저작자표시-비영리-변경금지 2.0 대한민국

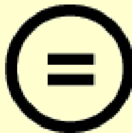
이용자는 아래의 조건을 따르는 경우에 한하여 자유롭게

- 이 저작물을 복제, 배포, 전송, 전시, 공연 및 방송할 수 있습니다.

다음과 같은 조건을 따라야 합니다:

저작자표시. 귀하는 원저작자를 표시하여야 합니다.

비영리. 귀하는 이 저작물을 영리 목적으로 이용할 수 없습니다.



변경금지. 귀하는 이 저작물을 개작, 변형 또는 가공할 수 없습니다.

- 귀하는, 이 저작물의 재이용이나 배포의 경우, 이 저작물에 적용된 이용허락조건을 명확하게 나타내어야 합니다.
- 저작권자로부터 별도의 허가를 받으면 이러한 조건들은 적용되지 않습니다.

저작권법에 따른 이용자의 권리는 위의 내용에 의하여 영향을 받지 않습니다.

이것은 이용허락규약(Legal Code)을 이해하기 쉽게 요약한 것입니다.

Disclaimer

dCollection

농학석사학위논문

식물병원성곰팡이 *Fusarium graminearum*의 CCAAT DNA 결합부위를 가진 전사조절인자 *FCT1*과 *FCT3*의 기능과 상호작용분석

Functional analysis of Transcription Factors containing CCAAT-DNA Binding domain in *Fusarium graminearum*

2017년 8월

서울대학교 대학원

농생명공학부 식물미생물전공

남 혜 진

Functional analysis of Transcription Factors containing CCAAT-DNA Binding domain in *Fusarium graminearum*

A dissertation submitted in partial

fulfillment of the requirement for

the degree of

MASTER OF SCIENCE

to the Faculty of

Department of Agricultural Biotechnology

at

SEOUL NATIONAL UNIVERSITY

By

Hyejin Nam

August, 2017

Fusarium graminearum 의 CCAAT DNA 결합부위를 가진 전사조절인자 *FCT1* 과 *FCT3* 의 기능과 상호작용분석

지도교수 이 인 원

이 논문을 농학석사학위논문으로 제출함

2017년 8월

서울대학교 대학원

농생명공학부 식물미생물학 전공

남 혜 진

남혜진의 석사학위논문을 인준함

2017년 6월

위 원 장 가 종 억

부 위원장 이 인 원

위 원 박 은 우

A THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Functional analysis of transcription Factors containing CCAAT-DNA Binding domain in *Fusarium graminearum*

UNDER THE DIRECTION OF DR. YIN-WON LEE

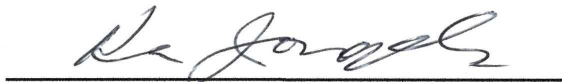
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF SEOUL NATIONAL UNIVERSITY

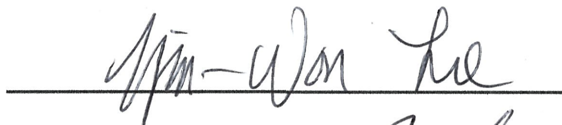
BY
HYEJIN NAM

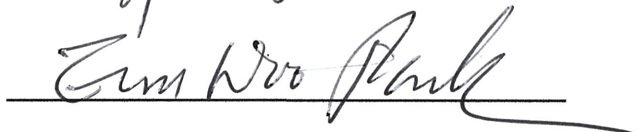
MAJOR IN PLANT MICROBIOLOGY
DEPARTMENT OF AGRICULTURAL BIOTECHNOLOGY

JUNE 2017

APPROVED AS A QUALIFIED THESIS OF YOONJI LEE
FOR THE DEGREE OF MASTER OF PHILOSOPHY
BY THE COMMITTEE MEMBERS

CHAIRMAN 

VICE CHAIRMAN 

MEMBER 

Abstract

Functional analysis of Transcription Factors containing CCAAT-DNA Binding domain in *Fusarium graminearum*

Hyejin Nam
Major in Plant Microbiology
Department of Agricultural Biotechnology
The Graduate School
Seoul National University

The homothallic ascomycete fungus *Fusarium graminearum* is an important plant pathogen of major cereal crops. The CCAAT sequence is present in roughly 30% of eukaryotic promoters. In filamentous fungi, the CCAAT sequence has been known to modulate the expression of several critical genes involved in various developmental stages. Eight transcription factors (TFs) containing CCAAT-DNA binding domain (*FCT1~8*) are in *F. graminearum*. Among them, we chose 2 TF

mutants, Δ*fct1* and Δ*fct3*, that have similar pleiotropic defects from a previously generated TF mutant library. Both deletion mutants have similar defects in mycelia growth, sexual reproduction, and virulence. Double deletion of *FCT1* and *FCT3* resulted in indistinguishable phenotypes compared to each single deletion mutant. Moreover, Fct1 and Fct3 physically interacted with each other and localized to nuclei, suggesting that Fct1 and Fct3 form protein complex for transcriptional regulation. This is the first study dealing with TFs containing CCAAT DNA-binding domain in plant pathogenic fungi and present weighty perception to understand molecular mechanisms underlying functions of CCAAT-binding factors in *F. graminearum.*

Key word : CCAAT, DNA binding domain, *Fusarium graminearum*, transcription factor

Student number: 2015-23132

CONTENTS

ABSTRACT 1

INTRODUCTION 7

MATERIALS AND METHODS 9

1. Fungal strains and media 9
2. Genetic manipulations, primers, and sequencing 11
3. Targeted deletion and complementation 15
4. Quantitative real time (qRT)-PCR 18
5. Cellular localization of Fct1 and Fct3 19
6. Sexual crosses 20
7. Virulence test and trichothecenes analysis 20
8. Yeast-two-hybrid assay 21

RESULTS 23

1. Identification of CCAAT-DNA binding transcription factors *FCT1* and *FCT3* 23
2. Vegetative growth and perithecial development 25
3. Cellular localization of Fct1 and Fct3 25
4. Expression of *FCT1* and *FCT3* 26

5. *FCT1* and *FCT3* are involved in pathogenicity during infection of wheat ··········· 31

6. *FCT1* and *FCT3* have an effect on the biosynthesis of trichothecenes ··········· 34

7. Fct1 interacts with Fct3 ··········· 36

DISCUSSION ··········· 38

REFERENCES ··········· 42

LIST OF TABLES

Table 1. *F. graminearum* strains used in this study-------------------------10

Table 2. Primers used in this study--12

LIST OF FIGURES

Figure 1. Targeted deletion and complementation of *FCT1* and *FCT3*-17

Figure 2. Distribution of *FCT1* and *FCT3* homologs in fungi -----------24

Figure 3. Vegetative growth on complete media (CM) and minimal media (MM) -27

Figure 4. Sexual development of the *F. graminearum* strains------------28

Figure 5. Cellular localization of Fct1 and Fct3----------------------------29

Figure 6. Expression of *FCT1* and *FCT3* in wild-type strain ------------30

Figure 7. Wheat head virulence and micrographs of hand sections-----32

Figure 8. Longitudinal sections of infected wheat heads -----------------33

Figure 9. Total trichothecenes (deoxynivalenol and 15-acetyl-deoxynivalenol) -----35

Figure10. Yeast two hybrid of Fct1 and Fct3 ------------------------------37

Introduction

The ascomycete fungus *Fusarium graminearum* is a prominent plant pathogen that causes *fusarium* head blight (FHB) in cereal crops and ear and stalk rot on maize (Goswami & Kistler, 2004), resulting in a severe yield losses and accumulation of mycotoxins (e.g., trichothecenes and zearalenone) that are harmful to animals and humans (Desjardins, 2006). *F. graminearum* produces sexual spores (ascospores) and asexual spores (conidia). In particular, sexual spores (ascospores) of *F. graminearum* are believed to be the primary inocula for FHB epidemics in cereal crops (Sutton, 1982). Ascospores are forcibly discharged from mature fruiting bodies (perithecia) into the air under milder temperatures and moderate moisture (Paulitz, 1996). To date, several genes related to sexual development have been studied in *F. graminearum*, and those genes are known to be closely related to disease development (Hou et al., 2002, Lee et al., 2012, Paulitz, 1996, Shim et al., 2006, Urban et al., 2003).

In the *F. graminearum* genome, 8 transcription factors containing CCAAT-DNA binding domain were identified at a previous work (Son et al., 2011a). Several deletion mutants of containing CCAAT-DNA binding domain, including FCT1 and FCT3 in *F. graminearum*, exhibited abnormality in fungal development, sexual development, pathogenicity and mycotoxins production. These data suggest that

the transcription factors containing CCAAT-DNA binding domain are required for normal development of *F. graminearum*.

The CCAAT sequence is one of the most common *cis*-elements present in the promoter regions of numerous eukaryotic genes. A statistical analysis of over 500 promoters revealed that the CCAAT sequence is an element present in approximately 30% of eukaryotic promoters (Bucher, 1990, Périer et al., 2000). The CCAAT sequence in filamentous fungi has been shown to modulate the expression of several genes, such as the *A. nidulans* acetamidase gene (*amdS*), the *A. oryzae* Taka-amylase A gene (*taa*), the *A. nidulans* penicillin biosynthesis genes (*ipnA* and *aatA*) (Brakhage et al., 1999, Tsukagoshi et al., 2001).

Previously, we performed genome-wide functional analyses of whole transcription factor genes in *F. graminearum* (Son et al., 2011b). In this study, we selected two genes that previously demonstrated a defect in perithecial development and further characterized its function in *F. graminearum* using a variety of techniques, including gene deletion and complementation. Our results demonstrate that *FCT1* and *FCT3* is required for conidiation, vegetative growth, sexual development, trichothecene production and pathogenicity. Furthermore, Yeast-two-Hybrid analyses of 2 genes revealed that both FCT isoform strongly interacts with each other.

Materials and Methods

1. Fungal strains and media

All strains used in this study are listed in Table 1. The wild-type strain Z-3639 (Bowden & Leslie, 1999) and mutants derived from the wild-type strain were maintained according to the *Fusarium* laboratory manual (Leslie & Summerell, 2008). A transgenic strain, mat1r (Son et al., 2011b), carrying both the *MAT1-1* deletion and histone H1 tagged with red fluorescence protein (RFP), was used in the co-localization study. The growth rates of the wild type and transgenic strains were measured in CM and minimal media (MM; 3 g KH_2PO_4, 0.5 g $MgSO_4$, 0.5 g KCl, 30 g sucrose, and 20 g agar per liter). Minimal liquid medium supplemented with 5mM agmatine (MMA) was used for trichothecenes analysis (Gardiner et al., 2009).

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

Strain	Genotype	Reference
Z-3639	Wild-type	(Bowden & Leslie, 1999)
Δfct1	Δ*tf170::GEN*	This study
Δfct3	Δ*tf172::GEN*	This study
FCT1c	Δ*tf170::TF170-GFP-HYG*	This study
FCT3c	Δ*tf172::TF172-GFP-HYG*	This study
Δfct1 Δfct3	Δ*tf170::GEN; Δtf172::GEN*	This study
mat1r	Δ*mat1-1-1::GEN; hH1::hH1-RFP-HYG*	(Son et al., 2011a)
mat1g	Δ*mat1-1-1::GEN; hH1::hH1-GFP-HYG*	(Hong et al., 2010)

2. Genetic manipulations, primers, and sequencing

Fungal genomic DNA was extracted according to the *Fusarium* laboratory manual (Leslie & Summerell, 2008). Total RNA was isolated from mycelia or perithecia ground in liquid nitrogen using the Easy-Spin Total RNA Extraction Kit (Intron Biotech, Seongnam, Korea). Standard protocols were followed for restriction endonuclease digestion, agarose gel electrophoresis, and DNA gel blot hybridization with 32P labeled probes (Sambrook et al., 1989). The PCR primers used in this study were synthesized at an oligonucleotide synthesis facility (Bionics, Seoul, Korea) (Table 2), diluted to 100 mM in sterilized water, and stored at 220uC. DNA sequencing was performed at the National Instrumentation Center for Environmental Management (Seoul National University, Seoul, Korea) and the sequences were compared against the *Fusarium* Comparative Database at the Broad Institute (http://www.broadinstitute.org/annotation/genome/fusarium_group).

Table 2. Primers used in this study.

Primer	Sequence (5'-3')	Purpose
Gen-For	CGACAGAAGATGATATTGAAGG	For amplification of *gen* cassette from pII99 vector
Gen-Rev	CTCTAAACAAGTGTACCTGTGC	
FCT1-5F	GGGAGGCCTTTCACCACGAC	For *FCT* gene deletion
FCT1-5R	gcacaggtacacttgtttagagCTTTTGCGCGAGCTGGATTTAC	
FCT1-3F	ccttcaatatcatcttctgtcgAGGAAGGAAAGGAAGACAGAGACGAG	
FCT1-3R	TCACCAACACAGTCGAACATCAGG	
FCT1-5N	GAGCATGCGTACCCCAGTATCCAT	
FCT1-3N	GGTGGTGATACGAAATGACGGAATG	
FCT1-with 5F	CGGGGATGGTCTCAGCACTTATG	
FCT3-5F	GCTGGCGCAATGGGGAAAAA	

FCT3-5R	gcacaggtacacttgtttagagTAGTGTTGTAAGGCATGGTGGTTGAT	
FCT3-3F	ccttcaatatcatcttctgtcgAAAATCACCAGAGACGCCAGAGCA	
FCT3-3R	TGGATGTGGCGTTGTTGGAGTT	
FCT3-5N	TGTACCATTCCCTTCTCCCCAACAG	
FCT3-3N	TTATCGAGAAACCAAGAGCAATCACTC	
FCT3-with 5F	ACGGGGCTTGCGGTCTAGCTT	
FCT3-5F GFP	FCT3-5F	For *FCT* gene complementation
FCT3-5R GFP	gaacagctcctcgcccttgctcacGCCTGACATGGTCACATCTC	
FCT3-3F GFP	cctccactagctccagccaagccTTACAAGTCGTAATGAACAGTGAC	
FCT3-3R GFP	FCT3-3R	
FCT3-5N GFP	FCT3-5N	
FCT3-3N GFP	FCT3-3N	

FCT1-bait-F	aacgcagagaggccattacggccATGGACAATGTGTCACCATTG	For Yeast two Hybrid assay
FCT1-bait-R	aacgcagagaggccgaggcggccaaATCGCTCTCATCCCCATCC	
FCT3-prey-F	aacgcagagaggccattacggccATGCCTTACAACACTACAGCTATTC	
FCT3-prey-R	aacgcagagaggccgaggcggccgCTAGCCTGACATGGTCACATCT	
RT_FCT1_For	GGAAAAGGAGAAGGATAAAGAGCATAAG	For qRT-PCR
RT_FCT1_Rev	CCAGCGTTCACAGTGTTTTCGT	
RT_FCT3_For	GATCGCCCTCTGCTCCAACA	
RT_FCT3_Rev	TGATGTGCGACAGCAGAGGC	

3. Targeted deletion and complementation

To elucidate the functions of *FCT1* and *FCT3* in *F. graminearum*. Targeted gene deletion and complementation strains were created by homologous recombination. Each gene open reading frame (ORF) in the *F. graminearum* wild-type strain Z-3639 was replaced with the geneticin resistance gene (*gen*) to create the *FCT1* and *FCT3* deletion mutant strain. To generate each gene′s complementation strain, gene was replaced on the wild-type allele of each gene fused to the hygromycin resistance gene (*hyg*). To generate the *FCT1* and *FCT3* double deletion mutant strain, open reading frame (ORF) in the *FCT1* complementation strain was replaced with the hygromicin resistance gene (*hyg*). Deletion and complementation were confirmed by Southern hybridizations.

A slightly modified double-joint (DJ) PCR strategy (Yu et al., 2004) was applied to construct fusion PCR products for targeted gene deletion. Firstly, the 5′ and 3′ flanking regions of *FCT1* were amplified from the wild-type strain using primer pairs FCT1-5F/ FCT1-5R and FCT1-3F/FCT1-3R, respectively. Secondly, a geneticin resistance cassette (*gen*) under the control of the *A. nidulans trpC* promoter and terminator was amplified from pII99 (Namiki et al., 2001) using the primer pair Gen-for/Gen-rev. Three amplicons (5′ flanking, 3′ flanking, and gen) were then fused by a second round of DJ PCR. Finally, a 4.9 kb DNA fragment was

amplified with the nested primer pair FCT1-5N/FCT1-3N using a second round PCR product as template. To complement the *FCT1* deletion mutant, a fusion construct was generated by DJ PCR, which included the *FCT1* ORF with its own promoter, the green fluorescent protein gene (*GFP*), the hygromycin resistance gene cassette (*hyg*), and the 3′ flanking region of the *FCT1* gene. The *FCT1* ORF with its own promoter was amplified with the primer pair FCT1-5F/FCT1- 5R GFP. The GFP-hyg was amplified from pIGPAPA, and the 3′ flanking region of the *FCT1* gene was amplified by primer pair FCT1-3F/FCT1-3R. The fusion construct was then transformed into the *FCT1* deletion mutant. Deletion and complementation of *FCT3* gene was produced using the same strategy (Figure 1).

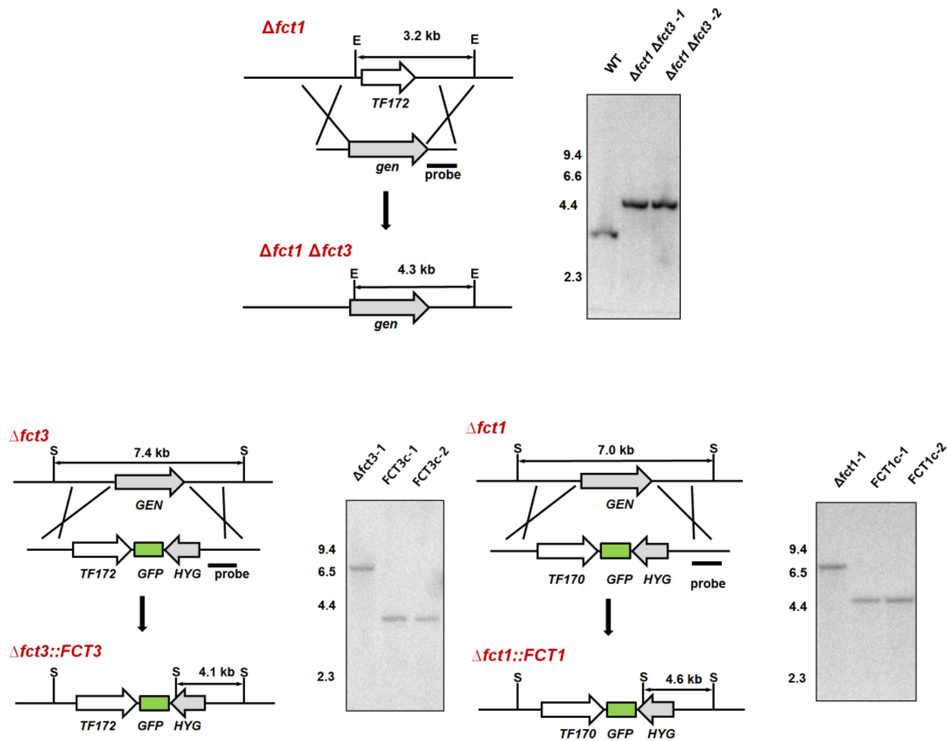


Figure 1. Targeted deletion and complementation of *FCT1* and *FCT3.*

WT, *F. graminearum* wild-type strain Z-3639; HK241*,* *FCT1* deletion mutant; HK242, fct1-derived strain complemented with *FCT1-GFP*; HK249, *FCT3* deletion mutant; HK250, fct3-derived strain complemented with *FCT3-GFP;* HK254, *FCT1* and *FCT3* double deletion mutant; S, Sfi1; E, EcoRⅤ; GEN, geneticin resistance gene cassette; HYG, hygromycin B resistance gene cassette. The sizes of DNA standards (in kilobasese) are indicated on the left of the blot.

4. Quantitative real time (qRT)-PCR

Total RNA was isolated from vegetative cultures at 5 day after inoculation and at 3, 5, and 7 day after sexual induction using an Easy-Spin Total RNA Extraction Kit (Intron Biotech, Seongnam, Korea). The first strand cDNA was synthesized with SuperScriptIII reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative realtime PCR (qRT-PCR) was performed by using SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) with primers (Table 1). The endogenous housekeeping gene cyclophilin (*CyP1*; locus ID: FGSG_07439.3) was used as an endogenous control for normalization (Kwon et al., 2009). The PCR was repeated three times with two replicates per run. The changes in fluorescence of the SYBR green dye in each cycle were monitored by the system software, and the threshold cycle (CT) above the background for each reaction was calculated. The gene expression was calibrated using the formula $2^{-\Delta\Delta CT}$ method as previously described (Lee et al., 2009). The CT value of CyP1 was subtracted from that of *FCT1* and *FCT3* to obtain a ΔC_T value. The ΔC_T values of *FCT1* and *FCT3* expression in the wild-type vegetative stage at 5 day was subtracted from the ΔC_T value of each sample to obtain a $\Delta\Delta C_T$ value. The *FCT1* and *FCT3* transcript level relative to the calibrator

were expressed as $2^{-\Delta\Delta CT}$. A Tukey test was conducted using SPSS 12.0 software (SPSS, Inc. Chicago, IL) to examine statistically significant differences ($p<0.05$) of $2^{-\Delta\Delta CT}$ among the mean values of the samples.

5. Cellular localization of Fct1 and Fct3

To investigate cellular localization of Fct1, we generated a strain carrying green fluorescent protein (*GFP*) fused to C-terminus of *FCT1*. A PCR fragment including GFP and HPH was amplified from the pIGPAPA plasmid with primers GFPF and HYG-F1. The 5′ flanking region, which is the MYT3 C-terminus without its own terminator sequence, was amplified from the wild-type strain with primers FCT1-5F and FCT1-5R. The 3′ flanking region of *FCT1* was amplified by primers FCT1-3F and FCT1-3R. These three resulting PCR products were fused as previously described (Son et al., 2011a). The resulting PCR product was used as a template with primers FCT1-gfpF and FCT3-gfpR for a final construct. Subsequently, the final PCR products were transformed into the wild-type strain. To observe co-localization of FCT1 with a nuclear protein, a resulting FCT1-gfp transformant was outcrossed with a mat1r strain that contains red fluorescent protein (RFP) fused to histone. H1 was generated as previously described (Yu et al., 2004). Strains

containing both FCT1::GFP and hH1::RFP were selected by antibiotic resistance and confirmed by PCR. Microscopic observation was performed by a DE/Axio Imager A1 microscope (Carl Zeiss, Oberkochen, Germany) with the filter set 38HE (excitation 470/40; emission 525/50) for GFP and the filter set 15 (excitation 546/12; emission 590) for RFP. *FCT3* strain for investigating cellular localization was produced using the same strategy.

6. Sexual crosses

From strains grown on carrot agar media (Leslie & Summerell, 2008) for 5 days, aerial mycelia were removed with 700 ml of 2.5% Tween 60 solution to induce fertilization. The plates were incubated under a near-UV light for 7 to 10 days. For outcrosses, mycelia of a female strain grown on carrot agar media were fertilized with 1 ml of conidial suspension (10^6/ml) obtained from male strain, which was induced in CMC. The heterothallic Δ*mat2* mutant, a deletion strain of MAT1-2, was used as a tester mutant for outcrosses (Lee et al., 2003) Perithecia and ascospores were observed 9 days after sexual crosses.

7. Virulence test and trichothecene analysis

For the virulence test, the point inoculation method was carried out previously described (Son et al., 2011a). The conidia suspension (10^5 conidia ml^{-1}) was prepared from CMC and then 10 ml of the conidia suspension from each strain was injected into a center spikelet of wheat head (cultivar; Eunpamil). After inoculation, wheat plants were incubated in a humidity chamber for 3 days and then transferred to a greenhouse. Spikelets with disease symptoms were counted 14 day after inoculation as previously described (Wang et al., 2011). The experiment was performed with five replicate inoculations per strain, and two independent mutant strains were used for the experiment. Trichothecenes analysis was performed as previously described (Son et al., 2011a). Briefly, MMA cultures were extracted with ethyl acetate, and the extracts were concentrated to dry. A portion of each extract was derivatized with Sylon BZT (BSA+TMCS+TMSI, 3:2:3 respectively, Supelco, Bellefonte, PA, USA) and analyzed with a Shimadzu QP-5000 gas chromatograph mass spectrometer (GC-MS, Shimadzu, Kyoto, Japan) with a selected ion-monitoring mode as previously described (Seo et al., 1996). Trichothecenes were quantified based on the biomasses produced by each strain. The experiment was repeated three times.

8. Yeast-two-hybrid assay

Y2H assays were conducted using the DUALhunter System (Dualsystems Biotech) in accordance with the manufacturer′s instructions. Full-length *FCT1* cDNA cloned into the pDHB1 vector was used as the bait vector, which was co-transformed with the generated Y2H cDNA library. *S. cerevisiae* NMY51 (*MAT a his3Δ200 trp1-901 leu2-3, 112 ade2 LYS2::*(*lexApo*)*4- HIS3 ura3*::(*lexApo*)*8-lacZ ade2*::(*lexApo*)*8-ADE2 GAL4*) was used as a host. A total of 100 positive clones identified by selection on selective medium without Leu, Trp, His or Ade (SD-L-T-H-A) were sequenced. Several truncated *FCT1* constructs were cloned into prey vectors, and full-length *FCT1* and *FCT3* inserted into pDHB1 were used as bait vectors. After co-transformation, a colony picked from the SD-Leu-Trp plates was grown in liquid SD-Leu-Trp, subjected to fourfold serial dilutions, and spotted on selective plates. Empty vector and pDL20-Alg5 (-) were included as negative controls, and pAl-Alg5 (+) was included as a positive control.

Results

1. Identification of CCAAT-DNA binding transcription factors *FCT1* and *FCT3*

There are 5 transcription factors that contain CCAAT-DNA binding domain in *F. graminearum*. Among them, 2 transcription factors produced a few protoperithecia with significant defects in vegetative growth and virulence. We designated FGSG_01182 as *Fusairum graminearum* CCAAT-DNA binding domain containing transcription factor 1 (*FCT1*), which has an open reading frame (ORF) of 1,060 base pairs (bp) with three introns, and is predicted to encode a 276-amino acid protein and FGSG_05304 as *Fusairum graminearum* CCAAT-DNA binding domain containing transcription factor 3 (*FCT3*), which has an open reading frame of 797 base pairs with three introns, and is predicted to encode a 182-amino acid protein. FCT1 and FCT3 has no distinct homolog with other genes containing CCAAT-DNA binding domain in filamentous fungi (Figure 2).

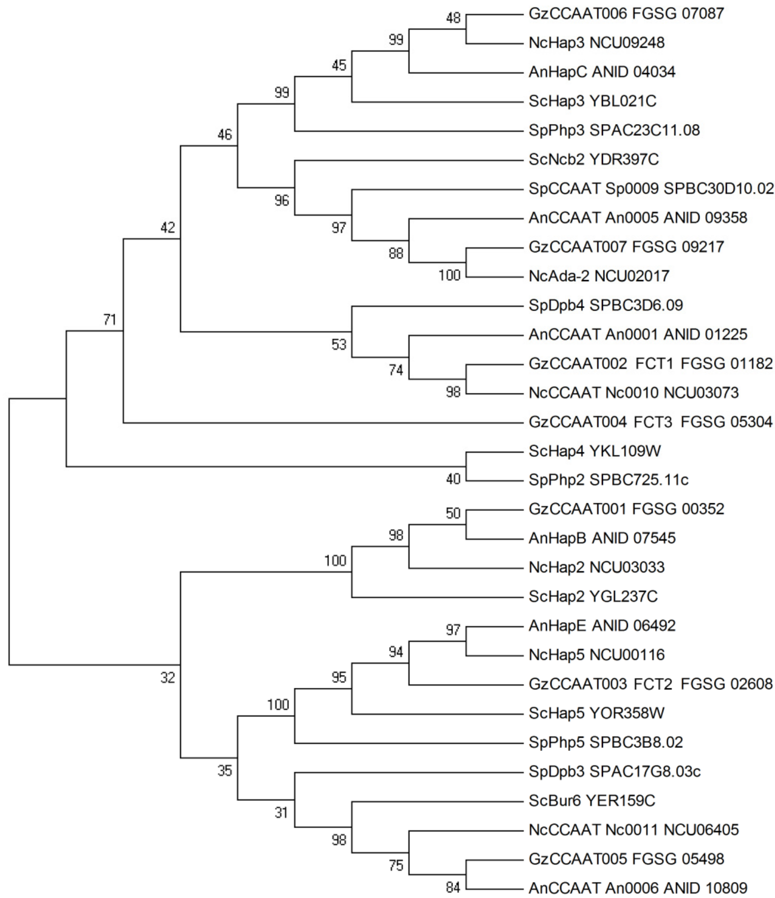


Figure 2. Distribution of *FCT1* and *FCT3* homologs in fungi.

Phylogenetic tree of *FCT1* and *FCT3* homologs in several fungal species. The alignment was performed with ClustalW, and the MEGA program, version 4.0. was used to perform a 1,000-bootstrap phylogenetic analysis using the neighbor-joining method.

2. Vegetative growth and perithecial development

To investigate the function of *FCT1* and *FCT3*, targeted gene deletion and complementation were performed (Figure 1). The deletion mutants poorly grew on CM and MM whereas the complemented strain fully restored growth defect (Figure 3).

In sexual development, *FCT1* and *FCT3* gene deletion mutant lost self-fertility, while the wild-type and complemented strains produced normal perithecia (Figure 4).

3. Cellular localization of Fct1 and Fct3

To examine Fct1 and Fct3 localization, the FCT1-GFP and FCT3-GFP fusion construct under the control of its native promoter was transformed into each gene deletion mutant. We selected each complementation strains carrying a single FCT-GFP copy and found a GFP signal in the nuclei of all of the examined strains. To confirm nuclear localization of FCT-GFP, FCT1comr (Δfct1::FCT1-GFP-hyg; hH1-RFP-gen) was generated by an outcross between mat1r and FCT1c. FCT1-GFP in the FCT1comr strain co-localized with hH1-RFP and was highly

fluorescent in mycelia. From perithecia by this outcrossing, we isolated 20 progeny expressed GFP and RFP in nucleus of the most developmental stages such as hyphae (Figure 5), indicating that Fct1 and Fct3 is continuously expressed in nuclei.

4. Expression of *FCT1* and *FCT3*

Expression of *FCT1* and *FCT3* increases markedly from vegetative stage to sexual stage until eight-fold higher expression than that of the wild-type strain (figure 6). Taken together, these results suggest that *FCT1* and *FCT3* play an important role during sexual development and maturation of perithecia.

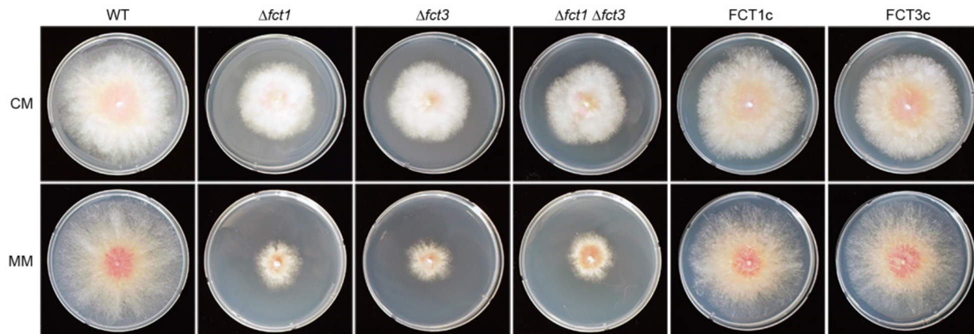


Figure 3. Vegetative growth on complete media (CM) and minimal media (MM).

Δ*fct1* and Δ*fct3* had defect in growth rate/ FCT1c and FCT3c showed similar phenotype compared with WT. Figures were taken 5 days after inoculation. WT, *F. graminearum* wild-type strain Z-3639; Δ*fct1, FCT1* deletion mutant; FCT1c, fct1-derived strain complemented with *FCT1-GFP*; Δ*fct3*, *FCT3* deletion mutant; FCT3c, fct3-derived strain complemented with *FCT3-GFP;* Δ*fct1* Δ*fct3*, *FCT1* and *FCT3* double deletion mutant.

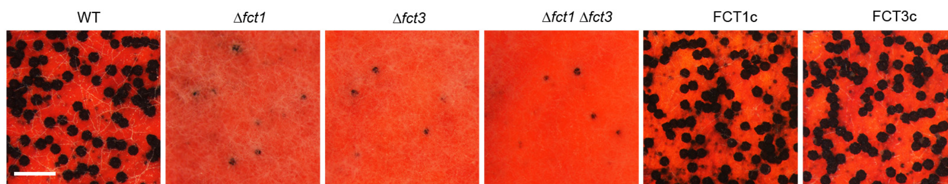


Figure 4. Sexual development of the *F. graminearum* strains.

Five-day old carrot agar culture was mock-fertilized to induce sexual reproduction and incubated for an additional 7 d**.** Scale bar = 200 ㎛.

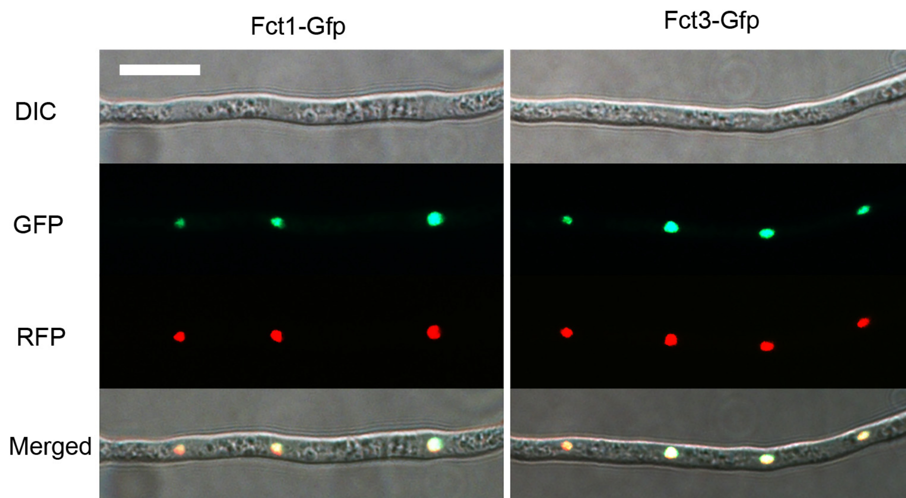


Figure 5. Cellular localization of Fct1 and Fct3.

Fct1 and Fct3 were fused with green fluorescent protein (Gfp), and histone H1 was fused with red fluorescent protein (Rfp). Co-localization of FCT1-GFP and hH1-RFP in germinated conidia. FCT3-GFP and hH1-RFP is also same. Scale bar = 20 ㎛.

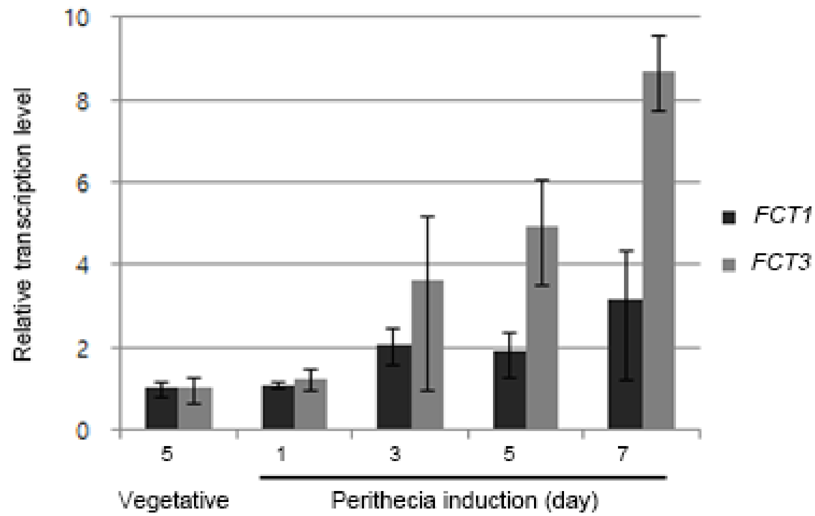


Figure 6. Expression of *FCT1* and *FCT3* in wild-type strain.

Transcript level of *FCT1* and *FCT3* was analyzed by quantitative real time-PCR (qRT-PCR) during the vegetative and sexual induction stages. WT, *F. graminearum* wild-type strain Z-3639.

5. *FCT1* and *FCT3* are involved in pathogenicity during infection of wheat

Our observations that the Δ*fct1* and Δ*fct3* strain grows slowly on CM and MM (Figure 3) led us to question whether the deletion of *FCT1* and *FCT3* affects *F. graminearum* pathogenicity during wheat infection. To evaluate pathogenicity on flowering wheat heads, conidial suspensions of each strain were point-inoculated on a spikelet and incubated in a greenhouse. The wild-type strain induced normal head blight symptoms, which manifests as discoloration, at 14 days after inoculation (Figure 7). In contrast, the Δ*fct1* and Δ*fct3* strain was restricted to infection sites, which were unable to spread from the rachis to reach adjacent spikelets on the head (Figure 8).

Figure 7. Wheat head virulence and micrographs of hand sections.

A center spikelet of each wheat head was injected with 10 μg of conidia suspension. Mock, negative control mock inoculated with 0.01 % Tween 20. Micrographs of hand sections after infection of wheat. Wheat spikelets were inoculated with conidia suspension from the strains expressing green fluorescent protein (GFP) in the cytoplasm. Infected wheat heads were dissected longitudinally at 6 days postinoculation and examined under a fluorescence microscope. GFP fluorescence represents hyphal growth spreading from the inoculation points. Arrowheads mark the inoculated spikelets.

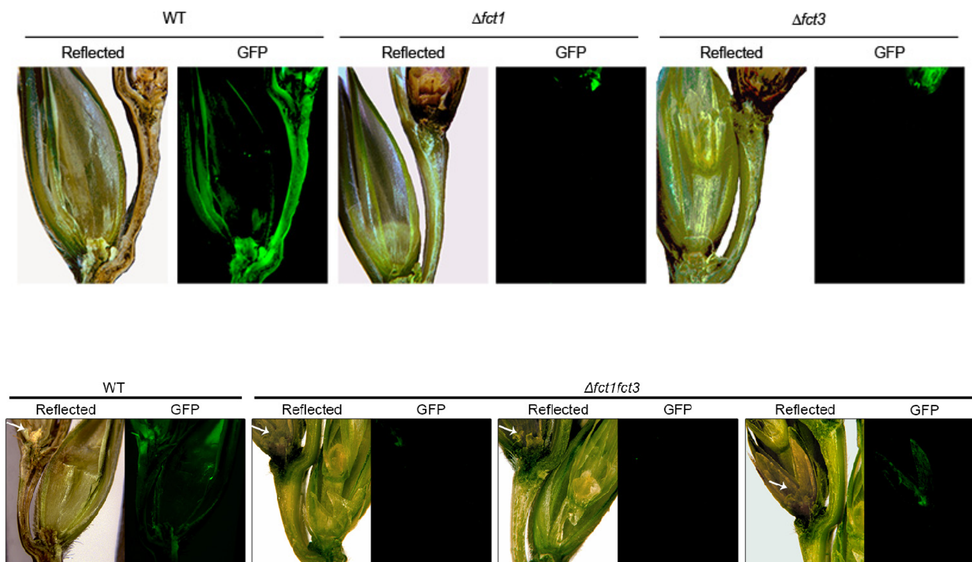


Figure 8. Longitudinal sections of infected wheat heads.

Wheat spikelets were inoculated with suspensions of conidia from green fluorescent protein (GFP)-expressing *F. graminearum* strains. Infected wheat heads were dissected 6 days after inoculation and examined by fluorescence microscopy. Spreading of the GFP signal represents spreading of hyphae from the points of inoculation. Arrowheads mark the inoculated spikelets.

6. *FCT1* and *FCT3* have an effect on the biosynthesis of trichothecenes

The level of trichothecene synthesized by both the *FCT1* and *FCT3* deletion mutant was significantly different than that of the wild-type and complementation strain. Transcription level of the trichothecene synthetic genes *TRI5* and *TRI6* was also significantly reduced in the deletion mutant (Figure 9)

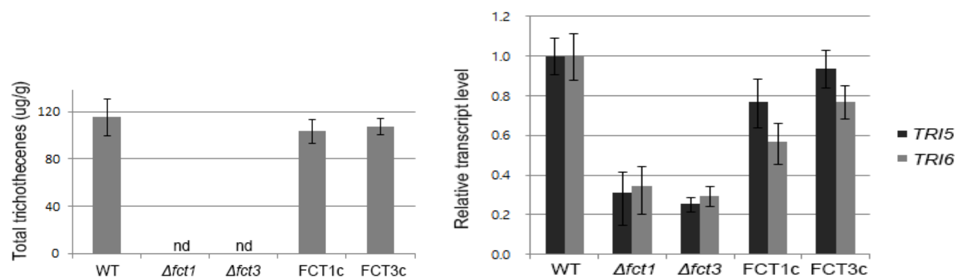


Figure 9. Total trichothecenes (deoxynivalenol and 15-acetyl-deoxynivalenol) production and transcriptional analyses of trichothecene synthetic genes.

(Left) Each strain was grown in MMA for 7 d. Trichothecenes were analyzed by GC-MS and quantified based on the biomass produced by each strain. (Right) Expression of *TRI5* and *TRI6* in the wild-type, *FCT1* and *FCT2* deletion, and *FCT1* and *FCT3* complementation strains. Gene transcription was analyzed by qRT-PCR 4 d after inoculation in MMA. WT, *F. graminearum* wild-type strain Z-3639; Δ*fct1*, *FCT1* deletion mutant; Δ*fct3*, *FCT3* deletion mutant; FCT1c, Δ*fct1*-derived strain complemented with *FCT1*; FCT3c, Δ*fct3*-derived strain complemented with *FCT3*.

7. Fct1 interacts with Fct3

Δ*fct1* and Δ*fct3*, that have similar pleiotropic defects from a previously generated TF mutant library. Both deletion mutants have similar defects in mycelia growth, sexual reproduction, and virulence. Double deletion of *FCT1* and *FCT3* resulted in indistinguishable phenotypes compared to each single deletion mutant. So, we predicted Fct1 and Fct3 interacts each other. To supporting that, we used a yeast two hybrid (Y2H) system to check the protein-protein interaction. A positive interaction in the yeast two-hybrid assay is indicated by yeast colony growth on medium lacking tryptophan, leucine and histidine, and alanine (Figure 10).

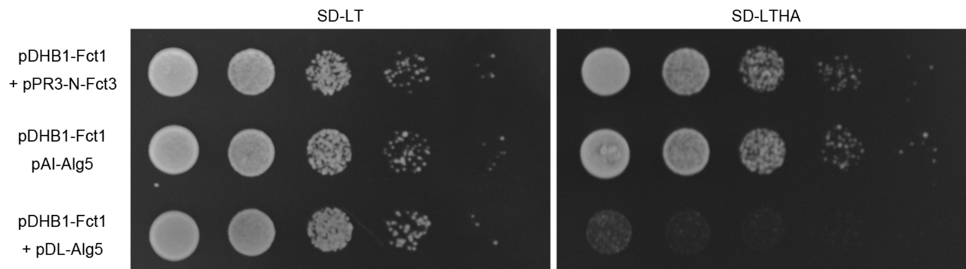


Figure 10. Yeast two hybrid of Fct1 and Fct3.

Interaction between Fct1 and Fct3. Vectors were co-introduced into yeast NMY51 strain in different combinations. pDHB1-FCT3/pAL-Alg5 protein interaction was used as a positive control and pDHB1-FCT1/pDL-Alg5 protein interaction was used as a negative control.

Discussion

In this study, we identified and characterized transcription factor containing CCAAT-DNA binding domain which has a specific function during sexual development in *F. graminearum.* In-depth phenotyping revealed that the *FCT1* and *FCT3* deletion mutant have defects in biological processes including vegetative growth, conidiation, toxin production, and virulence. We demonstrated that *FCT1* and *FCT3* plays an important role in *F. graminearum* on wheat heads. As previously mentioned.

Transcription factors belonging to the CCAAT-box binding factor family (also known as the Nuclear Factor Y) are present in all higher eukaryotes. In a statistical analysis of over 500 promoters, Bucher found that the CCAAT box is one of the most ubiquitous elements, being present in 30% of eukaryotic promoters (Bucher, 1990). Studies in plants have revealed that each subunit of this heterotrimeric transcription factor is encoded by a gene belonging to a multigene family allowing a considerable modularity. In most species, the CCAAT-binding factor (CBF), also known as the Nuclear Factor Y (NF-Y) or Hem Activator Protein (HAP) is composed of three subunit: NF-TA (also termed CBF-B, HAP2), NF-YB (CBF-A, HAP3), NF-YC (CBF-C, HAP5) (Laloum et al., 2013).

The CCAAT box is invariably flanked by at least one functionally important

promoter element. It has been shown that the distance between the two elements is important. In the yeast *S. cerevisiae*, CCAAT boxes are found in promoters of cytochromes and of other genes coding for proteins specifically activated by non-fermentable carbon sources (McNabb et al., 1997), and of genes involved in nitrogen metabolisms (Dang et al., 1996). In the filamentous fungus *A. nidulans*, CCAAT boxes are present in penicillin biosynthesis genes (Steidl et al., 1999). In higher eukaryotes, all sorts of promoters have CCAAT boxes: developmentally controlled and tissue-specific (Berry et al., 1992). It is increasingly evident that the prevalence of NF-Y sites in the latter is higher than among the general promoter data base (Laloum et al., 2013).

Members of the CCAAT-box binding gene family have diverse roles as transcriptional regulators for multiple cellular processes in animals and plants, including cell proliferation, apoptosis, differentiation, metabolic pathways, cell fate and identity, and stress responses (Oh & Reddy, 1999). Even though *FCT1* and *FCT3* has no distinct homolog with other genes containing CCAAT-DNA binding domain in filamentous fungi, Our results also showed that *FCT1* and *FCT3* is involved in diverse functions.

FCT1 and *FCT3* was found to be constitutively expressed from the vegetative stage to sexual reproduction, And expression of *FCT1* and *FCT3* increases markedly from vegetative stage to sexual stage until eight-fold higher expression than that of

the wild-type strain It means that that *FCT1* and *FCT3* regulate genes related sexual development. Also, Fct1 and Fct3 proteins were localized in nuclei during most developmental stages, suggesting the role of *FCT1* and *FCT3* as a transcriptional regulator.

NF-Y is a complex minimally composed of three subunits: NF-YA, NF-YB, NF0YC, all required for DNA-binding (McNabb et al., 1997, Sinha et al., 1996). The biochemistry of subunits association and the protein domains required have been studied in detail, as well as alterations induced on the DNA structure on binding (Liberati et al., 1999, Ronchi et al., 1996, Sinha et al., 1996). The NF-YB-NF-YC subunits form a tight dimer, which offers a complex surface for NF-YA association. Likewise we expected that two genes functions as a protein complex. Nevertheless, *FCT1* and *FCT3* has no distinct homolog with other genes containing CCAAT-DNA binding domain in filamentous fungi. The resulting dimer protein complex can then bind to DNA with high specificity and affinity an affinity: for most of the sites involved fungi life cycle overall.

In conclusion, we report that *FCT1* and *FCT3* is required in whole of cell cycle in *F. graminearum:* vegetative growth, sexual development, virulence and secondary metabolism. To our knowledge, this is the first study dealing with transcription factors containing CCAAT DNA-binding domain in plant pathogenic fungi and present weighty perception to understand molecular mechanisms underlying

functions of CCAAT-binding factors in *F. graminearum.*

More important things are the next. Compared with what is known about the structure and function of NF-Y animals, our knowledge about FCT genes in *F. graminearum* is lagging some way behind. However, studies on plant NF-Y genes in model and crop plants are accumulation and suggest that these genes play a role in a wide range of processes. So we have to characterize the functional specialization events and identify a CCAAT-DNA binding site and target genes in depth.

References

Berry M, Grosveld F, Dillon N, 1992. A single point mutation is the cause of the Greek form of Hereditary Persistence of foetal haemoglobin. *Nature: international weekly journal of science* **358**, 499-502.

Bowden RL, Leslie JF, 1999. Sexual Recombination in *Gibberella zeae*. *Phytopathology* **89**, 182-8.

Brakhage A, Andrianopoulos A, Kato M*, et al.*, 1999. HAP-like CCAAT-binding complexes in filamentous fungi: implications for biotechnology. *Fungal Genetics and Biology* **27**, 243-52.

Bucher P, 1990. Weight matrix descriptions of four eukaryotic RNA polymerase II promoter elements derived from 502 unrelated promoter sequences. *Journal of molecular biology* **212**, 563-78.

Dang V, Bohn C, Bolotin-Fukuhara M, Daignan-Fornier B, 1996. The CCAAT box-binding factor stimulates ammonium assimilation in *Saccharomyces cerevisiae*, defining a new cross-pathway regulation between nitrogen and carbon metabolisms. *Journal of bacteriology* **178**, 1842-9.

Desjardins AE, 2006. *Fusarium* mycotoxins: chemistry, genetics, and biology. American Phytopathological Society (APS Press).

Gardiner DM, Kazan K, Manners JM, 2009. Nutrient profiling reveals potent inducers of trichothecene biosynthesis in *Fusarium graminearum*. *Fungal Genetics and Biology* **46**, 604-13.

Goswami RS, Kistler HC, 2004. Heading for disaster: *Fusarium graminearum* on cereal crops. *Molecular plant pathology* **5**, 515-25.

Hong S-Y, So J, Lee J*, et al.*, 2010. Functional analyses of two syntaxin-like SNARE genes, *GzSYN1* and *GzSYN2*, in the ascomycete *Gibberella zeae*. *Fungal Genetics and Biology* **47**, 364-72.

Hou Z, Xue C, Peng Y, Katan T, Kistler HC, Xu J-R, 2002. A mitogen-activated protein kinase gene (MGV1) in *Fusarium graminearum* is required for female fertility, heterokaryon formation, and plant infection. *Molecular plant-microbe interactions* **15**, 1119-27.

Kwon S-J, Cho S-Y, Lee K-M, Yu J, Son M, Kim K-H, 2009. Proteomic analysis of fungal host factors differentially expressed by *Fusarium graminearum* infected with *Fusarium graminearum* virus-DK21. *Virus research* **144**, 96-106.

Laloum T, De Mita S, Gamas P, Baudin M, Niebel A, 2013. CCAAT-box binding transcription factors in plants: Y so many? *Trends in plant science* **18**, 157-66.

Liberati C, Di Silvio A, Ottolenghi S, Mantovani R, 1999. NF-Y binding to twin CCAAT boxes: role of Q-rich domains and histone fold helices. *Journal of molecular biology* **285**, 1441-55.

Lee J, Lee T, Lee YW, Yun SH, Turgeon BG, 2003. Shifting fungal reproductive mode by manipulation of mating type genes: obligatory heterothallism of *Gibberella zeae. Molecular microbiology* **50**, 145-52.

Lee J, Myong K, Kim J-E, Kim H-K, Yun S-H, Lee Y-W, 2012. FgVelB globally regulates sexual reproduction, mycotoxin production and pathogenicity in the cereal pathogen *Fusarium graminearum*. *Microbiology* **158**, 1723-33.

Lee S-H, Lee J, Lee S*, et al.*, 2009. GzSNF1 is required for normal sexual and asexual development in the ascomycete *Gibberella zeae*. *Eukaryotic cell* **8**, 116-27.

Leslie JF, Summerell BA, 2008. The *Fusarium* laboratory manual. John Wiley & Sons.

Mcnabb DS, Tseng K, Guarente L, 1997. The *Saccharomyces cerevisiae* Hap5p homolog from fission yeast reveals two conserved domains that are essential for assembly of heterotetrameric CCAAT-binding factor. *Molecular and cellular biology* **17**, 7008-18.

Namiki F, Matsunaga M, Okuda M*, et al.*, 2001. Mutation of an arginine biosynthesis gene causes reduced pathogenicity in *Fusarium oxysporum f. sp. melonis*. *Molecular plant-microbe interactions* **14**, 580-4.

Oh I-H, Reddy EP, 1999. The myb gene family in cell growth, differentiation and apoptosis. *Oncogene* **18**.

Périer RC, Praz V, Junier T, Bonnard C, Bucher P, 2000. The eukaryotic promoter database (EPD). *Nucleic acids research* **28**, 302-3.

Paulitz TC, 1996. Diurnal release of ascospores by *Gibberella zeae* in inoculated wheat plots. *Plant disease* **80**, 674-8.

Ronchi A, Berry M, Raguz S*, et al.*, 1996. Role of the duplicated CCAAT box region in gamma-globin gene regulation and hereditary persistence of fetal haemoglobin. *The EMBO journal* **15**, 143.

Sambrook J, Fritsch E, Maniatis T, 1989. Molecular cloning: a laboratory manual,

2nd edn. Cold Spring Laboratory Press. *New York*.

Seo J-A, Kim J-C, Lee D-H, Lee Y-W, 1996. Variation in 8-ketotrichothecenes and zearalenone production by *Fusarium graminearum* isolates from corn and barley in Korea. *Mycopathologia* **134**, 31-7.

Shim W-B, Sagaram US, Choi Y-E, So J, Wilkinson HH, Lee Y-W, 2006. FSR1 is essential for virulence and female fertility in *Fusarium verticillioides* and *F. graminearum*. *Molecular plant-microbe interactions* **19**, 725-33.

Sinha S, Kim I-S, Sohn K-Y, De Crombrugghe B, Maity SN, 1996. Three classes of mutations in the A subunit of the CCAAT-binding factor CBF delineate functional domains involved in the three-step assembly of the CBF-DNA complex. *Molecular and cellular biology* **16**, 328-37.

Son H, Lee J, Park AR, Lee Y-W, 2011a. ATP citrate lyase is required for normal sexual and asexual development in *Gibberella zeae*. *Fungal Genetics and Biology* **48**, 408-17.

Son H, Seo Y-S, Min K*, et al.*, 2011b. A phenome-based functional analysis of transcription factors in the cereal head blight fungus, *Fusarium graminearum*. *PLoS Pathog* **7**, e1002310.

Steidl S, Papagiannopoulos P, Litzka O*, et al.*, 1999. AnCF, the CCAAT Binding Complex of *Aspergillus nidulans*, Contains Products of the hapB, hapC, and hapE Genes and Is Required for Activation by the Pathway-Specific Regulatory GeneamdR. *Molecular and cellular biology* **19**, 99-106.

Sutton J, 1982. Epidemiology of wheat head blight and maize ear rot caused by *Fusarium graminearum*. *Canadian Journal of Plant Pathology* **4**, 195-209.

Tsukagoshi N, Kobayashi T, Kato M, 2001. Regulation of the amylolytic and (hemi-) cellulolytic genes in *aspergilli*. *The Journal of general and applied microbiology* **47**, 1-19.

Urban M, Mott E, Farley T, Hammond-Kosack K, 2003. The *Fusarium graminearum* MAP1 gene is essential for pathogenicity and development of perithecia. *Molecular plant pathology* **4**, 347-59.

Yu J-H, Hamari Z, Han K-H, Seo J-A, Reyes-Domínguez Y, Scazzocchio C, 2004. Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. *Fungal Genetics and Biology* **41**, 973-81.

Abstract in Korean

요약 (국문초록)

식물병원성곰팡이 *Fusarium graminearum* 의 CCAAT DNA 결합부위를 가진 전사조절인자 *FCT1* 과 *FCT3* 의 기능과 상호작용분석

남혜진

Fusarium graminearum 는 주요 곡류에 이삭마름병을 일으키고 인축에 유해한 독소를 생성하여 막대한 경제적 손실을 일으키는 식물병원성 곰팡이이다. 이 연구에서는 CCAAT DNA 결합부위를 가진 전사조절인자 (transcription factor, TF) *FCT1* 과 *FCT3* 의 기능분석연구를 수행하였다. CCAAT 염기서열은 진핵생물의 전체 프로모터의 약 30%에 존재한다. 특히 사상성 곰팡이에서는 여러 유전자의 발현을 조절한다고 알려져 있다. 이 연구에서는 기존 전사조절인자의 결손 돌연변이체에 해당 유전자를 재삽입 시킨 복원체와 이들 간의 상승작용에 대해서 알아보기

위하여 두 유전자를 모두 삭제한 이중결손 돌연변이체를 제작하였다. *fct1* 과 *fct3* 은 야생형 균주에 비해 영양생장과 유성생식에서 현격한 결함을 보였다. 특히 이들 결손변이체는 병원성을 완전히 잃어버렸다. 이러한 돌연변이 형질은 각 유전적 복원체에서 야생형과 동일한 정도로 회복된 것으로 보아 해당 유전자의 기능임을 확신할 수 있었다. 각 결손변이체의 형질 결함은 이중 결손변이체에서도 동일한 정도로 관찰되었다. 이를 통해 이 두 유전자는 동일한 유전적 신호전달 경로에서 작용하거나 두 단백질이 복합체로 작용할 것으로 예측할 수 있었다. 실제로 두 단백질간에 상호작용함을 Yeast Two Hybrid 실험을 통해 확인하였다. 두 단백질에 형광단백질을 표지하여 관찰한 결과, Fct1 과 Fct3 모두 핵에 위치하는 것으로 보아 두 단백질 모두 전사조절인자로 작용함을 예상할 수 있었다. 이 연구를 통해 CCAAT DNA 결합부위를 두 전사조절인자가 복합체를 형성하여 곰팡이의 생장, 유성생식, 병원성, 곰팡이독소 생합성에 중요한 역할을 함을 알 수 있었다.

주요어: CCAAT, DNA 결합부위, 붉은곰팡이, 전사조절인자

학번: 2015-23132