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WetA Is Required for Conidiogenesis and Conidium Maturation in the Ascomycete Fungus *Fusarium graminearum*

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***Fusarium graminearum*, a prominent fungal pathogen that infects major cereal crops, primarily utilizes asexual spores to spread disease. To understand the molecular mechanisms underlying conidiogenesis in *F. graminearum*, we functionally characterized the *F. graminearum* ortholog of *Aspergillus nidulans wetA*, which has been shown to be involved in conidiogenesis and conidium maturation. Deletion of *F. graminearum wetA* did not alter mycelial growth, sexual development, or virulence, but the *wetA* deletion mutants produced longer conidia with fewer septa, and the conidia were sensitive to acute stresses, such as oxidative stress and heat stress. Furthermore, the survival rate of aged conidia from the *F. graminearum wetA* deletion mutants was reduced. The *wetA* deletion resulted in vigorous generation of single-celled conidia through autophagy-dependent microcycle conidiation, indicating that WetA functions to maintain conidial dormancy by suppressing microcycle conidiation in *F. graminearum*. Transcriptome analyses demonstrated that most of the putative conidiation-related genes are expressed constitutively and that only a few genes are specifically involved in *F. graminearum* conidiogenesis. The conserved and distinct roles identified for WetA in *F. graminearum* provide new insights into the genetics of conidiation in filamentous fungi.**

Indefinite polarized filamentous growth is typical for many fungi. Hyphal growth requires the continuous uptake of organic matter, although mycelia can withstand undernourished conditions by translocating nutrients. Nonmotile fungi use spore formation as a strategy to escape from harsh conditions, such as nutrient deficiency. Spores are produced asexually or sexually, depending on the fungal species and environmental conditions. Various mechanisms of spore formation and diverse spore types have evolved, suggesting their importance in the life cycles of fungi (1).

The ascomycete fungi *Neurospora crassa* and *Aspergillus nidulans* have long been model organisms for fungal genetic studies. Decades of *A. nidulans* research have revealed the genetic and biochemical pathways for asexual sporulation, which have been summarized in several reviews (1–4). Studies have also dissected the molecular mechanisms of conidiogenesis in *Aspergillus* and *Penicillium* species, which are evolutionarily related to *A. nidulans* (5–12). These reverse genetic studies analyzed well-known regulators of conidiogenesis, such as the *fluffy* genes, which are activators of the central development pathway genes *brlA*, *abaA*, and *wetA* (2, 4, 13–16). The sensory and regulatory functions of velvet complexes have been well described (4, 17, 18), and their orthologs have been characterized in many fungi (19–27).

Fusarium graminearum (teleomorph *Gibberella zeae*) is an ascomycete fungus that causes *Fusarium* head blight (FHB) in wheat, barley, and rice, as well as ear and stalk rot in maize (28). FHB leads to many problems, including losses in grain yield and quality, as well as mycotoxicoses in humans and livestock (29). Recently, FHB epidemics have been rapidly reemerging throughout the world. In consequence, *F. graminearum* has become one of the most intensively studied fungal plant pathogens (30, 31). Ascospores and conidia of *F. graminearum* are produced by sexual and asexual reproduction, respectively (28). Ascospores are produced from specialized multicellular structures, called perithecia, and function as primary inocula (32, 33). Conidia are produced on infected crops and are required for secondary infection (33). Thus, asexual reproduction of *F. graminearum* is closely linked to the spread of disease in the field.

Many genes are reportedly involved in conidium production in *F. graminearum*. Most of these genes encode transcription factors (TFs) and kinases (34–40), and many play pivotal roles in major cellular processes. Knockout mutants of these genes were found to be pleiotropic and defective in sexual reproduction, vegetative growth, virulence, and conidiation. To our knowledge, there are only five *F. graminearum* transcription factors that specifically regulate conidium production (34, 41, 42). Since few conidiation-specific genes are known and there has been no systematic study of conidiation in *F. graminearum*, scant information regarding the molecular mechanism of conidium production is available.

We have used the *A. nidulans* model as a basis for characterizing *F. graminearum* orthologs of genes involved in acetyl coenzyme A synthesis (39, 43), the velvet complex (27), the G protein complex (44), and G protein signaling (45). These orthologs play species-specific roles in various aspects of development and virulence. Our work was particularly meaningful because we deepened the understanding of pathogenicity mechanisms by connecting well-known cellular processes with virulence in *F. graminearum*. In addition, we characterized the *abaA* ortholog, which was previously thought to be absent from *F. graminearum* (41). We found that AbaA is important for phialide formation and function and

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

Strain	Genotype	Source or reference
Z-3639	Wild-type *Fusarium graminearum*	46
hH1-GFP	*hH1*::*hH1-gfp-hyg*	47
mat1g	Δ*mat1-1-1*::*gen hH1*::*hH1-gfp-hyg*	47
Δ*mat2* mutant	Δ*mat1-2*::*gfp-hyg*	48
Δ*abaA* mutant	Δ*abaA*::*gen*	41
AbaA-OE	*abaA*::*gen*-$P_{ef1\alpha}$-*abaA*	41
HK144	Δ*wetA*::*gen*	This study
HK145	Δ*wetA*::*gen hH1*::*hH1-gfp-hyg*	This study
HK146	Δ*wetA*::*wetA-hyg*	This study
HK147	Δ*atg8*::*gen*	This study
HK148	Δ*mat1-2*::*gfp-hyg* Δ*wetA*::*gen*	This study
HK149	Δ*wetA*::*gen* Δ*atg8*::*gen*	This study
HK154	Δ*wetA*::*gen* Δ*abaA*::*gen*	This study
HK155	Δ*wetA*::*gen abaA*::*gen*-$P_{ef1\alpha}$-*abaA*	This study

that *abaA* overexpression causes pleiotropic phenotypes in *F. graminearum*. We also demonstrated that the AbaA-WetA pathway of *A. nidulans* is conserved in *F. graminearum* (41).

The aims of this study were to identify *wetA* in *F. graminearum* and to determine its role in fungal development. We used genetics, cytology, and biochemistry to characterize the *wetA* ortholog in *F. graminearum*. The results of this study expand our knowledge of conidiogenesis and conidium maturation in *F. graminearum*.

MATERIALS AND METHODS

Fungal strains and media. Transgenic strains were derived from the wild-type (WT) *F. graminearum* strain Z-3639 (46). The hH1-GFP (47), mat1g (47), Δ*mat2* (48), Δ*abaA* (41), and AbaA-OE (41) strains were used for outcrossing (Table 1). *F. graminearum* strains were maintained in complete medium (CM) and carnation leaf agar medium, according to the instructions in *The Fusarium Laboratory Manual*, and were stored as conidial suspensions in 20% glycerol at −70°C (28). Carboxymethyl cellulose medium (CMC) and yeast extract-malt extract agar (YMA) were used for conidium production, as described previously (49, 50). Other media used in this study were prepared and used according to the instructions in *The Fusarium Laboratory Manual* (28).

Nucleic acid manipulations, PCR primers, and DNA sequencing. For genomic DNA (gDNA) isolation, each strain was grown in 5 ml of liquid CM at 25°C for 3 days on a rotary shaker (150 rpm). The gDNA was extracted using a cetyltrimethylammonium bromide (CTAB) procedure (28). Restriction endonuclease digestion, gel electrophoresis, Southern blotting, and hybridization with ^{32}P-labeled probes were performed according to standard procedures (51). PCR primers used for this study (see Table S1 in the supplemental material) were synthesized by the Bionics oligonucleotide synthesis facility (Seoul, Republic of Korea). DNA sequencing was performed with an ABI 3730xl DNA analyzer by Macrogen, Inc. (Seoul, Republic of Korea). Sequences were input into the *Fusarium graminearum* Genome Database (52) and the *Fusarium* Comparative Database at the Broad Institute (http://www.broadinstitute.org/annotation/genome/fusarium_graminearum).

RACE-PCR. We determined the *wetA* open reading frame (ORF) using rapid amplification of cDNA ends (RACE)-PCR. The cDNA library used as the template was constructed in a previous study (53). DNA fragments were amplified with primer pairs WetA-RACE-4/WetA-RACE-6, pPRN3-N-For/WetA-RACE-7, pPRN3-N-For/WetA-RACE-3, pPRN3-N-For/WetA-RACE-2, pPRN3-N-Rev/WetA-RACE-1, and pPRN3-N-Rev/WetA-RACE-5 and were then directly sequenced.

Targeted gene deletion and fungal transformations. PCR products used for fungal transformations were constructed using a double-joint (DJ) PCR procedure (54). To delete the *wetA* ORF from the *F. graminearum* genome, the 5′ and 3′ flanking regions of *wetA* were amplified from the *F. graminearum* wild-type strain Z-3639 with primer pairs WetA-5F/WetA-5R and WetA-3F/WetA-3R, respectively (see Table S1 in the supplemental material). The Geneticin resistance gene cassette (*gen*), including the *A. nidulans trpC* promoter and terminator, was amplified from pII99 (55) with primers Gen-For and Gen-Rev. DJ PCR was performed to fuse the three amplicons. A final amplification was performed using the resulting PCR product as the template and primer pair WetA-5N/WetA-3N.

For complementation of the *wetA* deletion mutant, the wild-type *wetA* allele from *F. graminearum* strain Z-3639 was amplified with primer pair WetA-5F/WetA-3N-Com. The hygromycin resistance cassette (*hyg*) was amplified from pBCATPH using primer pair pBCATPH-hyg-F1/Gen-For (56). The resulting amplicons were fused by DJ PCR. The final PCR construct was obtained by nested PCR and was transformed into the *wetA* deletion mutant, as described previously (57).

Outcrosses and virulence tests. For self-fertilization, mycelia were grown on carrot agar for 5 days. They were then removed using the back of a surgical blade (no. 11; Feather Safety Razor, Osaka, Japan) and 2.5% sterilized Tween 60 (28). For outcrosses, female strains were fertilized with 1 ml of conidial suspensions from the corresponding male strains (48). After sexual induction, cultures were incubated under near-UV light (wavelength,365 nm; HKiv Import & Export Co., Ltd., Xiamen, China) at 25°C.

Virulence tests were performed using the wheat cultivar Eunpamil as described previously (53). In brief, 10 μl of a conidial suspension (1×10^5 conidia/ml) harvested from a 5-day-old culture grown in CMC was injected into a center spikelet of a wheat head at midanthesis. Plants were placed in a humidity chamber for 3 days and were then moved to a greenhouse for further incubation. Spikelets with disease symptoms were counted at 21 days after inoculation.

Conidium production and morphology. Conidium production was measured by counting the number of conidia produced after culturing of 10 μl of a conidial suspension (1×10^5 conidia/ml) in 5 ml of CMC for 5 days at 25°C on a rotary shaker (150 rpm). Counting was performed with a hemocytometer (Superior; Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). To obtain typically shaped conidia, we used the YMA induction method, as described previously (50). Briefly, *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 with Miracloth (Calbiochem, San Diego, CA) and were washed twice with distilled water. To induce conidiation, freshly harvested mycelia were spread on YMA and were incubated at 25°C under near-UV light (HKiv Import & Export Co., Ltd.) for 2 days. Conidia were harvested, and conidium morphology was observed using differential interference contrast (DIC) microscopy. DIC images were obtained with an Axio Imager A1 microscope (Carl Zeiss, Oberkochen, Germany), and AxioVision software (release 4.7; Carl Zeiss) was used to measure the dimensions of the conidia.

Specimen preparation and TEM. Specimens were prepared for electron microscopy as described previously (58). Briefly, 2 days after conidium induction, mature conidia were harvested and fixed. Specimens were dehydrated in an ethanol series (30%, 50%, 70%, and 80%) and were embedded in Spurr's resin (59). Ultrathin sections (thickness, 70 nm) were made using an ultramicrotome (MT-X; RMC, Tucson, AZ). Sections were stained with 2% uranyl acetate and Reynolds' lead citrate (60) and were examined using an energy-filtering Libra 120 transmission electron microscope (TEM) (Carl Zeiss, Oberkochen, Germany) operated at an accelerating voltage of 120 kV. Zero-loss energy-filtered images were recorded with a 4K slow-scan charge-coupled device camera (4000 SP; Gatan, Pleasanton, CA).

Chronological survival and necrotic cell death. An assay to examine the chronological survival of conidia was performed as described previously (61). Conidia were induced on YMA plates, which were incubated at 25°C in the dark. Survival rates were measured 2, 10, and 20 days after

conidium induction. Apoptotic or necrotic conidial cell death was distinguished by costaining with annexin V and propidium iodide (PI) (Clontech Laboratories, Inc., Palo Alto, CA) according to the manufacturer's instructions. Costained cells were examined using an Axio Imager A1 microscope (Carl Zeiss) with filter set 38 HE (excitation at 470 nm; emission at 525 nm) for annexin V and filter set 15 (excitation at 546 nm; emission at 590 nm) for PI.

Trehalose assay. The trehalose assay was performed as described previously, with slight modifications (5). In brief, freshly harvested conidia (2×10^8 conidia/ml, from a 2-day-old YMA culture) were washed three times with distilled water, resuspended in 400 μl of distilled water, and incubated at 100°C for 20 min. After centrifugation for 10 min at 11,000 × *g*, supernatants were transferred to new tubes containing 50 μl of 0.2 M sodium citrate (pH 5.5) and were incubated at 37°C for 8 h with and without 3 mU trehalase (T8778; Sigma-Aldrich, St. Louis, MO). Glucose produced by trehalase activity was measured by using a glucose assay kit (Sigma-Aldrich) according to the manufacturer's instructions. Samples without trehalase treatment served as negative controls, and the experiments were performed independently three times.

Stress tolerance tests. To evaluate mycelial growth under various chronic stress conditions, fungi were grown on potato dextrose agar (PDA) supplemented with 1 M NaCl, 10 mM H_2O_2, or 10 mg/liter sodium dodecyl sulfate (SDS). To examine acute oxidative stress tolerance, conidia from each strain were treated with 0, 0.025, 0.05, or 0.1 M H_2O_2 for 10 min. Heat stress was administered by incubating conidia at 50°C for 0, 10, 30, or 60 s. After treatments, conidial suspensions were incubated for 18 h at 25°C; then they were dropped onto glass slides to test for germination. All experiments were repeated twice, with three replicates for each sample.

RNA-sequencing and bioinformatics analysis. Total RNA was extracted with the Easy-Spin total-extraction kit (iNtRON Biotechnology, Seongnam, Republic of Korea). First-strand cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). RNA-sequencing libraries were created using the Illumina TruSeq RNA sample preparation kit, with no modifications to the standard low-throughput protocol. Sequencing was performed on an Illumina HiSeq 2000 instrument using the reagents provided in the Illumina TruSeq PE Cluster kit v3-cBot-HS and the TruSeq SBS kit v3-HS (200 cycles) kit.

Nucleotide sequence accession number. The data discussed in this publication were deposited in NCBI's Gene Expression Omnibus (62) and are accessible through GEO Series accession number GSE 46133 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46133). Relative transcript abundance was measured in reads per kilobase of exon per million mapped sequence reads (RPKM) (63). The $\log_2$ ratios of the RPKM values were used to identify differentially expressed genes.

RESULTS

Identification of *wetA* in *F. graminearum*. A BLASTp search for *A. nidulans* WetA (AnWetA) in the *F. graminearum* genome was performed using the *Fusarium* Comparative Database (http://www.broadinstitute.org/annotation/genome/fusarium_graminearum). The FGSG_10166.3 locus, encoding 243 amino acids (17% identity), was identified. However, FGSG_10166.3 was reannotated as FGSG_17727, which encodes 608 amino acids (24% identity), in the *F. graminearum* Genome Database (52). Further sequencing analysis of the cDNA revealed that *F. graminearum wetA* (Fg*wetA*) does not contain introns and that the deduced ORF from the *F. graminearum* Genome Database is correct. A previous comparative genomic analysis for regulators of *A. nidulans* conidiogenesis also showed that the *F. graminearum* genome contains a 608-amino-acid WetA ortholog (1). In addition, the Fg*wetA* transcript contains a ~500-bp 5′ untranslated region (5′ UTR) and a 1,000-bp 3′ UTR.

BLASTMatrix analysis (64) using AnWetA showed that WetA is highly conserved in the subphylum Pezizomycotina, whereas homologs with low similarity (E value, >1e−10) exist in Oomycota and Basidiomycota (Fig. 1A). Homologs in Pezizomycotina contain a highly conserved 61-amino-acid C-terminal region, whereas homologs in Oomycota and Basidiomycota do not have this conserved C-terminal region (Fig. 1B). FgWetA and AnWetA showed 24% identity in their global alignment, and both contained the conserved C-terminal region (Fig. 1C).

InterProScan did not predict any distinct domains for AnWetA and FgWetA. However, the PANTHER classification system predicted that AnWetA and FgWetA contain a protein ESC1/WetA-related (family identification [ID] PTHR22934) domain and function as DNA-binding proteins (65). AnWetA and FgWetA have detectable nuclear localization signals (NLSs) within the ESC1/WetA-related domains (66), and FgWetA contains an additional NLS near its N-terminal region (Fig. 1C).

Phenotypes of the *wetA* deletion mutant. To elucidate the function of *wetA* in *F. graminearum*, we deleted it from the wild-type *F. graminearum* strain Z-3639. The *wetA* ORF was replaced with *gen* via homologous recombination, and the replacement was confirmed by Southern blotting (see Fig. S1A in the supplemental material). For complementation of the resulting mutation, the wild-type *wetA* allele fused with *hyg* was introduced into the *wetA* deletion mutant. The complementation mutant was also confirmed by Southern hybridization (see Fig. S1B in the supplemental material). Repeated trials did not succeed in generating a complementation mutant carrying green fluorescent protein (GFP)-labeled WetA (WetA-GFP), an interspecies complementation mutant using *A. nidulans wetA*, or an overexpression mutant, as performed previously (41), suggesting that altered UTRs of *wetA* are crucial for its biochemical function. The radial growth, sexual development, and virulence of the *wetA* deletion mutants did not differ from those of the wild-type and complemented strains (Fig. 2A to C). However, the *wetA* deletion mutants produced significantly fewer conidia than the wild-type and complemented strains (Table 2). In addition, the conidia of the *wetA* deletion mutants were longer and contained fewer septa than those of the wild-type and complemented strains (Table 2).

To follow conidiogenesis *in vivo*, we generated strain HK145 (Δ*wetA*::*gen hH1*::*hH1-gfp-hyg*) through an outcross between mat1g (47) and HK144 (Δ*wetA*::*gen*). Dozens of ascospores were isolated, and their genotypes were confirmed by fluorescence microscopy, antibiotic resistance testing, and PCR screening. In the wild-type strain (hH1-GFP), phialides were produced from the hyphae (Fig. 2D), and each conidium budded from a mature phialide. Each young conidium obtained its nucleus from a phialide cell. A young conidium could mature with or without assistance from a phialide cell and became a mature conidium containing 2 to 6 cells (Fig. 2D). In contrast, the *wetA* deletion resulted in abnormal production of conidiophores and conidia. About 50% of the phialides appeared normal, but conidia from the *wetA* deletion mutants were longer than conidia from wild-type phialides (Table 2), and nuclei were not evenly distributed in the conidia. In addition, some short and single-celled conidia were also observed in the *wetA* deletion mutant (Fig. 2D).

Microcycle conidiation induced in the *wetA* deletion mutant. To determine the origin of the short and single-celled conidia, we observed conidiophores and conidium production on YMA over time. One day after conidium induction on YMA, most mutant conidia were longer and had fewer septa than wild-type conidia,

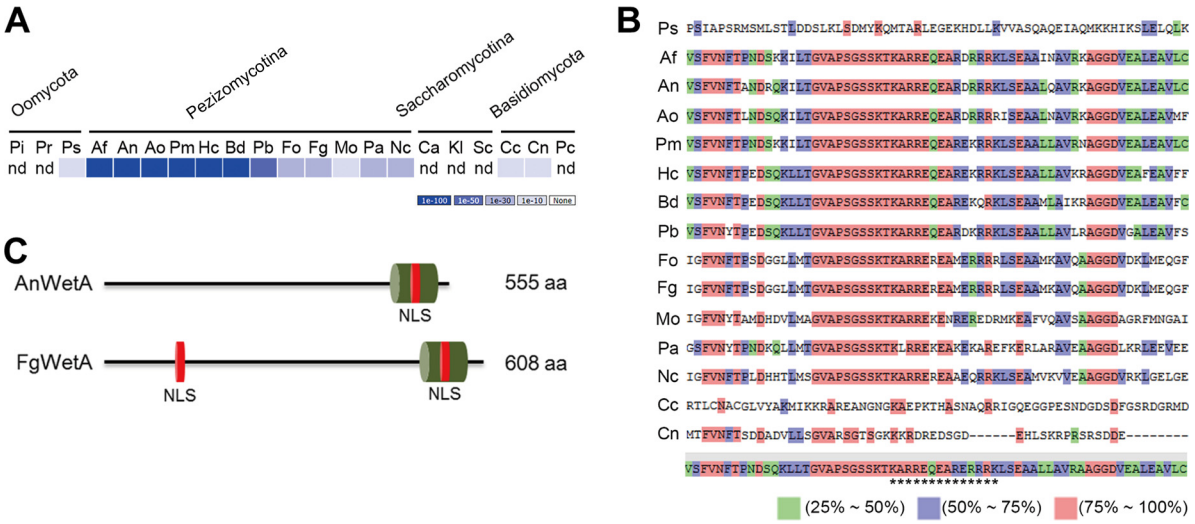

FIG 1 Identification of WetA homologs. (A) Homology-based distribution of WetA homologs from representative fungal species. The distribution image was constructed using the BLASTMatrix tool available on the Comparative Fungal Genomics Platform (http://cfgp.riceblast.snu.ac.kr/) (64). Pi, *Phytophthora infestans*; Pr, *Phytophthora ramorum*; Ps, *Phytophthora sojae*; Af, *Aspergillus fumigatus*; An, *Aspergillus nidulans*; Ao, *Aspergillus oryzae*; Pm, *Penicillium marneffei*, Hc, *Histoplasma capsulatum*; Bd, *Blastomyces dermatitidis*; Pb, *Paracoccidioides brasiliensis*; Fo, *Fusarium oxysporum*; Fg, *F. graminearum*; Mo, *Magnaporthe oryzae*; Pa, *Podospora anserina*; Nc, *Neurospora crassa*; Ca, *Candida albicans*; Kl, *Kluyveromyces lactis*; Sc, *Saccharomyces cerevisiae*; Cc, *Coprinus cinereus*; Cn, *Cryptococcus neoformans*; Pc, *Phanerochaete chrysosporium*; nd, not detected. (B) Comparison of the deduced amino acid sequence of the C-terminal region of *F. graminearum* WetA with the sequences of other fungal homologs. The stars under the consensus sequence indicate putative nuclear localization signals. The alignment was produced using the ClustalW algorithm. Residues that are 75 to 100% conserved are highlighted in pink; residues that are 50 to 75% conserved are highlighted in blue-violet; and residues that are 25 to 50% conserved are highlighted in green. (C) Comparison of *A. nidulans* and *F. graminearum* WetA orthologs. Schematic representations of the protein orthologs show the locations and alignment of the conserved domain. The green box represents the protein ESC1/WetA-related (family ID PTHR22934) domain predicted by the PANTHER classification system (65). NLS, nuclear localization signal predicted by NLStradamus (66).

but some single-celled conidia were also observed. However, the number of single-celled conidia produced from the *wetA* deletion mutant dramatically increased 2 days after conidium induction. At this stage, many of the long conidia produced from the *wetA* deletion mutants directly produced additional conidia without germination and mycelium formation; this process is called microcycle conidiation (Fig. 2E, white arrows). Furthermore, the cytoplasm of a long conidium was filled with vacuoles, the number of nuclei per conidium was markedly lower in the *wetA* deletion mutant than in the wild-type strain (Fig. 2E, black arrows), and about 30% of the long conidia fragmented to form short conidia (Fig. 2E, black arrowheads). Moreover, some single-celled conidia and some cells of long conidia lacked nuclei and cytoplasm (Fig. 2E, white arrowheads).

Chronological survival and necrotic cell death. We performed survival assays on aged conidia to investigate the role of *wetA* in fungal cell death. The viabilities of fresh conidia at 2 days after incubation were 100% for the wild-type and *wetA* deletion mutant strains (Fig. 3A). However, less than 70% and 10% of conidia from the *wetA* deletion mutants survived 10 and 20 days after incubation, respectively. Conidia and mycelia from the wild-type strain survived 50 days after incubation.

To determine the mechanism of cell death in the *wetA* deletion mutants, annexin V-PI costaining was performed 10 days after conidium incubation (Fig. 3B). Phosphatidylserine externalization is indicative of apoptotic cell death and is recognized by fluorescein isothiocyanate (FITC)-labeled annexin V (green fluorescence). Loss of membrane integrity is indicative of necrotic cell death and is recognized by PI (red fluorescence) (67). No conidial cells exhibiting the apoptotic marker (green fluorescence) were found in any of the strains observed. However, many of the aged conidial cells from the *wetA* deletion mutants were annexin V negative and PI positive, indicating that the *wetA* deletion resulted in vigorous necrotic cell death in aged conidia (Fig. 3B). In addition, many collapsed conidial cells were observed in the *wetA* deletion mutants after microcycle conidiation (Fig. 3B, white arrows).

Stress tolerance test and trehalose assay. To determine whether *wetA* is required for salt, oxidative, or detergent stress tolerance, each strain was inoculated onto PDA supplemented with 1 M NaCl, 5 mM H_2O_2, or 10 mg/liter SDS. The *wetA* deletion mutant, wild-type, and complemented strains grew similarly on these media (see Fig. S2 in the supplemental material). However, conidia from the *wetA* deletion mutants were more sensitive than wild-type conidia to acute H_2O_2 stress and high-temperature treatments (Fig. 3C and D). Because trehalose is known to be required for spore viability and stress resistance in aspergilli, such as *A. nidulans* and *A. fumigatus*, and because *wetA* is required for trehalose production in *A. fumigatus* (5, 18, 68), we measured trehalose production in each strain. All of the strains produced similar levels of trehalose per conidium (see Fig. S3 in the supplemental material).

TEM. TEM was performed to investigate the cell wall structure and subcellular components of the *wetA* deletion mutants. The cytoplasm of conidia from the *wetA* deletion mutants consisted mainly of vacuoles, whereas conidia from the wild-type and complemented strains contained various subcellular organelles in their cytoplasm (Fig. 3E). Many autophagic bodies were found in vacuoles of the *wetA* deletion mutants (Fig. 3E, white arrows), but they were absent in the wild-type and complemented strains. Cell wall

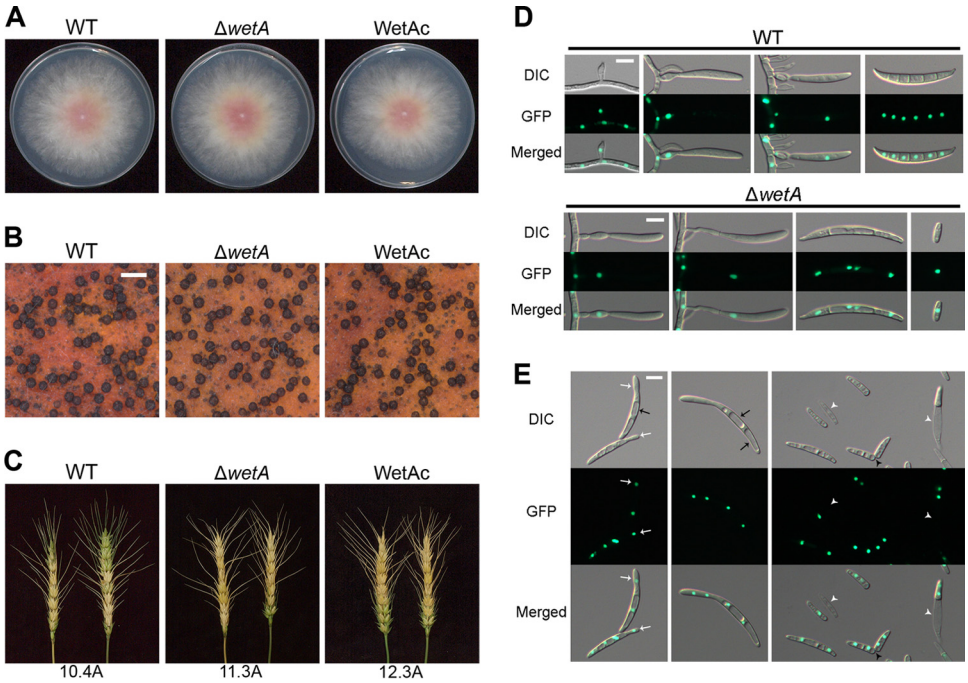

FIG 2 Phenotypic analyses of *F. graminearum* strains. (A) Mycelial growth of *F. graminearum* strains on complete medium. Pictures were taken 5 days after inoculation onto CM. WT, the *F. graminearum* wild-type strain Z-3639; Δ*wetA*, the *wetA* deletion mutant HK144; WetAc, the Δ*wetA* mutant-derived strain complemented with *wetA* (HK146). (B) Perithecium formation by *F. graminearum* strains on carrot agar. Pictures were taken 7 days after sexual induction on carrot agar. Bar, 500 μm. (C) Virulence on wheat heads. The center spikelet of each wheat head was injected with 10 μl of a conidium suspension. Pictures were taken 21 days after inoculation. Disease indexes (diseased spikelets per wheat head) followed by the same letter are not significantly different (P, >0.05) based on Tukey's test. (D) Morphologies of conidiophores from *F. graminearum* strains. CMC and YMA were used to produce conidia. Pictures were taken 1 to 3 days after the induction of conidiogenesis. Conidiophores and conidia of the wild-type strain and the *wetA* deletion mutant are shown. Fluorescent green dots represent hH1-GFP. DIC, differential interference contrast image; GFP, fluorescence microscopy image representing hH1-GFP; merged, overlays of the DIC and fluorescence microscopy images. Bars, 10 μm. (E) Morphologies of conidia from the *wetA* deletion mutant. YMA was used to induce conidium formation. Pictures were taken 2 to 4 days after the induction of conidiogenesis. White arrows indicate conidia produced by microcycle conidiation; black arrows indicate vacuoles; white arrowheads indicate fungal cells without nuclei or cytoplasm; and black arrowheads indicate fragmented conidia. Bar, 10 μm.

characteristics, such as structure and thickness, were similar for the *wetA* deletion mutant, wild-type, and complemented strains.

Autophagy-dependent microcycle conidiation in *wetA* deletion mutants. A recent study revealed that the *atg8* ortholog is essential for the generation of autophagic compartments in *F. graminearum* (69). Thus, we created a *wetA atg8* double deletion mutant, HK149 (Δ*wetA*::*gen* Δ*atg8*::*gen*), and compared conidia from this strain to conidia from the HK144 (Δ*wetA*), HK147 (Δ*atg8*), and wild-type strains. We deleted the *atg8* gene (locus ID FGSG_10740) in *F. graminearum* wild-type strain Z-3639. Successful gene replacement was confirmed by Southern hybridization (see Fig. S4 in the supplemental material). To obtain the *wetA atg8* double deletion mutant HK149, HK148 (Δ*mat1-2*::*gfp-hyg* Δ*wetA*::*gen*) had to be created. HK148 (Δ*wetA*::*gen*) was generated by outcrossing the Δ*mat2* strain with HK146. Thereafter, HK148 was fertilized with conidia from HK147 (Δ*atg8*::*gen*), and the resulting progeny were genotyped by fluorescence microscopy, antibiotic resistance testing, and PCR screening to select for strain HK149.

TABLE 2 Conidium production and morphology

Strain	Conidium production (10^6/ml)[a]	Conidium morphology[b]		
		Length (μm)	Width (μm)	No. of septa
WT	2.6 ± 0.13	44.6 ± 5.0	5.2 ± 0.59	4.1 ± 0.74
HK144 (Δ*wetA*)	1.7 ± 0.15*	71.5 ± 19.1*	5.3 ± 0.82*	3.0 ± 1.49*
HK146 (WetAc)	2.3 ± 0.19	43.4 ± 8.0	5.1 ± 0.42	4.1 ± 0.74

[a] Conidia were counted after incubation for 3 days on carboxymethyl cellulose medium (CMC). Asterisks indicate significance (P, <0.01) based on Tukey's test.
[b] Conidia were harvested from a 1-day-old yeast extract-malt extract agar (YMA) culture.

We compared the lengths of conidia 2 and 4 days after conidium induction on YMA (Fig. 4). At 2 days after induction, the wild-type and *atg8* deletion mutant strains produced normally shaped conidia, with lengths ranging from 39 to 63 μm (Fig. 4A). The lengths of conidia from the *wetA* deletion mutants ranged from 8 to 150 μm, with an average length of 48 μm. However, HK149 produced conidia with lengths ranging from 18 to 111 μm, with an average length of 60 μm.

At 4 days after induction, the conidia generated by microcycle conidiation in the *wetA* deletion mutants were shorter (mean length, 26 μm) than those observed at 2 days after induction. HK149 produced conidia with an average length of 51 μm. HK149 produced fewer, shorter, single-celled conidia compared to those of the *wetA* deletion mutants (Fig. 4B). The wild-type and *atg8* deletion mutant strains produced conidia with similar morphologies at 2 and 4 days after induction. In conclusion, microcycle conidiation induced in the *wetA* deletion mutant is highly dependent on autophagy.

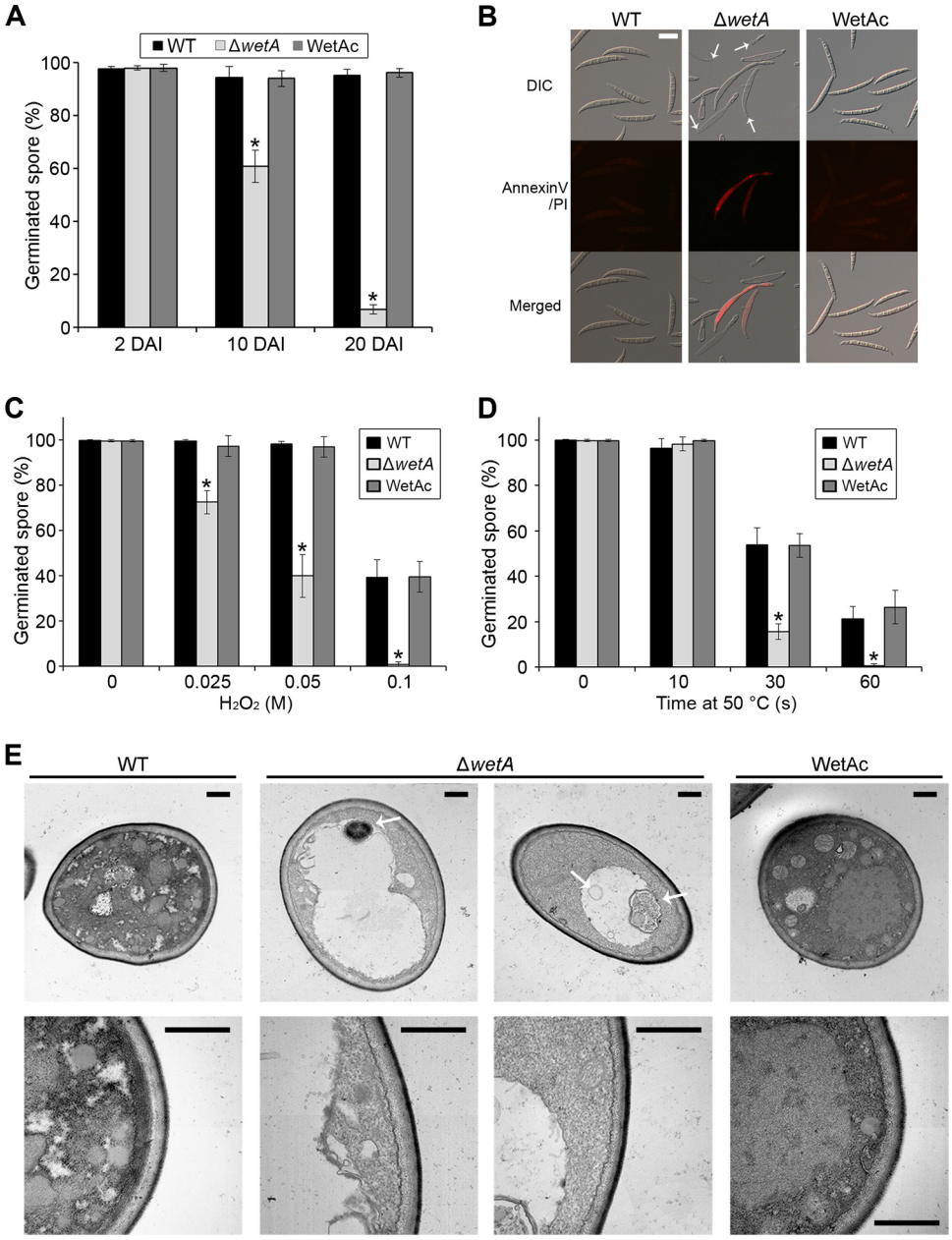

FIG 3 Assay of survival of conidia from *F. graminearum* strains. (A) Conidium production was induced on YMA, and strains were incubated for 2, 10, or 20 days. Harvested conidia were allowed to germinate, and the viabilities of the conidia were determined by the germination rate. An asterisk indicates a significant difference (P, <0.05) by Tukey's test. WT, *F. graminearum* wild-type strain Z-3639; Δ*wetA*, *wetA* deletion mutant HK144; WetAc, Δ*wetA*-derived strain complemented with *wetA* (HK146). DAI, days after conidium induction. (B) Annexin V (green)-propidium iodide (PI) (red) costaining of 10-day-old conidia to determine the type of cell death. White arrows indicate collapsed conidial cells after microcycle conidiation. DIC, differential interference contrast. Bar, 20 μm. (C) Tolerances of conidia to oxidative stress. (D) Tolerances of conidia to heat stress. (E) TEM images of conidia. Conidia were freshly harvested 2 days after induction. White arrows indicate autophagic bodies. Bars, 500 nm.

Genetic relationship between *abaA* and *wetA*. In our previous study, we predicted that the AbaA-WetA pathway is important for conidiation in *F. graminearum* (41). To analyze the genetic relationship between *abaA* and *wetA* in *F. graminearum*, we generated strains HK154 (Δ*wetA*::*gen* Δ*abaA*::*gen*) and HK155 (Δ*wetA*::*gen* *abaA*::*gen*-$P_{ef1\alpha}$-*abaA*) by outcrossing HK148 with the Δ*abaA* mutant and HK148 with AbaA-OE, respectively. As seen previously, the *abaA* deletion mutants produced immature phialides with abnormal morphology (41), which germinated and became thin hyphae. The AbaA-OE mutant produced abacus-like phialides and conidia (Fig. 5). The conidiophores of mutants HK154 and HK155 were indistinguishable from those of the Δ*abaA* and AbaA-OE strains, respectively. Moreover, the conidia of HK155 did not show vigorous microcycle conidiation or reduced viability. These findings suggested that the *abaA* deletion and overexpression mutations were epistatic to the *wetA* deletion in *F. graminearum*.

RNA-sequencing-based transcriptome analysis. We ob-

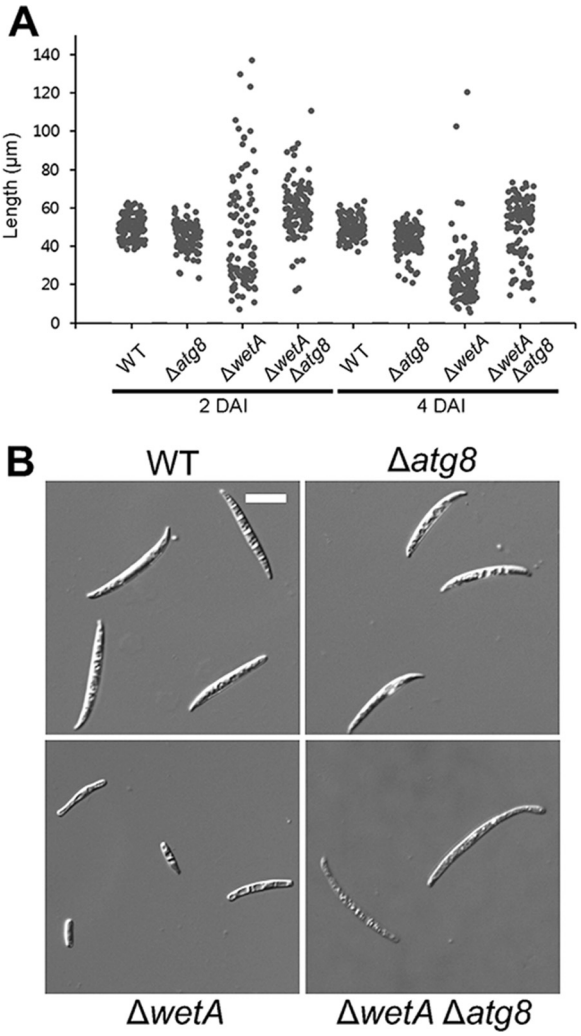

FIG 4 Lengths of conidia. (A) A total of 100 conidia were measured 2 and 4 days after conidium induction on YMA. Each conidium measured is represented as a spot. DAI, days after conidium induction; WT, *F. graminearum* wild-type strain Z-3639; Δ*atg8*, *atg8* deletion mutant HK147; Δ*wetA*, *wetA* deletion mutant HK144; Δ*wetA* Δ*atg8*, double deletion mutant HK149. (B) Pictures were taken 4 days after conidium induction. Bar, 20 μm.

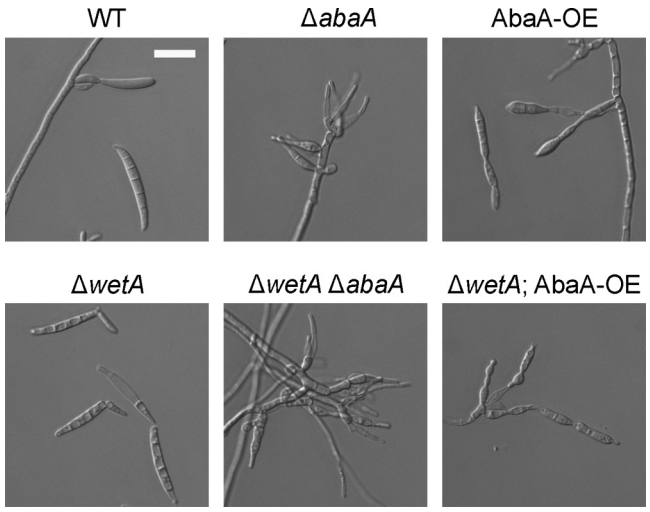

FIG 5 Morphologies of conidiophores from *F. graminearum* strains. Carboxymethyl cellulose medium was used to produce conidia. Pictures were taken 1 to 3 days after inoculation. WT, *F. graminearum* wild-type strain Z-3639; Δ*wetA*, *wetA* deletion mutant HK144; Δ*abaA*, *abaA* deletion mutant; Δ*wetA* Δ*abaA*, double deletion mutant HK154; AbaA-OE, transgenic strain in which the *abaA* promoter was replaced with the *ef1*α promoter; Δ*wetA* AbaA-OE, double mutant strain HK155. Bar, 20 μm.

tained and analyzed transcript profiles, which were generated from RNA-sequencing data, of wild-type and *wetA* deletion mutant strains at 0, 6, and 12 h after the induction of conidiogenesis. *F. graminearum* normally produces phialide cells 6 h after the induction of conidiogenesis, and the conidia mature 12 h after induction. Although most conidiophores develop synchronously, conidiation also occurs successively up to 12 h after the induction of conidiogenesis. Wild-type data were obtained from our previous study (41). Among the 13,820 genes recently reannotated (52), 693 genes with low RPKM values (<10) under all conditions tested were excluded, and 13,127 genes were selected for further analyses. Differentially expressed genes (DEG) were identified by 2-fold changes in RPKM values. RPKM values of zero were converted to 1 for the calculation of fold change.

To analyze our transcriptome data with regard to conidiation-related genes, we identified 96 conidiation-related genes in *F. graminearum* based on phenotypes described in the literature (see data set S1 in the supplemental material). Most of these genes have been characterized as TFs and kinases (34, 35). We identified 155 and 108 *F. graminearum* orthologs of conidiation-related genes in *A. nidulans* and *N. crassa*, respectively. *A. nidulans* and *N. crassa* genetic and phenotypic data were obtained from the *Aspergillus* Genome Database (http://www.aspergillusgenome.org/) and the *N. crassa* Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/) (70). In total, 319 putative conidiation-related genes were identified.

Genes that were down- or upregulated in the *wetA* deletion mutant 6 and 12 h after the induction of conidiogenesis were analyzed and were allocated to eight groups (groups 1 to 8), according to their expression patterns (see data set S1 in the supplemental material) (Fig. 6). A total of 1,137 genes were downregulated and 881 genes were upregulated 6 h after the induction of conidiogenesis in the *wetA* deletion mutant, whereas 624 genes were downregulated and 429 genes were upregulated in the *wetA* deletion mutant 12 h after the induction of conidiogenesis (Fig. 6). Among these, 150 genes (group 1) were downregulated and 117 (group 8) were upregulated throughout the conidiation process. The other five groups included genes that were regulated at specific stages of conidiation.

We categorized genes according to fluctuations in their transcript levels during wild-type conidiogenesis in order to identify genes involved in each step of the process (Table 3; see also data set S2 in the supplemental material). These gene groups were labeled A to I according to their transcript fluctuation patterns in the wild-type and *wetA* deletion mutant strains. Genes that were regulated in the *wetA* deletion mutant were also addressed for each group. Among the 319 putative conidiation-related genes identified, 15, 260, 22, 3, 10, 5, 1, 2, and 1 gene(s) were allocated to groups A, B, C, D, E, F, G, H, and I, respectively (see data set S3 in the supplemental material).

The FunCat (71) was also utilized to categorize proteins ac-

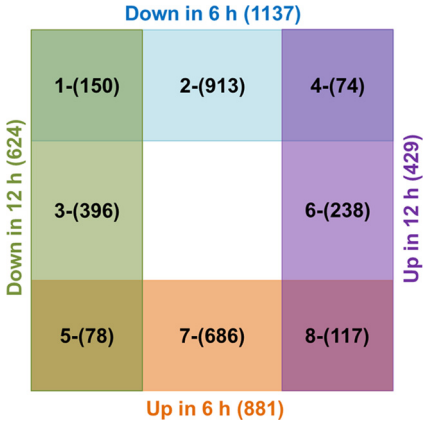

FIG 6 Numbers of genes differentially expressed in the *F. graminearum wetA* deletion strain and the wild-type strain. Down, genes that were downregulated more than 2-fold; Up, genes that were upregulated more than 2-fold. The number of genes differentially regulated in each group is given in parentheses after the group number (groups 1 to 8).

cording to their functions, and DEG in each functional category were also marked (see data set S1 and Table S2 in the supplemental material). The largest portion of genes included in "unclassified proteins" fluctuated greatly in the *wetA* deletion mutant throughout the sporulation process, and relatively many DEG were included in the metabolism and energy categories 6 and 12 h after induction of conidiogenesis.

DISCUSSION

In *A. nidulans*, several specialized structures originate from hyphae to support asexual sporulation. When signals for conidiation reach a hyphal cell, it differentiates into a foot cell, which elongates into a stalk with a vesicle forming at the stalk tip. Many metulae emerge from each vesicle, and each metula produces two conidiogenous phialides. Single-celled conidia are generated basipetally from each phialide cell (4). During this process, sequentially expressed developmental regulators precisely control cell differentiation, both temporally and spatially. BrlA is expressed during vesicle production and activates *abaA*, which is required for phialide formation and function (2, 13, 14). AbaA induces *wetA*, which is essential for conidium maturation (16, 72). AbaA is also known to activate other conidiation-specific genes, including *velB* and *vosA* (3, 9).

In *F. graminearum* conidiation, the fungus bypasses the formation of the foot cell, stalk, vesicle, and metulae. Single or multiple phialides arise from a hyphal cell and continuously generate multiseptate conidia (28, 40). Because *F. graminearum* has a much simpler conidiogenesis process, its genetic pathway for conidiogenesis is presumably different from the *A. nidulans* pathway. Our previous phenotypic analyses and those of others showed that the *brlA* and *fluffy* genes, which are upstream development activators, are not conserved in *F. graminearum* (13, 34, 41). However, *stuA* and *abaA* orthologs function similarly in *A. nidulans* and *F. graminearum* (40, 41). Thus, genetic variation between the two fungi is not explained solely by their morphological differences, and in-depth functional characterizations of well-studied orthologs are required in order to understand conidiogenesis in *F. graminearum*.

In this study, we characterized the functions of the *wetA* ortholog in *F. graminearum*. We found that WetA was specifically related to conidiogenesis and conidium maturation in *F. graminearum*. The *wetA* deletion mutants produced longer conidia with fewer septa. These conidia were sensitive to acute stresses, such as oxidative stress and heat stress. Aged conidia from the *wetA* deletion mutants had reduced survival rates. Interestingly, abnormal conidia from *wetA* deletion mutants generated single-celled conidia through autophagy-dependent microcycle conidiation. We also confirmed that *abaA* mutations were epistatic to the *wetA* deletion in this fungus.

WetA seems to have conserved roles for the separation and maturation of conidia in both *A. nidulans* and *F. graminearum*. Previous TEM observations demonstrated that in *A. nidulans wetA* mutants, the conidium wall is continuous with the innermost layer of the phialide wall (72). Conidia have been shown to be interconnected (5). Similarly, in Fg*wetA* deletion mutants, conidia were not separated and were longer than those from the wild-type strain. Improper maturation of conidia in *wetA* mutants resulted in conidia with fewer septa. Although the morphological defects of conidiophores differed between the *A. nidulans wetA* and *F. graminearum wetA* deletion mutants, we conclude that the differences result from morphological distinctions between wild-type conidiophores of the two fungi and that WetA functions similarly in the two fungi. Previous mutational analyses of *stuA* and *abaA* orthologs in *A. nidulans* and *F. graminearum* also demonstrated similar phenotypic differences, which presumably arose from innate morphological differences between the wild-type conidiophores (40, 41).

However, some *F. graminearum wetA* functions seem to be functionally differentiated from those of *A. nidulans*. *A. nidulans wetA* mutation led to altered cell wall composition and reduced trehalose accumulation (5, 72), whereas these phenotypes were normal in the *F. graminearum wetA* deletion mutant. According to recently proposed models representing patterns of gene evolution for regulators of conidiation, conidiation-related genes are categorized into gene groups that are functionally conserved across fungi, unique to a particular fungal clade, or functionally differentiated with the same biochemical function. Although *wetA* or-

TABLE 3 Expression pattern groups and their regulation in the *wetA* deletion mutant

Group	Fluctuation of transcript level[a]		No. of genes[b]:		
	0 → 6 h	6 → 12 h	Total	Upregulated in Δ*wetA* mutant	Downregulated in Δ*wetA* mutant
A	Decreased	Unchanged	1,028	45	42
B	Unchanged	Unchanged	8,046	57	138
C	Increased	Unchanged	1,160	19	57
D	Decreased	Increased	488	39	21
E	Unchanged	Increased	961	61	41
F	Increased	Increased	360	36	27
G	Decreased	Decreased	137	7	4
H	Unchanged	Decreased	580	10	19
I	Increased	Decreased	367	22	18

[a] "Decreased," "Increased," and "Unchanged" refer to at least 2-fold changes in RPMK values from the previous time point.
[b] Upregulated and downregulated genes in the Δ*wetA* mutant are genes with RPMK values higher and lower, respectively, than those of the wild-type strain at both 6 h and 12 h after conidium induction.

thologs of *A. nidulans* and *F. graminearum* have conserved roles in the separation and maturation of conidia, the same ancestral *wetA* gene likely has evolved to retain its original nature as a transcription factor in the two fungi but to have species-specific roles (73). One of the crucial regulators for conidiation, *flbD*/*rca-1*, also has a conserved biochemical function in *A. nidulans* and *N. crassa* but has distinct functions in each fungus (74). The functional differentiation of transcription factors such as WetA seems to be derived from the transcriptional rewiring caused by variable *cis*-regulatory elements (75, 76).

The most distinct phenotype of the *F. graminearum wetA* deletion mutants was the significant induction of the microcycle conidiation process, in which a fungal spore produces secondary spores directly and bypasses mycelium formation (77). Although this is difficult to observe in nature, numerous fungal species within diverse taxa perform microcycle conidiation during their life cycles (77). Nevertheless, there is scant information on this developmental process in *Fusarium*. Deletion of the *veA* ortholog gene in *Fusarium verticillioides* induced yeast-like growth similar to microcycle conidiation (19), and a *Fusarium solani* mutant with continuous microcycle conidiation has been reported (78). Microcycle conidiation is regarded as a key factor in the development of *Fusarium* keratitis disease by several *Fusarium* spp., such as *Fusarium oxysporum* and *F. solani*, because rapid fungal amplification is guaranteed by this process (79). However, microcycle conidiation had not been observed in *F. graminearum*, suggesting that this process is normally suppressed in *F. graminearum*.

In most species studied, microcycle conidiation is induced by physical and chemical stresses (80). Thus, this process may provide a significant survival advantage (77, 81, 82). A *wetA* deletion may trigger internal stresses in developing conidia that would then induce microcycle conidiation and the production of immature conidia. This assertion is supported by the reduced survival rate of aged conidia and the sensitivities of HK144 (Δ*wetA*) conidia to acute stress treatments. The ortholog of *veA* has also been found to be involved in the stress response of *F. verticillioides* (19).

Autophagy is a bulk degradation process that utilizes the vacuolar/lysosomal system in eukaryotes. It was recently shown to be important for fungal pathogenicity and development (83–85). In the *F. graminearum wetA* deletion mutants, we found enlarged vacuoles and many autophagic bodies inside the vacuoles (83). An *A. nidulans wetA* knockout mutant was found to induce the autolysis of conidia, resulting in a wettable phenotype (72). Because autophagy precedes autolysis and may function in the early onset of autolysis (86), our observation of autophagy in the *F. graminearum wetA* deletion mutants is not contradictory to the autolysis phenotype in the *A. nidulans wetA* deletion mutant. In addition, we found that the microcycle conidiation observed in the *F. graminearum wetA* deletion mutants was highly dependent on autophagy. Conidia from the *wetA* deletion mutants utilized autophagy for secondary conidium production, whereas wild-type conidia entered dormancy after maturation.

Although the life spans and stress tolerance phenotypes of conidia from *A. nidulans* and *F. graminearum* are similar, the causes of the phenotypes differ. Abnormal conidial phenotypes in an *A. nidulans wetA* mutant have been shown to result from altered cell wall composition and reduced trehalose accumulation (5, 72). However, the cell wall structures and trehalose levels did not differ between conidia from the *F. graminearum* wild-type and *wetA* deletion mutant strains. The causes of the reduced life span, and the increased sensitivities to acute stresses, of conidia from the *F. graminearum wetA* deletion mutant are likely twofold. First, these conidial cells contained organelles that were damaged by vigorous autophagy and therefore were highly sensitive to extracellular stresses. Second, continuous microcycle conidiation resulted in damaged or improperly separated conidia that leaked cytoplasmic fluid. Taken together, weak, immature, and damaged conidia generated by autophagy and microcycle conidiation have short life spans and are highly sensitive to acute stresses.

In this study, we investigated the molecular mechanisms underlying conidiogenesis in *F. graminearum* through next-generation sequencing-based genome-wide transcriptome analysis. Among 319 putative conidiation-related genes identified, ~82% (260 genes; group B) were constitutively expressed during conidiation (see data set S3 in the supplemental material). Moreover, expression patterns for 243 of these genes did not differ between the *wetA* deletion mutant and the wild-type strain. Because the transcripts of genes that are related to a particular function typically differ in abundance during distinct developmental stages, we conclude that the ~82% of genes that were constitutively expressed during conidiation are not specifically related to conidiation but are required for general functions during fungal development. For example, most TF knockout mutants showed pleiotropic defects in vegetative growth and sexual development, as well as in asexual conidiation, and only five TFs were found to be specific for conidiogenesis (34). Similarly, genes known to be related to conidiation, such as transducin-encoding *ftl1* (87), histone deacetylase-encoding *hdf1* (37), ATP citrate lyase-encoding *acl1* and *acl2* (53), topoisomerase-encoding *top1* (36), *FgstuA* (40), and *mes1* (38), are members of group B and also have pivotal roles in major cellular processes. The higher average RPMK value (10,506) for the 319 putative conidiation-related genes than for the whole genome (8,255) also supports our assertion that these genes have crucial roles in fungal development (34). Thus, it seems that very few genes are specifically required for conidiogenesis, and most known conidiation-related genes are also important for basic fungal physiology in *F. graminearum*.

Although we did not select certain genes from the RNA-sequencing data for the genetic analysis in this study, our transcriptomic analysis will provide good resources for the understanding of conidiogenesis in *F. graminearum*. As mentioned above, most of the conidiation-defective phenotypes reported before are derived from defective homeostasis of fungal cells. Therefore, additional novel conidiation-specific genes should be identified for better understanding of conidiogenesis in *F. graminearum*. We have identified stage-specific and *abaA*- and *wetA*-dependent genes in the current study and in previous studies, and these transcriptome data should facilitate the identification of conidiation-specific pathways and/or genes in *F. graminearum* (41).

In conclusion, we demonstrated that WetA has conserved roles in conidium separation and maturation in both *A. nidulans* and *F. graminearum*. We also showed that WetA of *F. graminearum* functions to maintain conidium dormancy by suppressing microcycle conidiation, a pathway that is highly dependent on autophagy. This is the first study that functionally characterized the *F. graminearum wetA* ortholog, and it expands our understanding of conidiogenesis and conidium maturation in filamentous fungi.

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