농학석사 학위논문

붉은곰팡이의 유성생식과정에 관여하는 winged-helix 전사조절인자의 유전적 상관관계 연구

A genetic network of winged-helix transcription factors regulating sexual reproduction in the homothallic fungus *Fusarium graminearum*

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황 보 아 람

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BY

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Abstract

A genetic network of winged-helix transcription factors regulating sexual reproduction in the homothallic fungus *Fusarium graminearum*

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Fusarium graminearum is a destructive plant pathogenic fungus causing Fusarium head blight (FHB) on various cereal crops. The infection of this fungus leads to severe yield losses and contamination of mycotoxins, such as trichothecenes and zearalenone, that are harmful to humans and animals. This fungus produces two types of spores through sexual and asexual reproduction. During sexual reproduction, ascospores are produced and discharged from the fruiting bodies

‘perithecia’, serving as the primary inocula in the following spring. In plant disease epidemiology, sexual reproduction is crucial in that it increases genetic diversity of population and provides overwintering structures such as perithecia and associated tissues. It has been known that mating-type factors act as master regulators in sexual reproduction, but there is still a limited understanding of the molecular mechanisms underlying sexual developmental stages. Winged-helix transcription factors (TFs) are key regulators of embryogenesis and cell differentiation in higher eukaryotes. In several fungal species, it also has been revealed that they are involved in fungal morphogenesis and development. In *F. graminearum*, there are 28 putative winged-helix transcription factors. The objectives of this study were i) to determine the roles of winged-helix genes and ii) to figure out their genetic relationship with mating-type genes during the sexual development in this fungus. In this study, nine winged-helix TFs that appeared to be important for sexual reproduction were investigated. Deletion, complementation, and overexpressing mutants were generated, and their phenotypes were analyzed. All deletion mutants were self-sterile and defective in perithecia formation and/or ascospore development. Extensive outcross analyses revealed that, four self-sterile mutants, Δ*gzwing15*, Δ*gzwing16*, Δ*gzwing20*, and Δ*gzwing27*, had both female and male fertilities same as mating-type gene mutants. Moreover, these four genes were expected to have essential roles in sexual reproduction by constructing genetic

networks with mating-type genes. The results of this study will contribute to the understanding of molecular genetic regulation of sexual development in fungi.

Keywords: *Fusarium graminearum*, Winged-helix transcription factors, sexual reproduction

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INTRODUCTION

The homothallic ascomycete fungus *Fusarium graminearum* is an important plant pathogen which causes Fusarium head blight (FHB) on cereal crops and ear rot on maize. Infection by this pathogen leads to severe economic losses and contamination of grains with mycotoxins, such as trichothecenes and zearalenone, which are harmful to humans and animals (Desjardins & Proctor, 2007). Sexual reproduction is an important developmental process for genetic diversity, stability, and pathogenicity in fungi (Lee et al., 2012; Son et al., 2011; Urban et al., 2003). Through sexual reproduction, *F. graminearum* produces sexual fruiting bodies, perithecia, which are required for survival under harsh conditions and dispersal of ascospores (Dill-Macky & Jones, 2000; Guenther & Trail, 2005; Lin et al., 2011). During the following flowering season, the ascospores are discharged into the air and serve as the primary inoculum, causing plant disease (Paulitz, 1996). To date, in *F. graminearum,* several genes related to sexual reproduction have been studied, and those genes such as *MAP1* (Urban et al., 2003), and *FgVelB* (Lee et al., 2012) were reported to be related to disease development. It has been known that mating-type (*MAT*) genes encode master regulators of sexual reproduction in ascomycete fungi, including *F. graminearum* (Lee et al., 2003). However, understanding of the molecular mechanisms underlying sexual developmental stages is still limited.

Winged-helix transcription factors (TFs), including members of the forkhead and the RFX subclasses, are known as core components of transcription systems in prokaryotes and eukaryotes (Teichmann et al., 2012). They are involved in diverse cellular responses and differentiation processes in many organisms. In *Saccharomyces cerevisiae*, Fkh1 and Fkh2 are regulators of genes involved in the G2 and M phases of the cell cycle (Zhu et al., 2000). And Hcm1 has a role in controlling S phase and chromosome segregation (Pramila et al., 2006). The *fkhF* and *fkhE* genes, which encode forkhead TFs in *Aspergillus nidulans*, are involved in asexual development (Park et al., 2010), and the FhpA is a regulator of sexual development (Lee et al., 2005). In the rice blast fungus *Magnaporthe oryzae*, *MoFKH1* and *MoHCM1* are involved in fungal development, pathogenicity, and stress response (Park et al., 2014).

In the previous study, 28 putative winged-helix TFs were identified in *F. graminearum*. Furthermore, several deletion mutants of these TFs showed defective phenotypes during sexual reproduction (Son et al., 2011). In this study, nine winged-helix TFs that appeared to be important for sexual reproduction were investigated. The objectives of this study were i) to characterize the roles of winged-helix TF genes and ii) to elucidate their genetic relationship with mating-type genes during the sexual development in *F. graminearum.* This study will

contribute to the understanding of winged helix TFs-mediated sexual development and provides insights into the genetic regulation of fungal sexual reproduction.

MATERIALS AND METHODS

I. Fungal strains and media

All the strains used in this study are listed in Table 1. The *F. graminearum* wild-type strain Z-3639 (Bowden & Leslie, 1999) and mutants derived from this strain were stored in 20% glycerol at -80°C. Culture media were prepared following the Fusarium laboratory manual (Leslie & Summerell, 2006).

II. Nucleic acid manipulations and genetic modifications

Fungal genomic DNA was prepared as previously described (Leslie & Summerell, 2006). The mycelia or perithecia in different stages were harvested, and Total RNA was isolated from mycelia ground in liquid nitrogen using an Easy-Spin Total RNA Extraction Kit (Intron Biotech, Seongnam, Republic of Korea). Restriction endonuclease digestion and agarose gel electrophoresis were performed following standard protocols (Russell & Sambrook, 2001). A North2South Biotin Random Prime Labeling Kit and a North2South Chemiluminescent Hybridization and Detection Kit (Thermo Scientific, Waltham, MA, USA) were used for Southern blot hybridization. DNA sequencing was performed by Macrogen (Seoul, Korea) and all the primers used in this study (Table 2) were synthesized at an oligonucleotide synthesis facility (Bioneer, Daejeon, Korea).

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

Strain	Genotype	Source or reference
Z-3639	*F. graminearum* wild-type	(Bowden & Leslie, 1999)
Δ*mat1;hH1-GFP*	Δ*mat1-1::gen; hH1::hH1-GFP-hyg*	(Hong et al., 2010)
Δ*gzwing11*	Δ*gzwing11::GEN*	(Son et al., 2011)
Δ*gzwing12*	Δ*gzwing12::GEN*	(Son et al., 2011)
Δ*gzwing13*	Δ*gzwing13::GEN*	(Son et al., 2011)
Δ*gzwing15*	Δ*gzwing15::GEN*	(Son et al., 2011)
Δ*gzwing16*	Δ*gzwing16::GEN*	(Son et al., 2011)
Δ*gzwing18*	Δ*gzwing18::GEN*	(Son et al., 2011)
Δ*gzwing19*	Δ*gzwing19::GEN*	(Son et al., 2011)
Δgzw*ing20*	Δgzw*ing20::GEN*	(Son et al., 2011)

Δgzw*ing27*	Δgzw*ing27::GEN*	(Son et al., 2011)
GzWING15c	Δ*gzwing15:: GzWING15-GFP-HYG*	This study
GzWING16c	Δ*gzwing16:: GzWING16-GFP-HYG*	This study
GzWING18c	Δ*gzwing18:: GzWING18-GFP-HYG*	This study
GzWING19c	Δ*gzwing19:: GzWING19-GFP-HYG*	This study
GzWING20c	Δ*gzwing20:: GzWING20-GFP-HYG*	This study
GzWING27c	Δ*gzwing27:: GzWING27-GFP-HYG*	This study

For targeted gene deletion, The fusion PCR products were generated via the double-joint (DJ) PCR strategy (Yu et al., 2004). The geneticin resistance cassette (GEN) was amplified from pII99 (Namiki et al., 2001), and the 5′ and 3′ flanking regions of the target gene were amplified from the genomic DNA of the wild-type strain. The three resultant amplicons were fused through a second round. The final constructs were generated in a third round of PCR using nested primers. The constructs were transformed into wild-type protoplasts, as described previously (Han et al., 2007).

For complementation of the deletion mutants, the GFP fusion construct was generated via the yeast gap repair approach, as described previously (Bruno et al., 2004). The ORF of each gene and 1 kb of its upstream promoter region were amplified and co-transformed with the Xho1-digested vector pDL2 (Zhou et al., 2011) into the yeast strain PJ69-4A. Subsequently, the GFP fusion vector was rescued from the yeast transformants and transformed into *Escherichia coli* DH10B. After verification through sequencing, the resulting vector was transformed into the deletion mutant.

Table 2. Primers used in this study

Primer	Sequence (5′ → 3′)	Description
Gen-for	CGACAGAAGATGATATTGAAGG	Forward and reverse primers for amplification of the geneticin cassette from the pII99 vector
Gen-rev	CTCTAAACAAGTGTACCTGTG	
GzWing011_5F	GATCTTTGAGTTACCGCGTCCTACAG	Forward and reverse primers for amplification of 5' flanking region of *GzWING11* with tail for the geneticin resistance gene cassette fusion
GzWing011_5R	gcacaggtacacttgtttagagCAGAGGGCTGCTGCGTCAAGT	
GzWing011_3F	ccttcaatatcatcttctgtcgAGCTCAAAGAGAGAAAGGCCAAAGAT	Forward and reverse primers for amplification of 3' flanking region of *GzWING11* with tail for the geneticin resistance gene cassette fusion
GzWing011_3R	CTTCCTTGTCCGCTGGTGTTTTATG	
GzWing011_5N	GCGATCTCTGACTGCGGCTGAT	Forward and reverse nest primers for third fusion PCR for amplification of *GzWING11* deletion construct
GzWing011_3N	CGTGGTTTTGCGGCGTAGG	
GzWing012_5F	TGGCCTTCATTGCTTCGGAGATAC	Forward and reverse primers for amplification of 5' flanking region of *GzWING12* with tail for the geneticin resistance gene cassette fusion
GzWing012_5R	gcacaggtacacttgtttagagGCTTGAGCAATGTATGGGTCTTGG	
GzWing012_3F	ccttcaatatcatcttctgtcgATTGGCGAAGCATTGATCAGTGA	Forward and reverse primers for amplification of 3' flanking region of *GzWING12* with tail for the geneticin resistance gene cassette fusion
GzWing012_3R	CCTGTCAAGATTGAAGGCGATTACC	
GzWing012_5N	AATGTCGTTGCGGTGGCTTGTC	Forward and reverse nest primers for third fusion PCR for amplification of
GzWing012_3N	CGCGGATCTTTCGACTTTTTACTG	

		GzWING12 deletion construct
GzWing013_5F	CAGGTGTTTAAAGGGACGGTTGG	Forward and reverse primers for amplification of 5' flanking region of *GzWING13* with tail for the geneticin resistance gene cassette fusion
GzWing013_5R	gcacaggtacacttgtttagagGCAAAGGCGAGAATAGGTAAGGTA	
GzWing013_3F	ccttcaatatcatcttctgtcgGCTCGAAAGGAAAGCTCAAGTCTA	Forward and reverse primers for amplification of 3' flanking region of *GzWING13* with tail for the geneticin resistance gene cassette fusion
GzWing013_3R	AACGAAGAGCATTGTTTGGTTGTTC	
GzWing013_5N	TAGTCGTCATACAGCGCCTCAAAA	Forward and reverse nest primers for third fusion PCR for amplification of *GzWING13* deletion construct
GzWing013_3N	TGCTTTGATGACATGTCTTGGTGAC	
GzWing015_5F	AGTTTCTCCTTCGACCCACACCTC	Forward and reverse primers for amplification of 5' flanking region of *GzWING15* with tail for the geneticin resistance gene cassette fusion
GzWing015_5R	gcacaggtacacttgtttagagAAGTGGAAGGGGATGACGGTAA	
GzWing015_3F	ccttcaatatcatcttctgtcgACGTGGCAAGACGACGAAATAA	Forward and reverse primers for amplification of 3' flanking region of *GzWING15* with tail for the geneticin resistance gene cassette fusion
GzWing015_3R	TGGCTCAGTGACGTTGTTTAGGAG	
GzWing015_5N	CATCAGTCTCGTCTCGCTTTGTCC	Forward and reverse nest primers for third fusion PCR for amplification of *GzWING15* deletion construct
GzWing015_3N	GCCTTTGAGCGACAGATAGACACC	
GzWing015-GFP F	tatagggcgaattgggtactcaaattggttAGTACTTACTACAGGCCATTGATACAGATG	Forward and reverse primers for amplification of *GzWING15* open reading frame for GFP fusion protein construction under native promoter
GzWing015-GFP R	cccggtgaacagctcctcgcccttgctcacAAATTGTGTTGAAAAACTTCGGGGTAAT	

GzWing016_5F	CCAATACGTAAGAGGAGCACAGTT	Forward and reverse primers for amplification of 5' flanking region of *GzWING16* with tail for the geneticin resistance gene cassette fusion
GzWing016_5R	gcacaggtacacttgtttagagCGGGGACGTTGTTGATTTAGG	
GzWing016_3F	ccttcaatatcatcttctgtcgCTCAGAACCAAGGAGAACGAGCAC	Forward and reverse primers for amplification of 3' flanking region of *GzWING16* with tail for the geneticin resistance gene cassette fusion
GzWing016_3R	CTTCTCGCCAAAGCTGATGACAC	
GzWing016_5N	GTGGCTTGTGACCCTGTGGACT	Forward and reverse nest primers for third fusion PCR for amplification of *GzWING16* deletion construct
GzWing016_3N	GCTTTGCTGCTGCTGATGGTGA	
GzWing016-GFP F	tatagggcgaattgggtactcaaattggttCAACATGCTTTAGGGTGAG TGACG	Forward and reverse primers for amplification of *GzWING16* open reading frame for GFP fusion protein construction under native promoter
GzWing016-GFP R	cccggtgaacagctcctcgcccttgctcacTAGCCCGCCTGGGGCGAA CT	
GzWing018_5F	TGAGACAGAGATTACAACCAACGGAAC	Forward and reverse primers for amplification of 5' flanking region of *GzWING18* with tail for the geneticin resistance gene cassette fusion
GzWing018_5R	gcacaggtacacttgtttagagATTGTGGGGGTGCCGTGTATT	
GzWing018_3F	ccttcaatatcatcttctgtcgGACCCAAGAGCTCATCAAAGAAAA AG	Forward and reverse primers for amplification of 3' flanking region of *GzWING18* with tail for the geneticin resistance gene cassette fusion
GzWing018_3R	AGCAGACCTAAAGCTTTAGCACAGAAGT	
GzWing018_5N	GCCCATTCTACAGGCCTTGCTATTT	Forward and reverse nest primers for third fusion PCR for amplification of *GzWING18* deletion construct
GzWing018_3N	TGGAAGAGGGTACAAATATCGTTGAGG	
GzWing019_5F	CAGCAGTAGAACCGGACATTAGGG	Forward and reverse primers for

GzWing019_5R	gcacaggtacacttgtttagagGGTTTAAGGGGAGACGTTTGATGA	amplification of 5' flanking region of *GzWING19* with tail for the geneticin resistance gene cassette fusion
GzWing019_3F	ccttcaatatcatcttctgtcgATTTTGTTTGCACACGAAGACGAC	Forward and reverse primers for amplification of 3' flanking region of *GzWING19* with tail for the geneticin resistance gene cassette fusion
GzWing019_3R	AAATGATTGTCGTGGTTAGCAAAGG	
GzWing019_5N	AAGGATGCCCCCAGTGTCAGAA	Forward and reverse nest primers for third fusion PCR for amplification of *GzWING19* deletion construct
GzWing019_3N	GACCGACGAGAATAAATGAGCCCAC	
GzWing020_5F	TTCTAGTGATAGTTAGTAGCTGTGCCTGAGTCA	Forward and reverse primers for amplification of 5' flanking region of *GzWING20* with tail for the geneticin resistance gene cassette fusion
GzWing020_5R	gcacaggtacacttgtttagagTAGATTATCGCGAATGCCGACTACT	
GzWing020_3F	ccttcaatatcatcttctgtcgTGTAAGGCGGCGTGTATGAGAAG	Forward and reverse primers for amplification of 3' flanking region of *GzWING20* with tail for the geneticin resistance gene cassette fusion
GzWing020_3R	GAACCGTGTCTTGCGTGATAGAAAG	
GzWing020_5N	ACGAGGCGAGAGGTAAGGTGAGTAA	Forward and reverse nest primers for third fusion PCR for amplification of *GzWING20* deletion construct
GzWing020_3N	GAAAGCAACATAGTCGCGACAAAGAG	
GzWing020-GFP F	tatagggcgaattgggtactcaaattggttTATGGCTGATGTGAGGAGTAGAGGTG	Forward and reverse primers for amplification of *GzWing16* open reading frame for GFP fusion protein construction under native promoter
GzWing020-GFP R	cccggtgaacagctcctcgcccttgctcacCAGGAATCCCTTTGTAAAACTG	
GzWing027_5F	ATCAAACCATGCGTCAATCAGTGT	Forward and reverse primers for amplification of 5' flanking region of *GzWING27* with tail for the geneticin
GzWing027_5R	gcacaggtacacttgtttagagTGTGTTGAAGTCAAGGACGGTGTT	

		resistance gene cassette fusion
GzWing027_3F	ccttcaatatcatcttctgtcgACCAGTCAAACAACCCCTCCTC	Forward and reverse primers for amplification of 3' flanking region of *GzWING27* with tail for the geneticin resistance gene cassette fusion
GzWing027_3R	GTCTGGGATGAATAGGATGATGGG	
GzWing027_5N	GCAGTGACAACGACTATATGCTCCAACAA	Forward and reverse nest primers for third fusion PCR for amplification of *GzWING27* deletion construct
GzWing027_3N	TGGACAACTTTCAAACGATGGATG	
GzWing027-GFP F	tatagggcgaattgggtactcaaattggttGTCCAACCAACATCGGCAC ATT	Forward and reverse primers for amplification of *GzWING27* open reading frame for GFP fusion protein construction under native promoter
GzWing027-GFP R	cccggtgaacagctcctcgcccttgctcacGAGTCCCTGGTTGTTCCAA TTGTAG	
GzWing011-RT-F	TGGAAGAATGGAAGACCTGGAATAAG	For real-time PCR of *GzWING11*
GzWing011-RT-R	TATTGATGGGTAGTTTTCTCGCAGC	
GzWing012-RT-F	TGAAGCTGATCTCGGCACTGAAAC	For real-time PCR of *GzWING12*
GzWing012-RT-R	GTGTTGAGATGGCTGCTGTTCGTT	
GzWing013-RT-F	TTCGAAGCTCATCAAACCAATCACT	For real-time PCR of *GzWING13*
GzWing013-RT-R	CTGTGGTTGTGTAAGGCTGAGACG	
GzWing015-RT-F	ATAAAATCCGAGCCAATGACTGCG	For real-time PCR of *GzWING15*
GzWing015-RT-R	CATGGCAATCAAGTCGGCGTAAC	
GzWing016-RT-F	CGGAGAAATCAACACAGGCAACAG	For real-time PCR of *GzWING16*
GzWing016-RT-R	TGGGCGAAGCGTAAGGGAATC	
GzWing018-RT-F	GCAACAGGTTCAAGAGAGGACGG	For real-time PCR of *GzWING18*
GzWing018-RT-R	ATTGTGGGGGTGCCGTGTATT	

GzWing019-RT-F	ACAAAGAGGATACGGTGACGACAACT	For real-time PCR of *GzWING19*
GzWing019-RT-R	GGACATGGAGGACATGGTCTGAGT	
GzWing020-RT-F	TAGTTCTTTTTCTGCCATCGGACAT	For real-time PCR of *GzWING20*
GzWing020-RT-R	TCGATGTTGGCGTTCCACTCTTC	
GzWing027-RT-F	GAATCAGCAACAGCGGTCACATC	For real-time PCR of *GzWING27*
GzWing027-RT-R	CCACTGCTTGGGGAACTTTGATA	
MAT1-RT-F	CTGGAAGAACTGGGCATCGTAA	For real-time PCR of *MAT1-1-1*
MAT1-RT-R	GATATTCTTGTGGCTGGCTACTTT	
UBH-RT-F	GTTCTCGAGGCCAGCAAAAAGTCA	For real-time PCR of *UBH1*
UBH-RT-R	CGAATCGCCGTTAGGGGTGTCTG	

III. Sexual development assay and genetic crosses

To induce sexual reproduction, the wild-type and mutants were incubated on carrot agar at 25°C for 5 days (Leslie & Summerell, 2006). Mycelia grown on carrot agar were mock-fertilized with 700 µl of a 2.5% Tween 60 solution to induce sexual reproduction and incubated under near-UV light (wavelength: 365 nm; Sankyo Denki, Tokyo, Japan) at 25°C for 7-10 days.

For outcrosses, the female strain grown on carrot agar was fertilized with 1 ml of conidial suspension from a male strain (106 conidia/mL) at 5 days after inoculation. All fertilized cultures were incubated at 25°C under near-ultraviolet light (wavelength, 365 nm; HKiv Import and Export Co., Ltd., Xiamen, China). Perithecia were observed 7 days after fertilization, and ascospores were observed 9 days after sexual induction. Microscopic observation of ascospore was performed with an M6 B microscope (Leica Microsystems, Wetzlar, Germany) with L5 fluorescence filter (excitation 480/40 nm; emission 527/30 nm)

IV. Microscopic observation

Microscopic observation of perithecia was performed with a DE/Axio Image A1 microscope (Carl Zeiss, Oberkochen, Germany). To observe the morphology of

asci and ascospore, the perithecia from each strain were dissected on slide glasses in a drop of 15% glycerol, and then the asci were softly flattened under a coverslip (Son et al., 2012). Fluorescence microscopic images were obtained using a DE/Axio Imager A1 microscope with the filter set38HE (excitation 470/40; emission 525/50) for GFP and the filter set 49 (excitation 356; emission 445/50) for CMAC.

V. Specimen preparation for scanning electron microscopy (SEM)

To observe the surface structure during sexual reproduction, the wild-type and deletion mutants were grown on carrot agar under near-UV light at 25°C for 7-10 days and specimen preparation for scanning electron microscopy. To preserve the structure, a vapor fixation procedure was used (Kim, 2008). The inoculated plates were placed upside down in a well-ventilated fume hood. The inside of the plate lid was filled with 2% (w/v) osmium tetroxide and kept closed at room temperature (22–24°C) for 8 hours. Agar blocks (approximately 1-2 mm thick) were then excised using a razor blade and mounted on a metal stub. They were sputter-coated with gold and observed with a scanning electron microscope (FE-SEM, Zeiss Sigma, Carl Zeiss, Germany) operating at an accelerating voltage of 4 kV.

VI. Reverse transcription PCR and quantitative real-time PCR

Total RNA was extracted from vegetative cultures at 5 days after inoculation and sexual cultures at 0, 1, 3, 5, and 7 days after sexual induction. First-strand cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) with Oligo(dT)20 primers. qRT-PCR was performed with the CFX Real-Time PCR System (Bio-Rad) using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). For normalization, the endogenous housekeeping gene ubiquitin C-terminal hydrolase (*UBH1*) was used as an internal control. Relative expression levels were calculated through the 2-ΔΔCT method (Livak & Schmittgen, 2001). qRT-PCR assays were repeated three times, each with three biological replicates.

RESULTS

I. Several winged-helix TFs are important for vegetative growth

Nine winged-helix gene deletion mutants were obtained by targeted gene deletion and confirmed through Southern hybridizations (Fig. 1). I compared vegetative growth of the Δ*gzwing11*, Δ*gzwing12*, Δ*gzwing13*, Δ*gzwing15*, Δ*gzwing16*, Δ*gzwing18*, Δ*gzwing19*, Δ*gzwing20*, Δ*gzwing27*, and the wild-type on complete medium (CM) and minimal medium (MM). As previously reported (Son et al., 2011), the growth rates of the Δ*gzwing15*, Δ*gzwing16*, Δ*gzwing18*, Δ*gzwing19,* and Δ*gzwing20* were significantly reduced in comparison with the wild type (Fig. 2), especially in Δ*gzwing19*. The results indicate that these five genes are important for the vegetative growth of *F. graminearum.* The defective phenotypes caused by deletion were restored in the complemented strains.

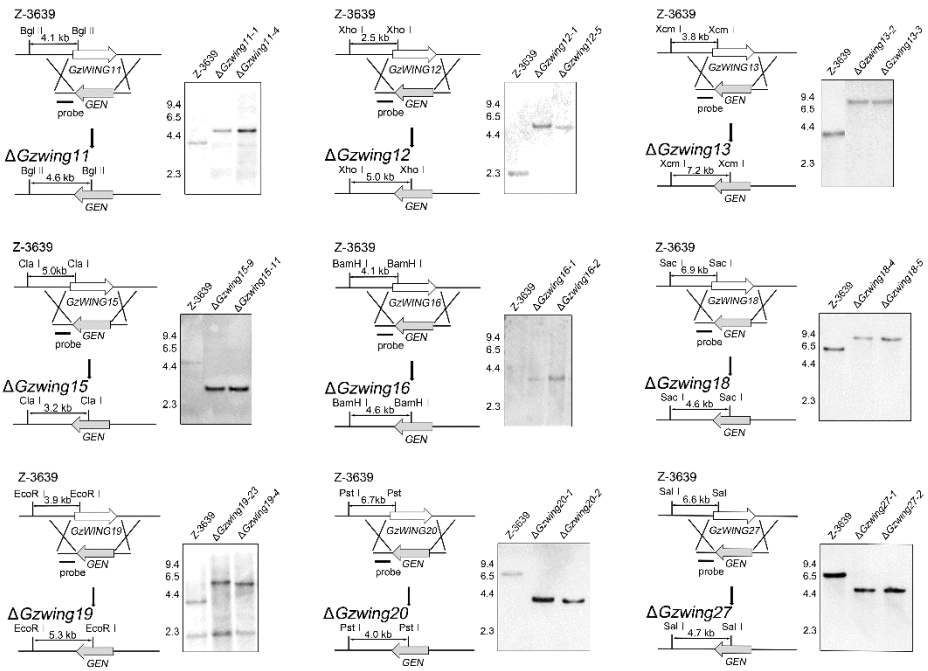

Figure 1. Targeted deletion of winged-helix genes.

Targeted gene deletion. Each of 9 genes was individually deleted from the genome of *F. graminearum* wild-type strain Z-3639. The restriction enzymes used for each blot and the size of the DNA standards (kb) are indicated to the left of the blot. *GEN*, geneticin resistance gene cassette.

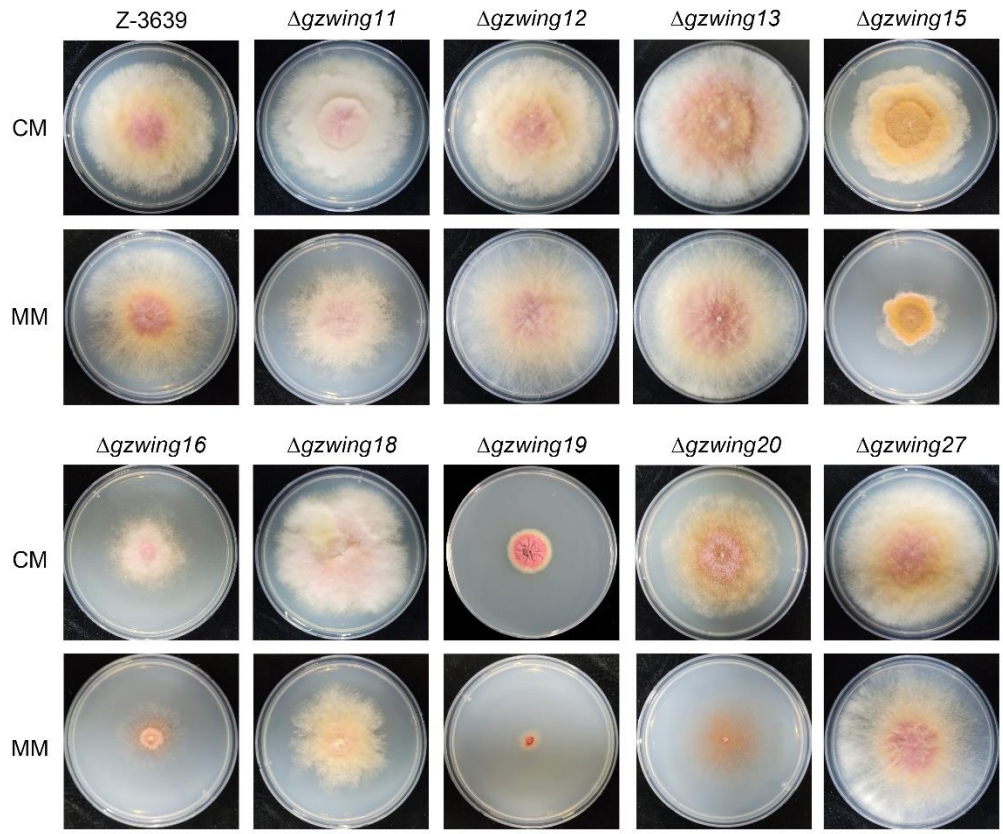

Figure 2. Vegetative growth of winged-helix gene deletion mutants.

Radial growth of the wild-type and mutants on complete medium (CM) and minimal medium (MM). Photographs were taken 5 days after inoculation.

II. Nine winged-helix TFs are involved in sexual reproduction

As described in previous studies, all the winged-helix deletion mutants were self-sterile and defective in perithecia formation and/or ascospore development. Seven days after sexual induction, Δ*gzwing11*, Δ*gzwing18*, Δ*gzwing19*, and Δ*gzwing20* mutants failed to produce perithecia (Fig. 3A), and Δ*gzwing13*, Δ*gzwing15*, Δ*gzwing16*, and Δ*gzwing18* mutants produced defective perithecia with no ascospore, while the wild-type strains produced mature perithecia and asci with ascospore (Fig. 3A and B).

A unique structure was observed in the sexual reproduction process of Δ*gzwing11* mutants (Fig. 4A and B). An immature perithecium-like shape was observed on the inside of the carrot agar, and the surface was covered with asexual spores, conidia (Fig. 4B). This showed that sexual and asexual structures were simultaneously observed during sexual reproduction.

Δ*gzwing12* mutants developed normal perithecia but produced an abnormal ascospore in comparison with the wild-type (Fig. 5A). Also, the number of septa present in each of the Δ*gzwing12* mutant ascospores was reduced compared with the wild-type (Fig. 5B). While 83% of the wild-type ascospores had 2 to 3 septa, 50% of the Δ*gzwing12* mutant ascospores had 0 to 1 septum.

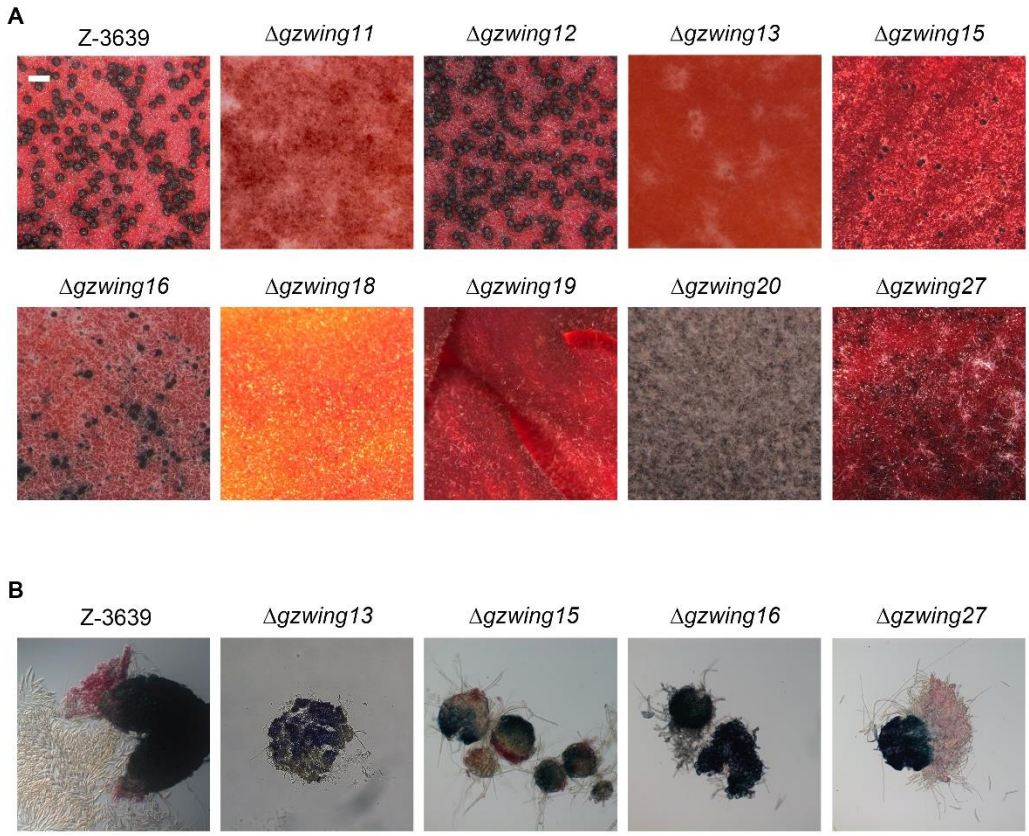

Figure 3. Sexual development of winged-helix gene deletion mutants.

A. Perithecia formation. Photographs were taken at 7 days after induction. Scale bars = 500 μm. B. Perithecia and ascospores. The structures were squeezed with a coverslip.

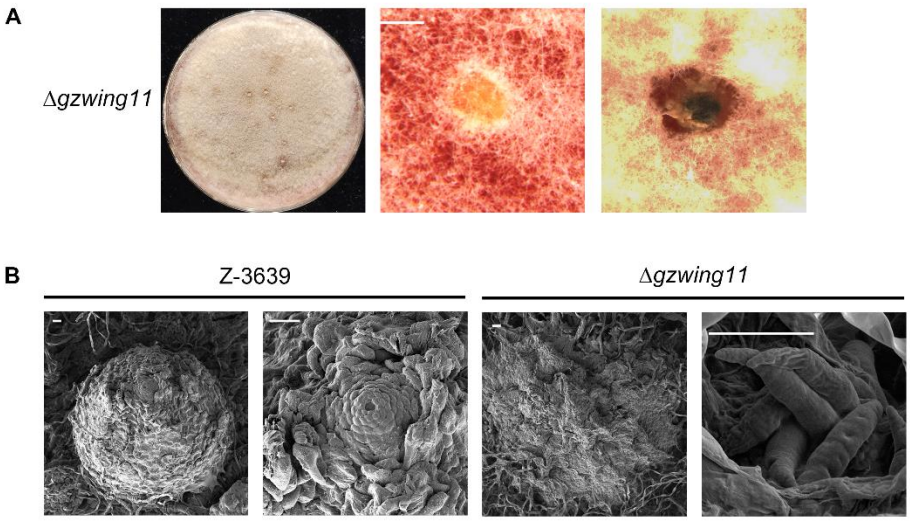

Figure 4. Sexual development of Δ*gzwing11* mutant.

A. Structures observed during induction of sexual reproduction. Photographs were taken at 10 days after induction. The inside of the carrot medium was observed after removing the surface of the agar using a surgical knife (right panel). Scale bars = 1000 μm. B. Scanning electron microscopic images of wild-type and Δ*gzwing11* mutant. Photographs were taken at 10 days after induction. Scale bars = 10 μm.

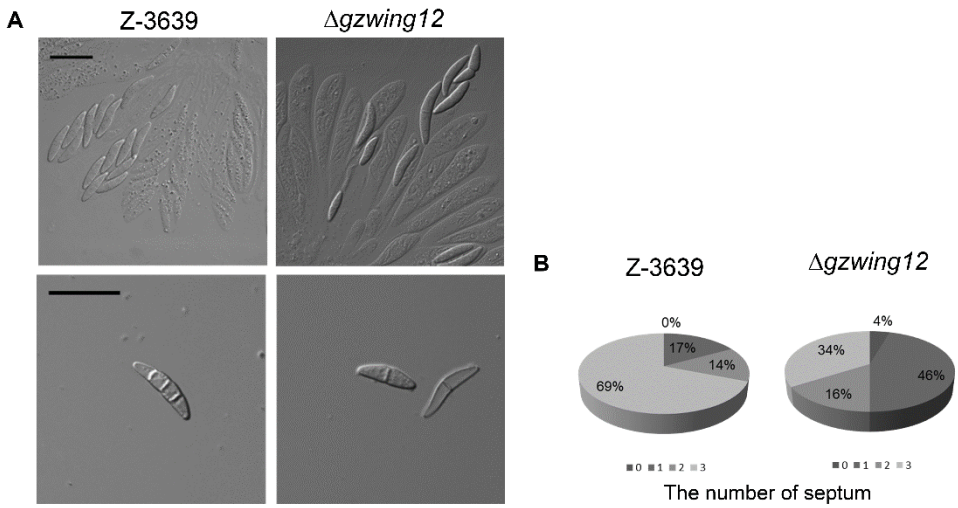

Figure 5. Ascospores of Δ*gzwing12* mutant.

A. Ascospores of wild-type and Δ*gzwing12* mutant. Scale bars = 20 μm. B. The number of septum of wild-type and Δ*gzwing12* mutant ascospores.

III. Δ*gzwing15*, Δ*gzwing16*, Δ*gzwing20*, and Δ*gzwing27* had both female and male fertilities

To determine whether self-sterile winged-helix deletion mutants retain the female and male ferilities, extensive outcross analyses were performed. When the Δ*gzwing15*, Δ*gzwing16*, Δ*gzwing20*, and Δ*gzwing27* mutants were mated as the female, normal mature perithecia were produced, and eight ascospores in each ascus showed 1:1 segregation for GFP signals according to Mendelian genetics (Fig. 6). Also, the same results were observed when all winged-helix deletion mutants were used as male strains (Fig. 7). These results suggest that *GzWING15, GzWING16, GzWING20*, and *GzWING27* are not tied to the role as female or male in fertility, but required for the self-fertility.

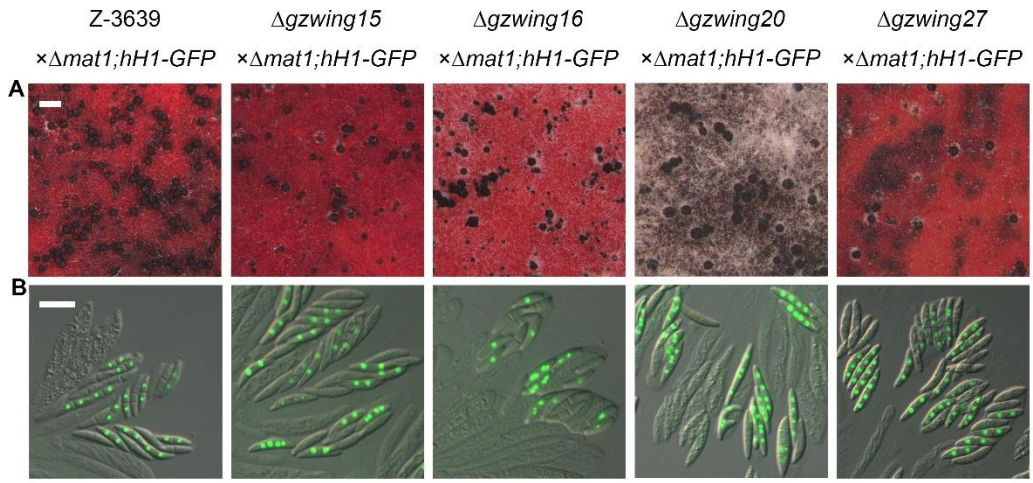

Figure 6. Analysis of female fertility of winged-helix gene deletion mutants.

A. For outcrosses, mycelia of the female strain grown on carrot agar media were fertilized with 1 ml of male strain conidial suspension. The heterothallic strain (Δ*mat1-1; hH1-GFP*) was used as a tester strain for outcrosses. Perithecia and ascospores were observed 10 days after fertilization. Scale bars = 500 μm. B. Eight ascospores of an ascus from Δ*gzwing15*, Δ*gzwing16*, Δ*gzwing20*, or Δ*gzwing27* × Δ*mat1-1;hH1-GFP* outcross segregated into 1:1 with and without GFP tagged histone H1. Scale bars = 20 μm.

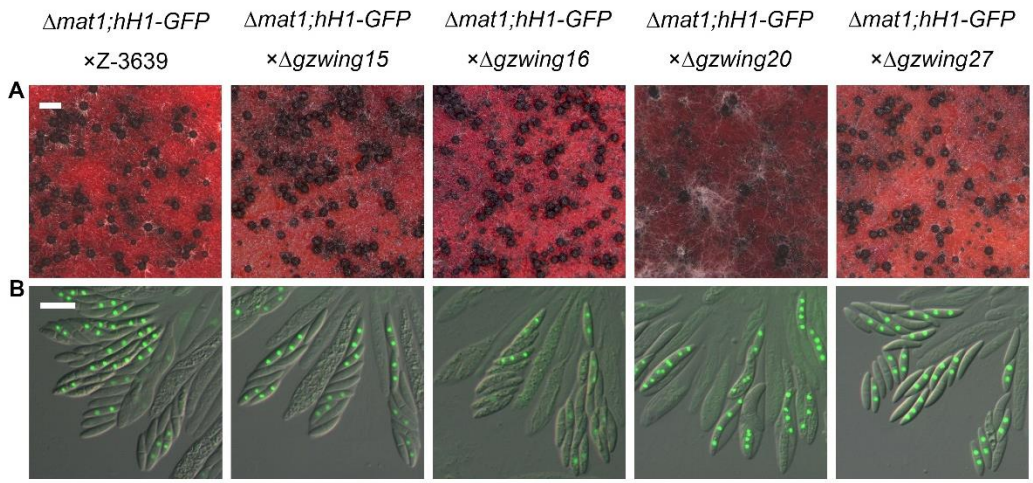

Figure 7. Analysis of male fertility of winged-helix gene deletion mutants.

A. The heterothallic strain (Δ*mat1-1; hH1-GFP*) was used as a female strain. Scale bars = 500 μm. B. Eight ascospores of an ascus from Δ*mat1-1*;*hH1-GFP* × Δ*gzwing15*, Δ*gzwing16*, Δ*gzwing20*, or Δ*gzwing27* outcross segregated into 1:1 with and without GFP-tagged histone H1. Scale bars = 20 μm

IV. Winged-helix genes regulate each other and mating-type genes during sexual reproduction

To determine the expression profiles of *GzWING15, GzWING16, GzWING20*, and *GzWING27* and to figure out their genetic relationship with mating-type genes during sexual development, the relative transcript levels of the genes were assayed by qRT-PCR. RNA was extracted from the aerial hyphae corrected 5-day-old carrot agar and fungal biomasses scrapped off carrot agar 0, 1, 3, 5, and 7 days after sexual induction. In the wild-type, the transcript levels of *MAT1-1-1* and *MAT1-2-1* peaked one day after sexual induction (Fig. 8A and B). In contrast, the transcript levels of *MAT1-2-1* continues to increase in the Δ*gzwing15*, Δ*gzwing16*, Δ*gzwing20*, and Δ*gzwing27* compared with that of the wild-type (Fig. 8B). Also, the expression pattern of *MAT1-1-1* in Δ*gzwing15* and Δ*gzwing27* was different from that of the wild-type during sexual development (Fig. 8A)

The transcript level of *GzWING27* peaked at the early stage of sexual development (1 day) and then gradually declined in wild-type (Fig. 8F). However, in the Δ*gzwing15*, Δ*gzwing16,* and Δ*gzwing20* mutants, the transcript level of *GzWING27* was continuously increased, suggesting that the expression of *GzWING27* may be negatively regulated by *GzWING15, GzWING16, GzWING20* in the late stage of sexual reproduction.

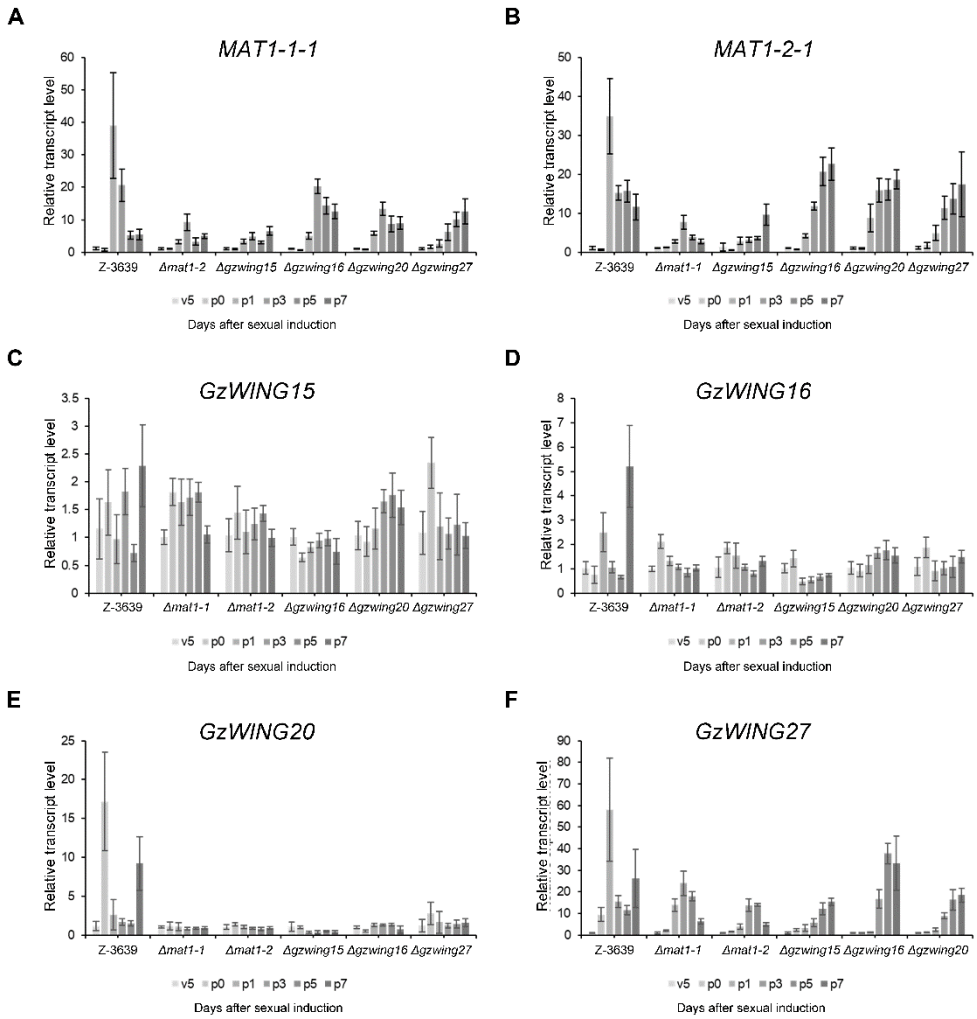

Figure 8. Genetic relationship between winged-helix genes and mating-type genes.

A-F. Transcript levels of winged-helix genes and mating-type genes during sexual development of wild-type and each mutant.

DISCUSSION

Sexual reproduction is an important developmental process for genetic diversity, stability, and pathogenicity in fungi (Lee et al., 2012; Son et al., 2011; Urban et al., 2003). However, understanding of the molecular mechanisms underlying sexual developmental stages is still limited.

Previous studies have reported that several putative winged-helix transcription factors are involved in sexual reproduction (Son et al., 2011). As reported, the deletion of nine winged-helix genes resulted in defects in sexual reproduction. Deletion of *GzWING13*, *GzWING15*, *GzWING16*, and *GzWING27* resulted in the formation of immature perithecia and failed to produce ascospores, suggesting that these genes are involved in perithecia development and maturation. The remaining deletion mutants did not produce even immature ascospores, so these four genes (*GzWING11*, *GzWING18*, *GzWING19*, and *GzWING20*) should be involved in early stage of sexual development. During the sexual reproduction of Δ*gzwing11*, perithecium-like structures (sexual reproduction structures) and conidia (asexual spores) were simultaneously observed. Pathogens generally display alternating sexual and asexual stages depending on the environment (McDonald & Linde, 2002a, 2002b). Therefore, *GzWING11* is expected to be involved in determining sexual and asexual stages. Half of the ascospores produced by the Δ*gzwing12*

mutants had an abnormal number of septa. This suggests that *GzWING12* may be involved in spore delimitation.

The sexual ability of *F. graminearum* is determined by master regulators called the mating-type (*MAT*) gene (Debuchy & Turgeon, 2006). As a homothallic fungus, *F. graminearum* carries *MAT1-1* and *MAT1-2* in a single nucleus for controlling sexual development. Thus, in this study, the Δ*mat1-1; hH1-GFP* strains were used to determine whether self-sterile winged-helix deletion mutants retain the female and male fertilities. Most self-sterile deletion mutants are female sterile. Interestingly, same as the mating-type gene (Kim et al., 2012; Lee et al., 2003), four winged-helix gene deletion mutants (Δ*gzwing15*, Δ*gzwing16*, Δ*gzwing20*, and Δ*gzwing27*) had both female and male fertilities.

The relationship between these genes was figured out through analysis of transcription levels during sexual reproduction. Transcription of *MAT1-2-1* continuously increased in Δ*gzwing15*, Δ*gzwing16*, Δ*gzwing20* and Δ*gzwing27*, and *MAT1-1-1* showed a similar pattern in Δ*gzwing15* and Δ*gzwing27*. Moreover, the expression of *GzWING27* continuously increased in Δ*gzwing15*, Δ*gzwing16*, and Δ*gzwing20*. These results indicate that *GzWING15, GzWING16, GzWING20*, and *GzWING27* negatively regulate *MAT1-2-1*, and *GzWING15* and *GzWING27* negatively regulate *MAT1-1-1*. Also, *GzWING15, GzWING16*, and *GzWING20*

negatively regulate *GzWING27*. In conclusion, these data suggest that winged-helix genes regulate each other and mating-type genes during sexual reproduction. And these four genes were expected to have essential roles in sexual reproduction by constructing genetic networks with mating-type genes. The results of this study will contribute to the understanding of molecular genetic regulation of sexual development in fungi.

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붉은곰팡이의 유성생식과정에 관여하는 winged-helix 전사조절인자의 유전적 상관관계 연구

황보아람

초록

붉은곰팡이(*Fusarium graminearum*)는 전 세계적으로 주요작물에 병을 일으켜 경제적 손실을 일으키고 인축에 유해한 곰팡이 독소를 생산하는 중요한 식물병원성 곰팡이이다. 붉은곰팡이는 유성생식을 통해 유전적 다양성을 획득하고 병 전파에 있어 일차전염원으로 작용하는 자낭포자를 생산한다. 유성생식과정에서 Mating-type 유전자가 주요 조절자 역할을 하는 것으로 알려져 있지만, 유성생식과정의 분자생물학적 메커니즘에 대한 연구는 여전히 부족하다. Winged-helix 전사조절인자는 진핵 생물에서 발생 및 세포분화의 주요 조절자이다. 붉은곰팡이는 28개의 winged-helix 전사조절인자를 가지고 있다고 밝혀져 있다. 본 연구에서

는 유성생식과정에 관련되어 있을 것으로 추정되는 9개의 전사조절인자의 기능을 조사하고, 유성생식과정에서 mating-type 유전자와의 유전적관계를 분석하였다. Winged-helix 유전자의 결손변이체, 형질복원체, 과발현체를 제작하여 이들의 표현형을 분석하였다. 모든 결손변이체는 유성생식과정에서 결함을 보였다. 또한 outcross를 통해 *GzWING15*, *GzWING16*, *GzWING20*, *GzWING27* 결손변이체가 mating-type 유전자와 동일하게 female과 male 생식능력을 모두 가지고 있음을 확인하였다. 또한 유성생식과정에서의 전사량 분석을 수행하여 winged helix 유전자와 mating-type 유전자의 관련성을 확인하였다. 본 연구의 결과를 통해 winged helix 유전자가 유성생식과정과 mating-type 유전자에 관련이 있음을 확인하였고, 이들이 유전적 네트워크를 구축하여 유성생식에서 중요한 역할을 할 것이라 추정할 수 있다.

주요어: 붉은곰팡이, Winged-helix 전사조절인자, 유성생식

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