농학 석사 학위논문

Fusarium graminearum 의
전사조절인자 FgZC248 의 기능연구

Transcription factor gene *fgzc248* is involved in DNA damage response in *Fusarium graminearum*

2014 년 8 월

서울대학교 대학원
농생명공학부 식물미생물학전공
부민민

A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

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BY

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The Graduate School of Seoul National University

August 2014

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UNDER THE DIRECTION OF
DR. YIN-WON LEE

SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF SEOUL NATIONAL UNIVERSITY

BY
MINMIN FU

MAJOR IN PLANT MICROBIOLOGY
DEPARTMENT OF AGRICULTURAL BIOTECHNOLOGY

AUGUST 2014

APPROVED AS A QUALIFIED THESIS OF MINMIN FU
FOR THE DEGREE OF MASTER OF SCIENCE
BY THE COMMITTEE MEMBERS

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MINMIN FU

Abstract

Fusarium graminearum is an important plant pathogen which causes *Fusarium* head blight in cereal crops. The maintenance of genome integrity plays important role in the survival or virulence of the plant pathogen since DNA acts as the carrier of genetic information. Transcription factors (TFs) orchestrate gene expression under the control of various cellular signaling pathways and therefore function as key mediators of cellular functions. To identify TFs related to DNA damage responses in *F. graminearum*, four different DNA damage agents were used to screen the previously generated *F. graminearum* transcription factor mutant library. Sixteen transcription factor gene deletion mutants showed altered sensitivity to at least one DNA damage agent. Among them, the *fgzc248* deletion mutant was specifically sensitive to hydroxyurea than the wild-type strain. Moreover, deletion

of *fgzc248* resulted in defects in virulence and sexual development. Result of the alkaline comet assay demonstrated that disruption of *fgzc248* function resulted in DNA damages in cells. Levels of transcript accumulation of DNA repair genes increased in the *fgzc248* deletion mutant compared with wild-type. This study is the first characterizing a novel transcription factor FgZC248 involved in DNA damage response and will provide new insights in mechanisms underlying DNA damage responses in *F. graminearum*.

Key word: DNA damage response, *Fusarium graminearum*, transcription factor, hydroxyurea.

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. Introduction

Fusarium graminearum (teleomorph: *Gibberella zeae*) is a plant pathogen which causes *Fusarium* head blight on major cereal crops (Bai and Shaner, 2004). It causes high yield and quality losses all over the world due to sterility of florets and formation of discolored, withered and light test-weight kernels (Goswami and Kistler, 2004). Contamination of the grain with fungal mycotoxins endowed with toxic effects to both animals and human beings also bring a lot of damage (Placinta *et al.*, 1999; McMullen *et al.*, 1997).

DNA functions as the carrier of genetic information and therefore genome integrity and stability are essential for all organisms (Goldman *et al.*, 2002). DNA is continually subjected to damage from both intrinsic and extrinsic sources and DNA damages results in single-strand and double-strand breaks, base damage, and DNA-protein crosslinks (Goldman and Kafer, 2003). If left unrepaired or incorrectly repaired, DNA damage will result in cell cycle arrest, cell death, loss of genetic information, and genomic instability (Stephen *et al.*, 2009; Goldman and Kafer, 2003). To deal with the fundamental problem of genomic erosion, a protective mechanism DNA damage-response (DDR) system has evolved to detect DNA damages and to promote repair of damaged DNA (Harper and Elledge, 2007; Rouse and Jackson, 2002; Harrison and Haber, 2006). Tolerance of genotoxic

lesions, cell-cycle checkpoint pathways, and DDR ensures the maintenance of genome integrity and stability by promoting a set of DNA repair mechanisms (Giglia-Mari *et al.*, 2010). Moreover, DNA replication, gene transcription, DNA repair, and cell cycle checkpoint should be interlinked to assure cell survival and normal regulation of cellular functions following DNA damages (Levitt and Hickson, 2002).

The objective for this study was to characterize TFs related to DNA damage responses in *F. graminearum*. I screened the previously generated *F. graminearum* transcription factor mutant library using four different DNA damage agents and found that Δ*fgzc248* is highly sensitive to hydroxyurea. To elucidate regulatory mechanisms of FgZC248, I analyzed transcriptome by RNA-seq. This is the first characterizing a novel transcription factor FgZC248 involved in DNA damage response and will provide new insights in mechanisms underlying DNA damage responses in *F. graminearum*.

II. Materials and Method

1. Strains and culture conditions

The *F. graminearum* strains used in this study were generated from previous study (Son *et al*., 2011). Cultures were maintained in complete media (CM; Leslie and Summerell, 2006) agar. Conidia were produced in carboxymethyl cellulose medium (CMC; Capellini and Peterson, 1965), or on yeast malt agar (YMA; Harris, 2005). All the media used in this study were prepared as described in the *Fusarium* laboratory manual (Leslie and Summerell, 2006). The growing temperature of the fungal strains was set at 25 ℃. All strains were stored as conidia and mycelia in 30% glycerol solution at -80 ℃.

DNA-damage agents used in the study were methyl methanesulfonate (MMS), hydroxyurea (HU), bleomycin (BLM), and camptothecin (CPT). The concentration of each DNA-damage agent was established in which the wild-type strain showed about one half of the radial growth observed on CM medium without any DNA damage agent (Table 1). CM supplemented with each DNA damage agent was used to screen 657 transcription factor gene deletion mutants. Statistical analyses were conducted using *R* statistical software packages (Core Team R, 2012).

Table 1 DNA damage agents used in the study

DNA damage agents	Mechanism	Results
MMS 0.1 μl/ml (Methyl methanesulfonate)	Methylates DNA	DNA synthesis defects
HU 10 mM (Hydroxyurea)	Inhibits ribonucleotide reductase (RNR)	S-phase arrest
BLM 10 mU/ml (Bleomycin)	Imitates gamma irradiation	Double-strand DNA breaks
CPT 0.4 μM (Camptothecin)	Locks topoisomerase I covalently onto the DNA	S-phase strand breaks

2. Nucleic acid manipulations , PCR primers and conditions

Genomic DNA was extracted from lyophilized mycelia according to the *Fusarium* laboratory manual (Leslie and Summerell, 2006). Restriction endonuclease digestion, agarose gel electrophoresis, Southern blotting, and hybridization with ^{32}P-labeled probes were performed following standard protocols (Sambrook and Russell, 2001). Total RNA was isolated from mycelia ground in liquid nitrogen using the Easy-Spin Total RNA Extraction Kit (Intron Biotech, Seongnam, Korea). PCR and quantitative real-time (qRT)-PCR primers used in this study were synthesized by oligonucleotide synthesis facility (Bionics, Seoul, South Korea) (Table 2 and 3). General PCR procedures were performed in accordance with the manufacturer′s instructions (TaKaRa Bio, Inc., Otsu, Japan).

Table 2 Fungal transformation primers used in this study

Primer	Sequence	Purpose
Gen-For	CGACAGAAGATGATATTGAAGG	For amplification of *gen* cassette from pII99 vector
Gen-Rev	CTCTAAACAAGTGTACCTGTGC	
FgZC248-5F	TGGACTCATCGGGCACTAACAAT	For *fgzc248* deletion
FgZC248-5R	GCACAGGTACACTTGTTTAGAGAGTTGGAAACAGGAGAAAGAAACAGAA	
FgZC248-3F	CCTTCAATATCATCTTCTGTCGCTCATTGTGCCTGATTCCCCC	
FgZC248-3R	AAGAAGCGTCCCCATACTCTAACACT	
FgZC248-5N	ATTGGACACCACTGTCCTGTTTCCCTA	
FgZC248-3N	TTGCCAAGAGCATGTTTCTGGACTTT	
pII99-G3	CAATAGCAGCCAGTCCCTTCCC	
pII99-G2	AGTGGATCCTCAGAAGAACTCGTCAA	

pIGPAPA-sGFP	GTGAGCAAGGGCGAGGAGCTG	
Hyg-F1	GGCTTGGCTGGAGCTAGTGGAGG	
FgZC248-ORF-Rev	GAACAGCTCCTCGCCCTTGCTCACGTCGGGGGAATCAGGCACAA	For *fgzc248* complementation
FgZC248-3F-GFP	CCTCCACTAGCTCCAGCCAAGCCGCAACTGCATGTAGCCACGACG	
pU,pBC-H3	CGTTATGTTTATCGGCACTTTGC	
pIGPAPA-H2	TCGCTCCAGTCAATGACCGC	

3. Genetic manipulations and fungal transformations

Double-joint (DJ) PCR strategy was applied to construct fusion PCR products for targeted gene deletion and complementation (Yu *et al.,* 2004). For gene deletion, the 5′ and 3′ flanking regions of *fgzc248* were amplified from the genomic DNA of the wild-type strain using primer pairs FgZC248-5F/FgZC248-5R and FgZC248-3F/FgZC248-3R, respectively. A geneticin resistance gene cassette (*gen*) under the control of the *Aspergillus nidulans trpC* promoter and terminator was amplified from pII99 (Namiki, 2001) using a primer pair Gen-for/Gen-Rev. Three amplicons (5′ flanking: *gen*: 3′ flanking amplicons) were mixed at 1:3:1 molar ratio and fused by second round of DJ-PCR. Finally, the fusion constructs were amplified with the nested primers using a second round PCR product as a template. For complementation, 5′ flanking region that included the *fgzc248* ORF with its own promoter was amplified from the genomic DNA of the wild-type strain using primers FgZC248-5F/FgZC248-ORF-Rev and 3′ flanking region was amplified from genomic DNA of wild-type with FgZC248-3F-GFP/FgZC248-3R, respectively. The *gfp-hyg* was amplified form pIGPAPA (Horwitz, 1999) using pIGPAPA-sGFP/Hyg-F1 primers. Three amplicons were then fused by a second round of DJ-PCR. Finally, the fusion constructs for transformation were amplified with the nested primers using a second round PCR product as a template (Lin *et al.*,

2011).

For fungal transformation, conidia were harvested 3-4 days after inoculation in CMC medium and freshly harvested conidia were inoculated into 50 ml YPG (1% yeast extract, 1% peptone, and 2% glucose) for 12 h at 25 ℃. Mycelia were harvested through sterilized filter paper and incubated in 35 ml NH_4Cl containing 10 mg/ml Driselase (Sigma-Aldrich, St. Louis, MO, USA) for 3 h at 30 ℃ with 50 rpm shaking to generate protoplasts. Polyethylene glycol (PEG)-mediated fungal transformation was applied for deletion and complementation. Fungal transformants carrying the *gen* or *hyg* were regenerated in regeneration medium and overlay with 1% water agar containing geneticin (150 μg/ml; Sigma-Aldrich, St. Louis, MO, USA) or hygromycin B (150 μg/ml; Calbiochem, La Jolla, CA, USA) respectively. After 2-5 days incubation, antibiotic-resistant colonies were selected for further studies.

4. Sexual crosses

For self-fertilization, cultures were grown on carrot agar plates for 5 days. Sexual reproduction was induced by removing aerial mycelia with sterile 2.5% Tween 60 solution (Leslie and Summerell, 2006).

5. Virulence test

For virulence test, the point inoculation on wheat cultivar Eunpamil′s heads was carried out as previously described (Lee *et al*., 2009). The conidia used for inoculation were harvested from CMC cultures and suspend in 0.01% Tween 20 solution at 10^6 spores/ml. After inoculation of 10 μl conidia suspension at the middle of the spikelet, inoculated plants were incubated in high humidity chamber at 25 ℃ for 3 days and transferred to a greenhouse for additional 11 days to check the disease symptoms. Five replicated inoculations per strain and three independent mutant strains were used in the experiment.

6. Quantitative real-time PCR (qRT)-PCR

Freshly harvested conidia from CMC were inoculated into 50 ml liquid CM and incubated for 24 h at 25 ℃ with shaking. Then, harvested mycelia were inoculated into CM or CM supplemented with HU (10 mM) for additional 2 h. Total RNA was isolated from mycelia that were ground in liquid nitrogen using an Easy-Spin total RNA extraction kit (Intron Biotech), each first-strand cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen).

Quantitative real-time PCR (qRT-PCR) was performed with SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and a 7500 real-time PCR system

(Applied Biosystems, Foster City, CA, USA) with corresponding primer pairs (Table 4). Ubiquitin C-terminal hydrolase (*ubh*; FGSG_01231.3) (Kim and Yun, 2011) was used as a reference gene. We compared the cycle threshold ($2^{-\Delta\Delta CT}$) to measure the transcript levels of target genes in different conditions (Livak and Schmittgen, 2001). The PCR was performed three times with two replicates per run.

7. Alkaline comet assay

Each strain was inoculated in 50 ml CM media and incubated at 25 ℃ for 5 days. Mycelia were harvested through sterilized filter paper and subcultured on YMA in dark to induce conidia production. Freshly harvested conidia were incubated in 50 ml YPG for 12 h with shaking. Mycelia were harvested through sterilized filter paper and incubated in 35 ml of 1M NH_4Cl containing 10 mg/ml Driselase at 30 ℃ to generate protoplast (Lee *et al.*, 2002). For HU treatment, the protoplasts were generated in Driselase solution supplemented with 10 mM HU. Protoplasts were collected by centrifugation after 4 h incubation, resuspended in 1 M NH_4Cl (2×10^4 cells/ml).

Low-gelling-temperature agarose (1%, Sigma-Aldrich) was molten in 1 M NH_4Cl and kept in 40 ℃. To improve agarose adhesion, we score the edges of dust-free

frosted-end microscope slides using a diamond-tipped pen. Then dipping the slides into molten 1% agarose and wiping one side clean and air-dry to prepare agarose-precoated slides. 0.4 ml of protoplasts suspension was mixed with 1.2 ml low-gelling-temperature agarose at 40 ℃ in a 5 ml plastic disposable tube. Then 1.2 ml agarose mixtures were poured to a pre-coated slide and allowed to gel. Slides were submerged in alkaline lysis condition to detect of DNA single-strand breaks, double-strand breaks and alkali-labile sites in the DNA, in accordance with a standard protocol. After overnight lysis in dark, the slides were washed 3 times in alkaline rinse solution for 20 minutes each to ensure removal of salt and detergent. After wash, submerge slides in fresh alkaline rinse solution in electrophoresis chamber. Electrophoresis for 25 min at a voltage of 0.6 V/cm. Remove slides from electrophoresis chamber and rinse in 400ml distilled water and stained in ethidium bromide solution, rinsed again with distilled water and dried in room temperature. Before fluorescence microscopy the dried agarose will be rehydrated. (Olive and Banath, 2006)

Use CometScore image analysis software to analysis the DNA damage in individual cell through the percentage of DNA in tail. The pictures we use for analysis were taken on an Axio Image A1 microscope with a CCD camera and the 550-nm/605-nm filter set.

8. RNA-seq analysis

Wild-type and Δ*fgzc248* conidia were harvested from CMC culture and inoculated into CM for 24 h with shaking. Then, harvested mycelia were divided and inoculated into CM and CM supplemented with HU (10 mM) for additional 2 h. Total RNA was extracted as stated above from mycelia that were ground in liquid nitrogen. RNA-sequencing libraries were constructed using the Illumina TruSeq RNA sample preparation kit in accordance to the standard low-throughput protocol. Samples were run on an Illumina HiSeq2000 instrument using the reagents provided in the Illumina TruSeq paired-end (PE) cluster kit V3-cBot-HS and the TruSeq SBS kit v3-HS (200 cycles).

Genome-wide transcript levels of genes were quantified in reads per kilobase of exon per million mapped sequence reads (RPKM) (Mortazavi *et al*. 2008). When the RPKM value was 0, it was changed to 1 to calculate the fold change of the transcript level. Genes for which differential transcript levels were detected were functionally characterized using the Munich Information Centre for Protein Sequences FunCat functional classification and annotation system (Ruepp *et al.*, 2004). Over- and underrepresentation analysis of FunCat categories was performed by using Pearson's χ^2 test. RNA-seq was performed three replications for each sample.

Table 3 Primers used in qRT-PCR

Primer	Sequence	Purpose
Ubh RT-F	GTTCTCGAGGCCAGCAAAAAGTCA	Forward and reverse primers for qRT-PCR of reference gene *Ubh*
Ubh RT-R	CGAATCGCCGTTAGGGGTGTCTG	
FgZC248 RT-F	CATGGTGTTCCTTGCGATTTCA	Forward and reverse primers for qRT-PCR of gene *fgzc248*
FgZC248 RT-R	AAGTTGACTGTGGGTGCTGGTGA	
FGSG_01132 RT-F	GGCTCATCAAAACCTGCGAAAAA	Forward and reverse primers for qRT-PCR of selected genes involved in DNA damage response and cell cycle.
FGSG_01132 RT-R	TCCCAGTAGCGGTGTCTTCATCC	
FGSG_05924 RT-F	GGGATAACCGTGGTGCCGAT	
FGSG_05924 RT-R	ATCCTCATCCTCCCCGTTCTCC	
FGSG_10050 RT-F	TGGCGACCTCAAGAAGCAGAAG	
FGSG_10050 RT-R	CCTCAGTGCGGTTGGGGTCA	
FGSG_13552 RT-F	TCATGGATCGAACGAGGCAACT	
FGSG_13552 RT-R	CTGTTGGCGTCGAGAAGAGGAA	
FGSG_16955 RT-F	GATCAAAACCCAACTCCAGCACAA	
FGSG_16955 RT-R	CGTTCTGGCGTGCTCTATTACTGG	
RNR RT-F	CTTGGACAGGGTAGTGGTTGACG	
RNR RT-R	TGGCAAATGTACAAGAAGGCAGAG	

III. Result

1. Screening of transcription factor gene deletion mutants in *F. graminearum*

I obtained 657 transcription factor gene deletion mutants from a previous study (Son *et al.*, 2011). All these mutants were inoculated on CM and CM supplemented with four DNA damage agents. Through the screening, 16 mutants showed altered sensitivity to at least one DNA damage agent (Table 4 and Fig. 1). Among them, there have several genes has already studied in other species as well as *F. graminearum* that are involved in DNA damage response (Table 4).

2. Identification of novel transcription factor gene *fgzc248* in *F. graminearum*

fgzc248 (locus ID: FGSG_01176) encodes 492 amino acids containing the fungal Zn(2)-Cys(6) binuclear cluster domain and FgZC248 is a conserved protein which have not been studied before. FgZC248 homologs are conserved in Pezizomycotina of Ascomycota, especially in class Sordariomycetes (Fig. 2).

3. Deletion and complementation

To investigate the function of *fgzc248*, target gene deletion and complementation were performed. All the genetic manipulations were confirmed by Southern blot analyses (Fig. 3). The deletion mutant showed a slight growth defect than wild-type.

4. Phenotypic analyses

In accordance with screening data, *fgzc248* deletion mutant showed little growth in CM supplemented with HU whereas the complemented strain fully restored growth defect (Fig. 4). In sexual development, Δ*fgzc248* mutant lost self-fertility, while the wild-type and complemented strains produced normal perithecia (Fig.5).

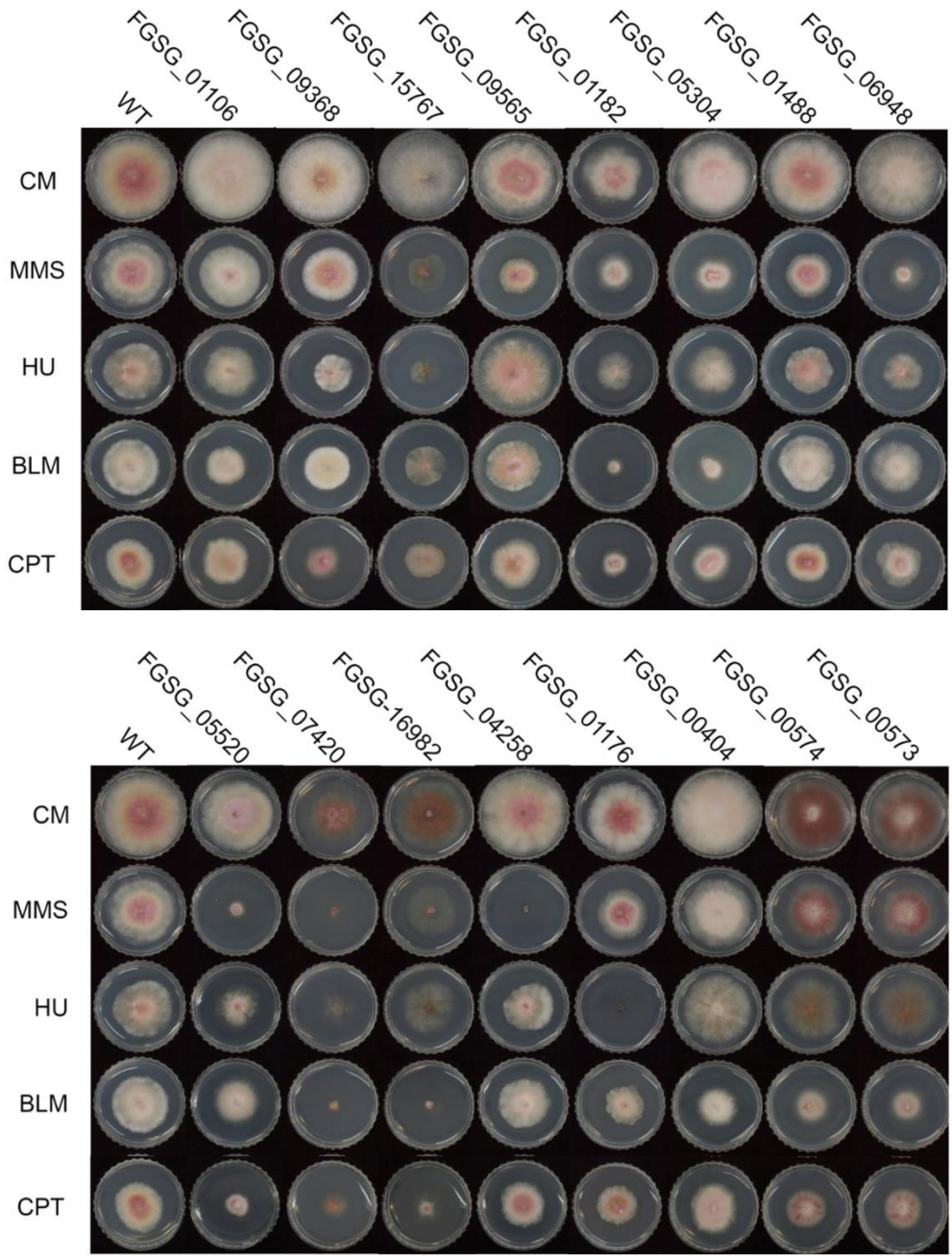

Fig. 1. Transcription factor gene deletion mutants involved in DNA damage responses in *F. graminearum*.

Table 4 Putative TFs involved in DNA damage responses

TF No.	Locus number	DNA damage agent				Description of the gene product
		MMS	HU	BLM	CPT	
56	FGSG_01106		S	S		Related to arsenite-resistance protein 2
117	FGSG_09368		S		S	Related to C2H2 zinc finger protein
135	FGSG_15767	S	S	S	S	Related to zinc finger protein SFP1
157	FGSG_09565	S	R			Probable siderophore regulation protein (GATA factor)
170	FGSG_01182	S	S	S	S	Conserved hypothetical protein
172	FGSG_05304	S	S	S	S	Conserved hypothetical protein
187	FGSG_01488	S	S			Conserved hypothetical protein
237	FGSG_06948	S	S			Related to tetratricopeptide repeat protein tpr1
347	FGSG_05520	S	S	S	S	Related to cullin homolog 4A
352	FGSG_07420 (*rfx1*)	S	S	S	S	Related to cephalosporin C regulator 1 (cpcR1 gene)
356	FGSG_16982			S	S	Related to myosin 2
392	FGSG_04258	S				Probable DNA repair protein UVS-2
641	FGSG_01176 (*fgzc248*)		S			Conserved hypothetical protein
694	FGSG_00404	R	R	S		Conserved hypothetical protein
695	FGSG_00574			S		Related to purine utilization positive regulator
696	FGSG_00573			S		Related to GAL4-like transcriptional activator

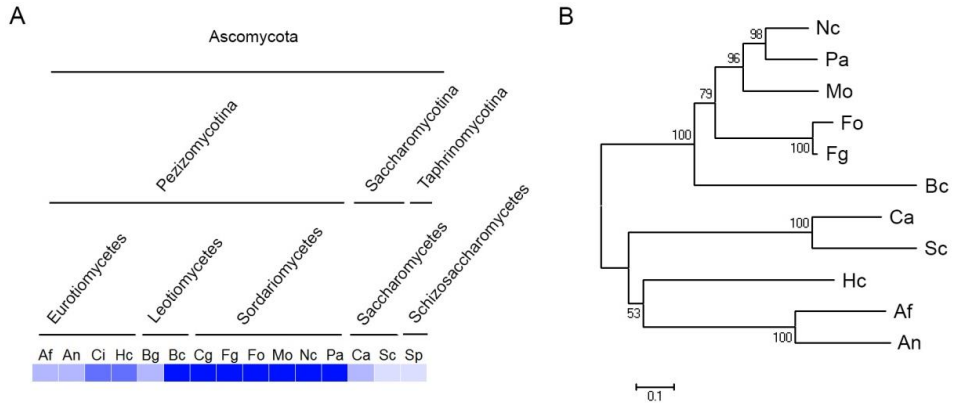

Fig. 2. Identification of FgZC248 and its distribution in fungi. (A) Distribution of FgZC248 in representative fungal species. The image was conducted by using BLASTMatrix tool that is available on the Comparative Fungal Genomic Platform (http://cfgp.riceblast.snu.ac.kr/). (B) Phylogenetic tree of FgZC248 homologs in several fungal species. ClustalW was used to perform the alignment, and the MEGA program version 4.0 was used to perform a bootstrap phylogenetic analysis using the neighbor joining method. Af: *Aspergillus fumigates*; An: *Aspergillus nidulans*; Ci: *Coccidioides immitis*; Hc: *Histoplasma capsulatum*; Bg: *Blimeria graminis*; Bc: *Botrytis cinerea*; Cg: *Colletotrichum graminicola*; Fg: *Fusarium graminearum*; Fo: *Fusarium oxysporum*; Mo: *Magnaporthe oryzae*; Nc: *Neurospora crassa*; Pa: *Podospora anserine*; Ca: *Candida albicans*; Sc: *Saccharomyces cerevisiae*; Sp: *Schizosaccharomyces pombe*.

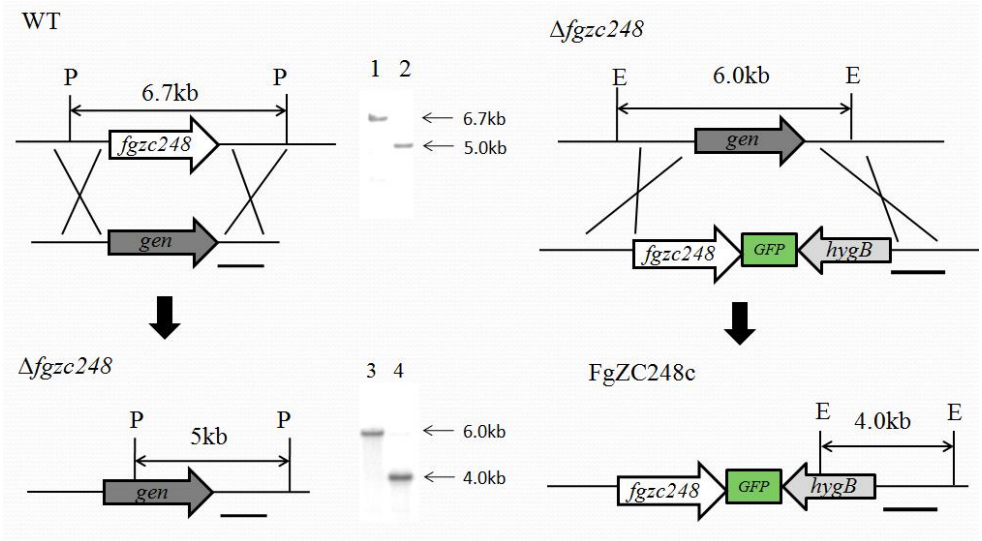

Fig. 3. Targeted deletion and complementation of *fgzc248*. WT, *G. zeae* wild-type strain GZ3639; Δ*fgzc248*, *fgzc248* deletion mutant; FgZC248com, Δ*fgzc248*-derived strain complemented with *fgzc248*; *gen*, genetic resistance gene cassette; *hygB*, hygromycin B resistance gene cassette; *GFP*, green florescence protein cassette; P, *Pst* I. E, *Eco*R V. Lane 1, WT; Lane 2, deletion strain using 3-flanking as the probe and enzyme *Pst* I. Lane 3, deletion strain; Lane 4, complementation strain using 3-flanking as the probe and enzyme *EcoR* V.

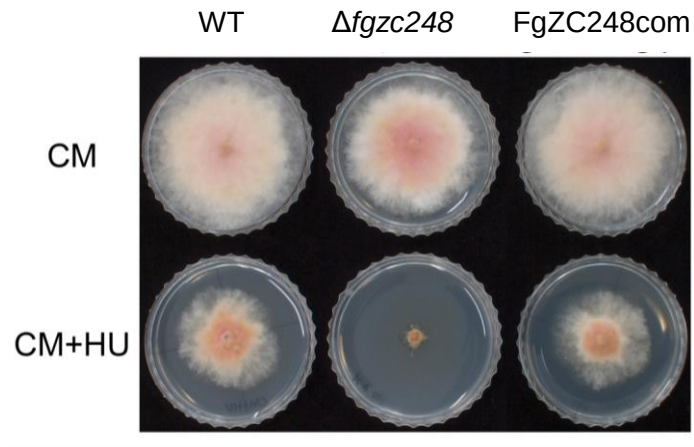

Fig. 4. Mycelia growth of wild-type, Δ*fgzc248* and complementation strains on CM and CM supplemented with 10 mM HU. Pictures were taken 3 days after inoculation. WT, *G. zeae* wild-type strain GZ3639; Δ*fgzc248*, *fgzc248* deletion mutant; FgZC248com, Δ*fgzc248*-derived strain complemented with *fgzc248*.

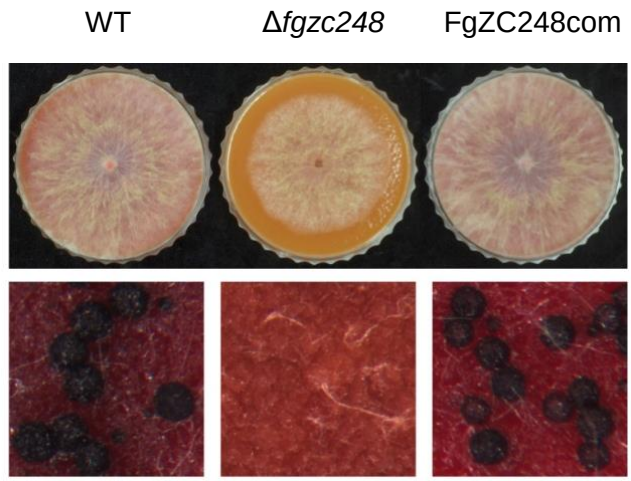

Fig. 5. Growth rate and perithecium formation of wild-type, Δ*fgzc248* mutant and complementation strains on carrot agar. Pictures were taken 5 days after inoculation and 7 days after sexual induction, respectively. WT, *G. zeae* wild-type strain GZ3639; Δ*fgzc248*, *fgzc248* deletion mutant; FgZC248com, Δ*fgzc248*-derived strain complemented with *fgzc248*.

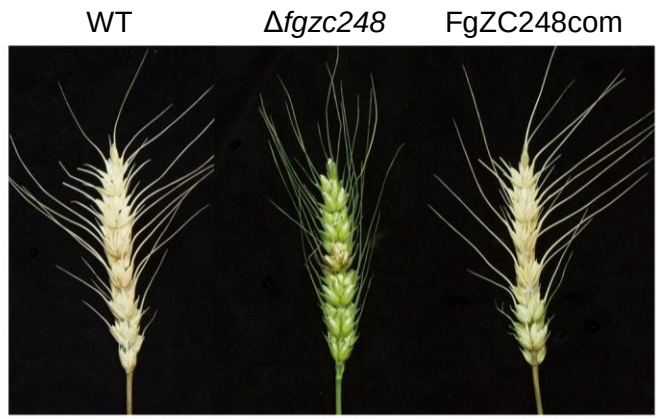

Fig. 6. Virulence of wild-type, Δ*fgzc248* mutant and complementation stains on wheat head. Conidia suspension of each strain was injected on a center of wheat head Asterisk indicates data differed significantly ($p<0.05$) based on Tukey′s test. WT, *G. zeae* wild-type strain GZ3639; Δ*fgzc248*, *fgzc248* deletion mutant; FgZC248com, Δ*fgzc248*-derived strain complemented with *fgzc248*.

5. Virulence analysis

The virulence of the fungal strains was examined by point inoculation of wheat spikelet. Fourteen days after inoculation, the wild-type and complemented strains colonized the injected spikelet and adjacent spikelets so that induced normal head blight symptoms (Fig. 6). However, Δ*fgzc248* only infected the inoculated spikelet but lost its ability to spread into the neighboring spikelets.

6. Accumulation of DNA damage in cells

Alkaline comet assay was performed for the analysis of the DNA single-strand breaks, double-strand breaks, and alkali-labile sites of DNA in each cell. The head of the comet shows undamaged DNA, while the tail of comet means the damaged DNA in each cell. Therefore, cells with more DNA damage show a higher percentage of DNA in tail. We tested the wild-type and Δ*fgzc248* strains with and without HU treatment (Fig. 7). HU can induce DNA damage in *F. graminearum* with the increase of comet tail in HU treated cells. DNA damage in HU-untreated Δ*fgzc248* cells and HU-treated wild-type cells was increased and showed similar DNA damage level ($P<0.01$). This result suggests that deletion of *fgzc248* triggered DNA damage in the absence of a DNA damage agent. HU-treated Δ*fgzc248* cells showed significantly increase in percentage DNA in tail compared to HU-treated

wild-type cells, which demonstrated DNA in deletion mutant is easy to get damaged when expose to HU than wild-type (P<0.01).

7. Effects of *fgzc248* on regulation of genome-wide transcript levels.

To know the effects of *fgzc248*, we analyzed transcriptome by using RNA-seq and qRT-PCR. Genes with transcript levels showing a 3-fold or greater difference between the wild-type and deletion mutant strains were categorized based on their predicted functions (Ruepp *et al*., 2004) (Table 6). The results showed that, among 13,820 genes, 898 genes (6.5%) were up-regulated in the Δ*fgzc248* strain while 522 genes (3.8%) were down-regulated, respectively.

In Δ*fgzc248*, the transcript level of 6 genes involved in cell cycle and DNA procession (including 2 genes involved in DNA repair) were up-regulated by 3-fold or more compared to the wild-type strain, demonstrating that deletion of *fgzc248* resulted in cell cycle abnormal as well as DNA damage. Consistent with the RNA-seq data, qRT-PCR analysis confirmed that the genes related with DNA repair and cell cycle were up-regulated in the Δ*fgzc248* (Table 6).

Since Δ*fgzc248* was highly sensitive to HU, which works as the inhibitor of ribonucleotide reductase (RNR), I analyzed the transcript level of *rnr* (Locus ID:

FGSG_05174) in cells under each condition. The transcript level of *rnr* was increased by about two-fold when HU was treated in the wild-type strain or *fgzc248* was deleted (Table 6). The transcript level of *rnr* in HU-treated Δ*fgzc248* was about four times higher than in HU-treated wild-type.

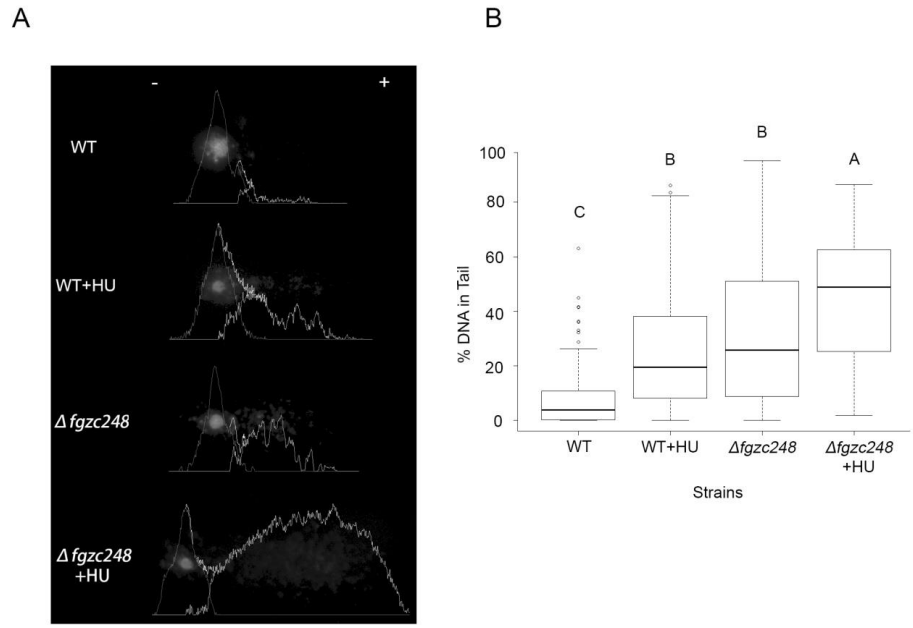

Fig. 7. Detection of increased DNA damage in Δ*fgzc248* strain and HU treated cells. (A) Photographs of comets and analysis of alkaline comet assay. DNA fragments generated from DNA single-strand breaks, double-strand breaks and alkali-labile sites in the DNA migrated toward the anode, creating the comet assay. Comets of the Δ*fgzc248* and HU treated wild-type, Δ*fgzc248* cells produced longer tails. Graphs show image analysis quantifying DNA contents of head and tail. (B) Box-plot of average percentages of DNA in comet tails. Hydroxyurea-treated Δ*fgzc248* cells showed significantly higher percentages of DNA in comet tail than wild-type and hydroxyurea-treated wild-type cells ($p<0.01$). And Δ*fgzc248* cells showed significantly higher percentages of DNA in comet tail than wild-type but similar with hydroxyurea-treated wild-type cells ($p<0.01$).

Table 5 Changes in transcription levels between Δ*fgzc248* strain and wild-type strain in functional categories

FunCat ID	FunCat category name	No. of genes in the genome	No. of genes up-regulated over 3-fold	No. of genes down-regulated over 3-fold
01	Metabolism	2322	121	79
02	Energy	503	22	15
10	Cell cycle and DNA procession	659	6	3
10.01.05.01	DNA repair	156	2	1
11	Transcription	718	4	6
12	Protein synthesis	370	0	3
14	Protein fate	920	15	7
16	Protein with binding function or cofactor requirement	1714	53	33
18	Regulation of metabolism and protein function	242	2	1
20	Cellular transport, transport facilities and transport routes	1390	63	51
30	Cellular communication/signal transduction mechanism	312	5	3
32	Cell rescue, defense and virulence	856	67	34
34	Interaction with the environment	606	27	24
40	Cell fate	240	2	2
41	Development	55	2	2
42	Biogenesis of cellular components	617	18	7
99	Unclassified proteins	9074	716	398
-	Total	13820	898	522

Table 6 Transcript levels of selected genes involved in DNA damage and cell cycle.

Locus Number	Fold change of transcript level in RNA-seq	Relative transcript level of indicated strain grown with or without HU				Description
		WT		Δ*fgzc248*		
		-HU	+HU	-HU	+HU	
FGSG_01132	2.2	1.0	2.6	1.8	2.8	Related to MUS81 protein, involved in the repair of UV-and methylation-induced DNA damage
FGSG_05924	4.4	1.0	6.2	4.5	11.3	Related to NAD+ ADP-ribosyltransferase
FGSG_10050	2.4	1.0	4.0	5.1	4.7	Related to mismatched base pair and cruciform DNA recognition protein
FGSG_13552	59.9	1.0	2.4	61.5	51.9	Related to NAD-dependent histone deacetylase (HDAC)
FGSG_16955	8.5	1.0	1.9	4	5.3	Related to deoxyribodipyrimidine photo-lyase
FGSG_05174 (*rnr*)	1.9	1.0	1.9	2.2	7.9	Ribonucleoside-diphosphate reductase large chain

IV. Discussion

In this study, 16 transcription factor genes were found to be involved in DNA damage responses in *F. graminearum* (Table 4 and Fig. 1). Four chemical reagents that can cause different kinds of DNA damage were chosen for the screening. Methyl methanesulfonate (MMS) is an alkylating agent that primarily methylates DNA on N^7-deoxyguanine and N^3-deoxyadenine, which will lead to DNA synthesis defects (Chang *et al.*, 2002). Hydroxyurea (HU) inhibits ribonucleotide reductase which is the enzyme to catalyze the formation of dNTPs and depletes nucleotides pool and thus leads to an S-phase arrest (Zhao *et al.*, 1998). Bleomycin (BLM) causes double-strand breaks which is a mimetic of gamma irradiation (Levin and Demple, 1996). Camptothecin (CPT) locks topoisomerase I covalently onto the DNA and prevent DNA re-ligation, thus causes strand breaks during S phase (Wan *et al.*, 1999).

Some of the screening data are consistent with the previous results reported in *F. graminearum* or other fungal species. Locus FGSG_09368 encodes a hypothetical protein related to MSN2 C_2H_2 zinc finger protein. In *Saccharomyces cerevisiae*, MSN2 controls expression of genes in response to DNA damage (Schmitt and McEntee, 1996). The product of locus FGSG_15767 is related to a zinc finger protein SFP1 which was reported to regulate G2/M transition during the mitotic

cell cycle and DNA-damage response in *S. cerevisiae* (Xu and Norris, 1998). Locus FGSG_05520 encodes a protein that is related to cullin homolog 4A which is known to be associated with the UV-damaged DNA-binding protein DDB (Shiyanov *et al.*, 1999). FGSG_07420 (*rfx1*) is crucial for maintenance of genome integrity and disruption of *rfx1* function caused spontaneous DNA double-strand breaks in *F. graminearum* (Min *et al*., 2014). Locus FGSG_04258 encodes probable DNA damage repair protein UVS-2/RAD18 that is involved in post-replication repair in *Neurospo*ra *crassa* and *S. cerevisiae* (Tomita *et al.*, 1993; Yoon *et al.*, 1995).

Sixteen TF mutants were highly sensitive to MMS, HU, BLM, and/or CPT suggesting that these TFs play important roles in different kind of DNA damages (Table 4). Interestingly, deletion mutant of FGSG_09565 showed increased sensitivity to MMS treatment and resistance to HU. The deletion mutant of a novel transcription factor gene FGSG_00404 is highly sensitive to BLM and resistant to both MMS and HU, demonstrating that single transcription factor can play different roles in response to different DNA damages. Five deletion mutants showed increased sensitivity to all DNA damage agents in the study. Two of them are novel transcription factors which are worthy for further study. Deletion strains of FGSG_04258 and FGSG_01176 (*fgzc248*) gene were more sensitive to only one

DNA damage agent. Since homologs for FGSG_04258 gene plays important roles in DNA damage response in other fungi (Tomita *et al.*, 1993; Yoon *et al.*, 1995), further study was carried out with a novel transcription factor FgZC248 containing the fungal Zn(2)-Cys(6) binuclear cluster domain for in-depth study (Table 4).

HU have been used both in prokaryotes and eukaryotes to study DNA damage-independent replication fork arrest (Lopes *et al*., 2001; Sogo *et al*., 2002). HU treatment leads to replication fork arrest and subsequent genomic instability (Ahmad *et al*., 1998). The alkaline comet assay was applied to determine whether Δ*fgzc248* cells accumulated DNA damage (Fig. 7). The DNA damage level of HU-treated wild-type cells is similar with the that of HU-untreated Δ*fgzc248* cells, and the transcript level of genes involved in DNA damage responses are increased in Δ*fgzc248* cells (Table 5 and 6), demonstrating that the disruption of the *fgzc248* resulted in DNA damage.

The primary cellular target of HU is ribonucleotide reductase (RNR) which is responsible for the synthesis of dNTPs (Fuchs and Karlstrom, 1973; Kren and Fuchs, 1987; Slater, 1973; Sneeden and Loeb, 2004). HU prevents dNTP pool expansion and drops dNTP level in yeast (Koç *et al.*, 2004) and survival of DNA damaged cells is highly dependent on increased dNTP levels in yeast (Chabes *et al.*, 2003). HU treatment decreases dNTP levels of the cell and leads to S-phase arrest.

Thus HU treatment resulted in disability of DNA repair. While *rnr* transcript level in HU-treated Δ*fgzc248* was 4-fold higher than the HU-treated wild-type, the Δ*fgzc248* strains did not grow in HU. We hypothesized that the dNTP level is still too low in HU-treated Δ*fgzc248* cells, despite of high transcript level of *rnr* gene.

Deletion of *fgzc248* resulted in defects in sexual development as well as virulence. The transcript level of FGSG_13552 which is related to NAD-dependent histone deacetylase (HDAC) has increased 60-fold in the Δ*fgzc248* compared to the wild-type strain (Table 6). A previous study showed that the level of histone acetylation is required for sexual development and virulence in *F. graminearum* (Son *et al.*, 2011). Acetylation increases DNA accessibility and is involved in DNA replication, histone deposition, and DNA repair. Histone acetylation is catalyzed by histone acetyl transferases (HATs), whereas the reverse reaction is carried out by histone deacetylases (HDACs) (Legube and Trouche, 2003). With the high expression of HDAC, the histone acetylation level may decrease and thus resulted in defects of sexual development and virulence.

The yeast SIR2, homolog of a protein encoded by FGSG_13552, belongs to class III HDAC and regulates cellular senescence by transcriptional silencing and repression of recombination at the targeted gene locus (Chang and Min, 2002). HDF1 histone deacetylase is the major class II HDAC and important for sexual

development and pathogenesis in *F. graminearum* (Li *et al*., 2011). Δ*hdf1* mutants showed similar phenotype with the Δ*fgzc248*. I hypothesized that the overexpression of class III HDAC *sir2* may have the similar effect with the deletion of class II HDAC *hdf1* gene. Since there are numerous HATs and HDACs even in a single organism (Kurdistani, 2003), the relationship with each class of HDAC and HATs need to be studied in further study.

In conclusion, the *fgzc248* gene is related to DNA-damage response and genome integrity in *F. graminearum*. Moreover, disruption of *fgzc248* function resulted in DNA damage in cells as well as multiple defects in virulence and sexual development. Transcriptomic analysis suggests that FgZC248 controls the genes involved in DNA-damage responses and a putative HDAC gene. Instrumental analysis of the dNTP levels in the *fgzc248* deletion mutant will confirm the hypothesis that reduced dNTP level causes DNA damage in the Δ*fgzc248* strain.

References

Ahmad, S.I., Krik, S.H., and Eisenstark, A. 1998. Thymine metabolism and thymineless death in prokaryotes and eukaryotes. Annu. Rev. Microbiol. 52: 591-625.

Bai, G.H., and Shaner, G.E. 2004. Management and resistance in wheat and barley to *fusarium* head blight. Annu. Rev. Phytopathol. 42: 135-161.

Bowden, R.L., and Leslie, J.F. 1999. Sexual recombination in *Gibberella zeae*. Phytopathol. 89: 357-369.

Cappellini, R.A., and Peterson, J.L. 1965. Macroconidium formation in submerged cultures by a non-sporulating strain of *Gibberella zeae*. Mycologia 57: 962-966.

Chabes, A., Georgieva, B., Domkin, V., Zhao, X., Rothstein, R., and Thelander, L. 2003. Survival of DNA damage in yeast directly depends on increase dNTP levels allowed by relaxed feedback inhibition of ribonucleotide reductase. Cell 112: 391-401.

Chang, M., Bellaoui, M., Boone, C., and Brown, G.W. 2002. A genome-wide screen for methyl methanesulfonate-sensitive mutants reveals genes required for S phase progression in the presence of DNA damage. Proc. NatL. Acad. Sci. U.S.A. 99:16934-16939.

Chang, K.T., Min, K.T. 2002. Regulation of lifespan by histone deacetylase. Aging Res. Rev. 1(3): 313-326.

Core Team R. 2008. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Fuchs, J.A., and Karlstrom, O. 1973. A mutant of Escherichia coli defective in ribonucleosidediphosphate reductase. 2. Characterization of the enzymatic defect. Eur. J. Biochem. 32:457-462.

Giglia-Mari, G., Zotter, A., and Vermeulen, W. 2010. DNA damage response. Cold Spring Harb. Perspect. Biol. by Cold Spring Harbor Laboratory Press.

Goldman, G.H. and Kafer., E. 2003. *Aspergillus nidulans* as a model system to characterize the DNA damage response in eukaryotes. Fungal Genet. Biol. 41:428-442.

Goldman, G.H., Mcguire, S.L., and Harris, S.D. 2002. The DNA damage response in filamentous fungi. Fungal Genet. Biol. 35:183-195.

Goswami, R.S., and Kistler, H.C. 2004. Heading for disaster: *Fusarium graminearum* on cereal crops. Mol. Plant Pathol. 5(6):515-525.

Harper, J.W., and Elledge, S.J. 2007. The DNA damage response: ten years after. Mol. Cell 28:739-745.

Harris, S.D. 2005. Morphogenesis in germinating *Fusarium graminearum* macroconidia. Mycologia 97: 880-887.

Harrison, J.C., and Haber, J.E. 2006. Surviving the breakup: the DNA damage checkpoint. Annu. Rev. Genet. 40: 209-235.

Horwitz, B.A., Sharon, A., Lu, S.W., Ritter, V., Sandrock, T.M., Yoder, O.C., and

Turgeon B.G. 1999. A G protein alpha subunit from Cochliobolus heterostrophus involved in mating and appreeorium formation. Fungal Genet. Biol. 26: 19-32.

Kim, H.K., and Yun, S.H. 2011. Evaluation of potential reference genes for quantitative RT-PCR analysis in *Fusarium graminearum* under different culture conditions. Plant Pathol. J. 27: 301-309.

Koç, A., Wheeler, L.J., Mathews, C.K., and Merrill, G.F. 2004. Hydroxyurea arrests DNA replication by a mechanism that preserves basal dNTP pools. J. Biol. Chem. 279: 223-230.

Kren, B., and J.A. Fuchs. 1987. Characterization of the *ftsB* gene as an allele of the nrdB gene in Escherichia coli. J. Bacteriol. 169:14-18.

Lee, S.-H., Lee, J., Lee, S., Park, E.-H., Kim, K.-W., Kim, M.-D., Yun, S.-H., and Lee, Y.-W. 2009. *GzSNF1* is required for normal sexual and asexual development in the ascomycete *Gibberella zeae*. Eukaryot. Cell 8:116-127.

Lee, T., Han, Y.-K., Kim, K.-H., Yun, S.-H., and Lee, Y.-W. 2002. *Tri13* and *Tri7* determine deoxynivalenol- and nivalenol-producing chemotypes of *Gibberella zeae*. Appl. Environ. Microb. 68(5): 2148-2154.

Legube, G., and Trouche, D. 2003. Regulation histone acetyltransferases and deacetylases. EMBO Rep. 4: 944-947.

Leslie, J.F., and Summerell, B.A. 2006. The *Fusarium* laboratory manual.

Blackwell Publishing, Ames, IA.

Levin, J.D., and B. Demple. 1996. In *vitro* detection of endonuclease IV-specific DNA damage formed by bleomycin in *vivo*. Nucleic Acids Res. 24: 885-889.

Levitt, N.C., and Hickson, I.D. 2002. Caretaker tumor suppressor genes that defend genome integrity. Trends Mol. Med. 8:179-186.

Li, Y.M., Wang C.F., Liu, W.D., Wang, G.H., Kang, Z.S., Kistler, H.C., and Xu, J.R. 2011. The HDF1 histone deacetylase gene is important for conidiation, sexual reproduction, and pathogenesis in *Fusarium graminearum*. Mol. Plant Microbe In. 24(4): 487-496.

Lin, Y., Son, H., Lee, J., Min, K., Choi, G.J., Kim, J.-C., and Lee, Y.-W. 2011. A putative transcription factor MYT1 is required for female fertility in the ascomycete *Gibberella zeae*. PLoS ONE 6(10): e25586.

Livak, K.J., and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\triangle\triangle CT}$ method. Methods 25: 402-408.

Lopes, M., Cotta-Ramusino, C., Pellicioli, A., Liberi, G., Plevani, P., Muzi-Falconi, M., Newlon, C.S., and Foiani, M. 2001. The DNA replication checkpoint response stabilizes stalled replication forks. Nature 412: 557-561.

McMullen, M., Jones, R., and Gallenberg, D. 1997. Scab of wheat and barley: a re-emerging disease of devastating impact. Plant Dis. 81, 1340–1348.

Min, K., Son, H., Lim, JY, Choi G.J., Kim, J.-C., Harris, S.D. and Lee, Y.-W. 2014. Transcription factor RFX1 is crucial for maintenance of genome integrity in *Fusarium graminearum*. Eukaryot. Cell 13: 427-436.

Mortazavi, A., Williams, B.A., McCue, K., Schaeffer, I., and Wold, B. 2008. Mapping and quantifying mammalian transcriptoms by RNA-Seq. Nat. Methods 5: 621-628.

Namiki, F., Matsunaga, M., Okuda, M., Inoue, I., Nishi, K., Fujita, Y., and Tsuge, T. 2001. Mutation of an arginine biosynthesis gene causes reduced pathogenicity in *Fusarium oxysporum* f. sp *melonis.* Mol. Plant Microbe. Interact. 14: 580-584.

Olive, P.L., and Banath, J.P. 2006. The comet assay: a method to measure DNA damage in individual cells. Nat. Protoc. 1:23-29.

Placinta, C.M., D′Mello, J.P.F., and Macdonald, A.M.C. 1999. A review of worldwide contamination of cereal grains and animal feed with *Fusarium* mycotoxins. Anim. Feed Sci. Tech. 78(1-2): 21-37.

Rouse, J., and Jackson, S.P. Interfaces between the detection, signaling, and repair of DNA damage. Science 197: 547-551.

Ruepp, A., Zollner, A., Maier, D., Albermann, K., Hani, J., Mokrejs, M., Tetko, I., Güldener, U., Mannhaupt, G., Münsterkötter, M., and Mewes, H.W. 2004. The FunCat, a functional annotation scheme for systematic classification of proteins from whole genomes. Nucleic Acids Res. 32: 5539-5545.

Sambrook, J., and Russell, D.W. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor laboratory Press, Cold Spring Harbor, NY.

Schmitt, A.P., and McEntee, K. 1996. Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in *Saccharomyces cerevisiae*. Proc. NatL. Acad. Sci. U.S.A. 93: 5777-5782.

Shiyanov, P., Nag, A., and Raychaudhuri, P. 1999. Cullin 4A Associates with the UV-damaged DNA-binding Protein DDB. Biol. Chem. 274: 35309-35312.

Sneeden, J.L., and Loeb, L.A. 2004. Mutations in the R2 subunit of ribonucleotide reductase that confer resistance to hydroxyurea. J. Biol. Chem. 279: 40723-40728.

Sogo, J.M., Lopes, M., and Foiani, M. 2002. Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. Science 297:599-602.

Son, H., Lee, J., Park, A.R., and Lee, Y.-W. 2011. ATP citrate lyase is required for normal sexual and asexual development in *Gibberella zeae*. Fungal Genet. Biol. 48(4): 408-417.

Son, H., Seo, Y.-S., Min, K., Park, A.R., Lee, J., Jin, J.M., Lin, Y., Cao, P., Hong, S.Y., Kim, E.-K., Lee, S.H., Cho, A., Lee, S., Kim, M., Kim, Y., Kim, J.E., Kim, J.C., Choi, G.J., Yun, S.H., Lim, J.Y., Kim, M., Lee, Y.H., Choi, Y.D., and Lee, Y.-W. 2011. A phenome-based functional analysis of transcription factors in the cereal head blight fungus, *Fusarium*

graminearum. PLoS Pathog. 7: e1002310.

Stephen, P., Jackson, and Bartek, J. 2009. The DNA-damage response in human biology and disease. Nature 461:1071-1078.

Tomita, H., Soshi, T., and Inoue, H. 1993. The *Neurospora uvs-2* gene encodes a protein which has homology to yeast RAD18, with unique zinc finger motifs. Mol. Gen. Genet. 238(1-2): 225-233.

Wan, S., Capasso, H., and Walworth, N.C. 1999. The topoisomerase I poison camptothecin generates a Chk1-dependent DNA damage checkpoint signal in fission yeast. Yeast 15: 821-828.

Xu, Z., and Norris, D. 1998. The *SFP1* gene product of Saccharomyces cerevisiae regulates G2/M transitions during the mitotic cell cycle and DNA-damage response. Genetics 150: 1419-1428.

Yoon, H.J., Lee, B.J., and Kang, H.S. 1995. The *Aspergillus uvsH* gene encodes a product homologous to yeast RAD18 and *Neurospora* UVS-2. Mol. Gen. Genet. 248(2): 174-181.

Yu, J.H., and Hamari, Z. 2004. Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. Fungal Genet. Biol. 41(11):973-981.

Zhao, X., Muller, E.G., and Rothstein, R. 1998. A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools. Mol. Cell 2, 329-340.

V. Abstract in Korean

요약 (국문초록)

Fusarium graminearum 의 전사조절인자 FgZC248 의 기능연구

부민민

식물병원성 곰팡이 *Fusarium graminearum* 의 전사조절인자들 중 DNA 손상반응에 관련된 전사조절인자를 찾기 위해 전사조절인자 삭제균주 library 를 스크리닝 하였다. 이를 위해 DNA 손상물질인 methyl methanesulfonate, hydroxyurea, bleomycin, campothecin 을 각각 처리한 조건에서 657 개의 전사조절인자 삭제균주들의 균사생장을 모두 관찰하였다. 그 결과 DNA 손상반응과 관련이 있는 16 개의 전사조절인자들이 발굴하였다. 그 중에서 전사조절인자 fgzc248 의 삭제균주는 오직 hydroxyurea 에서만 감수성을 보였고 Alkaline comet assay 를 수행하여 fgzc248 삭제균주는 세포내 DNA 손상이 축적된다는

것을 밝혀냈다. RNA-seq 을 통해 야생형과 전사체를 비교한 결과, DNA 수리 유전자들의 발현이 삭제균주에서 증가하였다.

주요어: 붉은곰팡이, DNA 손상반응, 전사조절인자

학번: 2012-24003