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FgFlbD Regulates Hyphal Differentiation Required for Sexual and Asexual Reproduction in the Ascomycete Fungus *Fusarium graminearum*[§]

Hokyoung Son[1], Myung-Gu Kim[1], Suhn-Kee Chae[2], and Yin-Won Lee[1]*

[1]*Department of Agricultural Biotechnology and Center for Fungal Pathogenesis, Seoul National University, Seoul 151-921, Republic of Korea*
[2]*Department of Biochemistry, Paichai University, Daejeon 305-343, Republic of Korea*

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Fusarium graminearum **is a filamentous fungal plant pathogen that infects major cereal crops. The fungus produces both sexual and asexual spores in order to endure unfavorable environmental conditions and increase their numbers and distribution across plants. In a model filamentous fungus, *Aspergillus nidulans*, early induction of conidiogenesis is orchestrated by the *fluffy* genes. The objectives of this study were to characterize *fluffy* gene homologs involved in conidiogenesis and their mechanism of action in *F. graminearum*. We characterized five *fluffy* gene homologs in *F. graminearum* and found that FlbD is the only conserved regulator for conidiogenesis in *A. nidulans* and *F. graminearum*. Deletion of *fgflbD* prevented hyphal differentiation and the formation of perithecia. Successful interspecies complementation using *A. nidulans flbD* demonstrated that the molecular mechanisms responsible for FlbD functions are conserved in *F. graminearum*. Moreover, *abaA-wetA* pathway is positively regulated by FgFlbD during conidiogenesis in *F. graminearum*. Deleting *fgflbD* abolished morphological effects of *abaA* overexpression, which suggests that additional factors for FgFlbD or an AbaA-independent pathway for conidiogenesis are required for *F. graminearum* conidiation. Importantly, this study led to the construction of a genetic pathway of *F. graminearum* conidiogenesis and provides new insights into the genetics of conidiogenesis in fungi.**

Keywords: conidiogenesis, *Fusarium graminearum*, *flbD*, *fluffy* genes

Introduction

Fusarium graminearum is an important fungal plant pathogen that causes Fusarium head blight (FHB) on cereal crops such as wheat, barley, and rice. FHB reduces grain yield and quality, and it may result in contamination with one or more harmful mycotoxins (Leslie and Summerell, 2006). Sexual spores (ascospores) and asexual spores (conidia) function as the primary and secondary inocula, respectively (Trail *et al.*, 2002; Guenther and Trail, 2005). Conidia are mainly responsible for spreading FHB during the cropping season since ascospores are only produced under specific environmental conditions (Guenther and Trail, 2005). Conidia are produced on conidiophores, a specialized type of fungal hyphae. Conidiogenesis requires complex cellular differentiation and is under precise spatial, temporal, and genetic control. The morphology of conidiophores and conidia are important fungal taxonomic characteristics (Alexopoulos *et al.*, 1996), especially in the genus *Fusarium* (Leslie and Summerell, 2006).

Living organisms recognize various environmental conditions and can alter their physiological processes to adapt to them. For example, fungi have developed complex signal transduction networks to recognize and respond to diverse environmental cues. These conserved signal transduction pathways in fungi include GTP binding proteins (G proteins), mitogen-activated protein kinase (MAPKs), protein kinase A/cyclic AMP (PKA/cAMP) signaling, and calcium signaling (Lengeler *et al.*, 2000).

The signal transduction pathways orchestrating conidiogenesis have been extensively studied in *Aspergillus nidulans*. Initiation of conidiogenesis, conidiophore formation, and conidia maturation are affected by sequentially expressed developmental regulators that are involved in at least two parallel signaling pathways in *A. nidulans* (Park and Yu, 2012; Lee *et al.*, 2013). One of the two pathways is mediated by heterotrimeric G protein signaling that can repress conidiation via PKA/ cAMP signals. G protein-coupled receptors (GPCR) recognize extracellular environmental cues that lead to the release of Gα subunits from heterotrimeric complexes. In *A. nidulans fadA* and *ganB* encode Gα subunits that function as repressors and activators for conidiation and vegetative growth, respectively (Adams *et al.*, 1998; Lengeler *et al.*, 2000; Chang *et al.*, 2004; Yu, 2006).

The second signaling pathway related to conidiogenesis is a genetic pathway that consists of upstream and central regulators. In this pathway, FluG is required for production of a diorcinol-dehydroaustinol adduct, known as an extracellular sporulation-inducing factor (ESID). Accumulation of this diffusible factor in hyphal cells leads to repression of *sfgA*, which is required to activate vegetative growth and suppress

*For correspondence. E-mail: lee2443@snu.ac.kr; Tel.: +82-2-880-4671; Fax: +82-2-873-2317
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asexual sporulation (Seo *et al.*, 2003, 2006; Rodríguez-Urra *et al.*, 2012). Subsequently, a series of *fluffy* genes, *flbB*, *flbC*, *flbD*, and *flbE*, initiate conidiophore formation (Lee and Adams, 1994; Adams *et al.*, 1998; Etxebeste *et al.*, 2010; Park and Yu, 2012; Rodríguez-Urra *et al.*, 2012). FlbC is a transcription factor containing C_2H_2 zinc finger DNA-binding domain that directly induces *brlA* expression (Kwon *et al.*, 2010a). FlbB physically interacts with FlbE and FlbD, and the resulting FlbB/FlbE and FlbB/FlbD complexes induce the expression of *flbD* and *brlA*, respectively (Garzia *et al.*, 2009, 2010; Kwon *et al.*, 2010b). FlbB has a basic leucine zipper (bZip) domain and FlbD has a cMyb DNA-binding domain, which suggests that they have a role as transcriptional regulators (Garzia *et al.*, 2010). After conidiogenesis initiation, central transcriptional regulators, such as BrlA, AbaA, and WetA, sequentially regulate genetic and biochemical processes for conidiophore formation and conidia maturation (Adams *et al.*, 1998).

There is crosstalk between the two parallel signaling pathways involved in conidiogenesis. For example, as a regulator of G protein signaling, FlbA functions to repress FadA activity (Yu *et al.*, 1996). Additionally, *sfgA* represses *flbA* as well as other *fluffy* genes, and therefore acts as a mediator that connects the two pathways (Seo *et al.*, 2003). PkaA, a downstream protein kinase of G protein signaling, also affects the FluG-dependent conidiogenesis pathway by negatively regulating *brlA* (Shimizu and Keller, 2001).

Recently, we found that an AbaA-WetA pathway is conserved in *F. graminearum,* but BrlA does not exist. This is in contrast to *A. nidulans*, in which the pathway of central regulators for conidiogenesis includes BrlA, AbaA, and WetA (Son *et al.*, 2013, 2014). Based on our previous results, we wondered which upstream regulators of conidiogenesis known in *A. nidulans* regulate AbaA-WetA pathway in *F. graminearum*. To this end we identified homologs of *fluffy* genes and characterized their functions in conidiogenesis in *F. graminearum*. Based on the results from this study, in combination with results from previous studies, we constructed regulatory genetic networks governing conidiogenesis in *F. graminearum* (Son *et al.*, 2013, 2014). These results give new insight into the genetic pathway for the early induction of conidiogenesis in *F. graminearum*.

Materials and Methods

Fungal strains and media

The wild-type *F. graminearum* strain Z-3639 (Bowden and Leslie, 1999), and all of the transgenic strains are described in Table 1. Strains hH1-GFP (Hong *et al.*, 2010), Δ*mat1* (Lee *et al.*, 2003), mat1g (Hong *et al.*, 2010), mat1r (Son *et al.*, 2011a), Δ*fgflbC* (previously designated as Δ*gzzc2h047*) (Son *et al.*, 2011b), Δ*fgflbD* (Son *et al.*, 2011b), and AbaA-OE (Son *et al.*, 2013) were constructed in previous studies (Table 1). A transgenic strain, mat1r, carrying both a *mat1-1-1* deletion and a histone H1 tagged with red fluorescence protein (RFP), was used for co-localization, as previously described (Son *et al.*, 2011a). Yeast malt agar (YMA) (Harris, 2005) and carboxymethyl cellulose (CMC) medium (Cappellini and Peterson, 1965) were used for induction of asexual sporulation. Nitrate nonutilizing (*nit*) mutants were generated on minimal medium supplemented with 1%, 2%, and 5% chlorate (MMC; Correll *et al.*, 1987). Other media used in this study were made and used according to the

Table 1. ***F. graminearum*** **strains used in this study**

Strain	Genotype	Source or reference
Z-3639	*Fusarium graminearum* wild type	Bowden and Leslie (1999)
WT *nit1*	*nit1*	This study
WT NitM	*nit5*	This study
A4	*Aspergillus nidulans* wild type	FGSC[a]
hH1-GFP	*hH1::hH1-gfp-hyg*	Hong *et al.* (2010)
Δ*mat1*	Δ*mat1-1-1::gen*	Lee *et al.* (2003)
mat1g	Δ*mat1-1-1::gen hH1::hH1-gfp-hyg*	Hong *et al.* (2010)
mat1r	Δ*mat1-1-1::gen hH1::hH1-rfp-hyg*	Son *et al.* (2011a)
HK156	Δ*fgfluG::gen*	This study
HK157	Δ*fgflbB::gen*	This study
HK158	Δ*fgflbE::gen*	This study
Δ*fgflbC*	Δ*fgflbC::gen*	Son *et al.* (2011b)
Δ*fgflbD*	Δ*fgflbD::gen*	Son *et al.* (2011b)
Δ*fgflbD nit1*	Δ*fgflbD::gen nit1*	This study
HK159	Δ*fgflbC:: fgflbC-gfp-hyg*	This study
HK160	Δ*fgflbD::gen hH1::hH1-gfp-hyg*	This study
HK161	Δ*fgflbD::fgflbD-gfp-hyg*	This study
HK162	Δ*fgflbD::*An*flbD-hyg*	This study
HK165	Δ*fgflbD::flbD-gfp-hyg hH1::hH1-rfp-gen*	This study
Δ*abaA*	Δ*abaA::gen*	Son *et al.* (2013)
AbaA-OE	*abaA::gen-*$P_{ef1\alpha}$*-abaA*	Son *et al.* (2013)
HK166	Δ*fgflbD::gen abaA::gen-*$P_{ef1\alpha}$*-abaA*	This study

[a] FGSC; Fungal Genetics Stock Center, University of Missouri, USA

Fusarium Laboratory Manual (Leslie and Summerell, 2006). The wild-type and transgenic strains were stored in a 20% glycerol stock at -80°C.

Nucleic acid manipulations and PCR primers

For genomic DNA isolation, fungal strains were grown in liquid complete medium (CM) at 25°C on a rotary shaker (150 rpm) for 3 days, and subsequently the mycelial mass was harvested and lyophilized. DNA was extracted with a cetyltrimethylammonium bromide protocol (Leslie and Summerell, 2006). Total RNA was extracted using an Easy-Spin Total RNA Extraction Kit (iNtRON Biotech, Korea) and following the manufacturer's instructions. Restriction endonuclease digestion, agarose gel electrophoresis, gel blotting, and DNA blot hybridization with ^{32}P-labeled probes were performed in accordance with standard techniques (Sambrook and Russell, 2001). The PCR primers (Supplementary data Table S1) used in this study were synthesized by a commercial oligonucleotide synthesis facility (Bionics, Korea).

Genetic manipulations and fungal transformations

Fusion PCR products were constructed by using a double-joint (DJ) PCR procedure previously described by Yu *et al.* (2004). For targeted deletion of the open reading frame (ORF) of *fgfluG*, two PCR primer sets FgFluG-5F/FgFluG-5R and FgFluG-3F/FgFluG-3R amplified the 5′- and 3′-flanking regions, respectively, of the *fgfluG* from Z-3639 genomic DNA. A 1.9 kb amplicon containing the geneticin resistance gene (*gen*) was amplified from pII99 (Namiki *et al.*, 2001) with the Gen-For and Gen-Rev primers. The 5′- and 3′-flanking regions and *gen* were mixed, and the second and final rounds of DJ PCR were carried out. Thereafter, final PCR products were directly transformed into protoplasts of the wild-type *F. graminearum* strain Z3639 with a previously described polyethylene-glycol-induced genetic transformation method (Han *et al.*, 2007). Deletion of *fgflbB* and *fgflbE* was performed similarly.

To complement the *fgflbC* and *fgflbD* deletion mutants, DNA fragments carrying the corresponding native promoter and ORF were fused with a *gfp-hyg* amplicon containing the green fluorescent protein (GFP) gene and the hygromycin resistance gene, which was amplified from the pIGPAPA vector with pIGPAPA-sGFP F/HYG-F1 primers (Horwitz *et al.*, 1999). After the third round of DJ PCR, each construct was transformed directly into protoplasts of the corresponding deletion mutant. For protoplasting of the *fgflbC* deletion mutant, conidia produced following growth in CMC medium were inoculated into YPG liquid medium (3 g yeast extract, 10 g peptone, and 20 g glucose per L). The *fgflbD* deletion mutants do not produce conidia, so hyphal fragments were used for YPG inoculation instead of conidia.

Interspecies complementation of the *fgflbD* deletion mutant was performed as previously described (Son *et al.*, 2013). The *flbD* ORF was amplified using AnFlbD-For/AnFlbD-Rev-hyg primers derived from *A. nidulans* FGSC A4 genomic DNA. The *flbD* ORF was fused with PCR products amplified with FgFlbD-5F/FgFlbD-5R-AnFlbD and FgFlbD-3F/FgFlbD-3R primer pairs from *F. graminearum* wild-type strain genomic DNA. The *hyg* gene was amplified with pBCATPH-comp-3 R/Gen-for primers from the pBCATPH vector (Kim *et al.*, 2005). Additional transformation procedures were the same as those used for complementation of the *fgflbD* deletion mutant.

Conidia production and morphology

Conidia production was measured by counting the number of conidia produced after incubating four freshly growing CM agar blocks (5 mm × 5 mm) in 30 ml of CMC medium in an Erlenmeyer flask for 3–5 days at 25°C on a rotary shaker (150 rpm). Counting was performed with a hemocytometer (Superior-Marienfeld, Germany).

The conidia induction method was used to obtain typically shaped conidia (Harris, 2005; Lee *et al.*, 2009). Briefly, after *F. graminearum* strains were incubated in 50 ml of CM for 3 days at 25°C on a rotary shaker (150 rpm), mycelia were harvested by filtration through Miracloth (CalBiochem, USA) and washed twice with distilled water. Freshly harvested mycelia were spread on YMA and incubated at 25°C under near UV light (wavelength: 365 nm; HKiv Import & Export Co., Ltd., China) to induce conidiation. Conidia morphology was evaluated on conidia harvested from a 2-day old YMA.

Quantitative real time (qRT)-PCR

Total RNA was extracted from the wild-type and Δ*fgflbD* strains grown in the 3-day old CM liquid culture, YMA cultures at 6 h and 12 h after asexual induction as previously described (Son *et al.*, 2014). First-strand cDNA was synthesized with SuperScriptIII reverse transcriptase (Invitrogen, USA) and qRT-PCR was performed with a SYBR Green Supermix (Bio-Rad, USA) and a 7500 real-time PCR system (Applied Biosystems, USA). The endogenous housekeeping gene cyclophilin (*cyp1*; Broad Institute ID: FGSG_07439.3) was used for normalization (Lin *et al.*, 2011). The cycle threshold (ΔC_T) value of gene expression was subtracted from the ΔC_T value of each sample to obtain a $\Delta\Delta C_T$ value. The transcript level relative to the calibrator was expressed as $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). qRT-PCR assays were repeated three times with two biological replications.

Outcrosses, mycelial growth, and virulence test

Mycelia grown for 5 days on carrot agar were mock-fertilized with 2.5% Tween 60 solution to induce sexual reproduction (homozygous selfings) as previously described (Leslie and Summerell, 2006). For heterozygous outcrosses, mycelia of the female strain grown on carrot agar plates were fertilized with 1 ml of a male strain conidia suspension (1×10^6 conidia/ml) or hyphal fragments. After sexual induction, fertilized cultures were incubated for 7 days under near UV light (HKiv Import and Export Co., Ltd.) at 25°C.

Microscopy

Microscopic observation was performed with a DE/Axio Imager A1 microscope (Carl Zeiss) using filter set 38HE (excitation 470/40; emission 525/50) for GFP, filter set 15

(excitation 546/12; emission 590) for RFP. AxioVision Rel. 4.7 software (Carl Zeiss) was used to measure conidia dimensions.

Results

Identification of fluffy genes in *F. graminearum*

Seven *fluffy* genes, *fluG*, *flbA*, *flbB*, *flbC*, *flbD*, *flbE*, and *tmpA* have been well characterized in *A. nidulans* (Adams *et al.*, 1998; Soid-Raggi *et al.*, 2006). A previous comparative genomic analysis looking for homologs of *A. nidulans* conidiogenesis regulators in the *F. graminearum* genome found homologs for *fluG*, *flbA*, *flbC*, and *flbD* (Fischer and Kües, 2006). *A. nidulans flbC* and *flbD* are homologs of *F. graminearum fgflbC* and *fgflbD*, respectively (Son *et al.*, 2011b). Further BLASTp analysis of *A. nidulans* FluG, FlbB, and FlbE against the *F. graminearum* Genome Database (http://mips.helmholtz-muenchen.de/genre/proj/FGDB/, Wong *et al.*, 2011) identified genes encoding *fgfluG* (Locus ID: FGSG_10043), *fgflbB* (Locus ID: FGSG_01313), and *fgflbE* (Locus ID: FGSG_09567) (Supplementary data Table S2). Since the FlbA ortholog (FgFlbA) was functionally characterized as a regulator of G protein signaling in a previous study (Park *et al.*, 2012), it was excluded from this study. TmpA also was excluded in this study because TmpA is involved in an independent pathway with other *fluffy* genes (Soid-Raggi *et al.*, 2006). The ORFs of *fgfluG*, *fgflbB*, and *fgflbE* from the wild-type strain Z-3639 were replaced with the *gen* gene by homologous recombination and the replacement was confirmed by Southern blot analysis (Supplementary data Fig. S1).

Homologs of *flbC* and *flbD* are required for conidia production

All *F. graminearum* strains were incubated in CMC medium to evaluate conidiogenesis. Deletion of homologs for *fluG* (*fgfluG*), *flbB* (*fgflbB*), and *flbE* (*fgflbE*) did not alter conidia production or morphology (Figs. 1A and 2, and Supplementary data Table S3). However, the *fgflbC* deletion mutants produced approximately half as many conidia as did the wild-type strain. Additionally, deletion of *fgflbD* completely abolished conidia production in CMC medium. To confirm the genetic requirements of *fgflbC* and *fgflbD* for *F. graminearum* conidiation, we performed a complementation assay. Each construct was inserted into the native locus and exact genetic replacements were confirmed using Southern hybridizations (Supplementary data Fig. S2A and C). Both complementation strains (HK159 and HK161) had restored the defective conidia production caused by Δ*fgflbC* and Δ*fgflbD* mutations (Supplementary data Fig. S2B and D).

For interspecies genetic complementation, the *flbD* ORF from *A. nidulans* driven by the native *fgflbD* promoter from *F. graminearum* was introduced into a *F. graminearum fgflbD* deletion mutant, which resulted in strain HK162. The genetic composition of the complemented mutants was confirmed by Southern hybridization (Supplementary data Fig. S3). The HK162 strain had similar conidiogenesis capabilities as the wild type (Supplementary data Fig. S2D).

Since *fgflbD* deletion mutants do not have male and female fertilities, the *hH1-gfp-hyg* construct from the hH1-GFP strain was amplified with the hH1-GFP-5N and hH1-GFP-3N primers and transformed directly into a Δ*fgflbD* strain to generate HK160. The hH1-GFP strain carrying the wild-type allele of *fgflbD* produced normal phialides and conidia (2-6-septate) continuously emerged from mature phialides (Fig. 1B, a white arrow). Deletion of *fgflbD* abolished phialide formation in CMC. Moreover, the Δ*fgflbD* mutant did not produce conidia even on media that strongly favor their production, e.g., YMA (Fig. 2A) and carnation leaf-piece agar (CLA) (Fig. 2B); instead, only vigorous mycelial growth occurred. However, the wild-type and complemented strains all produced sporodochia when cultured on these media (Fig. 2, arrows).

Developmental defects of the *fgflbD* deletion mutant

Deletion of *fgflbD* increased radial growth (Fig. 3A) and resulted in a "fluffier" mycelium than produced by the wild-type and other strains (Fig. 3B). In the wild-type culture,

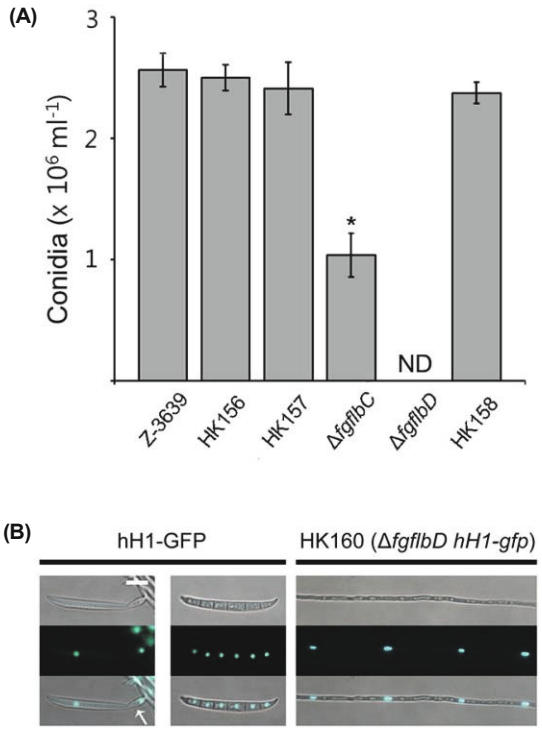

Fig. 1. Asexual development of *F. graminearum* strains. (A) Conidia production by *F. graminearum* strains. Conidiation was measured by counting the number of conidia produced in 5-day-old carboxymethyl cellulose (CMC) cultures. An asterisk indicates data that differed significantly ($P < 0.05$) based on Tukey's test. ND, not detected; Z-3639, *F. graminearum* wild-type strain; HK156, the *fgfluG* deletion mutant; HK157, the *fgflbB* deletion mutant; Δ*fgflbC*, the *fgflbC* deletion mutant; Δ*fgflbD*, the *fgflbD* deletion mutant; HK158, the *fgflbE* deletion mutant. (B) Strains were grown in CMC medium 1 to 3 days after conidiogenesis induction. A white arrow indicates a phialide producing conidium. Merged images present differential interference contrast (DIC) images and histone H1 tagged with GFP. hH1-GFP, the strain carrying the wild-type allele of *fgflbD* and *hH1-gfp*; HK160, the strain carrying both *fgflbD* deletion and *hH1-gfp*. Scale bar = 10 μm.

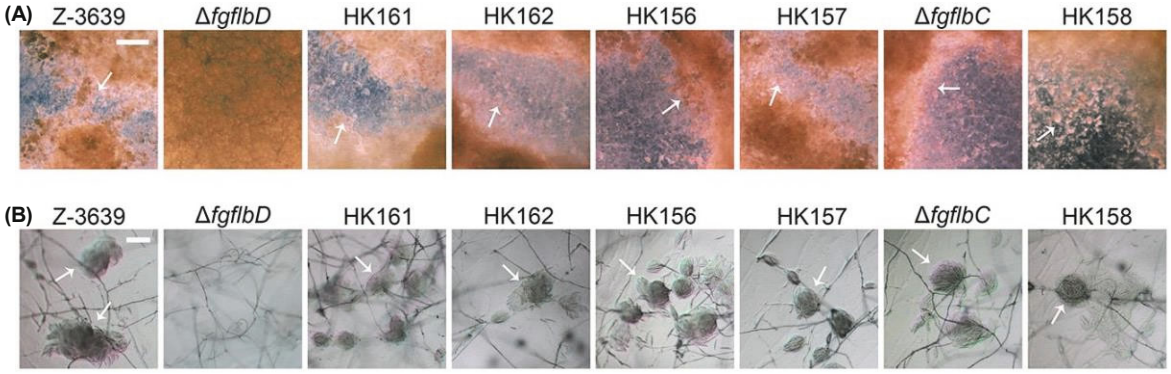

Fig. 2. Conidia production on media that strongly favor their production. (A) Production of sporodochia on yeast malt agar (YMA) 2 days after conidiogenesis induction. White arrows indicate sporodochia. Scale bar = 500 μm. (B) Production of sporodochia on carnation leaf-piece agar (CLA) 7 days after conidiogenesis induction. White arrows indicate sporodochia. Scale bar = 50 μm. Z-3639, *F. graminearum* wild-type strain; Δ*fgflbD*, the *fgflbD* deletion mutant; HK161, Δ*fgflbD*-derived strain complemented with *fgflbD* of *F. graminearum*; HK162, Δ*fgflbD*-derived strain complemented with *flbD* of *A. nidulans* (An*flbD*). HK156, the *fgfluG* deletion mutant; HK157, the *fgflbB* deletion mutant; Δ*fgflbC*, the *fgflbC* deletion mutant; HK158, the *fgflbE* deletion mutant.

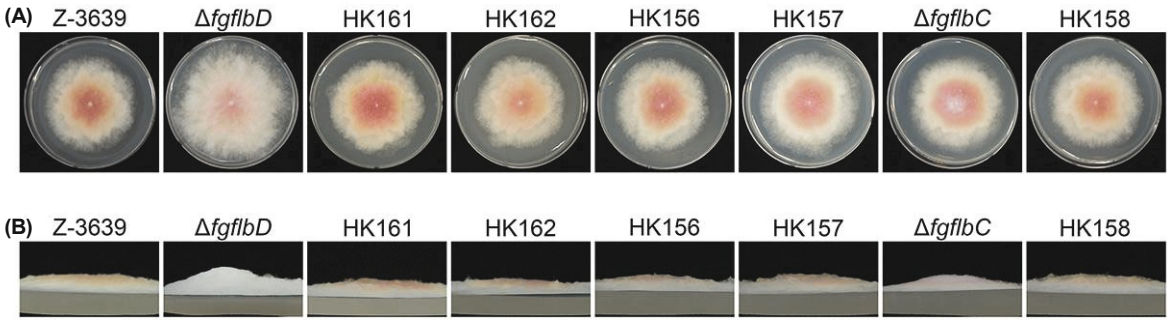

Fig. 3. Mycelial growth on CM. Pictures were taken 3 days after inoculation onto CM. The pictures were taken from the upper (A) and the side (B) of the plates. Z-3639, *F. graminearum* wild-type strain; Δ*fgflbD*, *fgflbD* deletion mutant; HK161, Δ*fgflbD*-derived strain complemented with *fgflbD* of *F. graminearum*; HK162, Δ*fgflbD*-derived strain complemented with *flbD* of *A. nidulans* (An*flbD*). HK156, the *fgfluG* deletion mutant; HK157, the *fgflbB* deletion mutant; Δ*fgflbC*, the *fgflbC* deletion mutant; HK158, the *fgflbE* deletion mutant.

mycelial growth reduced after sexual induction and many perithecia were produced (Fig. 4A). In contrast, the Δ*fgflbD* mutants did not produce any perithecia, and mycelia covered the culture surface 2 days after sexual induction. Although *F. graminearum* is a homothallic fungus, the Δ*mat1* mutants are heterothallic (Lee *et al.*, 2003) and require a partner for

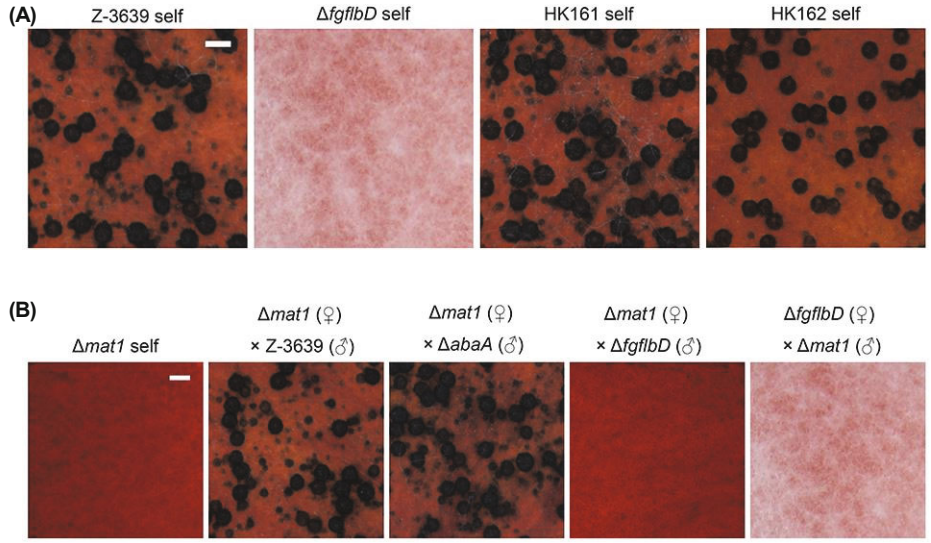

Fig. 4. Fertility tests of the *F. graminearum* strains. (A) Selfings of the wild-type and *fgflbD* mutant strain. (B) Outcrosses to determine male and female fertility of the *fgflbD* deletion mutant. Each female and male strain is denoted as "♀" and "♂", respectively. Pictures were taken 7 days after sexual induction on carrot agar. Scale bar = 500 μm. Z-3639, *F. graminearum* wild-type strain; Δ*fgflbD*, the *fgflbD* deletion mutant; HK161, Δ*fgflbD*-derived strain complemented with *fgflbD* of *F. graminearum*; HK162, Δ*fgflbD*-derived strain complemented with *flbD* of *A. nidulans* (An*flbD*); Δ*mat1*, the *mat1-1-1* deletion mutant; Δ*abaA*, the *abaA* deletion mutant.

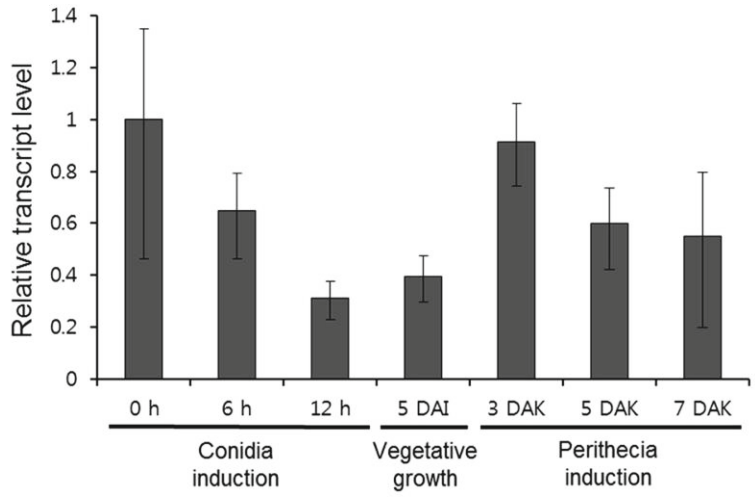

Fig. 5. Relative transcript accumulation of *fgflbD* in the wild-type Z-3639. The transcript level of *fgflbD* was analyzed by quantitative real time-PCR (qRT-PCR). mRNA was extracted from the wild-type strain Z-3639 grown in the 3-day old CM liquid culture, YMA cultures at 6 h and 12 h after asexual induction, 5-day old carrot agar cultures without sexual induction, and 3-, 5-, and 7-day old carrot agar after sexual induction. DAI, days after inoculation; DAK, days after sexual induction.

sexual reproduction. Therefore, Δ*mat1* mutants were used for outcrossing analyses. Since the Δ*fgflbD* mutants do not produce conidia, hyphal fragments were used for spermatization. The wild type and the non-conidia producing Δ*abaA* mutant both had normal male fertility (Fig. 4B). However, the *fgflbD* deletion mutants neither have male nor female fertilities.

Heterokaryon formation test

To examine the mechanism of sterility of *fgflbD* deletion mutants, we generated independent nitrate auxotrophic mutants that have mutations in the *nit1* gene encoding the nitrate reductase (*nit1*) and the *nit5* gene encoding the molybdenum-containing cofactor (NitM). Mycelial plugs of the wild type and *fgflbD* deletion mutant strains were inoculated on MMC and incubated at 25°C for 2 weeks. The cultures were observed periodically to select fast-growing sectors from the initial colony (Correll *et al.*, 1987; Bowden and Leslie, 1992). Because chlorate included in MMC is reduced to chlorite by nitrate reductase, the wild-type strain grew poorly. However, nitrate nonutilizing (*nit*) mutants cannot reduce chlorate to chlorite, so the *nit* mutants are chlorate-resistant. Fast growing sectors were transferred to CM agar and each *nit* mutant was identified as either *nit1*, *nit3*, or NitM based on growth patterns on MM amended with one of four different nitrogen sources (nitrate, nitrite, hypoxanthine, and ammonium) (Leslie and Summerell, 2006). We used these mutants to test the capability of *fgflbD* deletion mutant strains for cell fusion and heterokaryon formation. Similar to the positive control pairing (WT *nit1* × WT NitM), coculturing the mutants carrying both the *fgflbD* deletion and the *nit1* mutation (*fgflbD nit1*) with the WT NitM strain resulted in vigorous mycelial growth, suggesting that *fgflbD* deletion mutants retain a capacity for hyphal fusion and heterokaryon formation (Supplementary data Fig. S4). Two pairings, WT *nit1* × WT *nit1* and WT *nit1* × Δ*fgflbD nit1*, were used as negative controls.

Transcript profiles of *fgflbD*

The *fgflbD* transcript level in the wild-type strain was determined by qRT-PCR. In the wild-type strain, *fgflbD* is expressed constitutively during conidiogenesis and sexual development, but after conidia induction *fgflbD* transcript accumulation decreases (Fig. 5).

Subcellular localization of FgFlbD-GFP proteins

HK161 (*Δfgflbd::fgflbD-gfp-hyg*) was generated to examine FgFlbD localization. HK165 (*Δfgflbd::fgflbD-gfp-hyg hH1::hH1-rfp-gen*), which carries both *fgflbD-gfp* and *hH1-rfp*, was isolated as the progeny of a cross between mat1r (Son *et al.*, 2011a) and HK161. HK165 were selected by genotyping through fluorescence microscopy, testing antibiotic resistance, and screening by PCR. FgFlbD-GFP co-localized with hH1-RFP and was highly fluorescent in mature conidia (Fig. 6). However, the GFP signals were blurred after germination and were barely visible 2 h later. GFP signals from cells in the mycelia, phialides, and young conidia were weak.

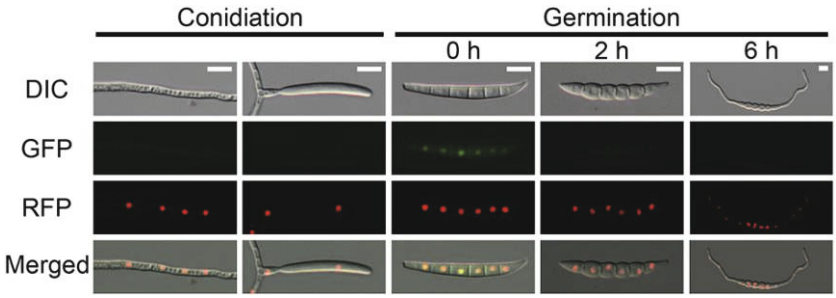

Fig. 6. Cellular localization of FgFlbD. FgFlbD was fused with GFP, and histone H1 was fused with RFP. The GFP signals were highly fluorescent in the nuclei of mature conidia. The GFP signals became blurred after germination and almost undetectable in hyphal cells. Scale bar = 10 μm.

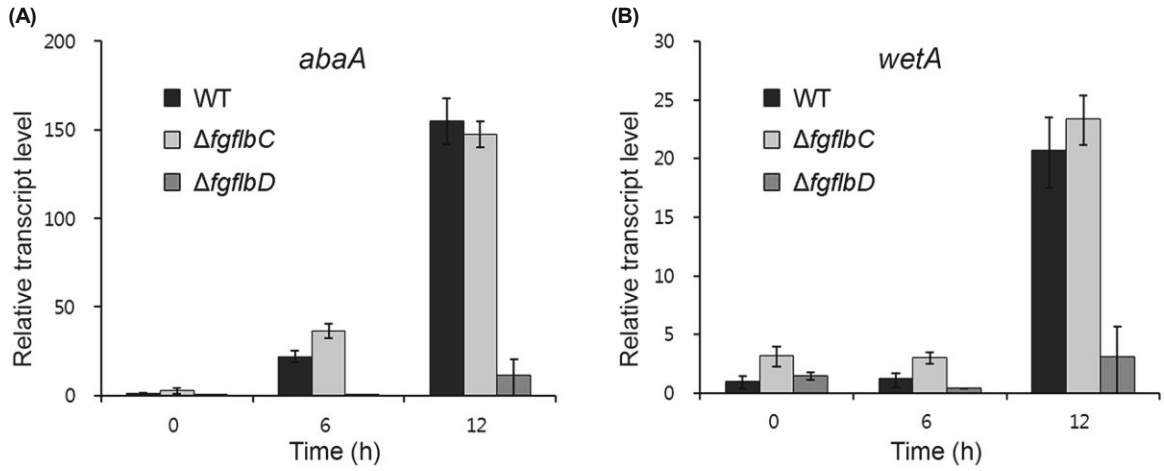

Fig. 7. Relative transcript accumulation of *abaA* and *wetA*. The transcript levels of *abaA* (A) and *wetA* (B) were analyzed by quantitative real time-PCR during the conidia induction stage in the wild-type, Δ*fgflbC*, and Δ*fgflbD* strains. Z-3639, *F. graminearum* wild-type strain; Δ*fgflbC*, the *fgflbC* deletion mutant; Δ*fgflbD*, the *fgflbD* deletion mutant.

Regulatory relationships among *fgflbD*, *wetA*, and *abaA*

The AbaA-WetA pathway is conserved in both *A. nidulans* and *F. graminearum* (Son *et al.*, 2013, 2014). *fluffy* genes, such as *flbC* and *flbD,* are amongst the upstream development activators required to activate the *brlA-abaA-wetA* pathway in *A. nidulans* (Adams *et al.*, 1998). To determine whether *fgflbC* and *fgflbD* are upstream regulators of *abaA* and *wetA* in *F. graminearum,* we checked the transcript levels of *abaA* and *wetA* in the Δ*fgflbC* and Δ*fgflbD* strains. In the wild-type strain, the expression level of *abaA* was induced 6 h after conidiogenesis and increased until 12 h (Fig. 7A); *wetA* transcript dramatically increased 12 h after induction of conidiogenesis (Fig. 7B). The transcript levels of *abaA* and *wetA* in the *fgflbC* deletion strain were unchanged in comparison to wild type. In contrast, the *fgflbD* deletion strains accumulated almost no transcripts of either *abaA* or *wetA* (Fig. 7). These results suggest that *fgflbC* does not regulate the AbaA-WetA pathway and that *fgflbD* is the only conserved upstream development activator in *F. graminearum.*

A mutant that overexpresses *abaA* produces abnormal phialides and conidia with abacus-like morphologies (Son *et al.*, 2013). To better understand genetic relationship between FgFlbD and AbaA, we also overexpressed *abaA* in a strain carrying a *fgflbD* deletion (Supplementary data Fig. S5). Neither the *fgflbD* single deletion mutants nor the double mutants that also overexpress *abaA* produced any conidiophores (Fig. 8). Deletion of *fgflbD* prevents *abaA* overexpression effect.

Discussion

In this study, we characterized homologs of the *A. nidulans fluffy* genes in *F. graminearum* to identify upstream regulators of conidiogenesis. We found that only FlbD is a conserved regulatory protein for conidiogenesis in both fungi. Our hypothesis that FlbD has conserved roles in both fungi is supported by experiments showing successful interspecies complementation. Additionally, FlbD is required for sexual reproduction in both *F. graminearum* and *A. nidulans* (Arratia-Quijada *et al.*, 2012).

Based on the results from our previous (Son *et al.*, 2013, 2014) and current studies, we propose a genetic regulatory pathway governing conidiogenesis in *F. graminearum* where only FgFlbD is required to activate the *abaA-wetA* signaling cascade (Fig. 9). In contrast, a complex regulatory and physical interaction mechanism among the *fluffy* genes and their protein products govern activation of the *brlA-abaA-wetA* pathway in *A. nidulans*. In both fungi, downstream

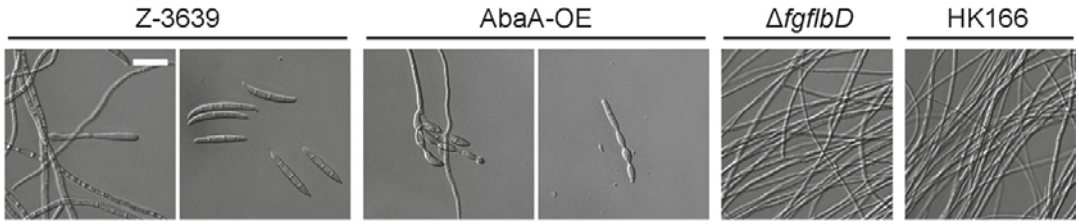

Fig. 8. Hyphal morphogenesis of *F. graminearum* strains grown in CMC. Pictures were taken 3 days after inoculation. A mutant that overexpresses *abaA* produces abnormal phialides and conidia with abacus-like morphologies (Son *et al.*, 2013). Neither the *fgflbD* single deletion mutants nor the double mutants that also overexpress *abaA* produced any conidiophores. Deletion of *fgflbD* prevents *abaA* overexpression effect. Z-3639, *F. graminearum* wild-type strain; AbaA-OE, an overexpression strain in which the native *abaA* promoter was replaced with the *ef1α* promoter; Δ*fgflbD*, the *fgflbD* deletion mutant; HK166, the strain carrying both *fgflbD* deletion and *abaA* sequences controlled by *ef1α* promoter instead of the native *abaA* promoter. Scale bar = 20 μm.

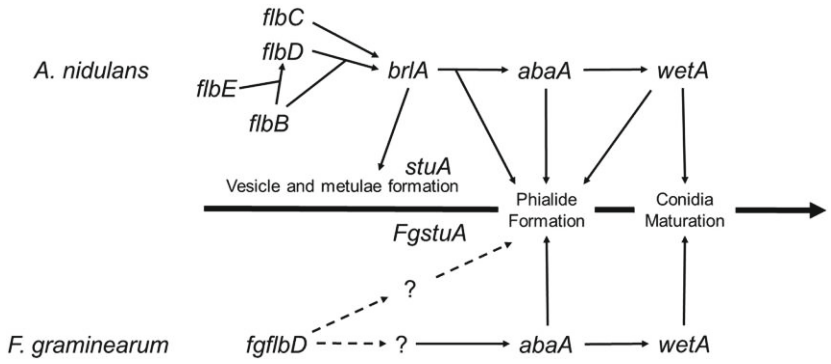

Fig. 9. Comparison of genetic pathways for conidiogenesis in *A. nidulans* and *F. graminearum*. In *A. nidulans*, FlbB physically interacts with FlbD and FlbE, and the resulting FlbB/FlbE and FlbB/FlbD complexes induce the expression of *flbD* and *brlA*, respectively. BrlA is an activator of the AbaA-WetA pathway. AbaA and WetA are required for phialide formation and conidia maturation, respectively. In *F. graminearum*, the AbaA-WetA pathway is similar to that of *A. nidulans*, except a *brlA* ortholog does not exist. Amongst the *fluffy* genes, only *fgflbD* has a conserved role for regulation of the AbaA-WetA pathway. The genetic network model for *A. nidulans* is slightly modified from Park and Yu (2012).

regulators such as AbaA and WetA have conserved roles in phialide formation and conidia maturation, respectively (Son *et al.*, 2013, 2014). In addition, the *stuA* orthologs have common roles in phialide formation and function in both fungi (Lysøe *et al.*, 2011).

Comparing genes present in the conidiogenesis pathway in multiple fungal genera can provide new insights into the evolution of regulatory pathways for conidiogenesis in fungi. When we examined *A. nidulans* and *F. graminearum*, we found that most of the *A. nidulans* conidiogenesis pathway upstream of *abaA* is not conserved in *F. graminearum*. For example, there is no *brlA* ortholog in *F. graminearum* and most *fluffy* gene homologs, except *fgflbD*, do not have a conserved regulatory function in both fungi; however, although the *flbD* orthologs of *F. graminearum* and *A. nidulans* both have crucial functions for conidiogenesis and have similar regulatory natures, the *N. crassa flbD* ortholog, *rca-1*, has no role in conidiogenesis in *N. crassa* (Shen *et al.*, 1998). Furthermore, *brlA* also is absent in *N. crassa* (Fischer and Kües, 2006), although in this species, FL is thought to have a role analogous to BrlA (Bailey and Ebbole, 1998). The *F. graminearum* homolog to *fl* in *N. crassa*, *gzzc256*, is not required for conidiogenesis (Son *et al.*, 2011b). Interestingly, *rca-1* from *N. crassa* fully complements an *Aspergillus flbD* mutation (Shen *et al.*, 1998). Thus, some of the early regulators of conidiogenesis, e.g., *brlA* and *fl*, may remain clade/species specific through gain-of-function in a particular clade/species or loss-of-function in all other fungi during fungal evolution (Ebbole, 2010). Some genes, such as *flbD*/*rca-1*, have evolved to retain their molecular function across fungal species, but have clade/species specific roles. However, some downstream regulators are functionally conserved across multiple fungal species. For example, the *stuA* orthologs have common functions in *F. graminearum* (Lysøe *et al.*, 2011), *F. oxysporum* (Ohara and Tsuge, 2004), and *N. crassa* (Aramayo *et al.*, 1996). Orthologs of *medA* are functionally conserved in *F. oxysporum* (Ohara *et al.*, 2004) and *Magnaporthe oryzae* (Lau and Hamer, 1998). Thus, early regulators of conidiogenesis are more evolutionally divergent than the downstream regulators.

FlbD functions differently in *A. nidulans* than in *F. graminearum*. A deletion of *flbD* in *A. nidulans* delays conidiation (Wieser and Adams, 1995) and production of abnormal fruiting bodies (Arratia-Quijada *et al.*, 2012) while in *F. graminearum* the deletion of *fgflbD* completely blocks phialide production in all conidia inducing conditions and prevents perithecia formation. Additionally, hyphal cells in the *F. graminearum* Δ*fgflbD* mutant did not differentiate into any specialized reproductive cells. Thus, *fgflbD* plays a more important role in hypal cell morphogenesis in *F. graminearum* than in *A. nidulans* and this is additionally supported by the expression patterns of *flbD* orthologs. *F. graminearum fgflbD* transcript levels were high during both asexual and sexual development, suggesting that FgFlbD functions constitutively throughout the fungal life cycle (Fig. 5). Furthermore, constitutive expression of *F. graminearum fgflbD* demonstrates that FgFlbD is required for the entire life cycle of *F. graminearum* and FgFlbD-dependent transcriptional regulation is determined by additional factors. This claim is supported by the subcellular localization pattern of FgFlbD. FgFlbD accumulated in nuclei only at resting conidia, where FgFlbD is not required and general transcriptional activity should be absent (Fig. 6). We suggest that the nuclear FgFlbD in conidia may only function when additional factors are expressed and/or translocated into nuclei. On the other hand, *A. nidulans flbD* transcripts were generally undetectable during sexual development and highly expressed during mycelial growth (Garzia *et al.*, 2010). Therefore, in *A. nidulans* the dosage level of nuclear FlbD correlates with the formation of the transcriptional complex that activates *brlA* expression (Garzia *et al.*, 2010). The different phenotypic defects of *flbD* mutations in *A. nidulans* and *F. graminearum* could also result from differences in *cis*-regulatory elements that alter transcriptional processes (Gasch *et al.*, 2004; Tuch *et al.*, 2008).

In conclusion, we report that *flbD* has conserved regulatory functions for conidiogenesis in both *A. nidulans* and *F. graminearum*. FgFlbD is responsible for hyphal differentiation in both sexual and asexual reproductive processes in *F. graminearum*. We also identified *abaA* and *wetA* as conidiation-related genes under control of FgFlbD. Furthermore, this study analyzed the role of *fluffy* genes in conidiogenesis in *F. graminearum*. These results provide new insights into the evolutionary aspects of the regulatory pathway for conidiogenesis in fungi.

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