ELP3 Is Involved in Sexual and Asexual Development, Virulence, and the Oxidative Stress Response in *Fusarium graminearum*

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Fusarium graminearum is an important fungal plant pathogen that causes serious losses in cereal crop yields and mycotoxicoses in humans and livestock. In this study, we characterized an insertion mutant, Z39R9282, with pleiotropic defects in sexual development and virulence. We determined that the insertion occurred in a gene encoding an ortholog of yeast elongator complex protein 3 (ELP3). Deletion of *elp3* led to significant defects in sexual and asexual development in *F. graminearum*. In the *elp3* deletion mutant, the number of perithecia formed was reduced and maturation of perithecia was delayed. This mutant also produced morphologically abnormal ascospores and conidia. Histone acetylation in the *elp3* deletion mutant was reduced compared with the wild type, which likely caused the developmental defects. Trichothecenes were not produced at detectable levels, and expression of trichothecene biosynthesis genes were significantly reduced in the *elp3* deletion mutant. Infection of wheat heads revealed that the *elp3* deletion mutant was unable to spread from inoculated florets to neighboring spikelets. Furthermore, the *elp3* deletion mutant was more sensitive to oxidative stress than the wild type, and the expression of putative catalase genes was reduced. We demonstrate that *elp3* functions in sexual and asexual development, virulence, and the oxidative stress response of *F. graminearum* by regulating the expression of genes involved in these various developmental processes.

The homothallic ascomycete fungus *Fusarium graminearum* is an important plant pathogen of major cereal crops such as wheat, maize, barley, and rice (Leslie and Summerell 2006). Epidemics of Fusarium head blight (FHB) caused by *F. graminearum* result in serious losses in wheat and barley yields. Infected grains are commonly contaminated with mycotoxins such as trichothecenes and zearalenone that can lead to mycotoxicoses in humans and livestock (Desjardins and Proctor 2007).

The random insertion mutagenesis method called restriction enzyme-mediated integration (REMI) was first described in *Saccharomyces cerevisiae* (Schiestl and Petes 1991) and was

later used to identify pathogenicity genes in several plant-pathogenic fungi, including *Cochliobolus heterostrophus*, *Ustilago maydis*, and *Magnaporthe grisea* (Bölker et al. 1995; Lu et al. 1994; Sweigard et al. 1998). REMI has been used to identify genes involved in *F. graminearum* sexual development and virulence (Han et al. 2004; Kim et al. 2009; Seong et al. 2005; Wang et al. 2011).

For pathogenic fungi to successfully colonize their hosts, various transcriptional regulatory mechanisms must control gene expression in response to environmental perturbations such as osmotic and oxidative stress. Post-translational modifications of chromatin such as histone acetylation are closely associated with transcriptional activation (Wolffe and Pruss 1996). Acetylation of lysine residues within the N-terminal tails of histone proteins changes chromatin structure by neutralizing positively charged nucleosomes and, consequently, increasing the accessibility of transcription factors to their target genes (Grunstein 1997). Histone acetylation is mediated by histone acetyltransferases (HAT) and is reversed by histone deacetylases (HDAC). HAT transfer the acetyl groups from acetyl-coenzyme A to the lysine residues of histones, whereas HDAC remove the acetyl groups (Kuo and Allis 1998). The general control nonderepressible 5 (GCN5)-related acetyltransferase (GNAT) superfamily is one of the conserved HAT families (Carrozza et al. 2003), and members of the GNAT family have similar three-dimensional structures and catalytic domains (Vetting et al. 2005).

Elongator protein 3 (ELP3), a member of the GNAT family, is the catalytic subunit of the Elongator complex. Elongator complex was first identified in *S. cerevisiae* as a multisubunit complex associated with elongating RNA polymerase II (RNAPII) (Otero et al. 1999); it was later found to be composed of six subunits, ELP1 to ELP6. In yeast, purified ELP3 acetylated histones in vitro, and the HAT activity of ELP3 was essential for its function in vivo (Wittschieben et al. 1999, 2000). Mammalian ELP3 was also purified and its ability to acetylate histones H3 and H4 was verified (Hawkes et al. 2002).

Previous studies revealed that *elp3* was required for the activation of a group of stress-inducible genes in eukaryotes (Creppe and Buschbeck 2011; Han et al. 2007a; Nelissen et al. 2010; Wittschieben et al. 2000). In *S. cerevisiae*, loss of *elp3* reduced the activation of genes involved in the adaptation to stressful growth conditions (Wittschieben et al. 2000). In *Arabidopsis*, *elp3* was required for the transcription of stress-induced and auxin-related genes (Chen et al. 2006; Nelissen et al. 2010). Using human cells, *elp3* was found to regulate the heat shock response gene, *hsp70* (Han et al. 2007a). Although

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the biochemical function of *elp3* is conserved in eukaryotes, *elp3* target genes vary by species.

In this study, we identified the *F. graminearum* ortholog of *S. cerevisiae elp3* using a forward genetics approach. The *F. graminearum elp3* deletion mutant showed defects in sexual and asexual development, trichothecene production, virulence, and the oxidative stress response. We hypothesized that the pleiotropic defects seen in the *elp3* deletion mutant was caused by reduced histone acetylation and that *elp3* might play a role in regulating pathogenicity genes in *F. graminearum*. The objectives of this study were to characterize the roles of *elp3* in *F. graminearum* and to identify the stress-inducible genes regulated by *elp3*. Our results demonstrated that *elp3* functioned in sexual and asexual development, virulence, and regulation of oxidative stress-induced genes in *F. graminearum*.

RESULTS

Identification of *elp3*.

A REMI mutant named Z39R9282 exhibited defects in sexual development and virulence relative to the wild-type strain (Fig. 1A). This mutant also had reduced aerial mycelia and excess accumulation of red pigments (Fig. 1A). Southern hybridization of Z39R9282 *Bgl*III-digested-genomic DNA using pBCATPH as a probe revealed that single insertion of the vector had occurred. Plasmid rescue was utilized to identify the insertion site, and it was found to be at the *Hind*III site located 274 bp downstream of the start codon of FGSG_02040.3, which encodes an ortholog of yeast ELP3 (Fig. 1B; Supplementary Fig. S1A). ELP3 contains a HAT domain, a putative S-adenosylmethionine-binding domain, and iron-sulfur cluster

domains. ELP3 is highly conserved among eukaryotes, and a phylogenetic analysis of ELP3 orthologs revealed that they clustered into distinct groups according to their phyla. Fungal orthologs of ELP3 clustered into two groups corresponding to the subphyla Pezizomycotina and Saccharomycotina (Fig. 1C).

Targeted deletion and complementation.

To elucidate the functions of *elp3* in *F. graminearum*, targeted gene deletion and complementation strains were created by homologous recombination. The *elp3* open reading frame (ORF) in the *F. graminearum* wild-type strain Z-3639 was replaced with the geneticin resistance gene (*gen*) to create the *elp3* deletion mutant strain. To generate the *elp3* complementation strain, *gen* was replaced on the wild-type allele of *elp3* fused to the hygromycin resistance gene (*hyg*). Southern hybridizations were performed on *Sal*I-digested genomic DNA from these strains using the 5' flanking region of *elp3* as a probe, and these hybridizations confirmed successful gene deletion and complementation (Supplementary Fig. S2). When cultured on complete media (CM), the *elp3* deletion mutant produced sparse aerial mycelia and more red pigments compared with the wild-type and complementation strains (Fig. 2A). However, there was no significant difference in radial growth among the strains (Table 1). The *elp3* deletion mutant showed indistinguishable phenotypes with an REMI mutant Z39R9282 in sexual and asexual development, virulence, and toxin production (data not shown).

Sexual and asexual development.

When self-fertilized, the *elp3* deletion mutant produced fewer perithecia compared with the wild-type and complemen-

Fig. 1. Identification of *elp3*. **A**, Phenotypes of the Z39R9282 mutant. Mycelial growth on complete media (CM), perithecia formation on carrot agar, and virulence on wheat heads. Pictures were taken 5 days after inoculation on CM, 7 days after sexual induction on carrot agar, and 21 days after inoculation on wheat heads. Scale bar = 500 μ m. Arrowheads mark the inoculated spikelets. **B**, Schematic showing where the pBCATPH vector inserted into the Z39R9282 genome. **C**, Phylogenetic tree of ELP3 orthologs in eukaryotes by ClustalW alignment. MEGA program version 5.2 was used to perform a 1,000-bootstrap phylogenetic analysis using the neighbor joining method. WT = the *Fusarium graminearum* wild-type strain Z-3639 and H = *Hind*III.

tation strains (Fig. 2B). Perithecia maturation was also delayed but not abolished in the *elp3* deletion mutant. Seven days after sexual induction, perithecia produced asci with mature ascospores in the wild-type strain, whereas immature

perithecia with young asci were observed in the *elp3* deletion mutant. Ten days after sexual induction, several asci containing mature ascospores were observed in the *elp3* deletion mutant (Fig. 2C). The *elp3* deletion mutant produced ascospore cirrhi (Fig. 2B) and discharged ascospores (Fig. 2D), similar to the wild-type strain. The reduced number of discharged ascospores of the *elp3* deletion mutant was due to fewer perithecia (Fig. 2D).

Although both the wild-type and the *elp3* deletion mutant strains produced and discharged ascospores, their morphologies differed (Fig. 3A). To visualize the nucleus in the *elp3* deletion mutant, we generated strain YJ19 ($\Delta elp3::gen\ hhl1::hhl1-gfp-hyg$) by outcrossing mat1g (Hong et al. 2010) and the *elp3* deletion mutant. In the wild-type strain, the majority of ascospores were four celled and the individual cells were uninucleate and of equal size. In contrast, the ascospores in the *elp3* deletion mutant were mainly two celled and each cell tended to contain two nuclei (Fig. 3B). The mean ascospore length of the *elp3* deletion mutant (21.9 μm) was similar to that of the wild-type strain (20.8 μm) regardless of the number of cells (Fig. 3A). The germinability of ascospores was not affected by the deletion of *elp3* (Table 1). Binucleate two-celled ascospores from the *elp3* deletion mutant germinated on CM without changes in septation (Supplementary Fig. S3).

Conidia production and germination of the *elp3* deletion mutant did not differ from the wild-type and complementation strains (Table 1). The average length of conidia produced from the *elp3* deletion mutant on yeast extract-malt extract agar (YMA) was approximately 20% longer than conidia produced from the wild-type strain ($P < 0.01$). The mean length of conidia from the *elp3* deletion mutant was 59.2 μm , while that of the wild-type strain was 47.8 μm (Fig. 3C). Despite the increased length of the conidia in the *elp3* deletion mutant, the number of septa was similar to the wild type (Fig. 3C).

Trichothecene production and virulence.

The production of trichothecenes was analyzed in minimal media containing 5 mM agmatine (MMA) by gas chromatography mass spectrometry. Deoxynivalenol and 15-acetyldeoxynivalenol, major trichothecenes produced in MMA, were not detected in the *elp3* deletion mutant (Fig. 4A), whereas trichothecene production by the complementation strain did not differ from that of the wild-type strain. The quantitative real-time polymerase chain reaction (qRT-PCR) results showed that transcription of the trichothecene biosynthesis genes *tri5* and *tri6* was significantly reduced in the *elp3* deletion mutant compared with the wild-type strain (Fig. 4B).

In a virulence test, 21 days after wheat head inoculation, the *elp3* deletion mutant caused significantly reduced disease symptoms ($P < 0.01$), whereas the wild-type and complementation strains caused typical head blight symptoms (Fig. 4C).

Fig. 2. Vegetative growth and sexual development of *Fusarium graminearum* strains. **A**, Mycelial growth of *F. graminearum* strains. Pictures were taken 5 days after inoculation on complete media (CM) and minimal media (MM). WT = the *F. graminearum* wild-type strain Z-3639, $\Delta elp3$ = the *elp3* deletion mutant (YJ17), and ELP3c = the $\Delta elp3$ mutant-derived strain complemented with *elp3* (YJ18). **B**, Perithecia formation by *F. graminearum* strains. Cirrhi (indicated with white arrow) were observed in the WT and complementation strains 10 days (D) after sexual induction. Thirteen days after sexual induction, the *elp3* deletion mutants formed cirrhi. Pictures were taken 10 and 13 days after sexual induction on carrot agar. Scale bar = 500 μm . **C**, Morphologies of asci rosettes. Microscopic observations were performed 7 and 10 days (D) after sexual induction. Scale bar = 50 μm . **D**, Forcible discharge of ascospores from perithecia produced on carrot agar. A semicircular agar block covered with mature perithecia was placed on a coverslip.

In wheat heads inoculated with the *elp3* deletion mutant, the spread of the disease within a spike was blocked and only artificially inoculated spikelets showed necrosis. To visualize the spread of mycelia on wheat heads during infection, strains constitutively expressing green fluorescent protein (GFP) were injected into the center spikelets of wheat heads. Six days after inoculation with the *elp3* deletion mutant that constitutively expresses GFP (YJ19), hyphae were seen throughout the rachis but failed to spread to the adjacent spikelet (Fig. 5), whereas inoculation with the wild-type GFP-expressing strain (hH1-GFP) led to the spread of hyphae to the adjacent spikelet through rachis node.

Stress sensitivity assay.

Previous studies on *elp3* in other eukaryotes revealed that it regulated the expression of stress-inducible genes (Chen et al. 2006; Han et al. 2007a; Wittschieben et al. 2000). To determine whether *elp3* is required for the stress response in *F. graminearum*, strains were inoculated onto CM supplemented with various stress agents, including osmotic stress agents (NaCl, KCl, and D-sorbitol), an oxidative stress agent (H₂O₂), cell-wall-damaging agents (sodium dodecyl sulfate [SDS] and Congo red),

and fungicides (fludioxonil, iprodione, and benomyl), and their sensitivities to these agents were evaluated. In addition, sensitivity to high-temperature stress was tested. Hyphal growth of the *elp3* deletion mutant was severely inhibited by oxidative stress compared with the wild-type strain (Fig. 6A) but none of the other stress conditions affected hyphal growth of the *elp3* deletion mutant (Supplementary Figs. S4 and S5), indicating that *elp3* was specifically involved in the oxidative stress response.

Identification of catalase genes in *F. graminearum*.

To investigate the expression of oxidative stress-induced genes in the *elp3* deletion mutant, we first identified catalase genes in *F. graminearum*. Five putative monofunctional catalase genes (FGSG_06554.3, FGSG_06733.3, FGSG_16526.3, FGSG_02881.3, and FGSG_06596.3) were previously identified by BLASTp searches using *Cryptococcus neoformans* catalase sequences (Giles et al. 2006). We identified two bifunctional catalase-peroxidase genes (FGSG_02974.3 and FGSG_12369.3) by BLASTp searches in the *Fusarium* Comparative Database using *M. oryzae* catalase-peroxidase sequences (Skamnioti et al. 2007). We designated these seven *F. graminearum* catalase genes *fca1* to *fca7* (Table 2).

Table 1. Radial growth, ascospore germination, and conidiation in *Fusarium graminearum* strains^w

Strain	Radial growth (cm) ^x	Ascospore germination (%) ^y	Conidia	
			Germination (%) ^y	Production (number of conidia/ml) ^z
Z-3639	9.8	69.0	48.3	2.7 × 10 ⁶
YJ17	9.8	70.3	47.7	2.7 × 10 ⁶
YJ18	10.0	68.3	50.3	2.6 × 10 ⁶

^w All data were repeated twice with three replications. Values are not significantly different ($P < 0.05$) based on a Tukey's test.

^x Radial growth was measured after 5 days incubation on complete media.

^y Percentage was measured after 8 h of incubation.

^z Number of conidia were counted after 3 days of incubation in carboxymethyl cellulose.

Fig. 3. Morphologies of ascospores and conidia. **A**, Length (dark gray) and number of septa (light gray) of ascospores from *Fusarium graminearum* strains. Error bars indicate the standard deviations. An asterisk indicates a significant difference ($P < 0.01$, Tukey's test). WT = the *F. graminearum* wild-type strain Z-3639, *Δelp3* = the *elp3* deletion mutant (YJ17), and ELP3c = the *Δelp3* mutant-derived strain complemented with *elp3* (YJ18). **B**, Microscopic observations of ascospores. Fluorescent green dots represent hH1-green fluorescent protein (GFP). DIC = differential interference contrast image, GFP = fluorescence microscopy image, Merge = overlays of the DIC and fluorescence microscopy images. Scale bar = 10 μm. **C**, Length (dark gray) and number of septa (light gray) of conidia on YMA. **D**, Microscopic observations of conidia. Scale bar = 10 μm.

Expression and functional analyses of catalase genes in *F. graminearum*.

We performed qRT-PCR to measure transcript levels of the putative catalase genes in the wild-type and *elp3* deletion mutant strains under oxidative stress. In the wild-type strain, catalase gene transcripts were upregulated in 10 mM H₂O₂. However, in the *elp3* deletion mutant, catalase gene transcripts, except *fca1*, were either downregulated or unchanged in 10 mM H₂O₂ (Fig. 6B). The qRT-PCR results indicated that the *elp3* deletion mutant was unable to regulate catalase gene transcription in response to oxidative stress.

To further investigate the functions of the putative catalase genes in *F. graminearum*, we generated deletion mutants of these genes through homologous recombination (Table 2; Supplementary Fig. S6) and assayed the sensitivities of these deletion mutants to 5 mM H₂O₂. Only the *fca6* and *fca7* deletion mutant strains exhibited increased sensitivities to oxidative stress compared with the wild-type strain (Fig. 6C). Hyphal growth of the *fca7* deletion mutant was significantly reduced in the presence of H₂O₂, suggesting that FCA7 plays a major role in degrading extracellular H₂O₂ during vegetative growth.

We then created an *fca7*-overexpression strain (*fca7::gen-P_{ef1α}-fca7*, YJ28) in which *fca7* expression was controlled by

the elongation factor 1 α (*ef1 α*) promoter (Fig. 7A). qRT-PCR was used to quantify *fca7* transcripts in the wild-type and *fca7*-overexpression strains and *fca7* overexpression was confirmed in strain YJ28 (Fig. 7B). We tested the *fca7*-overexpression strain for sensitivity to oxidative stress and found that it was

Fig. 5. Longitudinal sections of infected wheat heads. Wheat spikelets were inoculated with suspensions of conidia from green fluorescent protein (GFP)-expressing *Fusarium graminearum* strains. Infected wheat heads were dissected 6 days after inoculation and examined by fluorescence microscopy. Spreading of the GFP signal represents spreading of hyphae from the points of inoculation. Arrowheads mark the inoculated spikelets. WT = the *F. graminearum* wild-type strain Z-3639, Δ *elp3* = the *elp3* deletion mutant YJ17, and Reflected = reflected light.

Fig. 4. Trichothecene production and virulence of *Fusarium graminearum* strains. **A**, Total trichothecene (deoxynivalenol and 15-acetyldeoxynivalenol) production by *F. graminearum* strains. Each strain was grown in minimal media containing 5 mM agmatine (MMA) for 7 days. Trichothecenes were analyzed by gas chromatography mass spectrometry and were quantified based on the biomass of each strain. WT = the *F. graminearum* wild-type strain Z-3639, Δ *elp3* = the *elp3* deletion mutant YJ17, ELP3c = the Δ *elp3* mutant-derived strain complemented with *elp3* (YJ18), and nd = not detected. **B**, Transcript levels of *tri5* and *tri6* in the WT and *elp3* deletion mutant strains. Transcript levels were analyzed by quantitative real-time polymerase chain reaction 4 days after inoculation in MMA. An asterisk indicates a significant difference ($P < 0.01$, Tukey's test). **C**, Virulence on wheat heads. The center spikelet of each wheat head was injected with 10 μ l of a conidial suspension, and pictures were taken 21 days after inoculation. Arrowheads indicate the inoculated spikelets. Significantly different ($P < 0.01$, Tukey's test) disease indices (diseased spikelets per wheat head) are designated with different letters. Mock = mock-inoculated with 0.01% of Tween 20.

more tolerant to H₂O₂ than the wild-type strain (Fig. 7C). To determine whether the oxidative stress sensitivity of the *elp3* deletion mutant was due to the reduced expression of *fca7*, we generated strain YJ29 ($\Delta\text{elp3}::\text{gen fca7}::\text{gen-}P_{\text{efl}\alpha}\text{fca7}$) by outcrossing the heterothallic strain carrying the *elp3* mutation (strain YJ20; $\Delta\text{mat1-1}::\text{gen } \Delta\text{elp3}::\text{gen}$) with strain YJ28 (*fca7::gen-}P_{\text{efl}\alpha}\text{fca7}) and confirmed the genotype of strain YJ29 by PCR. The oxidative stress sensitivity of the *elp3* de-*

letion mutant strain was partially restored by overexpression of *fca7* (Fig. 7C).

Western blot analysis of histone acetylation.

To investigate whether the deletion of *elp3* affected histone H3 acetylation in *F. graminearum*, we measured acetylation of lysine 14 in H3 (H3K14Ac) in the wild-type and *elp3* deletion mutant strains by Western blot analysis (Fig. 8A). We chose

Table 2. *Fusarium graminearum* strains used in this study

Strain	Genotype	Reference
Z-3639	<i>F. graminearum</i> wild type	Bowden and Leslie 1999
Z39R9282	REMI mutant	This study
YJ17	$\Delta\text{elp3}::\text{gen}$	This study
YJ18	$\Delta\text{elp3}::\text{elp3-hyg}$	This study
hH1-GFP	<i>hH1::hH1-gfp-hyg</i>	Hong et al. 2010
Δmat1	$\Delta\text{mat1-1}::\text{gen}$	Lee et al. 2003
mat1g	$\Delta\text{mat1-1}::\text{gen hH1::hH1-gfp-hyg}$	Hong et al. 2010
YJ19	$\Delta\text{elp3}::\text{gen hH1::hH1-gfp-hyg}$	This study
YJ20	$\Delta\text{mat1-1}::\text{gen } \Delta\text{elp3}::\text{gen}$	This study
YJ21	$\Delta\text{fca1}::\text{gen}$	This study
YJ22	$\Delta\text{fca2}::\text{gen}$	This study
YJ23	$\Delta\text{fca3}::\text{gen}$	This study
YJ24	$\Delta\text{fca4}::\text{gen}$	This study
YJ25	$\Delta\text{fca5}::\text{gen}$	This study
YJ26	$\Delta\text{fca6}::\text{gen}$	This study
YJ27	$\Delta\text{fca7}::\text{gen}$	This study
YJ28	<i>fca7::gen-}P_{\text{efl}\alpha}\text{fca7}</i>	This study
YJ29	$\Delta\text{elp3}::\text{gen fca7}::\text{gen-}P_{\text{efl}\alpha}\text{fca7}$	This study

Fig. 6. Sensitivity of *Fusarium graminearum* strains to oxidative stress and transcriptional analyses of catalase genes. **A**, Mycelial growth of *F. graminearum* strains on complete media (CM) supplemented with 5 mM H₂O₂. WT = the *F. graminearum* wild-type strain Z-3639, Δelp3 = the *elp3* deletion mutant (YJ17), and ELP3c = the Δelp3 mutant-derived strain complemented with *elp3* (YJ18). **B**, Transcript levels of catalase genes in the WT and *elp3* deletion mutant strains. Total RNA was isolated from the WT and *elp3* deletion mutant strains grown for 3 h in CM only or in CM supplemented with 10 mM H₂O₂, and gene transcripts were analyzed by quantitative real-time polymerase chain reaction. **C**, Mycelial growth of WT and catalase deletion mutant strains on CM supplemented with 5 mM H₂O₂. Δfca1 = the *fca1* deletion mutant (YJ21), Δfca2 = the *fca2* deletion mutant (YJ22), Δfca3 = the *fca3* deletion mutant (YJ23), Δfca4 = the *fca4* deletion mutant (YJ24), Δfca5 = the *fca5* deletion mutant (YJ25), Δfca6 = the *fca6* deletion mutant (YJ26), and Δfca7 = the *fca7* deletion mutant (YJ27).

lysine 14 of H3 because, in yeast, it is one of the main acetylation sites of the Elongator complex (Winkler et al. 2002). Although relative histone acetylation level was highest 3 days after sexual induction for both the wild type and the *elp3* deletion mutant, histone acetylation was markedly reduced in the *elp3* deletion mutant (Fig. 8B).

DISCUSSION

In this study, we identified a gene encoding an ortholog of yeast ELP3 in *F. graminearum* using forward genetics. To elucidate the functions of ELP3 in *F. graminearum*, we created an *elp3* deletion mutant and analyzed its phenotypes. We found that *elp3* was involved in diverse biological processes, including sexual and asexual reproduction, trichothecene production, and virulence in *F. graminearum*. We also found that *elp3* played a role in the oxidative stress response by regulating catalase gene expression.

In *F. graminearum*, hundreds of genes required for sexual and asexual reproduction must be tightly regulated in time and space (Hallen et al. 2007; Qi et al. 2006; Son et al. 2013, 2014). Histone acetylation is generally important for the regulation of gene expression and is associated with gene activation, whereas deacetylation is associated with transcriptional repres-

sion (Grunstein 1997; Kuo and Allis 1998; Struhl 1998). We found that the level of histone acetylation was lower in the *elp3* deletion mutant than in the wild-type strain. We previously reported that reduced histone acetylation contributed to defects in sexual development in *F. graminearum* (Son et al. 2011). Thus, the reduced acetylation in the *elp3* deletion mutant contributed to the sexual and asexual developmental defects in the *elp3* deletion mutant.

Perithecium formation is a complex cellular differentiation process mediated by diverse signaling pathways. Inappropriate expression of genes involved in cell proliferation and differentiation resulted in defects in perithecium development in *F. graminearum* (Lin et al. 2011, 2012). Deletion of *elp3* affected the production and maturation of perithecia, indicating that *elp3* was required for proper gene expression during perithecium formation. The *elp3* deletion mutant also produced abnormal ascospores. In the wild-type strain, an ascospore develops from a single uninucleate cell. Following the first mitosis, a medial septum is formed, resulting in a two-celled ascospore. The nucleus in each cell undergoes a second mitosis, and the resulting two nuclei are immediately separated by an additional septum. Thus, fully developed ascospores contain four uninucleate cells (Headrick et al. 1988). The *elp3* deletion mutant produced ascospores containing two dinucleate cells, indi-

Fig. 7. Sensitivity of the *fca7* overexpression strain to oxidative stress. **A**, The *ef1 α* promoter was inserted into the natural promoter of *fca7*. The left panel shows how the *fca7* overexpression strain was constructed and the right panel confirms insertion by Southern analysis. WT = the *Fusarium graminearum* wild-type strain Z-3639, fca7OE = overexpression strain in which the natural *fca7* promoter was replaced with the *ef1 α* promoter (YJ28), X = *Xba*I, and *gen* = geneticin resistance gene. Lane 1, Z-3639; lanes 2 and 3, isolates of the *fca7* overexpression strain. The DNA size standards (kb) are indicated on the left of the blot. **B**, Transcript levels of *fca7* in the WT and *fca7* overexpression strains. Total RNA was isolated from the WT and *fca7* overexpression strains grown for 3 h in complete media (CM) only or in CM supplemented with H₂O₂, and transcript levels were analyzed by quantitative real-time polymerase chain reaction. **C**, Mycelial growth of fungal strains on CM supplemented with 5 mM H₂O₂. Δ *elp3* = the *elp3* deletion mutant (YJ17) and Δ *elp3*-fca7OE = the *elp3* deletion mutant that overexpresses *fca7* (YJ29).

cating a failure to progress through the second mitotic division. When assessing asexual development, we found that the *elp3* deletion mutant produced longer conidia compared with the wild type. Defects in cell cycle progression during conidiogenesis in *F. graminearum* frequently results in elongated conidia (Min et al. 2014; Son et al. 2014). Deletion of *wetA*, a *F. graminearum* transcription factor known to be involved in conidiogenesis, resulted in longer conidia (Son et al. 2014). Taken together, these results indicate that the defects in sexual and asexual development of the *elp3* deletion mutant result from impairments in cell cycle progression.

The *elp3* deletion mutant exhibited reduced virulence on wheat heads, and several *F. graminearum* genetic studies have shown that trichothecenes are virulence factors for plant disease (Desjardins et al. 1996; Maier et al. 2006; Proctor et al. 1995). Thus, we hypothesized that reduced trichothecene production might contribute to the reduced virulence of the *elp3* deletion mutant. In the *elp3* deletion mutant, the transcript levels of the trichothecene biosynthesis genes *tri5* and *tri6* were significantly lower than in the wild-type strain. These observations suggested that *elp3* played a role in virulence by regulating trichothecene biosynthesis gene expression.

In many organisms, ELP3 has been found to regulate genes involved in stress responses (DeFraia et al. 2013; Han et al. 2007a; Nelissen et al. 2005; Walker et al. 2011; Wittschieben et al. 2000). In yeast, an *elp3* deletion mutant showed a delayed response to high temperature and salt stress as well as reduced activation of several genes, including *gal1-10* and *pho5*, which are required for survival under stressful growth conditions (Wittschieben et al. 2000). In plants, ELP3 has been found to be required for basal plant immunity (DeFraia et al. 2013) and, in human cells, ELP3 regulates *hsp70* expression by acetylating histones H3 and H4 in heat-shock conditions (Han et al. 2007a). Although the role of *elp3* in the stress response is conserved in eukaryotes, the subset of stress-inducible genes has diverged during evolution (Creppe and Buschbeck 2011).

We tested the sensitivity of the *elp3* deletion mutant to diverse stress conditions and found that *F. graminearum* *elp3* was mainly involved in the oxidative stress response. Oxidative stress is a stress commonly encountered by phytopathogenic fungi. During infection, fungal pathogens encounter toxic environments generated by the hosts' defense mechanisms. The oxidative burst, a rapid production of reactive oxygen species (ROS), is one of the host's earliest responses to pathogen infection (Torres et al. 2006; Wojtaszek 1997). Several pathogens produce ROS-scavenging enzymes to protect themselves from the plant-derived oxidative stress (Schouten et al. 2002); catalase, which metabolizes H_2O_2 , is one of these antioxidant enzymes. We found that, in *F. graminearum*, *elp3* was required for catalase gene expression under oxidative stress conditions.

Seven putative catalase genes, *fca1* to *fca7*, have been identified in *F. graminearum* through BLAST searches, and we generated deletion mutants for all seven genes. Among these mutants, only the *fca6* and *fca7* deletion mutants showed increased sensitivity to extracellular H_2O_2 . Deletion of the other catalase genes did not affect sensitivity to oxidative stress, which may be due to the overlapping and redundant antioxidant functions of these genes. In *C. neoformans*, single and quadruple catalase mutants did not exhibit oxidative-stress phenotypes, indicating that *C. neoformans* possessed a robust and redundant antioxidant defense system (Giles et al. 2006). Because *fca7* primarily contributed to the extracellular H_2O_2 resistance phenotype, we constructed an *elp3* deletion mutant that overexpressed *fca7*. We found that the oxidative stress sensitivity of the *elp3* deletion mutant was partially comple-

mented by overexpression of *fca7*. Thus, *elp3* may confer resistance to oxidative stress by regulating the expression of *fca7*.

In this study, we observed that the *elp3* deletion mutant had reduced catalase activity under oxidative stress and exhibited reduced virulence on wheat heads. In *Claviceps purpurea*, deletion of the transcription factor *cptf1*, a general regulator of catalase activity, resulted in reduced virulence (Nathues et al. 2004). In *M. oryzae*, it was reported that the catalase gene *catB* was involved in its pathogenicity (Skamnioti et al. 2007). In addition, the catalase-peroxidase gene *cpxB* in *M. oryzae* played a role in fungal defense against H_2O_2 accumulated in epidermal cells during early infection (Tanabe et al. 2011).

In a previous study, *F. graminearum* HDAC genes *hdf1*, *hdf2*, and *hdf3* were characterized (Li et al. 2011). The deletion of the HDAC genes led to the opposite results for pigmentation, conidial morphology, and response to oxidative stress than the *elp3* deletion. Unlike the *hdf1* deletion mutant, which formed whitish colonies on potato dextrose agar (Li et al. 2011), the *elp3* deletion mutant displayed more red pigmentation than the wild-type strain. The conidia produced by the *elp3* deletion mutant were longer than those produced by the wild-type strain, whereas the $\Delta hdf1 \Delta hdf2$ double mutant produced shorter conidia (Li et al. 2011). In the *hdf1* deletion mutant, sensitivity to H_2O_2 was reduced and the putative catalase genes were upregulated (Li et al. 2011), whereas the *elp3* deletion mutant was more sensitive to H_2O_2 and catalase gene expression was reduced. Because HAT and HDAC have opposite functions (Kuo and Allis 1998), these observations raised the possibility that *elp3* cooperated with HDAC genes to maintain the appropriate level of histone acetylation during these biological processes. In yeast, the phenotype of a $\Delta elp3 \Delta gcn5$ double mutant was partially restored by mutation of specific

Fig. 8. Western blot of whole cell extracts of *Fusarium graminearum* strains. **A**, Whole cell extracts were prepared from carrot agar cultures. WT = the *F. graminearum* wild-type strain Z-3639, $\Delta elp3$ = the *elp3* deletion mutant (YJ17), α -H3 = primary antibody against histone H3, and α -acH3 = primary antibody against histone H3 acetylated at Lys14. Numbers above the blots indicate the number of days of incubation after sexual induction. **B**, Levels of α -acH3 and α -H3 binding were quantified using Image Studio software, and the data are presented as the ratio of α -acH3/ α -H3 binding. Error bars represent the standard deviations of three independent experiments.

HDAC genes, demonstrating the balance between histone acetylation and deacetylation (Wittschieben et al. 2000).

In conclusion, we have shown that *F. graminearum elp3* is involved in diverse biological processes, including sexual and asexual reproduction, secondary metabolite production, virulence, and the oxidative stress response. This article demonstrated that the defects in the *elp3* deletion mutant were associated with reduced histone acetylation and demonstrated that *elp3* was required for activation of genes involved in oxidative stress, a stress that pathogens encounter upon plant invasion.

MATERIALS AND METHODS

Fungal strains and culture media.

The *F. graminearum* wild-type strain Z-3639 (Bowden and Leslie 1999) and mutants derived from this strain were used in this study (Table 2). All strains were stored as conidial suspensions in 20% glycerol at -70°C . Culture media used in this study were prepared following the *Fusarium* laboratory manual (Leslie and Summerell 2006). For conidia production, carboxymethyl cellulose (CMC) liquid culture media (Cappellini and Peterson 1965) and YMA media (Harris 2005) were used. MMA was used for trichothecene production (Gardiner et al. 2009).

Nucleic acid manipulations, primers, PCR conditions, and DNA sequencing.

Genomic DNA was extracted from mycelia powder as previously described (Leslie and Summerell 2006). Total RNA was extracted from mycelia in liquid nitrogen using the Easy-Spin Total RNA Extraction Kit (Intron Biotech, Seongnam, Korea). Standard techniques were used for restriction endonuclease digestion, Southern hybridization with ^{32}P -labeled probes, agarose gel electrophoresis, and plasmid rescue (Sambrook and Russell 2001). The PCR primers used in this study (Supplementary Table S1) were synthesized by an oligonucleotide synthesis facility (Bionics, Seoul, Korea). PCR was performed according to manufacturer's instructions (Takara Bio Inc., Otsu, Japan). DNA sequencing was performed at the National Instrumentation Center for Environmental Management (Seoul National University, Seoul, Korea) and sequences were compared against the *Fusarium* Comparative Database at the Broad Institute.

Genetic manipulations and fungal transformations.

The double-joint (DJ) PCR method was used to generate fusion PCR products for the creation of targeted gene deletion, complementation, and overexpression strains (Yu et al. 2004). To create deletion strains, *gen* was amplified from pII99 (Namiki et al. 2001), and the 5' and 3' flanking regions of target genes were amplified from the wild-type strain. The three amplicons (5' flanking, 3' flanking, and *gen*) were fused by DJ PCR as previously described (Hong et al. 2010). For complementation of the *elp3* deletion mutant, a wild-type copy of *elp3* was fused with *hyg* from pBCATPH (Gritz and Davies 1983). The fusion construct was transformed into the deletion mutant as previously described (Han et al. 2007b). To construct the *fca7* overexpression mutant, a fusion construct containing the 5' flanking region of *fca7*, the *fca7* ORF, and the *gen-P_{efl}* region from pSKGEN (Lee et al. 2011) was created and transformed into the wild-type strain.

Conidia production and germination.

Fungal strains were incubated in 50 ml of CM for 72 h at 25°C on a rotary shaker (150 rpm). Mycelia were harvested and washed twice with sterile distilled water. To induce conidiation, harvested mycelia were spread onto YMA and incubated for 48 h at 25°C under near-UV light (wavelength: 365 nm;

HKiv Import & Export Co., Ltd., Xiamen, China). Conidia were collected in sterile distilled water, filtered through cheesecloth, washed with sterile distilled water, and collected by centrifugation (5,000 rpm, 25°C , 5 min). Conidia production was measured by counting the number of conidia produced after inoculating 5 ml of CMC with 10 μl of conidia suspension (10^5 conidia/ml) and incubating for 72 h at 25°C on a rotary shaker (150 rpm). The rate of conidia germination was determined as previously described (Lee et al. 2009b). In brief, 10 ml of CM was inoculated with 1 ml of conidia suspension (10^6 conidia/ml) and incubated for 2, 4, 6, 8, and 12 h. The number of germinating conidia was counted at each time point.

Sexual crosses.

For self-fertilizations, fungal strains were grown on carrot agar plates at 25°C for 5 days. The mycelia grown on carrot agar plates were mock fertilized with 700 μl of a 2.5% Tween 60 solution (Leslie and Summerell 2006) to induce sexual reproduction. After sexual induction, cultures were incubated under near-UV light (wavelength: 365 nm; HKiv Import & Export Co., Ltd.) at 25°C . Perithecia and ascospores were observed 7 to 10 days after fertilization. For outcrosses, mycelia of heterothallic female strains grown on carrot agar plates for 5 days were fertilized with 1 ml of a male strain conidia suspension. After sexual induction, cultures were incubated under near-UV light at 25°C for 7 days. Ascospores were discharged onto 2% water agar plates. Ascospores were isolated and pure-cultured in CM.

Ascospore discharge and germination.

Ascospore discharge was observed using small acrylic chambers (1 by 2.5 by 5 cm) as previously described (Trail et al. 2005). A semicircular agar block (9.5 mm in diameter) covered with mature perithecia was placed on a coverslip and incubated in the chamber for 24 h.

Ascospore germination tests were performed as previously described (Lee et al. 2009b). Ascospores were harvested by placing the carrot agar upside down and allowing the mature perithecia to discharge ascospores on petri dish covers. Ascospore suspension (1 ml at 10^6 conidia/ml) was incubated in 10 ml of CM for 2, 4, 6, 8, and 12 h. The number of germinating ascospores was counted at each time point. The experiments were performed twice with three replicates.

Virulence assays and trichothecene analysis.

Virulence tests were performed using the susceptible wheat 'Eunpamil' as previously described (Lee et al. 2009a). Conidial suspensions (10 μl at 10^6 conidia/ml) harvested from CMC were injected into center spikelets of wheat heads at midanthesis. After inoculation, plants were incubated in a humidity chamber for 3 days and then transferred to a greenhouse. Spikelets displaying symptoms of FHB were counted 21 days after inoculation.

Six days after inoculation, wheat spikes infected with GFP-tagged strains were evaluated. Freehand longitudinal sections of spikes were cut using a clean scalpel (Baldwin et al. 2010). Sectioned spikes were viewed with reflected light and fluorescent light (excitation 470 and emission 525) using a SteREO Lumar V12 microscope (Carl Zeiss, Oberkochen, Germany).

Trichothecene production was measured as previously described (Son et al. 2011). In brief, MMA cultures were extracted with ethyl acetate and the extracts were dehydrated. Each dehydrated extract was derivatized with trimethylsilylating reagent (BSA + TMCS + TMSI, 3:2:3; Supelco, Bellefonte, PA, U.S.A.) and analyzed on a Shimadzu QP-5000 GC-MA (Shimadzu, Kyoto, Japan) using the selective ion-monitoring mode as previously described (Seo et al. 1996).

Stress sensitivity assays.

To evaluate the effects of diverse stressors on mycelial growth, agar plugs from actively growing cultures were transferred to CM agar plates supplemented with osmotic stress agents (1.5 M D-sorbitol, 1.2 M NaCl, and 1.2 M KCl), an oxidative stress agent (5 mM H₂O₂), cell-wall-damaging agents (SDS at 5 mg/liter and Congo red at 60 mg/liter), and fungicides (fludioxonil at 0.023 mg/liter, iprodione at 8.6 mg/liter, and benomyl at 0.65 mg/liter). Plates were incubated at 25°C for 5 days. For the temperature sensitivity test, the wild-type, *elp3* deletion mutant, and complementation strains were grown in CM at 25, 30, and 32°C for 5 days in the dark. At least two independent tests were performed for each assay and each strain was tested in triplicate.

Identification of catalase genes *F. graminearum*.

To identify putative catalase and catalase-peroxidase genes in *F. graminearum*, a BLASTp search with *M. oryzae* catalase and catalase-peroxidase sequences (Skamnioti et al. 2007) was performed in the *Fusarium* Comparative Database.

qRT-PCR analysis.

Total RNA was extracted from wild-type and *elp3* deletion mutant strains grown for 3 h in CM only or in CM supplemented with 10 mM H₂O₂. cDNA was synthesized from the total RNA using SuperScriptIII reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.). qRT-PCR was performed using SYBR Green Super mix (Bio-Rad, Hercules, CA, U.S.A.), a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, U.S.A.), and primer pairs specific for catalase genes. The cyclophilin gene (*CyP1*; FGSG_07439.3) was used as the endogenous control for normalization (Kwon et al. 2009). qRT-PCRs were performed three times, and the transcript level of each target gene was calculated as previously described (Livak and Schmittgen 2001).

To analyze transcription of the trichothecene biosynthesis genes *tri5* and *tri6*, we incubated conidia from wild-type and *elp3* deletion mutant strains in MMA media for 5 days, isolated total RNA from each strain, and performed qRT-PCR as described above.

Microscopic observations.

Spore morphology was observed by differential interference contrast (DIC) microscopy. DIC images were obtained with a DE/Axio Imager A1 microscope (Carl Zeiss) equipped with an UPlanApo ×100 objective lens (1.3 numerical aperture; Carl Zeiss). AxioVision software (release 4.7; Carl Zeiss) was used to measure spore dimensions. Chitin staining was conducted by adding Calcofluor white stock solution (10 mg/ml; Sigma, St. Louis) to ascospore samples on slide glasses as previously described (Son et al. 2012). Ascospores were observed using filter set 49 (excitation 356; emission 445/50). To visualize GFP expression in spores, microscopy was performed with the DE/Axio Imager A1 microscope (Carl Zeiss) using the 38H (excitation 470/40; emission 525/50) filter set.

Western blotting.

Whole cell extracts (WCE) were prepared as previously described (Son et al. 2011). In brief, mycelia harvested from carrot agar cultures were ground in liquid nitrogen and extracted with 1 ml of breaking buffer (50 mM Tris at pH 8.4, 192 mM glycine, and 0.1% SDS) supplemented with 1 mM phenylmethylsulfonyl fluoride, a protease inhibitor. WCE were separated on 15% SDS polyacrylamide gels and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). Western blot analyses were performed using chemiluminescence (Amersham ECL Select Western Blotting Detec-

tion Reagent; Amersham Pharmacia Biotech) according to the manufacturer's instructions. Primary rabbit anti-histone H3 (Cell Signaling Technology, Beverly, MA, U.S.A.) and anti-acetyl-Lys-4 H3 antibodies (07-353; Upstate Biotechnology, Inc., Lake Placid, NY, U.S.A.) were used at a 1:1,000 dilution. Anti-rabbit secondary antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech) was used at a 1:4,000 dilution. The blocking buffer was composed of 5% dry nonfat milk in 0.1% Tween 20-Tris-buffered saline (pH 7.6). The detection method used was chemiluminescence and bands were visualized and quantified with Image Studio software (version 2.0; Li-Cor Biosciences, Lincoln, NE, U.S.A.).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Fusarium Comparative database:
www.broadinstitute.org/annotation/genome/fusarium_graminearum