regulate the expression of oxidative stress-related genes in *S. cerevisiae* (Lee *et al.*, 1999). A novel bZIP TF, Zif1, has also been reported in *F. graminearum* (Wang *et al.*, 2011). In this study, we identified five new TFs (*GzbZIP007*, *GzZC086*, *GzZC236*, *GzHOME001* and *GzC2H010*) that are involved in the oxidative stress response in this fungus. We proposed a hypothetical simplified genetic network of TF genes in the oxidative stress response (Fig. 6C). Because studies of genetic networks governing the oxidative stress response have been limited because of the lack of TF mutants, further studies of these TFs in filamentous fungi, including *F. graminearum*, will expand our understanding of the molecular mechanisms underlying the oxidative stress response in this type of fungus.

We found that multiple TFs (*FgAP1*, *FgSKN7*, *GzZC086*, *GzZC236*, *GzHOME001* and *GzC2H010*) co-regulated the expression of *FCA7* in response to oxidative stress, reflecting a complex network of transcriptional activators of antioxidant genes. A cooperative role of these TFs has been reported in previous studies (Calvo *et al.*, 2012; Mulford and Fassler, 2011; Shalaby *et al.*, 2014). In *S. cerevisiae* and *C. heterostrophus*, Yap1 and Skn7 cooperate to activate the expression of antioxidant genes in response to oxidative stress (Mulford and Fassler, 2011; Shalaby *et al.*, 2014). In *F. graminearum*, the genetic relationship between orthologues of Skn7 and Atf1 has been investigated (Jiang *et al.*, 2015). We further identified the putative TF binding sites in the upstream regions (–500 to −1 bp) of *FCA7* using MatchTM software (Kel *et al.*, 2003). The promoter of *FCA7* was found to contain two C_2H_2 zinc finger protein-binding regions (GCCCC and TTGGC) and several binding motifs (ACCTG, GCTGT, CCTGT, etc.) for homeobox proteins, suggesting that *FCA7* might be under the direct regulation of GzC2H010 (C_2H_2 zinc finger) and/or GzHOME001 (homeobox).

We also found that three TFs (*ZIF1*, *GzZC086* and *GzbZIP007*) may be involved in alternative antioxidant systems, such as the non-enzymatic antioxidant response (Apel and Hirt, 2004). Non-enzymatic antioxidants include low-molecular-weight compounds, such as glutathione, ascorbate and cysteine. Homologues of *CYS-3* have been reported to regulate sulfur metabolism, which is involved in the biosynthesis of amino acids, such as cysteine and methionine (Kong *et al.*, 2015; Marzluf, 1997). The roles of cysteine and methionine residues in proteins in antioxidant functions have been reported in several studies (Fauchon *et al.*, 2002; Levine *et al.*, 2000; Pócsi *et al.*, 2004). Thus, the increased H_2O_2 sensitivity of the *gzbzip007* deletion mutant may be a result of defects in sulfur metabolism. In *M. oryzae*, deletion mutants of Mo*METR*, an orthologue of *CYS-3*, showed methionine auxotrophy and hypersensitivity to H_2O_2 (Kong *et al.*, 2015).

In conclusion, our work reveals that *F. graminearum* possesses a robust antioxidant system that is involved in the maintenance of the cellular ROS balance during cell differentiation and proliferation, and that major peroxidases are involved in the oxidative stress response in this species. We suggest that Fca7 is particularly important for pathogen–host interactions and that multiple TFs co-regulate the expression of *FCA7* under oxidative stress conditions.

EXPERIMENTAL PROCEDURES

Fungal strains and culture media

The *F. graminearum* wild-type strain Z-3639 (Bowden and Leslie, 1999) and transgenic strains derived from this strain were used in this study (Table S1). The catalase deletion mutants (*fca1–fca7*) and TF deletion mutants used in this study have been derived previously (Lee *et al.*, 2014; Son *et al.*, 2011b). Putative peroxidase genes were identified from the Fungal Peroxidase Database (fPoxDB; http://peroxidase.riceblast.snu.ac.kr) (Choi *et al.*, 2014). The protein sequences of these genes were obtained from the MIPS *Fusarium graminearum* database (FGDB; http://mips.helmholtz-muenchen.de/genre/proj/FGDB/) (Wong *et al.*, 2011). All strains were stored as mycelial suspensions in 20% glycerol solution at −80 °C. The culture media used in this study were prepared following the *Fusarium* laboratory manual (Leslie and Summerell, 2006). To induce conidial production, carboxymethyl cellulose (CMC) and yeast malt agar (YMA) were used as described previously (Cappellini and Peterson, 1965; Harris, 2005). MM containing 5 mM agmatine (MMA) was used for trichothecene production (Gardiner *et al.*, 2009).

Nucleic acid manipulations, Southern blotting and PCR

Genomic DNA was extracted from mycelial powder according to the *Fusarium* laboratory manual (Leslie and Summerell, 2006). Total RNA was

extracted from mycelia ground in liquid nitrogen using the Easy-Spin Total RNA Extraction Kit (iNtRON Biotech, Seongnam, South Korea). Standard protocols were used for restriction endonuclease digestion, agarose gel electrophoresis, Southern blotting and hybridization with ^{32}P-labelled probes (Sambrook and Russell, 2001). The primers for PCR and qRT-PCR used in this study (Table S3, see Supporting Information) were synthesized by an oligonucleotide synthesis facility (Bionics, Seoul, South Korea). PCR procedures were performed according to the manufacturer's instructions (TaKaRa Bio, Inc., Ostu, Japan).

Targeted gene deletion

The double-joint (DJ) PCR strategy was used to construct fusion PCR products for targeted gene deletion (Yu *et al.*, 2004). To create deletion strains, the 5′ and 3′ flanking regions of the target genes were amplified from the genomic DNA of the wild-type strain, and *GEN* was amplified from pII99 using the primer pair Gen-for/Gen-Rev. Three amplicons (5′ flanking region, 3′ flanking region and *GEN*) were fused in a second round of DJ PCR. Finally, fusion constructs were amplified using nested primers to generate split markers. The resulting constructs were transformed into the wild-type strain as described previously (Son *et al.*, 2011a). Southern hybridization was performed to confirm single-copy integration.

Vegetative growth, conidiation and sexual development

Radial growth rates on CM and MM were measured 5 days after inoculation with freshly grown culture plugs from MM. Conidial production was measured by counting the number of conidia after incubating culture plugs from CM in 5 mL of CMC for 3 days at 25 °C on a rotary shaker (200 rpm).

For self-fertilization, fungal strains were grown on carrot agar plates for 5 days. To induce sexual reproduction, aerial mycelia were removed with sterile 2.5% Tween 60 solution (Leslie and Summerell, 2006). For outcrosses, mycelia of heterothallic female strains grown on carrot agar plates for 5 days were fertilized with 1 mL of a conidial suspension from male strains. After sexual induction, all cultures were incubated under near-UV light (wavelength, 365 nm; HKiv Import & Export Co., Ltd., Xiamen, China) at 25 °C.

Virulence assays and trichothecene analysis

For the virulence test, the point inoculation method was performed using the susceptible wheat cultivar Eunpamil as described previously (Son *et al.*, 2011a). Ten microlitres of conidial suspension (10^6 conidia/mL) harvested from CMC were injected into the middle of the spikelet. After inoculation, the plants were incubated in a high-humidity chamber for 3 days and transferred to a glasshouse. The number of spikelets displaying symptoms of FHB was determined 21 days after inoculation. More than five replicated inoculations per strain and two independent mutant strains were used in the experiment.

Total trichothecene production (deoxynivalenol and 15-acetyl-deoxynivalenol) was measured as described previously (Son *et al.*, 2011a). Cultures grown in MMA were filtered through cheesecloth, and filtrates were extracted with an ethyl acetate–methanol solution. The dehydrated extracts were derivatized with Sylon BTZ (N,O-bis(trimethylsilyl)acetamide + trimethylchlorosilane + trimethylsilylimidazole, 3 : 2 : 3; Supelco, Bellefonte, PA, USA), and the derivatized products were analysed using a Shimadzu QP-5000 gas chromatograph-mass spectrometer (Shimadzu, Kyoto, Japan). The total trichothecene concentration was quantified based on the biomass produced by each strain in MMA. The experiment was repeated five times.

Oxidative stress sensitivity assays

To evaluate the effects of oxidative stress on the mycelial growth of the peroxidase deletion mutants, 10 mM H_2O_2 was used. Agar plugs from actively growing cultures were transferred to CM with or without supplementation with H_2O_2, and the plates were incubated at 25 °C for 5 days. To screen 657 TF deletion mutants (Son *et al.*, 2011b), CM supplemented with 10 mM H_2O_2 was used. At least two independent tests were performed for each assay, and each strain was tested in triplicate.

qRT-PCR analysis

Conidial suspensions (10^6 conidia/mL) harvested from YMA were inoculated into 50 mL of liquid CM and incubated for 24 h at 25 °C on a rotary shaker (200 rpm). Total RNA was extracted from strains grown for an additional 30 min in CM only or in CM supplemented with 5 mM H_2O_2. cDNA was synthesized from total RNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). qRT-PCR was performed using SYBR Green Super Mix (Bio-Rad, Hercules, CA, USA) and a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA), using primer pairs specific for peroxidase genes (Table S3). The cyclophilin gene (*CYP1*; FGSG_07439) was used as a reference gene. qRT-PCR was performed three times with two replicates per run, and the transcript level of each target gene was calculated as described previously (Livak and Schmittgen, 2001).

To measure the transcript levels of the trichothecene biosynthetic genes, *TRI5* and *TRI6*, we incubated conidia from the wild-type and peroxidase deletion mutant strains in MMA for 4 days, isolated total RNA from each strain and performed qRT-PCR as described above.

Peroxidase enzyme activity assay and H_2O_2 assay

Fungal strains grown for 24 h in 50 mL liquid CM were incubated for an additional 30 min in CM supplemented with 5 mM H_2O_2. Crude proteins were extracted from harvested mycelia (ground in liquid nitrogen) of wild-type and peroxidase deletion mutant strains using 1 mL of potassium phosphate buffer (250 mM, pH 7.0) supplemented with 1 mM phenylmethylsulfonyl fluoride, a protease inhibitor. The protein concentration was determined colorimetrically by the Bradford assay (Bio-Rad). Total peroxidase enzyme activities were measured using the Quantichrom Peroxidase Assay (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions. The quantification of H_2O_2 in semi-dried mycelia was determined using an Amplex Red hydrogen peroxide/peroxidase assay kit (Invitrogen).

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SUPPORTING INFORMATION

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

Table S1 *Fusarium graminearum* strains used in this study.
Table S2 Summary of the peroxidase mutant phenotypes.

Table S3 Primers used in this study.

Fig. S1 Targeted gene deletion. Each peroxidase-encoding gene (A–X) was deleted individually from the genome of the *Fusarium graminearum* wild-type (WT) strain Z-3639. *GEN*, geneticin resistance gene cassette. The sizes of the DNA standards (in kilobases) are indicated to the left of the blot.

Fig. S2 Total trichothecene production of wild-type (WT) and *FCA7* overexpression (FCA7oe) strains. Each strain was grown in agmatine (MMA) for 7 days. Trichothecenes were analysed by gas chromatography-mass spectrometry (GC-MS) and quantified based on the biomass of each strain.

Fig. S3 Mycelial growth on complete medium (CM) supplemented with 4 mM H_2O_2 of *gzbzip007* mutant strains carrying an *FCA7* deletion or with *FCA7* overexpression (*FCA7*oe). The photographs were taken 4 days after inoculation. WT, wild-type.

Fig. S4 Virulence on wheat heads. The centre spikelet of each wheat head was injected with a conidial suspension of one of the fungal strains. The photographs were taken 21 days after inoculation. The arrowheads indicate the inoculated spikelets. 'Mock' indicates wheat heads that were mock inoculated with 0.01% Tween 20. WT, wild-type.