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Discovery of essential genes in *Fusarium graminearum* for the establishment of RNAi-based disease control

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김 시 은

Discovery of essential genes in *Fusarium graminearum* for the establishment of RNAi-based disease control

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Sieun Kim

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김 시 은

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위 원 장 이 용 환 (인)

부 위 원 장 손 호 경 (인)

위 원 김 국 형 (인)

위 원 오 창 식 (인)

위 원 이 정 관 (인)

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Discovery of essential genes in *Fusarium graminearum* for the establishment of RNAi-based disease control

UNDER THE DIRECTION OF DR. HOKYOUNG SON

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

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SIEUN KIM

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BY THE COMMITTEE MEMBERS

CHAIRMAN	Yong-Hwan Lee
VICE CHAIRMAN	Hokyoung Son
MEMBER	Kook-Hyung Kim
MEMBER	Chang-Sik Oh
MEMBER	Jungkwan Lee

ABSTRACT

Discovery of essential genes in *Fusarium graminearum* for the establishment of RNAi-based disease control

Sieun Kim

Program in Plant Microbiology

Department of Agricultural Biotechnology

The Graduate School

Seoul National University

Fusarium graminearum is an important plant pathogenic fungus that causes Fusarium head blight (FHB) in major cereal crops such as wheat and barley worldwide. With the lack of available resistant cultivars and emergence of fungal strains exhibiting reduced sensitivity to chemical fungicides, there is a need to establish a sustainable disease control strategy with new antifungal targets. Spray-induced gene silencing (SIGS) is a promising fungal disease control method involving external application of double-stranded RNAs (dsRNAs) targeting fungal virulence genes or essential genes. In this study, I identified and characterized lipase genes and essential genes in *F. graminearum*, investigating their functional roles and evaluating their potential as antifungal targets via SIGS approach.

Lipases are enzymes that catalyze the hydrolysis of long-chain tri-, di-, and

monoglycerides into free fatty acids and glycerol. During the infection process, plant pathogenic fungi secrete extracellular lipases to degrade the plant cuticle layers composed of waxes and lipid polymers. A total 86 putative lipase-encoding genes were identified and individually deleted from *F. graminearum* genome. Comprehensive functional characterization of 86 putative lipase-encoding genes revealed that most lipase mutants were normal in the assessed phenotypes, including virulence, indicating a significant functional redundancy of lipases in *F. graminearum*. Among these lipases, FgLip1 and Fgl1 acted as core extracellular lipases that are essential for the decomposition of extracellular lipid sources. Furthermore, I identified three lipase-regulatory transcription factors (TF) and one histone acetyltransferase that play a crucial role in the transcriptional regulation of *FgLIP1* and *FGL1*. Notably, Gzzc258 was identified as a key lipase regulator involved in the induction of lipase activity during sexual developmental stages.

Essential genes, which are vital for the survival of the organism, have been targeted by commercial antifungal drugs due to their potential to completely inhibit pathogen growth. To identify *F. graminearum* essential genes, I selected essential gene candidates that had previously failed to be deleted. Among them, I failed to obtain deletion mutants for 13 genes with multiple independent transformations. For validation of their essentiality, conditional suppression mutants were generated using a conditional promoter system and their growth was assessed under repressive conditions. Since the previously established P_{ZEAR} system, which is activated by zearalenone or the estrogenic compound β-estradiol, was not applicable to several

putative essential genes, I developed a versatile conditional gene expression system using the *F. graminearum* copper-responsive 1 (*FCR1*) promoter (P_{FCR1}). P_{FCR1} effectively drove copper-dependent expression of heterologous genes, such as green fluorescent protein gene and *FgENA5*. This conditional gene expression system also enabled the generation of the suppression mutants of *FgIRE1,* an essential gene of *F. graminearum,* which allowed its functional characterization.

The essentiality of 11 genes were confirmed using a conditional promoter replacement strategy with either P_{ZEAR} or $P_{FCR1.}$ Additionally, two genes that could not be deleted through a CRISPR/Cas9-mediated approach were also identified as essential genes. To investigate the potential of external application of dsRNAs targeting these essential genes for plant protection, I sprayed dsRNAs on the barley leaves prior to fungal inoculation. *Fg10360*-dsRNA-, *Fg13150*-dsRNA-, and *Fg06123*-dsRNA-sprayed barley leaves exhibited significantly reduced lesion size compared to the water- or *GFP*-dsRNA-treated leaves, suggesting that they could be promising targets for RNA interference (RNAi)-based disease control in *F. graminearum.* Taken together, this study provides resources for RNAi-based antifungal targets in plant pathogenic fungi, also presenting comprehensive database of fungal lipases and conditional promoter system as genetic tool in *F. gramineaurum*.

Keywords: *Fusarium graminearum*, essential gene, lipase, conditional promoter, RNA interference

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CHAPTER I

Genetic and transcriptional regulatory mechanisms of lipase activity in the plant pathogenic fungus *Fusarium graminearum*

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ABSTRACT

Lipases, which catalyze the hydrolysis of long-chain tri-, di-, and monoglycerides into free fatty acids and glycerol, participate in various biological pathways in fungi. In this study, we examined the biological function and regulatory mechanisms of fungal lipases via two approaches. First, we performed a systemic functional characterization of 86 putative lipase-encoding genes in the plant pathogenic fungus *Fusarium graminearum*. The phenotypes were assayed for vegetative growth, asexual and sexual reproduction, stress responses, pathogenicity, mycotoxin production, and lipase activity. Most mutants were normal in the assessed phenotypes, implying overlapping roles for lipases in *F. graminearum*. In particular, FgLip1 and Fgl1 were revealed as core extracellular lipases in *F. graminearum*. Second, we examined the lipase activity of previously constructed transcription factor (TF) mutants of *F. graminearum* and identified three TFs and one histone acetyltransferase that significantly affect lipase activity. The relative transcript levels of *FgLIP1* and *FGL1* were markedly reduced or enhanced in these TF mutants. Among them, Gzzc258 was identified as a key lipase regulator which is also involved in the induction of lipase activity during sexual reproduction. To our knowledge, this study is the first comprehensive functional analysis of fungal lipases and provides significant insights into the genetic and regulatory mechanisms underlying lipases in fungi.

INTRODUCTION

The filamentous ascomycete fungus *Fusarium graminearum* is a causal agent of Fusarium head blight (FHB) on wheat and barley and ear rot on maize (Leslie and Summerell, 2006). *F. graminearum* infection leads to significant yield losses and mycotoxin contamination (e.g., deoxynivalenol (DON) and zearalenone (ZEA)) on the infected grains, which threatens food and feed safety (Goswami and Kistler, 2004; Desjardins and Proctor, 2007). To understand the molecular mechanisms underlying various biological processes and virulence of *F. graminearum*, a number of studies have been conducted through large-scale reverse genetic approaches (Son et al., 2011b; Wang et al., 2011; Yun et al., 2015; Shin et al., 2017; Lee et al., 2018; Dilks et al., 2019; Jiang et al., 2019; Jiang et al., 2020). However, major outbreaks of FHB continue to occur (McMullen et al., 2012; Ma et al., 2020), and studies on fungal development and pathogenicity are still needed.

Lipases (EC 3.1.1.3) are a class of carboxyl ester hydrolases (EC 3.1.1.-) that break the ester linkages of long-chain tri-, di-, and monoglycerides and produce free fatty acids and glycerol, while 'true' esterases (EC 3.1.1.1) catalyze the hydrolysis of simple esters and short-chain fatty acids (Bornscheuer, 2002). These enzymes also catalyze the synthesis and transesterification of the ester bonds to generate new esters (Singh and Mukhopadhyay, 2012). Fungal lipases are mostly extracellular and are significantly induced by environmental factors such as nitrogen and carbon sources, especially in the presence of a lipid source (Gupta et al., 2004; Singh and Mukhopadhyay, 2012). Besides utilization of extracellular lipid sources,

fungal lipases participate in various biological processes, such as appressorium formation (Thines et al., 2000), autophagy (Teter et al., 2001; Nguyen et al., 2011), cell cycle progression (Kurat et al., 2009), and membrane lipid synthesis (Zanghellini et al., 2008).

Plant pathogenic fungi secrete diverse extracellular enzymes during infection, such as cutinases and lipases to degrade plant cuticle layers composed of waxes and lipid polymers called cutin (Kolattukudy, 1985; Sunna and Antranikian, 1997; Voigt et al., 2005). Previous studies of *Botrytis cinerea* and *Alternaria brassicicola* showed that the addition of antilipase antibodies to a conidial suspension led to reduced lesion formation (Commenil et al., 1998; Berto et al., 1999). Pre-treatment of wheat leaves with Lip1 resulted in reduced epicuticular wax crystalloids on the leaf surfaces in *Blumeria graminis* (Feng et al., 2009). In *F. graminearum*, the secreted lipase Fgl1 has been characterized as a virulence factor that represses the vascular callose deposition of the wheat by releasing free fatty acids (Voigt et al., 2005; Blümke et al., 2014). Another secreted lipase, FgLip1, and several feruloyl esterases in *F. graminearum* were reported to be dispensable for pathogenicity (Feng et al., 2005; Balcerzak et al., 2012). Lipase mutants in *Fusarium oxysporum*, *B. cinerea*, and *Magnaporthe oryzae* also retained full virulence on the host plants (Reis et al., 2005; Wang et al., 2007; Bravo-Ruiz et al., 2013).

In *F. graminearum*, several studies have reported the importance of lipid metabolism in the sexual developmental stages. Gene expression profiling suggested that lipid biosynthesis occurs in early sexual development, followed by lipid oxidation processes (Guenther et al., 2009; Sikhakolli et al., 2012). Palmitoyl-2-

oleoyl-3-linoleoyl-rac-glycerol was reported to be a major lipid required for sexual development in this fungus (Lee et al., 2011). Also, Fpo1, a negative regulator of perithecium development in *F. graminearum*, was reported to regulate fatty acid metabolism, and the mutant showed enhanced perithecium formation with excess lipid accumulation during sexual development (Shin et al., 2020).

The aims of this study were (i) to functionally characterize putative lipase-encoding genes for vegetative growth, asexual and sexual reproduction, stress response, pathogenicity, mycotoxin production, and lipase activity, and (ii) to identify transcription factors (TFs) regulating the lipase activity in *F. graminearum*. In this study, we constructed a genome-wide knockout mutant library of 86 putative lipase-encoding genes in *F. graminearum* and functionally characterized all of the mutants. We identified three TFs and one histone acetyltransferase regulating the lipase activity via screening the extracellular lipase activity of previously constructed TF mutants (Son et al., 2011b). We also demonstrated the relationship between sexual reproduction and lipase activity in *F. graminearum*. Our findings and genetic resources provide insights into the genetic and regulatory mechanisms of the lipase activity underlying fungal development.

MATERIALS AND METHODS

I. Strains and culture conditions

The *F. graminearum* wild-type strain Z-3639 (Bowden and Leslie, 1999) was used as a parental strain for transformation experiments. All strains were stored as mycelial suspensions in 20% glycerol solution at -80 °C. TF deletion mutants, and the complementation and overexpressing strain of *MYT3* used in this study have been constructed previously (Son et al., 2011b; Kim et al., 2014). The culture media were prepared following the *Fusarium* laboratory manual (Leslie and Summerell, 2006). Conidial production was induced in carboxymethyl cellulose (CMC) medium or on yeast malt agar (YMA) (Cappellini and Peterson, 1965; Harris, 2005). For lipase activity assay, MM supplemented with 1% olive oil as a sole carbon source was emulsified with Sonics VCX750 ultrasonic processor (Sonics & Materials, Newtown, Connecticut, USA). The growth temperature for fungal strains was set at 25°C.

II. Nucleic acid manipulations, Southern blotting and PCR

The genomic DNA was extracted as previously described (Leslie and Summerell, 2006). Total RNA was extracted from mycelia ground in liquid nitrogen using the Easy-Spin Total RNA Extraction Kit (Intron Biotech, Seongnam, Republic of Korea). Standard protocols were used for restriction endonuclease digestion and agarose gel electrophoresis (Sambrook and Russell, 2001). Southern blot hybridization was performed using the North2South Biotin Random Prime Labeling

Kit and the North2South Chemiluminescent Hybridization and Detection Kit (Thermo Scientific, USA), or by following standard techniques (Sambrook and Russell, 2001). The PCR primers (Table 1) were synthesized by an oligonucleotide synthesis facility (Bionics, Seoul, Republic of Korea).

Table 1. Primers used in this study.

Primers used to generate deletion mutants

Gene name	Oligo name	Sequence (5' to 3')
FGL1	05906-5F	GTACGATTGGAGCGCATTTGATTAG
	05906-5R	gcacaggtacacttgtttagagGCTGGCCTGGGATATCCTGACTTA
	05906-3F	ccttcaatatcatcttctgtcgCTCACGCCAACCGCTCATCA
	05906-3R	GCAACGAGGAGCAACAATCAGA
	05906-5N	CGAGTACCCCGTCCAAAACCT
	05906-3N	CCATGGAGCGAACCTTTTGAGT
	05906 WITH 5F	ACAAGCTGTGACAAGATGGGAACTC
Fg08945	08945-5F	AGCGCTTGGATGATTCGGAGAC
	08945-5R	gcacaggtacacttgtttagagaTCCGATTGGGAATTGCTTTAGACA
	08945-3F	ccttcaatatcatcttctgtcgaTCGGATTACGCGATAGGTTCTGTG
	08945-3R	AGTGTCAATGAGGCAAGGCTGTG
	08945-5N	CAGAGCACCAGTACCGACGACAG
	08945-3N	CATGCTTTGGTGCTTGGTTCGT
	08945 WITH 5F	CGGCCCTTGAACTGAGACGAA
FGL3	04818-5F	AATGTCATCCGCCGATCCTATAAA
	04818-5R	gcacaggtacacttgtttagagTAGTCGCCAGCTGAAAGAAACAAC
	04818-3F	ccttcaatatcatcttctgtcgCTGCTCAGGTTGGTGGGTTCTAA
	04818-3R	GGTGACGGTTGTAGGAGGCTGTAA
	04818-5N	CGTTCAGCATCACATCGTCCAG
	04818-3N	GGTGGAGGACTTGGGTGATGATT
	04818 WITH 5F	TGGTGAAGTGAGCTAGTGGGAAGG

FGL2	01240-5F	TGCAGACGCACCTCCAGACTTT
	01240-5R	gcacaggtacacttgtttagagaGAATCCGCAAGTTTGAACCGC
	01240-3F	ccttcaatatcatcttctgtcgaCGCAGACTGGGGTAGAGGACG
	01240-3R	GACGACACGCAAAGCTTCCATC
	01240-5N	CGGACTCGTTATTCTCTCTGCCC
	01240-3N	TTTGGTCCCGCTCGCTGAA
	01240 WITH 5F	GCTTGGGTTGGGGCTGTGTT
Fg02082	02082-5F	CAACGCAGTGAGCATGAATCCTT
	02082-5R	gcacaggtacacttgtttagagaTGGGTGGAGGAGGCTGAGGG
	02082-3F	ccttcaatatcatcttctgtcgaCTCGTGGAAATAAACAACCCCGT
	02082-3R	AGCGGAGATACCCAGGAAAATGTT
	02082-5N	CATGGGGCATGCATCGTCAG
	02082-3N	CCCATGAACTCGTATGAGCCCAA
	02082 WITH 5F	CGGGTCTGTTATCGAGGGTTTGT
Fg12119	12119-5F	TAGCCTTCATCCTGGCACGGTA
	12119-5R	gcacaggtacacttgtttagagaGCGAGAGCCGGTAGGATAAGCTT
	12119-3F	ccttcaatatcatcttctgtcgaCTTGAAAACAACGGCTTTGGATGG
	12119-3R	CCGATTCCACTACTCTTATGCCCA
	12119-5N	CGCACGTCGGCTCTGTCAATC
	12119-3N	CTCCGAGTCTTCAGGTGGGTTGTC
	12119 WITH 5F	GCTTGGACAGCCGTATTCTGAAAC
Fg02360	02360-5F	CCTTCTTGGCGGTTGGTAGTCTCA
	02360-5R	gcacaggtacacttgtttagagaGGCGTTTCTCATTCTTGCTTGGA
	02360-3F	ccttcaatatcatcttctgtcgaTACTAAGACGGCACCTGAAAACGG

	02360-3R	CGTTGCTCTTGGCCGTATCTTTTA
	02360-5N	CGTAGTCTACCGTCATCGCTGAGG
	02360-3N	CGCGTGGGACTGGAGCAACT
	02360 WITH 5F	AAAAGGCGCTGGGGCAAGG
Fg03612	03612-5F	AACGGCTTCTCAGGGTCAAACATC
	03612-5R	gcacaggtacacttgtttagagCTGCCGAACAAGGAATGAAGTATAGAC
	03612-3F	ccttcaatatcatcttctgtcgTGTTTCTGGTCACGAGTTTCCGA
	03612-3R	CCGAGCTGGCGTGGTCTTTG
	03612-5N	GCTAGACTGATGCCGAGTGTTCCA
	03612-3N	ATTCGATCCCCGCTCTGTTTCA
	03612 WITH 5F	GGCGAATGTCTGGGAACAGCA
Fg03530	03530-5F	GGACGGCCTTGACGAGTGCTA
	03530-5R	gcacaggtacacttgtttagagAATTCATGAGCGTCGCTACTGGA
	03530-3F	ccttcaatatcatcttctgtcgCAGTTGTGTCACCCCCCGATT
	03530-3R	GCTGGGCGTCTCAAGAAATCAC
	03530-5N	CAGACGAACAGCGTTGAAACCC
	03530-3N	GGAGGAGACAGCCGGTTTGAC
	03530 WITH 5F	TCGCAGCCTGAACACCTCCA
Fg11578	11578-5F	GGAAGTGTTTGGCATAGGGAATGA
	11578-5R	gcacaggtacacttgtttagagTGGCGATACTTTGACCAGCAGC
	11578-3F	ccttcaatatcatcttctgtcgAAAACCGCTAGTGCCACCTGTG
	11578-3R	GATAGAAAATAATAATAGGCAGAT
	11578-5N	TCTCCAGGAGAGTTGCCGTCTTG
	11578-3N	GGTATACCCTACTTTTCACTTTT

	11578 WITH 5F	CATAATACGGAGGCTCGGAAGACC
Fg11229	11229-5F	ATTTCCGCATAACATACGACACCG
	11229-5R	gcacaggtacacttgtttagagaTGGCGAAAAATCTCCGAAGTGTAG
	11229-3F	ccttcaatatcatcttctgtcgaGCCTGTCAAGATGAGCGAGAGTGA
	11229-3R	CAATTGTGTTGCGAGGGTATGTCC
	11229-5N	CGCTCTGAATATTTCCTCGGTGG
	11229-3N	TCAAATGGTAACGCCCCAGAAAT
	11229 WITH 5F	AGCCCGTTGAGCCTGTTCCTT
Fg09358	09358-5F	CCCCGATCCCGACCCTACTT
	09358-5R	gcacaggtacacttgtttagagaGGTGGTGAAGTACGGTGAAGGGA
	09358-3F	ccttcaatatcatcttctgtcgaATTCGCCAGTTCTCGGGATGA
	09358-3R	AAAACGCCGAGCCTATGGGTAAAA
	09358-5N	TTATGCTACTGGCGAGCCTGTCA
	09358-3N	ACAGCACTTCCATGATGTCGCC
	09358 WITH 5F	GCACAGAGGCCAATTACCCAAGA
Fg04848	04848-5F	ACGGTGGATGGAGCTAGGTAAGGA
	04848-5R	gcacaggtacacttgtttagagTCATCAAAACCCCGGATTACAAC
	04848-3F	ccttcaatatcatcttctgtcgTGTGGGGATACCTCACTAGTATAGCG
	04848-3R	TGGCGCAACAACCATGATACAAT
	04848-5N	GACTCTGTCATGCTCGACGGTTG
	04848-3N	TTGACGTCCTTTGCTTGATTCACA
	04848 WITH 5F	CGTCGTTGGCGCCACTTCT
Fg13219	13219-5F	GGAGCTCGAAATCATCGCAGATAC
	13219-5R	gcacaggtacacttgtttagagaGCGAAAAGTGCCAAAACATAGCG

	13219-3F	ccttcaatatcatcttctgtcgaAGACCCCAACGGTGGTGACAG
	13219-3R	CTCCGGCCAGTAACCTTCTTCG
	13219-5N	CCGGCCCTGGTCTCATCG
	13219-3N	GTTTGTGTTGAAGGGGTCTGTTGG
	13219 WITH 5F	TCTTGACTGCCTCTCCAGGTTCAG
Fg11112	11112-5F	CAAGAACCATGCTTCGTCGGAG
	11112-5R	gcacaggtacacttgtttagagCATCCGAATTGCCTAAATGGGTAA
	11112-3F	ccttcaatatcatcttctgtcgCGGCCGGTTATTTATCAAGTATAGC
	11112-3R	TGATGATGGACGTTTCTCTGAGGA
	11112-5N	CCATCTGTAGCCACGACTTCATCA
	11112-3N	GAGCCCATTGAGATGCTTGAGAT
	11112 WITH 5F	GGGTTGACTCTACCAACAAAGCGA
Fg00968	00968-5F	TGGCGATCTTCTGCTGAGAGGA
	00968-5R	gcacaggtacacttgtttagagaGCGGAGCTTCTTACGAGGCATAG
	00968-3F	ccttcaatatcatcttctgtcgaACGAGGGCTATATTGTTGTCCGC
	00968-3R	GGGTTTCAACACATGGCAGACTTT
	00968-5N	GTAGAAAACAGGCGCCTCATTCAG
	00968-3N	TGCCGAGTGGAAACAATCATAACG
	00968 WITH 5F	CTGGAAAGGTGCCTGTGAGTCG
Fg07261	07261-5F	CCGGCATGATTGGAGGGTGTA
	07261-5R	gcacaggtacacttgtttagagaGGAATTCTCACGGCCATTTGAAG
	07261-3F	ccttcaatatcatcttctgtcgaCACTGGCGATTAAACTGGGTGGT
	07261-3R	TAGCGAGCGTTCCATCATAAGACC
	07261-5N	AGGAGATGTGACGGAACGGATGT

	07261-3N	GGATCCCAAAAGTTCCTCGCC
	07261 WITH 5F	TTGGAAAGAGAAACCCTCCTGAAGAT
Fg04677	04677-5F	GAATGAGCCTGCCACACCACT
	04677-5R	gcacaggtacacttgtttagagACCCTGGATGTTGAAGGTGTGAA
	04677-3F	ccttcaatatcatcttctgtcgTCGTCACATTCACTGTGGAAGTCA
	04677-3R	TCCCCTGAGTCTTGTGTCCGTAGT
	04677-5N	ATCGACCTGTTCCATAAGCAGTCC
	04677-3N	ACTATCAACAAGGGCACCAACGG
	04677 WITH 5F	TGTGTTGGCGGTATTTTGCGTAGT
Fg03129	03129-5F	CCTTGTCAGAAACAGTGATGCCG
	03129-5R	gcacaggtacacttgtttagagaGAACCTGCTTCGATTCCCTTGTATA
	03129-3F	ccttcaatatcatcttctgtcgaGCTGGTCATGGAACGAGATAAGGTG
	03129-3R	CGCTGCCGCTGAGAACGA
	03129-5N	CGCATAATGACGATCCGACTCAC
	03129-3N	TGTGGCGCTAGTTATTGAGTGTGC
	03129 WITH 5F	AGAACCCAAGCCGACAATCAACTG
Fg01223	01223-5F	GATGGTGTTGGTTGCAAGAGAAGA
	01223-5R	gcacaggtacacttgtttagagCGATGCCGCGAAATGAAGATA
	01223-3F	ccttcaatatcatcttctgtcgGACAACAAACCGGGTCATTCATC
	01223-3R	GATGATGTCCTGATCCTTTCGCTC
	01223-5N	ACTGGGTAGGCCTGTGCTTGTTTA
	01223-3N	AATGTTGCCAGCACCTGTCGTAA
	01223 WITH 5F	GGCCGTGTCTTGTCGAGGTAGG
Fg11280	11280-5F	TGGACAACGTCTCTGTCAGGATGA

	11280-5R	gcacaggtacacttgtttagagTATACGCGCAACAATCATAAGCAC
	11280-3F	ccttcaatatcatcttctgtcgGAGGATGTGTTGGAGTCCGAAAAT
	11280-3R	CCATGAAGGCGACGTTAAGGATAA
	11280-5N	TCTGCTAGGTGTCTCTTGGTGCG
	11280-3N	ACGCAGGACAAGGATGGATGAA
	11280 WITH 5F	AGTAGGGGCTTTGAGGTATGAGAGGA
Fg02634	02634-5F	CCAGATTCGGTGTCATTTCGTTTTA
	02634-5R	gcacaggtacacttgtttagagaTGTATCCTTCCTCCTGACTCCGC
	02634-3F	ccttcaatatcatcttctgtcgaTACCCTGTGGACGATGATGAGCC
	02634-3R	GGGGGTGGTGCTGCGTGT
	02634-5N	AAGAACACGCGTATTGCTGGTCA
	02634-3N	CAATCAGTCGATGGTTGTAAGGTGC
	02634 WITH 5F	TGAAGATAGAATAACCTCGGACGCC
Fg00520	00520-5F	ACACATCAGGCGGGTTGCTAACT
	00520-5R	gcacaggtacacttgtttagagaGCAGGGACATGATTAGTTAGAAACGA
	00520-3F	ccttcaatatcatcttctgtcgaTGGAAGCATCGATAAACATTAGCCC
	00520-3R	CGTCTCTTGTGTTTTCAGCGGC
	00520-5N	AAAACGCCGAGGCTAAGATGGT
	00520-3N	CGCATGGCATAACTTTCTCTAACCC
	00520 WITH 5F	GCGCGGCTTCTTCCCAGTC
Fg08139	08139-5F	TCCTATGGGAGTGTCACGATTGC
	08139-5R	gcacaggtacacttgtttagagTCGATGGTTTGTCAAGGTAGGATG
	08139-3F	ccttcaatatcatcttctgtcgGTGCCTGAAAACTGCCTCTCAA
	08139-3R	ACTGAATTCATCGCCATCGTCTTC

	08139-5N	TCCAACAATGCACTCAGCAGAAAT
	08139-3N	GAGCTTGATCGTGACATTGTGTGG
	08139 WITH 5F	ACATGCCATCAGCCGACACG
FgTGL4	07555-5F	CCAACCAATGTCAAGCAAAAAGGA
	07555-5R	gcacaggtacacttgtttagagaCGGACATTGCGACAGCCTACG
	07555-3F	ccttcaatatcatcttctgtcgaGCGTGCGGGCTGGATACTATG
	07555-3R	AGATGCCCTTGCCCCAGTTCT
	07555-5N	CTCGGAGAAATAGCCACTGTCGTC
	07555-3N	GAGGGAGAAGGAAGTGTAGTCGCC
	07555 WITH 5F	TTGACTTTGTAGGAGGTTTCGCAGA
Fg11174	11174-5F	AAAGCTAGCGTCGCTGAGTTGTG
	11174-5R	gcacaggtacacttgtttagagGCATCATGCTGCACGTAGTTCC
	11174-3F	ccttcaatatcatcttctgtcgTCCACCAGTATTGTGTCCTTTTCG
	11174-3R	CACGGTTTGCGAGAATTGAGTTG
	11174-5N	TTAACGTCTTGGCATCCCAGTCAT
	11174-3N	ACCAGGACCCGATCTAACACAGG
	11174 WITH 5F	CCCACTCTTTCCATGACATTCTTGA
Fg11429	11429-5F	TGAATCGCAACTATGGGACCTATGG
	11429-5R	gcacaggtacacttgtttagagaGGTTTTTGCGCTTTTGGACACG
	11429-3F	ccttcaatatcatcttctgtcgaAGGAAGACTATGGCTGATCCAAATGA
	11429-3R	CGTCGTCTTCATGAGTAGGCACAAC
	11429-5N	GTCCGAATCCACCTCCTCCTGA
	11429-3N	CTGCTGGCTGCTGGTAAGAGAAAC
	11429 WITH 5F	GATACCACCAGTGCTTGTGCCAC

Fg05297	05297-5F	CAGATCGTGCATGTTTCGTGTTG
	05297-5R	gcacaggtacacttgtttagagTGGCTGATGATGAAGATGCTGC
	05297-3F	ccttcaatatcatcttctgtcgGCGACGAGCGAGTATCTGACCT
	05297-3R	TCAGCATCAAGCCACCCTATCTG
	05297-5N	AGCACATTATCAAGCAGGAGACGA
	05297-3N	AGTGGTTGTGGGGTGATGAATAGT
	05297 WITH 5F	CATGTGTAAGGCTCATGAACGGTG
Fg06645	06645-5F	TCGTTTTCGGGTTCAAGTGTTAGG
	06645-5R	gcacaggtacacttgtttagagaCTGGCCGGAGCGAAACATAAG
	06645-3F	ccttcaatatcatcttctgtcgaGATCGTTAACAGCAATTTTCAAGCG
	06645-3R	CGGTGGACAGGCATGTGAGTCTTA
	06645-5N	ACTTGCCACACTCGATCGTTTGA
	06645-3N	CGTAATGGCGTTGAAAGACTGGTT
	06645 WITH 5F	GACGATGACATTGCGAACGAGTTT
Fg00208	00208-5F	TGATGTAAGTCCAGGTGGCAGTGA
	00208-5R	gcacaggtacacttgtttagagaCTCCATGTTGGCTTCGGCG
	00208-3F	ccttcaatatcatcttctgtcgaCTGGCAAACTGGATCATCTCGC
	00208-3R	CTTCGGTTTCCATGGAACAACG
	00208-5N	TGCCCACGTTGCATTCTACTCG
	00208-3N	ACTCCTCCACAAGCGTCCCATTA
	00208 WITH 5F	CGCGCCTCCAAAGTACCGTC
Fg03062	03062-5F	CCACAGCCATCAATCGGTCGT
	03062-5R	gcacaggtacacttgtttagagaACCACTATGGGTTCATTCTGTTCGTT
	03062-3F	ccttcaatatcatcttctgtcgaAAGTGATACAAGCTCGAAGTGGCG

	03062-3R	TACTTGTTATCCAGGTTGGTGCCC
	03062-5N	TAAATCGCTGAAACTGAACCCGTC
	03062-3N	GCACCAACCTGATGCTACACCAC
	03062 WITH 5F	AAGGTCATGAAAAGCCCATACAGGT
Fg08150	08150-5F	GCCATTACCCAACGCACGAA
	08150-5R	gcacaggtacacttgtttagagACATGCGTCCAGACAGCTACAGAG
	08150-3F	ccttcaatatcatcttctgtcgGTTCGGCGTGCTTCAAAGAATAC
	08150-3R	ACACATTGGTGGGACGCTTCTC
	08150-5N	TGGGTGTCTTGTACTCAACGACGA
	08150-3N	ACTGTTGAGTGGACGCCTGGTATT
	08150 WITH 5F	CGATTCAGCAGCGGAGATACCC
Fg10693	10693-5F	CCCCAGATAAGAATCTCCCGTGTT
	10693-5R	gcacaggtacacttgtttagagaAGCTAGAGCCAGCAATACCACAGAGT
	10693-3F	ccttcaatatcatcttctgtcgaCCATTGAAGGGGGTAGCAGGTTT
	10693-3R	TCCACGAAAAATGCCAGGAACTAC
	10693-5N	GTCGCCATTGCTACCCCTCAC
	10693-3N	TGCCCCAACCAGATTTCATAGTGT
	10693 WITH 5F	ATGCGTTAGATGGGGTGTGGG
Fg02823	02823-5F	TCGTCGTGTCATCAACCCTCG
	02823-5R	gcacaggtacacttgtttagagaCATCATCTGCTTTGCTCTGCGTG
	02823-3F	ccttcaatatcatcttctgtcgaTTCTGTCTGTAGCCGCGATGGTTT
	02823-3R	CCGGTACGAAGAAAATGGGAGACT
	02823-5N	TGCCTTTGGGAACTCTGCTCTATG
	02823-3N	AGGAACGACGACGGCTAACAGTG

	02823 WITH 5F	AGCATCAAAGGCCTTCCAGACAC
Fg03875	03875-5F	TCAAGGCGAGCGCTACAGGA
	03875-5R	gcacaggtacacttgtttagagaGGCCAGTCTTGTTGTTGCGTGA
	03875-3F	ccttcaatatcatcttctgtcgaCATCAGTGTACCATCCTGAACCGC
	03875-3R	GAAACATCCAAATCGCGGTTACAT
	03875-5N	GACCCCTGGTGCATATGTGGCT
	03875-3N	GAGCACGCGGTCCCAAAGTC
	03875 WITH 5F	GAAGATGGAACGGTCAAACGCC
Fg01969	01969-5F	CGCGAAAAATACAAGGTGCCATAA
	01969-5R	gcacaggtacacttgtttagagaGAGGGAGCCCCGGTAGTGTCT
	01969-3F	ccttcaatatcatcttctgtcgaTGTCCGTCTGGGCGAGGG
	01969-3R	GGGCGATCAGGATTCCACTCAC
	01969-5N	GCGCCTCGATTGTTTGTCCTC
	01969-3N	CACCTCAACCTCTCCCTTCTCCTC
	01969 WITH 5F	CGTCGACCGTTGTTGCTCTTCT
Fg02073	02073-5F	AATTCATTGGACGTGCTGGACCT
	02073-5R	gcacaggtacacttgtttagagCCAAGTGGCAAAGGGGAAATAA
	02073-3F	ccttcaatatcatcttctgtcgTTGCAGCGTTAGCAGGATTCATC
	02073-3R	AGGGGCATTTGAAGGCAGGA
	02073-5N	GACAGATGGCAGTGCAGTGGATT
	02073-3N	TTGACCCAAACCGCATAAACA
	02073 WITH 5F	CCATGCGCCTCAAAATATAACCAT
FgPLD1	09917-5F	AAATAAGGGGTGGGAGGCATACG
	09917-5R	gcacaggtacacttgtttagagaATGCCGTTCGAGGAGTGAGGTC

	09917-3F	ccttcaatatcatcttctgtcgaGCGTTGCACCCCTGGCTATC
	09917-3R	TCCGGGAGCAGAAGACCTAAACC
	09917-5N	ACGGCGTACAAGATAGCTGAACCA
	09917-3N	GCCTTGCTTCCTGTTTCCCTCTTA
	09917 WITH 5F	GCGTCCGGCGAACCTGAG
FgPLD3	06175-5F	CCAACTGTGCCCTGTGACGG
	06175-5R	gcacaggtacacttgtttagagGTGAGATGCAGGATGGGTAACAAT
	06175-3F	ccttcaatatcatcttctgtcgGCGCTCAACAGTCTCACTGATGTG
	06175-3R	TTGGTTTTGCCTGGGTTGATG
	06175-5N	TCGGCCATGAGCTGTCAATAGT
	06175-3N	CCATCGGCTTTGTCATTGCTG
	06175 WITH 5F	CCGGATGTCTGAAGACACGAATGT
FgPLD2	01973-5F	GACACTGTGTTTCGGGACTGGATT
	01973-5R	gcacaggtacacttgtttagagaGGTACGCTTGTGTTCCGCCAT
	01973-3F	ccttcaatatcatcttctgtcgaAGATGGCGGAATGGGGACTTC
	01973-3R	GAGGTCCGAAGGCAGGAATAAGAA
	01973-5N	GGCGGAGAGAGAGAGACCAGAGTC
	01973-3N	CGCTTCTCATCGCAAACCTCAT
	01973 WITH 5F	TGAGAGCGACACACAAATCACTGAC
Fg09004	09004-5F	AGGGCTGGGCACACCGACT
	09004-5R	gcacaggtacacttgtttagagaAGAGCTCCAGCACCGTTTGAGTT
	09004-3F	ccttcaatatcatcttctgtcgaGGTGGGTGGAGCGTTGTAAGATT
	09004-3R	ATCCTCCGGTTTCTTAGCCTCCA
	09004-5N	ATCTTGCTGTTGTCATTGGTGCTA

	09004-3N	AGTGCTGCGGATGGCTAAACG
	09004 WITH 5F	GAATTGGGCCGGTGTTTGAATAA
Fg06404	06404-5F	CCAACGCAGGGTCATCATCACTA
	06404-5R	gcacaggtacacttgtttagagaGCGATCGTTGGACATAGCAGAAGT
	06404-3F	ccttcaatatcatcttctgtcgaGAAGCAGCTGCCCCCTAAGAACTA
	06404-3R	GTCCTGCTCGCAATTTTCATTCTG
	06404-5N	TCCGAGGGTTGTTGGTGAAAGTT
	06404-3N	GCGAGAATGGCCTTGCGTTAC
	06404 WITH 5F	AAATACGAGCGGGGCGATTCT
Fg07372	07372-5F	TCGCGGAGATTCGTTCGTATGT
	07372-5R	gcacaggtacacttgtttagagGAGCGGGGCTTGAGTGTTACTTG
	07372-3F	ccttcaatatcatcttctgtcgTTGCCGATAAGCTCAGGAACGA
	07372-3R	ACGCTGCGATATGGAAGACGATAA
	07372-5N	ACAAGCATGCAGACACGACCAG
	07372-3N	CGATAACGAAAACTGGGATGAAAG
	07372 WITH 5F	GTGGGGACACGAGGAGGCAT
Fg03358	03358-5F	CCACATCTCGAAATCTCAACAGGG
	03358-5R	gcacaggtacacttgtttagagCCATTGAAGTTGCACCGACCA
	03358-3F	ccttcaatatcatcttctgtcgGTGGTTGTCTGGTGAGGAGGTCTA
	03358-3R	TGTTGCACTCCTTCACACACTCGT
	03358-5N	AAGCGCTGTTGTGTCGTTCCA
	03358-3N	AGCCGAACTCACTCAAATAACGAA
	03358 WITH 5F	TGGACGTAATGCCCGTTTCAATA
Fg09154	09154-5F	CGAGATGCCTACGTCTTGATGGAA

	09154-5R	gcacaggtacacttgtttagagaGCGGTGGTGAGTAAAAGATAGGTG
	09154-3F	ccttcaatatcatcttctgtcgaGCGGAGCAATCGTGAATGAAGGTT
	09154-3R	GCGTCCTATCTTAGGCCGACTTTT
	09154-5N	GGCGCTGTGTTCAGGTTCTATACG
	09154-3N	TCCAAAAGCATATATACGCCGCC
	09154 WITH 5F	CATGGAGTAGGATGGCGGTGTG
Fg09256	09256-5F	AGGAAGAGTGGGATTTGGTTAGGA
	09256-5R	gcacaggtacacttgtttagagCCTTGGGTAGGTAGGTGTAAGTGG
	09256-3F	ccttcaatatcatcttctgtcgCAATAATTATTTTCCAAATCACCCC
	09256-3R	TGATCTCGACCCTTCTCTTTACCC
	09256-5N	GAAAGCACATGAACAACCGAAGC
	09256-3N	CGTGCTAAAACCTCATCCGACAGT
	09256 WITH 5F	TGTTTTGTTTCCATTTCTGGTGAGTG
Fg08571	08571-5F	CTGCTGTGTCAAATCATCTGCCC
	08571-5R	gcacaggtacacttgtttagagaAGGGAATAGCCATCGCAACAATC
	08571-3F	ccttcaatatcatcttctgtcgaGGAAGAGACAGTGGCTCGGAAAA
	08571-3R	CGCATACATACCGCAGGATGAAAT
	08571-5N	GCACACGAAGACGACAAGGGAG
	08571-3N	CATGGCACCTCCTAAGGCTTGAA
	08571 WITH 5F	AGAGATAGTGGAAACCTACGGGCG
TRI8	03532-5F	GGTTGTGAGAGCCCTGCGAAA
	03532-5R	gcacaggtacacttgtttagagaCGCCAACCAAGCCCAACC
	03532-3F	ccttcaatatcatcttctgtcgaTTGACTTCTGGAGATCTGCCTGGT
	03532-3R	CGCTATGTAGGACTCGTAGGCAATG

	03532-5N	GCCATTGTTGAGGAGTCGTAAAGC
	03532-3N	TGCGTCGAGATTTTAGGCGTAGC
	03532 WITH 5F	TGTTCCAGGCTCGGTATTCTCAAA
Fg03486	03486-5F	TCCAAAGCATGTCATCAGCCTATCT
	03486-5R	gcacaggtacacttgtttagagaAGCCAAGCCTGAGACGCCAT
	03486-3F	ccttcaatatcatcttctgtcgaCATATGTATCGCCCGCTGGAAAG
	03486-3R	GAAGCAACAGCAGCAGAAGCCAT
	03486-5N	CAAAGCGCTTCTTCATCGTGTCA
	03486-3N	CGTAGGTGACAATGGTAAGCCCG
	03486 WITH 5F	TTGGTGGTAGTGTTTGCTCGTGAA
Fg03846	03846-5F	GAGGAGGGCCAATTAGGACACAG
	03846-5R	gcacaggtacacttgtttagagCCGTCGACGTTATAACTGTAGTGCG
	03846-3F	ccttcaatatcatcttctgtcgTCTGATTGTTGGCCGTTAGTGTCT
	03846-3R	TGCCCAGCTTCCAGACTACACC
	03846-5N	TACAAAGGGCGGCAAGAGTGAA
	03846-3N	CCACCGTTCACCAGAGATGATTG
	03846 WITH 5F	GCACAGTACCCGGTTCTTCATCCT
Fg03911	03911-5F	CGGAAACGGACCTGATGAGAAGTA
	03911-5R	gcacaggtacacttgtttagagaGAGCGAGGACTACTGGAAGGACG
	03911-3F	ccttcaatatcatcttctgtcgaGGGAGACCTACCTCGCCTTGACT
	03911-3R	GCGCTTTGGGTGCCTTTACG
	03911-5N	TGAGTGGCTTGAGGAGTTTTGGC
	03911-3N	GCAAAGCGAAAAAAGGGGATAGAA
	03911 WITH 5F	GACGAGCAGTGAATTGTGGGAGTG

Fg01571	01571-5F	ATAACCTGATTGGAGCGTTGTTGC
	01571-5R	gcacaggtacacttgtttagagTGGATGGATAGTGGCTGAGGTG
	01571-3F	ccttcaatatcatcttctgtcgACCGCTGATATGCCCAACTTTTA
	01571-3R	ACTTACTCAATCGGGCAACTCGG
	01571-5N	CCCGACAATCACAATCACAAGTCA
	01571-3N	CAACAAAGGGGTAGAACACGGGTA
	01571 WITH 5F	CTCGGAGGCCATCGCATAA
LIP1	01603-5F	TGCATTGGGCTTCACTTCTGATG
	01603-5R	gcacaggtacacttgtttagagAGTCCCGCATTCTCCATCCTAC
	01603-3F	ccttcaatatcatcttctgtcgAAACTCAACCCGCTAACCATCC
	01603-3R	GCGGCCACTCTGCGTAAGTCT
	01603-5N	GGGGCTGTCGATATGGTCCTGT
	01603-3N	TTCAACCAATCTCCCCCGTCTAA
	01603 WITH 5F	TCATGATGGCACCTCGGAAA
Fg01711	01711-5F	GACACGACCGGATGGTAGCAAG
	01711-5R	gcacaggtacacttgtttagagGTTAATTCCCCGCGACCAACT
	01711-3F	ccttcaatatcatcttctgtcgTGCGACAGTGAGGCATAGTCAAA
	01711-3R	CCGAGTGGCCAGCATCATACC
	01711-5N	GCAGTCAGCAGCCGGTTTTCTA
	01711-3N	GCATTCTGTTCTACGGTGTCGGTC
	01711 WITH 5F	ACCAACAGGAGGAACAGCATAAGG
FAEC2	02133-5F	GATGCGCTGGTCCTTTTCTGC
	02133-5R	gcacaggtacacttgtttagagTCGGGCCTGCTTGAACTGA
	02133-3F	ccttcaatatcatcttctgtcgCCGATCATGTGCCGTAACAGA

	02133-3R	TAATCGTGGCGACAATAACAGCAG
	02133-5N	TGACCAGCTGTTTGTTGGAGATTG
	02133-3N	GGCCAACAGCAATAAGACCGAG
	02133 WITH 5F	GCCAGTAAAAATATCTTGCCAGTG
Fg02944	02944-5F	CGAGACGCAATAAACGTTCCAGA
	02944-5R	gcacaggtacacttgtttagagTGCGAGGTTTAGCAATGTCTCAT
	02944-3F	ccttcaatatcatcttctgtcgGGCATGACCTGCACACTAAACAC
	02944-3R	GCCACCCCTTGTCCATTCACT
	02944-5N	TTGTTCAGTACACCGGTTTGAGCA
	02944-3N	GCAGCGACTCCTTTACCGTTAGC
	02944 WITH 5F	AAGAACCCCAAAGCTCCAATACG
FAEC3	02987-5F	TGACGAAAACGATCCCACACAG
	02987-5R	gcacaggtacacttgtttagagTGGAACCAAGCACCAGAATGTC
	02987-3F	ccttcaatatcatcttctgtcgTGTGTGCGCATGATGTTGATTAG
	02987-3R	CTCACCCAACTGGCACTCAAGAC
	02987-5N	TCTTCGGAAGCGGTCTCACTGT
	02987-3N	ACCTCATCCAATATGCTGCTGTCA
	02987 WITH 5F	CATCATGGCCCGTGTTCGTA
Fg03217	03217-5F	GGGTTGCACTGCAGTTTACGGT
	03217-5R	gcacaggtacacttgtttagagTTTAATGAGTTGCTTGTGGTCGC
	03217-3F	ccttcaatatcatcttctgtcgACTCGGACTGCATCCATCACTC
	03217-3R	AAGAGCTTGCAACAATGTTTCACG
	03217-5N	CGGGCCGGAGCGAGACTA
	03217-3N	CTGCAAATCCTCGATTCGGTCTC

	03217 WITH 5F	GGGTCCCAGAGATGTTCAGTGC
Fg03243	03243-5F	TCAACAGCAGGCCATTCTAACTCG
	03243-5R	gcacaggtacacttgtttagagaCGCAGTGACGAGGCTTAGAAGAGA
	03243-3F	ccttcaatatcatcttctgtcgaTTGCTAGCGAACAGTTTGGAAGGT
	03243-3R	GTGTCATTGACAGCTCGGTGGG
	03243-5N	TCGTCTGATGTCGTGTCTCCTGC
	03243-3N	ACCCGAAGCTGATAAACAAACGC
	03243 WITH 5F	CGAGGGTACCGTTCTTGACTTTGA
Fg03601	03601-5F	CCTTGATGGATGGTATGTTGCGTC
	03601-5R	gcacaggtacacttgtttagagaGGCAAGCGCTGCAAACAGTAAT
	03601-3F	ccttcaatatcatcttctgtcgaCGAGTCAAGAGCTGCAGCAAAAGT
	03601-3R	CCACGAAATCGAACAGGCTCAG
	03601-5N	TTGCTGTCAAGCTGTTGATGTTTCA
	03601-3N	ACGACCTACATTAGTGCTGACGGC
	03601 WITH 5F	TCGTACACCGAGCCATTCAGAGA
Fg03687	03687-5F	CGCGCAAATCATATGGAGAATGT
	03687-5R	gcacaggtacacttgtttagagTCACATAGGCTCAGCGGTTCTTTA
	03687-3F	ccttcaatatcatcttctgtcgGGAGTCATCCCTGGAAGCAAAA
	03687-3R	GCCTCTTCACGAATAAAGCCATCA
	03687-5N	CCGAACTGCCAAGTCCTGCTAA
	03687-3N	TCGCCGAGGACTTTTATCAGGTC
	03687 WITH 5F	AGGATGAGGTGGGAGAGCAGATG
Fg03738	03738-5F	GCATATGGTTCTCCAGACGGTGA
	03738-5R	gcacaggtacacttgtttagagGGCAACGCGGTGTTTGTATTT

	03738-3F	ccttcaatatcatcttctgtcgAGCTAGTGGGAACCAGAAAGGG
	03738-3R	CTTGTCTACTTCGTGCAGGGGCT
	03738-5N	GCTGGAAGCGCTGCTGTGAC
	03738-3N	GCCGATACCACATCTCTCCGAA
	03738 WITH 5F	TCCGCATTCTTAGACACCCATACA
Fg04657	04657-5F	CGGAAAATGTTGCTTGATACGAGA
	04657-5R	gcacaggtacacttgtttagagAATGCCTCGGTTGATTGGTAGTG
	04657-3F	ccttcaatatcatcttctgtcgTGCGCTCAGTGTTGTTCCATAAGG
	04657-3R	CTCCTTCCCCATCACATCCAGT
	04657-5N	CCAGGCAAATCTCAATCAACAGG
	04657-3N	CTATCACCCATACTGCGGACCTC
	04657 WITH 5F	ATATCTTTCCCCTCCCCAATGACC
Fg05597	05597-5F	TGTGTTGGTAGATGGCGGTAGAGG
	05597-5R	gcacaggtacacttgtttagagaGACGAGCCTTGCGATTCTTGTG
	05597-3F	ccttcaatatcatcttctgtcgaGATGAGTCTCAATCCAGCAGCAGC
	05597-3R	CGGCACGTCCCACAGTGATTC
	05597-5N	TGAAAACAAAAACAAAACCGTGAGG
	05597-3N	GATGGGCCCAGGTTCACGAT
	05597 WITH 5F	GCCTCGACAGCAACGCCG
Fg05935	05935-5F	CCCAACAGCTTGAATTGTCGGTAG
	05935-5R	gcacaggtacacttgtttagagaGCGCGGTAGTCAGAAGTTTGAGTG
	05935-3F	ccttcaatatcatcttctgtcgaGGGCAAGACGGAGGAGCAATC
	05935-3R	TGTCGGAGCCAAACTTCTTCTACG
	05935-5N	GAGTGGCATTCAGCGATGGGT

	05935-3N	TGTCGGTCGTACTTTTGAGGGAGA
	05935 WITH 5F	GAATGGAAGTTGTGGTGCCGACTA
Fg06437	06437-5F	TTTGCAGGCAAGCCAGTCATAG
	06437-5R	gcacaggtacacttgtttagagGAGAACACGCCAAATGTAAGGAAAG
	06437-3F	ccttcaatatcatcttctgtcgGTGCCGCAAGGACATACAGGA
	06437-3R	TTCAGATCGTTTTTATGCCGTATTG
	06437-5N	GACAAATGGGTGCAGTTCTCCTAT
	06437-3N	ATTTGTGCCGAATGTCTACCCTCT
	06437 WITH 5F	CGCGCTCCATATGACTCTGACTT
Fg06807	06807-5F	TCCGTTCCTTTATGACCCCTTTAG
	06807-5R	gcacaggtacacttgtttagagCTGTGCCTTGGTTTTGGTTATTGT
	06807-3F	ccttcaatatcatcttctgtcgTATCTTGATAAACAGGCATCCGACA
	06807-3R	AGACGGTATCCCTGGCATTAGTGT
	06807-5N	GCGAGCACTTCGGAATAAATGAC
	06807-3N	TATACTGGCCATGGACCTAATCTGAA
	06807 WITH 5F	ACGGCCCTGAAGAAGATGGTAA
Fg08192	08192-5F	GACGCGTCTTTGGTGTTGTTGT
	08192-5R	gcacaggtacacttgtttagagGCTAGTGCTGTGTTGATGAGAGTGC
	08192-3F	ccttcaatatcatcttctgtcgCTCAACCCAAATCCCTTCTCCATA
	08192-3R	GGGGTCACTCAGCACTTCAAATACT
	08192-5N	CAAGTTCGGCGTAATCCTGTAGC
	08192-3N	GCACTTGGTCTGGTACTCCCTCTTA
	08192 WITH 5F	TGCTGTTGGGTACGCATAGTCTG
Fg08746	08746-5F	GTGACGACGATTCTGATGATGACTC

	08746-5R	gcacaggtacacttgtttagagTCCGCCAAGAAAGCAAGTAATGA
	08746-3F	ccttcaatatcatcttctgtcgTGGCGCAATAGGTGGGATAGAG
	08746-3R	GTGACTCTTGCGACGGAATGTAAA
	08746-5N	GATAGCGATGAGGACTGGGAATAGAG
	08746-3N	GTGGCCCTGACCCGAAATCTA
	08746 WITH 5F	CCCCTTCTTGCTTTCGCTCATAA
Fg09122	09122-5F	CGTTGTTTCTGATGCTTGTCCTG
	09122-5R	gcacaggtacacttgtttagagTGAGTGCACTTGCCGAAACCTA
	09122-3F	ccttcaatatcatcttctgtcgTCCAATTTCGTTATCGGCAGTTTA
	09122-3R	ATTGGAGGAACTTGGTGGGAGAA
	09122-5N	ACGTTGTTCCCTGGTTTTCTCTGT
	09122-3N	CCCGACATCCCTGGTTTCAA
	09122 WITH 5F	TCCATGGATGAAAACAAGAACAGG
Fg09181	09181-5F	CGCAAGAGGCTGTGTCCGTG
	09181-5R	gcacaggtacacttgtttagagaGCAAGAAGAACAGCACCCAGCAT
	09181-3F	ccttcaatatcatcttctgtcgaGACAGCGAAATGCCTACTTGGATG
	09181-3R	ATGCTCTTTCTTCCGTAACCTGCG
	09181-5N	TCGAAGAAACGCAGCAGCAAAT
	09181-3N	TTCTTTCCTCGGTTGCTTGCTTC
	09181 WITH 5F	CGTCGGACCAAGTTTCGTTCAG
Fg09264	09264-5F	CGCGGATCTCGAGACAGTTATG
	09264-5R	gcacaggtacacttgtttagagAAGCCCAAATGATTCAAGTATGTCC
	09264-3F	ccttcaatatcatcttctgtcgAGCATTGTGGAAGTAAGCGAAGTG
	09264-3R	CCGTCAGCAGCAGTGGTTGTT

	09264-5N	AGAAGGCGGCAGTTCAGTCG
	09264-3N	GACCGGCAGAGGGGACATTAC
	09264 WITH 5F	GCGGAGACAGGCGATTATGA
Fg10020	10020-5F	CCTCATCCCAGCATCTTGACTTGT
	10020-5R	gcacaggtacacttgtttagagaGGAAGACACGATGGAGTGTTGTGG
	10020-3F	ccttcaatatcatcttctgtcgaTTGCTTTGTTCAGTCACTTGTGCG
	10020-3R	TGGTGCCTGTAATATCCCTCGTTC
	10020-5N	ACGAGGTCGGCAAGGAGTTAGTG
	10020-3N	ACCTGCCTGCACTACCACGGA
	10020 WITH 5F	TGACAAGAGTTGGAACGGGCG
Fg10308	10308-5F	GGGTCGATTTTCCGTTTGAGTG
	10308-5R	gcacaggtacacttgtttagagCGGAGTTTCGGGTTGCGTAA
	10308-3F	ccttcaatatcatcttctgtcgGATTTGGATTACAATGAGACGGGC
	10308-3R	GCCCAACTTACCCCGAATCTTAT
	10308-5N	CAATGAAAGGCCGTACAGTCGTT
	10308-3N	AAAGTAGGCGAGAGTGACGGTAGG
	10308 WITH 5F	ATCTTTCCACCCTTCTTTTCAATG
Fg10713	10713-5F	GTGGATGGAAGGTACGTCAGAGGA
	10713-5R	gcacaggtacacttgtttagagCCAGTTCAGAGTTATGCGGGGTAG
	10713-3F	ccttcaatatcatcttctgtcgTTGGGACCCCCTTTGTATCTGTG
	10713-3R	GCATAGGTGGTCGATAGGGGAGAG
	10713-5N	CGATTAGGTGAATGCAACGAGGAG
	10713-3N	GGTGATGCATGCCAGGGTTAGA
	10713 WITH 5F	CGGTAGCGAAAGGCGTTGTGT

Fg11555	11555-5F	CAATGCCCTTCAGATCGTTCGT
	11555-5R	gcacaggtacacttgtttagagCGTACCTGGTCCTGGCATCGT
	11555-3F	ccttcaatatcatcttctgtcgGCTAATCTGTGTAAGGATGCCGC
	11555-3R	TCAACCAGAGACCCTTCGCTACAT
	11555-5N	GGTCACATCAGCCCACGAGTTG
	11555-3N	AACCATACAAGCCATCTCGGACAA
	11555 WITH 5F	CGCCGAAATCTCCTCACGAC
Fg11562	11562-5F	CACGGATCCACCAGAACGAACT
	11562-5R	gcacaggtacacttgtttagagGGAGTCCGAACCGTGGCTGTAT
	11562-3F	ccttcaatatcatcttctgtcgCGGGGCAATACTGCACTTGAGACT
	11562-3R	TCATTGCATCTTCCAACTGTCCAC
	11562-5N	ACATTGGGGCCAGGGTATCATC
	11562-3N	TGAAGTAAGTGACACCGCAAATGA
	11555 WITH 5F	CGTACCCCTCGCCCACAG
FAEC1	12548-5F	CATCAAGGAAGCCGCGTAGTCA
	12548-5R	gcacaggtacacttgtttagagCGTGGGCAATAAGCGAGGACTA
	12548-3F	ccttcaatatcatcttctgtcgTTGATCGAGGTGGCGGCTATTA
	12548-3R	GGGCTGGGTCTTTGTCGTCTTATC
	12548-5N	ACGGTAGTCTGGGTGGGATGC
	12548-3N	TTCCCTCAACGGTAGAAAAGAAAGA
	12548 WITH 5F	GAAACCGAACCCAGCACCGTA
Fg12678	12678-5F	CAAGAGGACCGCGAAGATGAACT
	12678-5R	gcacaggtacacttgtttagagTCGGTGGGCGCTGTATATTTAGAC
	12678-3F	ccttcaatatcatcttctgtcgCGGGGAGGAGCTAAAGAGGAATA

	12678-3R	AATCTCATGGTGGGTGGTTGGTTA
	12678-5N	TGGAACGAGATCCCAACACTTACG
	12678-3N	GTTTTCCAAGCCAAGAGATGTGACC
	12678 WITH 5F	CTGCGAAGAAACCTCAACAATCC
Fg13493	13493-5F	GGTCTTGGTGGTCATTTCAGTGC
	13493-5R	gcacaggtacacttgtttagagTCTGCCTTGGATCAGCGTTTACTC
	13493-3F	ccttcaatatcatcttctgtcgCAAGAAGACGACTATGGGAGGCAA
	13493-3R	GCTTCACAGCTTACAATGGCGTC
	13493-5N	ATTATGCTTGCGCGTTGGCTTA
	13493-3N	GTCATGAGGGTGGTGTCTTTGTCC
	13493 WITH 5F	TGGAATCATGGTCGTTGCTAAGT
Fg13655	13655-5F	GCTGGAAACCCTTCTCGAATGACT
	13655-5R	GCAGAGCTACAAGGGGCGGT
	13655-3F	gcacaggtacacttgtttagagAGAGTTAGAGCGATGGGCAGCAC
	13655-3R	ccttcaatatcatcttctgtcgACCAGGGTTTTGGGTGAAGAATCT
	13655-5N	TTGGCCGATGAATCCGTTGA
	13655-3N	CGAGTCGATGATATCCAGCACCA
	13655 WITH 5F	GCTAACTTTGACGATGCCTGATAG
Fg13982	13982-5F	TACCCGTTGCTCGCATTGTTAC
	13982-5R	gcacaggtacacttgtttagagCCAGCGTTGACAGTCCCGTCT
	13982-3F	ccttcaatatcatcttctgtcgCAGGGTTTTGGGTGAAGAAT
	13982-3R	GCGGCAACGATGACTGGAAA
	13982-5N	TCAGCGATATGGCGAGGGACTA
	13982-3N	AGAGCACCCCGCAAAACATAGAT

	13982 WITH 5F	TCTTTTACGCCATCCTTCTTATCA
Fg07080	07080-5F	TCGACAGAGCTGAAAGGGTAGTGA
	07080-5R	gcacaggtacacttgtttagagGGTTAAACGCTGGACGTATCAGGT
	07080-3F	ccttcaatatcatcttctgtcgCAAGCGCTCGTCACGGTAATCTA
	07080-3R	GCGGCCACAGGTGAAAGAGAAT
	07080-5N	AAATGGCCGAAGAGTTGCGATA
	07080-3N	TGAGACTATCGAACAGCATACCCCA
	07080 WITH 5F	TACCATTATCATTGCCCTTGTCAT
FGL4	03209-5F	CGGACTTGCGTTTCTTGGAATC
	03209-5R	gcacaggtacacttgtttagagCCATGACGATCGCCATTACCA
	03209-3F	ccttcaatatcatcttctgtcgCCTACATACTTCAACAGCAACGACAG
	03209-3R	CTGAATCTAAACTCTGGCAACCCC
	03209-5N	AACCCGAAGATAGACTACCACCCG
	03209-3N	GCATACCAGGAACGTTGGTCATAA
	03209 WITH 5F	CATCCATGCATCCACAGTCGTAG
Fg09099	09099-5F	CTGGCTGGCACTGCATACGAC
	09099-5R	gcacaggtacacttgtttagagTTAATCCCTCCAGACCGGAAAATA
	09099-3F	ccttcaatatcatcttctgtcgTCCTGGTGCCTCTTAAACATCCTT
	09099-3R	TACTGCAGCTGTTCATCTTACGGC
	09099-5N	GAGGGATAGAAGCCCCTGTAGAATG
	09099-3N	GTACAAACCGAGCTGCACAACGA
	09099 WITH 5F	AAAGGGGGTCTTGGAATAGGAGC
Fg03095	03095-5F	CCCTCTCGATGCCGTCTTTGA
	03095-5R	gcacaggtacacttgtttagagGCCACGAATGCCAAGACTATCAGT

	03095-3F	ccttcaatatcatcttctgtcgCTCATGTTGCTCGTCACCACTTC
	03095-3R	TCTCAAGGTGAGGTGCAGATGTGT
	03095-5N	CCATCGACTTCAACGCTCAGGTA
	03095-3N	GACAATGACTCTGCCGATACCCTT
	03095 WITH 5F	GAAATTGAACCCTTGCCGTGTAGA
	Gen-For	CGACAGAAGATGATATTGAAGG
	Gen-Rev	CTCTAAACAAGTGTACCTGTGC
	Gen-G3	GGGAAGGGACTGGCTGCTATTG
	Gen-G2	GCAATATCACGGGTAGCCAACG
	Gen with 5F	GTTGCCTAGTGAATGCTCCGTAACA

Primers used to generate complemented strains and overexpressing strains

Purpose	Oligo name	Sequence (5' to 3')
*FGL1*com	FGL1-COM-F	tatagggcgaattgggtactcaaattggttTGCAGCACTGCCCGTATTGTAG
	FGL1-COM-R	aacagctcctcgcccttgctcacgttaattaagcctccgcctccgcctccTGATGAGCGGTTGGCGTG
*FGL1*oe	FGL1-OE-F	tttcgtaggaacccaatcttcaaaATGCGTCTCCTGTCACTCCTCTC
	FGL1-OE-R	= FGL1-COM-R
	FGL1-OENOG-R	cgagaaactaggccagcagtagacacATGGCGTACGGATGGTTATGAG
*LIP1*com	LIP1-PRO-F	TGCATTGGGCTTCACTTCTGATG
	LIP1-ORF-R	cgaccgggaaccagttaacaaCATGAGTCGAGAATGAATGCTGTTT
	HYG-F	TAACCCACTCGTGCACCCAAC
	HYG-R	TTGTTAACTGGTTCCCGGTCG
	LIP1-PRO-N	GGGAAGTTCAGGTACGGGAGTTTT
	HYG-F1	GGCTTGGCTGGAGCTAGTGGAGG

*LIP1*oe	LIP1-OE-F	tttcgtaggaacccaatcttcaaaATGAGATTCTCTGGTTTCGTCTCTG
	LIP1-OE-R	cccggtgaacagctcctcgcccttgctcacTACCACCAAAGCACCGGCAT
*FgSAS3*com	FgSAS3-COM-F	tatagggcgaattgggtactcaaattggttAGGGTGCCTCTGCCTTGTCTGT
	FgSAS3-COM-R	cccggtgaacagctcctcgcccttgctcacAATGTCCTCCCCTTCGGCATCA
*FgSAS3*oe	FgSAS3-OE-F	tttcgtaggaacccaatcttcaaaACAATGGAAGAGGATCGTCAACAAG
	FgSAS3-OE-R	= FgSAS-COM-R
*GzZC258*com	GzZC258-COM-5F	GATACGCACGCGAATCTCCTGTC
	GzZC258-COM-5R	cagctcctcgcccttgctcacGTGTCTCTTCATCACTGCCACACC
	GzZC258-COM-3F	cctccactagctccagccaagccTACGACGGTGTGGCAGTGATGAAG
	GzZC258-COM-3R	CGCCAAGCAACACCCATACAATC
	GzZC258-COM-5N	GCCTTTATCCCACAATCCACTTCC
	GzZC258-COM-3N	AAACCCACAGCTGCTCCCACAAA
	HYG-F1	GGCTTGGCTGGAGCTAGTGGAGG
	pIGPAPA-sGFP F	GTGAGCAAGGGCGAGGAGCTG
	Hyg-H3	CGTTATGTTTATCGGCACTTTGC
	Hyg-H2	GCTGCTCCATACAAGCCAACC
*GzZC258*oe	GzZC258-OE-5F	GATACGCACGCGAATCTCCTGTC
	GzZC258-OE-5R	gatagtggaaaccgacgccccAGTGTATTGTAGTTGAGGTTTTGCGC
	GzZC258-OE-F	tatcacaaaaggaacccaatcttcaaagCGTATCATGTCCAACGAAGATCCTT
	GzZC258-OE-R	GAAATAGCCAGGTCCGGGGG
	GzZC258-OE-5N	GCCTTTATCCCACAATCCACTTCC
	GzZC258-OE-RN	TCACCAGGGGAAGAATATGCT
	Neo-F	GGGGCGTCGGTTTCCACTATC
	EF-R	CTTTGAAGATTGGGTTCCTTTTGTGATA

	Gen-G3	GGGAAGGGACTGGCTGCTATTG
	Gen-G2	GCAATATCACGGGTAGCCAACG
*GzZC066*com	GzZC066-COM-F	tatagggcgaattgggtactcaaattggttCCTTGGCCGGTTAATCACGA
	GzZC066-COM-R	aacagctcctcgcccttgctcacgttaattaagcctccgcctccgcctccTGGTTGAGCTGGGTTTTCGG
*GzZC066*oe	GzZC066-OE-F	tttcgtaggaacccaatcttcaaaATGCTCACCAAGGGACCCCT
	GzZC066-OE-R	= GzZC066-COM-R

Primers used for qRT-PCR assays

Gene name	Oligo name	Sequence (5' to 3')
FGL1	FGL1-RT-F	ATCGTCGTCTCGTTCCGTGGTA
	FGL1-RT-R	GAGATCTCGTTCCAGGCATTCTG
LIP1	LIP1-RT-F	GGTGGCTGACAATATTGCTTCCT
	LIP1-RT-R	TCATGATGGCACCTCGGAAA
GzBZIP017	GzBZIP017-RT-F	CAACAACCACCTCAGCAACCTTCT
	GzBZIP017-RT-R	TCTGAGCACCGTTTCTACCCTGA
GzC2H018	GzC2H018-RT-F	GCATGGACTTTGTTAACCCTCTCA
	GzC2H018-RT-R	GAATCGCTGTAATGGAGGTTGTTG
GzC2H045	GzC2H045-RT-F	TCTTGATGGCCTTGACGGATTC
	GzC2H045-RT-R	CGGTGTTGCTAAGCTGAGATTGG
MYT3	MYT3-RT-F	CAGCAGCCACAACAGGAAGACATA
	MYT3-RT-R	GATTCCAAGGACTTCTCGGGTTTC
FgSAS3	FgSAS3-RT-F	CAGGAACCACGACGACGAAGATA
	FgSAS3-RT-R	GGTGCTGCAGTTTCTTCATCATCA
GzZC007	GzZC007-RT-F	GGACGAGAACGATCCAGAAGAAAA

	GzZC007-RT-R	AACACTAGTTGCGTTCCCTGCTTAC
GzZC066	GzZC066-RT-F	AAGAAAAGAGGACGACCACGACTG
	GzZC066-RT-R	TATCGTTGGTTCATGTGCTCTCCT
GzZC115	GzZC115-RT-F	GGATATCGTCATGAAGCCCAAGTT
	GzZC115-RT-R	GCGTACATTCTCAGGCAGTGGATA
GzZC157	GzZC157-RT-F	AGCGTGCAATGGAAAACCTGTC
	GzZC157-RT-R	ACCCCAGCGAATAGAATGTGAAAG
GzZC258	GzZC258-RT-F	AACTTTCGCCAGCTGCTATGTATG
	GzZC258-RT-R	ACATTCTGGCCTGCATACTGGTG
GzZC302	GzZC302-RT-F	ATCAAGCTGCAGGACAAAGTTAGG
	GzZC302-RT-R	TTCCGCTACTAGGCCCCAGATAC
GzZC303	GzZC303-RT-F	TGCCAGGACATTCTCGTTCACT
	GzZC303-RT-R	GCGCTCGTCGAAGTTGAAATAGTA
UBH	UBH-RT-F	GTTCTCGAGGCCAGCAAAAAGTCA
	UBH-RT-R	CGAATCGCCGTTAGGGGTGTCTG
Primers are categorized based on their applications.		

III . Genetic manipulations and fungal transformations

The double-joint (DJ) PCR method (Yu et al., 2004) was used to generate the fusion PCR products required for gene deletion, complementation, and overexpression. Fungal transformation was performed as previously described (Son et al., 2011a).

To construct deletion mutants, the 5' and 3' flanking regions of the target genes were amplified from the genomic DNA, and the geneticin resistance gene cassette (*GEN*) was amplified from pII99 (Namiki et al., 2001). Three fragments were fused by the DJ PCR method, and the final constructs were amplified using the nested primers. The resulting amplicons were transformed into the fungal wild-type protoplasts and the mutants were confirmed by Southern hybridization with a *GEN* probe or a flanking region probe or a probe located in the ORF region.

For complementation of the *gzzc258* deletion mutant, the 5' flanking region, including the open reading frame (ORF) with its native promoter, and the 3' flanking region, were amplified from the genomic DNA of the wild-type strain. The *GFP-HYG* construct carrying the green fluorescent protein (*GFP)* gene and hygromycin resistance gene cassette (*HYG*) was amplified from pIGPAPA (Horwitz et al., 1999). The three amplicons were fused and the final constructs were obtained using nested primers. The resulting amplicons were introduced into the *gzzc258* deletion mutant as previously described (Han et al., 2007) and the mutants were confirmed by Southern blot analysis (Figure 1).

To generate the *GzZC258* overexpression mutant, the 5′ flanking region and ORF of GzZC258 were amplified from the genomic DNA, and the *GEN-P* $_{ef1\alpha}$

construct carrying *GEN* and the elongation factor 1α promoter ($P_{EF1\alpha}$) were amplified from pSKGEN (Lee et al., 2011). The resulting products were fused and the final PCR constructs were amplified with nested primers. The fusion constructs were used to transform fungal wild-type protoplasts. Transformants constructed via DJ PCR and homologous recombination methods were all confirmed by Southern blot analysis (Figure 1).

To complement the *fglip1* deletion mutant, the ORF of *FgLIP1* and its own promoter were amplified and fused with *HYG* amplified from pIGPAPA. The fusion construct was randomly integrated into the *fglip1* deletion mutant.

For complementation of the *fgsas3* deletion mutant, the *FgSAS3-GFP* fusion construct was generated via yeast gap repair approach (Bruno et al., 2004). The ORF of *FgSAS3* and its native promoter were amplified from the genomic DNA of the wild-type strain. The resulting construct and Xho1-digested pDL2 were co-transformed into the yeast strain PJ69-4A (Zhou et al., 2011) using the Alkali-Cation Yeast Transformation Kit (MP Bio, Seoul, Republic of Korea). The *FgSAS3-GFP* fusion vector obtained from the yeast transformants was transformed into *Escherichia coli* DH10B. After verification by sequencing, the plasmid DNA was extracted with the DNA-spin Plasmid DNA Purification Kit (Intron Biotech, Seongnam, Republic of Korea) and used to transform the *fgsas3* deletion mutant. The complementation of the *gzzc066* and *fgl1* deletion mutants was performed via the same strategy.

To overexpress *FgSAS3*, the ORF was amplified and co-transformed with Xho1-digested pDL2 into the yeast strain PJ69-4A as described above. The *RP27-FgSAS3-GFP* fusion vector carrying the *M. oryzae* ribosomal protein 27 promoter

(Bourett et al., 2002) was obtained from the yeast transformants. The subsequent process was carried out in the same way. The verified vectors were transformed into the deletion mutant or the wild-type strain. The overexpression mutant of *GzZC066* was generated using the same strategy, and for *FGL1* and *FgLIP1,* overexpressing strains were constructed without the *GFP* construct.

To overexpress *FGL1* and *FgLIP1* in the TF deletion mutants, *FGL1* and *FgLIP1* overexpression vectors were transformed into the TF deletion mutants, respectively. All overexpressing strains were confirmed by qRT-PCR.

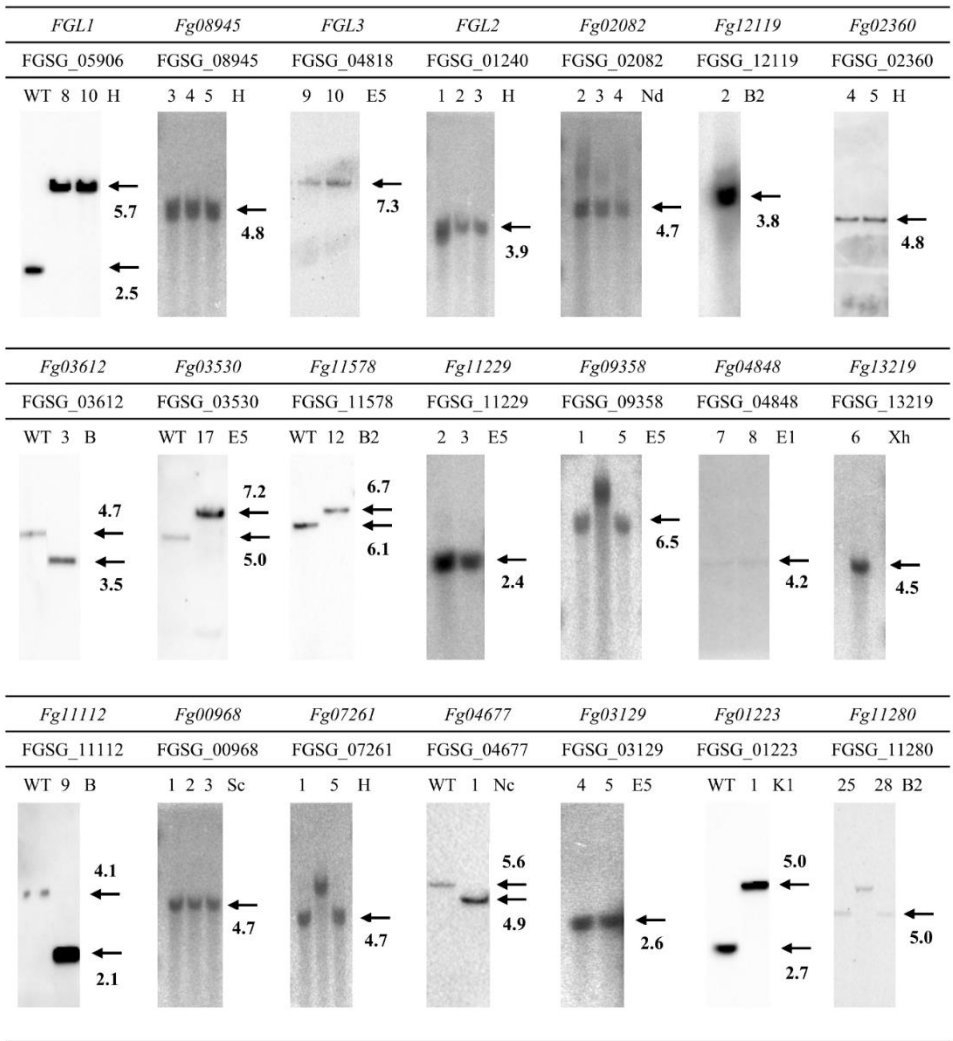
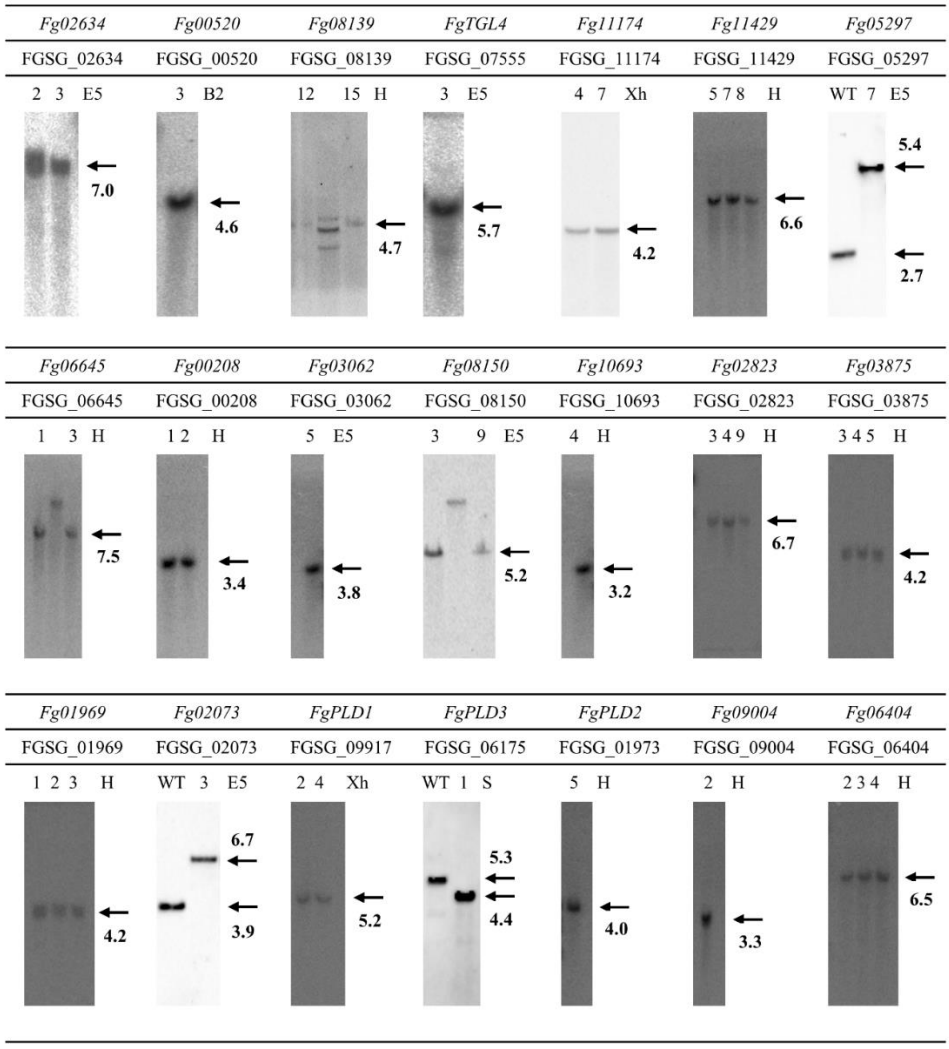


Figure 1. Confirmation of lipase deletion mutants by Southern blot analysis. The restriction enzymes used for each blot and the size of the DNA standards (kb) are indicated on the right of each blot. H, HindIII; E1, EcoRI; E5, EcoRV; B, BamHI; B1, BglI; B2, BglII; S, SalI; Sc, SacI; Nd, NdeI; Xh, XhoI; Xb, XbaI; P1, PstI; C1, ClaI; K1, KpnI; A1, AccI; Ss, SspI; WT, *F. graminearum* wild-type strain Z-3639.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4.6
Fg08139
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12 15 H
4.7
FgTGL4
FGSG_07555
3 E5
5.7
Fg11174
FGSG_11174
4 7 Xh
4.2
Fg11429
FGSG_11429
5 7 8 H
6.6
Fg05297
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WT 7 E5
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Fg08150
FGSG_08150
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5.2
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4 H
3.2
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FGSG_02823
3 4 9 H
6.7
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FGSG_03875
3 4 5 H
4.2
Fg01969
FGSG_01969
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4.2
Fg02073
FGSG_02073
WT 3 E5
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3.9
FgPLD1
FGSG_09917
2 4 Xh
5.2
FgPLD3
FGSG_06175
WT 1 S
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4.4
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FGSG_01973
5 H
4.0
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FGSG_09004
2 H
3.3
Fg06404
FGSG_06404
2 3 4 H
6.5

Fg07372	*Fg03358*	*Fg09154*	*Fg09256*	*Fg08571*	*TRI8*	*Fg03486*
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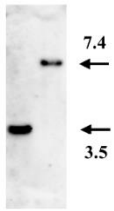	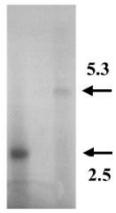	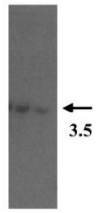	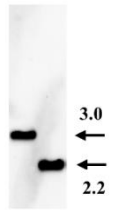	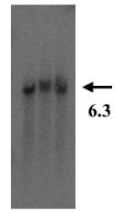	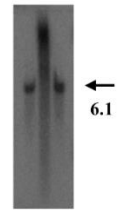	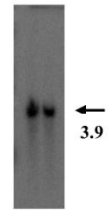

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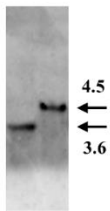	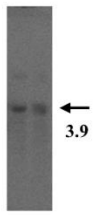	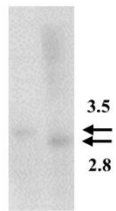	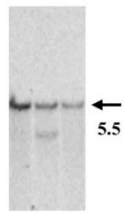	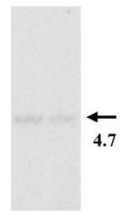	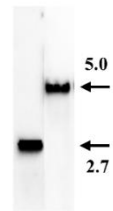	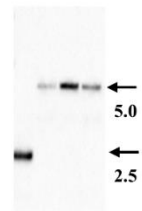

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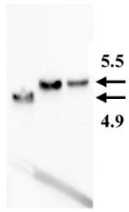	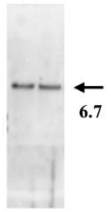	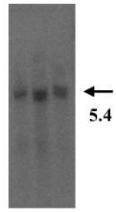	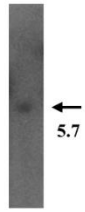	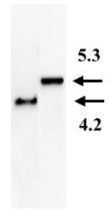	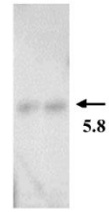	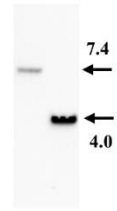

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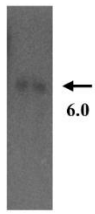	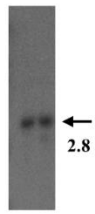	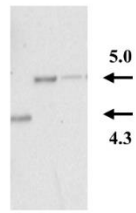	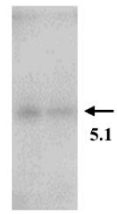	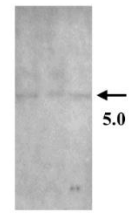	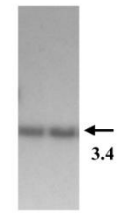	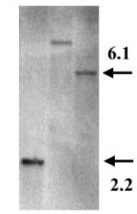

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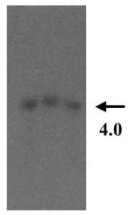	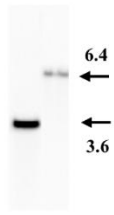	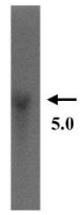	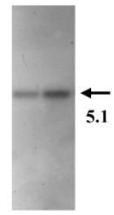	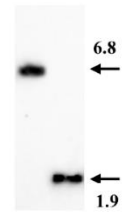	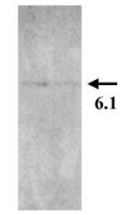	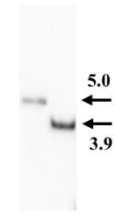

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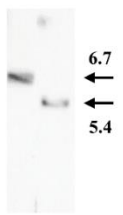	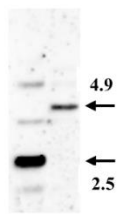	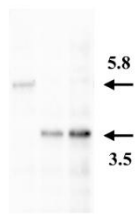	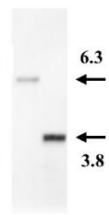	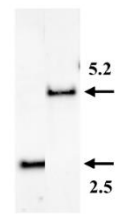	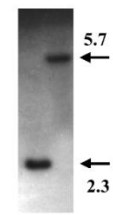	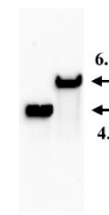

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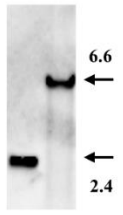
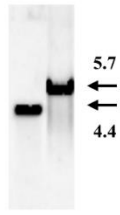
	

IV. Vegetative growth, conidiation, and sexual development

Radial growth and colony morphology were assayed on CM and MM 4-5 days after inoculation. For conidiation assays, a fresh mycelia plug from CM was inoculated in 5 mL of CMC for 5 days on a rotary shaker (200 rpm). The number of conidia was measured with a hemacytometer.

For sexual development, fungal strains were grown on carrot agar for 5 days, and aerial mycelia were removed with 0.4 mL of a sterile 2.5 % Tween 60 solution (Leslie and Summerell, 2006). The plates were then incubated under near-UV light (wavelength: 365 nm; Sankyo Denki, Tokyo, Japan). The number of perithecia, maturation, ascospore morphology, and ascospore discharge were observed after 7-9 days.

For outcrosses, the female strain was fertilized with 1 mL of a conidial suspension from the male strain 5 days after inoculation on carrot agar.

V. Stress response

Stress responses were evaluated by assaying vegetative growth on CM supplemented with various stress agents: oxidative stress (6 mM hydrogen peroxide and 40 μM menadione), osmotic stress (1 M NaCl, 1 M KCl, 1.5 M Sorbitol, 4 mM $FeSO_4$), pH stress (pH 4 and pH 11), cell wall stress (60 mg/L Congo Red and 100 mg/L sodium dodecyl sulfate), DNA synthesis inhibition (7 mg/L iprodione), inhibition of mitogen activated protein kinase (0.02 mg/L fludioxonil), inhibition of meiosis and intracellular transportation (0.65 mg/L benomyl), and azole fungicide (0.025 mg/L tebuconazole) (Son et al., 2011b).

VI. Virulence test and mycotoxin analysis

The point inoculation method was performed to assay fungal virulence as previously described (Son et al., 2011a). Conidia were harvested from CMC cultures and 10 μl of each suspension (10^5 conidia/mL) was injected into the center spikelet of a wheat head (cultivar: Eunpamil). The inoculated wheat plants were incubated in a humid chamber for 3 days and grown in a greenhouse for an additional 18 days. The number of diseased spikelets was measured 21 days after inoculation.

The DON and ZEA extraction was performed as previously described (Park et al., 2012). A fresh mycelial plug was inoculated on 1.5 g of rice substrate for 3 weeks. The rice culture was harvested, ground, and mixed vigorously with 6 mL of 84% acetonitrile for 30 min. After phase separation, the upper phase was filtered through a 0.45 μm syringe filter. Reverse-phase HPLC on a Prominence HPLC system (Shimadzu, Japan) with a C18 column was used for the analysis with a simple modification of the previous methods (Kim et al., 2005; Ok et al., 2018). For ZEA detection, the mobile phase was 70 % aqueous methanol, and the flow rate was 1 mL min^{-1}. For DON detection, the mobile phase was 10 % aqueous acetonitrile (ACN), and the flow rate was 1 mL min^{-1}. A gradient elution program was applied as follows: after 10% ACN was maintained for 11 min, it linearly increased to 30% ACN at 12 min. It was linearly decreased from 30% ACN at 12 min to 10% ACN at 18 min. Subsequently, 10% ACN was held for 17 min for re-equilibration of the column before injection of the next sample, giving a total run time of 35 min. The diode-array detection was apllied (ZEA and DON was detected at wavelength 235 nm).

VII. Lipase activity assay

Lipase activity was detected using rhodamine B (Kouker and Jaeger, 1987). MM supplemented with 1% olive oil (v/v) as a sole carbon source was emulsified, and rhodamine B was added to a final concentration of 0.0005% (w/v). The fresh mycelia plug was inoculated on the rhodamine B plates and the fluorescence was observed 2 days after inoculation under UV light. For screening the lipase activity of TF mutants, vitamin stock solution (Leslie and Summerell, 2006) was added to the medium to clearly observe the fluorescence.

For the quantitative lipase activity assay, a para-nitrophenyl palmitate (pNPP) assay was performed (Winkler and Stuckmann, 1979). The mycelia were cultured in lipase-inducing conditions, and a portion of culture supernatant was mixed with 200 µl of the reaction buffer containing 0.79 mM pNPP, 0.1% (v/v) Triton X-100, 0.1% (w/v) gum arabic, and 50 mM tris-Cl, pH 7.5. The plate was incubated at 37 °C. The amount of released para-nitrophenol (pNP) was measured spectrophotometrically at 410 nm. One unit (U) of lipase activity was defined as 1 µmol of pNP released per minute. For mycelia harvested from carrot agar cultures, the tissue was ground in liquid nitrogen and the whole cell extract was obtained with extraction buffer [50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 5 % Glycerol, 1% NP-4, 1mM EDTA, 1 mM PMSF, 2 µg/mL Leupeptin, 2 µg/mL Pepstatin A, 20 µg/mL Aprotinin]. After centrifugation at 13000 rpm for 20 min, the supernatant was used for the lipase activity assay as described above. Total protein concentration was determined using the Bradford method (Bradford, 1976).

Triacylglycerol lipase activity was measured with a lipase assay kit (MAK046, Sigma). One unit (U) of lipase was defined as the amount of enzyme needed to liberate 1 μmol of glycerol from triglycerides per minute.

To analyze the lipolysis product, 1 mL of reaction mixture containing 10 μl triolein, 50 mM tris-Cl buffer, pH 7.5, and 40 U of culture supernatant estimated by pNPP assay was incubated at 37 °C for 20 h on a rotary shaker (200 rpm). Total lipids were extracted with chloroform/methanol (2:1, v/v) and applied to silica gel 60 TLC plates (Merck, Darmstadt, Germany). The plates were developed with a solvent mixture of hexane/diethyl ether/acetic acid (70:30:1, v/v/v). The spots were visualized using para-anisaldehyde stain and compared with reference standards from Sigma (Supelco mono-, di- and tri-glycerol mix, 1787-1AMP; oleic acid, O1383). The amount of free glycerol in the reaction mixture was measured using the free glycerol assay kit (ab65537, Abcam).

To analyze the amount of oleic acids liberated during the hydrolysis of triolein, the culture supernatant with 50 U of lipase was mixed with 40 mM triolein dissolved in isooctane in 50 mM tris-Cl buffer, pH 7.5. The reaction was initiated by adding 40 mM of triolein dissolved in isooctane to form an aqueous/isooctane biphasic system. The reaction mixture was incubated at 37 °C for 0, 4, 12, 20, 30, 35, 40, and 48 h with vigorous mixing. Total lipids were extracted with acetonitrile:acetone (90:10, v/v), and a portion of the lipid extracts was used to analyze the number of oleic species. The Chiralpak IA column (Daicel Chemical Ind., Osaka, Japan) was loaded on a Waters Alliance e2695 separation module-HPLC (Waters, Milford, MA, USA) and Alltech ELSD 2000 instrument (Alltech Co., Deerfield, MA, USA) system following the previously described method (Lee et al.,

2022).

VIII. Visualization of lipids and microscopic observation

Conidial suspensions were inoculated in CM at 2×10^5 conidia per milliliter, and mycelia were harvested 24 h after incubation on a rotary shaker (200 rpm). The mycelia were recultured in MM, MM without a carbon source, and MM supplemented with olive oil as a sole carbon source for 6, 12, and 24 h. The harvested mycelia were stained with Nile red solution (0.01 mg/mL in acetone) for 5 min (Seong et al., 2008). Microscopic observation was performed with a Leica DM6 B microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Leica DMC6200 camera using the fluorescent filter Y3 (part no. 11504169). The perithecia were imaged 8 days after sexual induction with a Zeiss SteREO Lumar.V12 microscope (Carl Zeiss, Germany).

IX. qRT-PCR

Total RNA was prepared using the Easy-Spin Total RNA Extraction Kit (Intron Biotech, Seongnam, Republic of Korea). First strand cDNA was synthesized from total RNA with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). qRT-PCR was carried out with SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and a with the CFX Real-Time PCR System (Bio-Rad) using the corresponding primers (Table 1). The endogenous housekeeping gene ubiquitin C-terminal hydrolase (*UBH*) was used as a reference gene. The PCR was repeated three times with two biological replicates. The relative transcript levels of target genes

were calculated as previously described (Seong et al., 2008).

Conidial suspensions were inoculated in CM at 2×10^5 conidia per milliliter. Mycelia were harvested 24 h after incubation on a rotary shaker (200 rpm) and washed twice with distilled water. The mycelia were recultured in MM supplemented with olive oil as a sole carbon source. Total RNA was isolated at indicated time points, and the relative transcript levels were analyzed by qRT-PCR. For overexpressing strains, conidial suspensions were inoculated in CM at 5×10^5 conidia per milliliter, and total RNA was extracted after 24 h cultivation. The constitutive expression of each gene was confirmed via qRT-PCR.

RESULTS

I. Identification and deletion of putative lipase-encoding genes in *F. graminearum*

We identified putative lipases in *F. graminearum* based on conserved lipase and esterase domains using InterProScan (Quevillon et al., 2005) and Pfam (Finn et al., 2008) and manually added 9 putative lipases based on MIPS *F. graminearum* genome database (Mewes et al., 2004). A total of 86 putative lipases were identified and classified based on Pfam terms (Mistry et al., 2021) and NCBI Conserved Domains Database (CDD) search (Lu et al., 2020) (Table 2 and Table 3) in which 57% of them belong to the α/β hydrolase fold enzymes, while 19% to the SGNH hydrolases, and the rest to families with phospholipase domains.

To investigate the biological functions of these lipases in *F. graminearum*, we disrupted all 86 putative lipase-encoding genes via homologous recombination and split-marker method. The open reading frame (ORF) of each gene was replaced with a geneticin resistance gene cassette (*GEN*), and the transformants were screened by geneticin resistance and diagnostic PCR. The deletion mutants were further confirmed by Southern blot hybridization (Figure 1). We characterized phenotypic changes in vegetative growth, conidiation, sexual reproduction, stress response, mycotoxin production, virulence, and lipase activity for deletion mutants. Most mutants had no defects in the assessed phenotypes, which implies a significant functional redundancy of lipases in *F. graminearum*. The phenotypic data are

summarized in Table 3.

Table 2. Classification of putative lipases in *F. graminearum.*

Superfamily	Family	Description	Number
Abhydrolase	Lipase_3	Lipase (class 3)	5
	LIP	Secretory lipase	3
	Abhydro_lipase	Partial alpha/beta-hydrolase lipase region	1
	Abhydrolase_1	alpha/beta hydrolase fold	1
	Abhydrolase_2	Phospholipase/Carboxylesterase	5
	Abhydrolase_3	alpha/beta hydrolase fold	7
	Coesterase	Carboxylesterase	16
	Tannase	Tannase and feruloyl esterase	6
	DUF676	Putative serine esterase (DUF676)	4
	PGAP1	PGAP1-like protein	1
SGNH_hydrolase	Lipase_GDSL	GDSL-like Lipase/Acylhydrolase	3
	Lipase_GDSL_2	GDSL-like Lipase/Acylhydrolase	13
Patatin_and_cPLA2	Patatin	Patatin-like phospholipase	10
Phospholip_A2_3	Phospholip_A2_3	Prokaryotic phospholipase A2	1
PLA2_B	PLA2_B	Lysophospholipase catalytic domain	6
PLDc	PLDc	Phospholipase D Active site motif	2
	PLDc_2	PLD-like domain	2
Total			86

Table 3. Phenotypes of 86 lipase gene deletion mutants.

Mycelia growth, sexual development, conidiation, toxin production, virulence, and lipase activity

Locus ID	Gene name	Family	Mycelia growth		Sexual development				Conidiation	Toxin production		Virulence	Lipase activity
			CM	MM	NP	PM	AF	AD		ZEA	DON		
FGSG_05906	*FGL1*	Lipase (class 3)	4[a]	4[b]	4[c]	4[d]	4[e]	4[f]	4[g]	4[h]	4[h]	4[i]	4[j]
FGSG_08945	*Fg08945*	Lipase (class 3)	4	4	4	4	4	4	4	4	4	4	4
FGSG_04818	*FGL3*	Lipase (class 3)	4	4	4	4	4	4	4	4	4	4	4
FGSG_01240	*FGL2*	Lipase (class 3)	4	4	4	4	4	4	4	4	4	4	4
FGSG_02082	*Fg02082*	Lipase (class 3)	4	4	4	4	4	4	4	4	4	4	4
FGSG_12119	*Fg12119*	Lipase_GDSL	4	4	4	4	4	4	4	4	4	4	4
FGSG_02360	*Fg02360*	Lipase_GDSL_2	4	4	4	4	4	4	4	4	4	4	4
FGSG_03612	*Fg03612*	Lipase_GDSL	4	4	4	4	4	4	4	4	4	4	4
FGSG_03530	*Fg03530*	Lipase_GDSL_2	4	4	4	4	4	4	4	4	4	4	4
FGSG_11578	*Fg11578*	Lipase_GDSL_2	4	4	4	4	4	4	4	4	4	4	4
FGSG_11229	*Fg11229*	Lipase_GDSL_2	4	4	4	4	4	4	4	4	4	4	4
FGSG_09358	*Fg09358*	Lipase_GDSL	4	4	4	4	4	4	4	4	4	4	4
FGSG_04848	*Fg04848*	Lipase_GDSL_2	4	4	4	4	4	4	4	4	4	4	4
FGSG_13219	*Fg13219*	Lipase_GDSL_2	4	4	4	4	4	4	4	4	4	4	4
FGSG_11112	*Fg11112*	Lipase_GDSL_2	4	4	4	4	4	4	4	4	4	4	4
FGSG_00968	*Fg00968*	Lipase_GDSL_2	4	4	4	4	4	4	4	4	4	4	4
FGSG_07261	*Fg07261*	Lipase_GDSL_2	4	4	4	4	4	4	4	4	4	4	4
FGSG_04677	*Fg04677*	Lipase_GDSL_2	4	4	4	4	4	4	4	4	4	4	4
FGSG_03129	*Fg03129*	Lipase_GDSL_2	4	4	4	4	4	4	4	4	4	4	4
FGSG_01223	*Fg01223*	Lipase_GDSL_2	4	4	4	4	4	4	4	4	4	4	4
FGSG_11280	*Fg11280*	Lipase_GDSL_2	4	4	4	4	4	4	4	4	4	4	4

FGSG_02634	*Fg02634*	Patatin	4	4	4	4	4	4	4	4	4	4	4
FGSG_00520	*Fg00520*	Patatin	4	4	4	4	4	4	4	4	4	4	4
FGSG_08139	*Fg08139*	Patatin	4	4	3	3	4	4	4	4	4	4	4
FGSG_07555	*FgTGL4*	Patatin	4	4	4	4	4	4	4	4	4	4	4
FGSG_11174	*Fg11174*	Patatin	4	4	4	4	4	4	4	4	4	4	4
FGSG_11429	*Fg11429*	Patatin	4	4	4	4	4	4	4	4	4	4	4
FGSG_05297	*Fg05297*	Patatin	4	3	3	3	4	4	4	4	4	4	4
FGSG_06645	*Fg06645*	Patatin	4	4	4	4	4	4	4	4	4	4	4
FGSG_00208	*Fg00208*	Patatin	4	3	4	4	4	4	4	4	4	4	4
FGSG_03062	*Fg03062*	Patatin	4	4	4	4	4	4	4	4	4	4	4
FGSG_08150	*Fg08150*	PLA2_B	4	4	4	4	4	4	4	4	4	4	4
FGSG_10693	*Fg10693*	PLA2_B	4	4	4	4	4	4	4	4	4	4	4
FGSG_02823	*Fg02823*	PLA2_B	4	4	4	4	4	4	4	4	4	4	4
FGSG_03875	*Fg03875*	PLA2_B	4	4	4	4	4	4	4	4	4	4	4
FGSG_01969	*Fg01969*	PLA2_B	4	4	4	4	4	4	4	4	4	4	4
FGSG_02073	*Fg02073*	PLA2_B	4	4	4	4	4	4	4	4	4	4	4
FGSG_09917	*FgPLD1*	PLDc	1	1	0	0	0	0	2	6	6	0	4
FGSG_06175	*FgPLD3*	PLDc_2	4	4	4	4	4	4	4	4	4	4	4
FGSG_01973	*FgPLD2*	PLDc	4	4	4	4	4	4	4	4	4	4	4
FGSG_09004	*Fg09004*	PLDc_2	4	4	4	4	4	4	4	4	4	4	4
FGSG_06404	*Fg06404*	Abhydrolase_2	4	4	4	4	4	4	4	4	4	4	4
FGSG_07372	*Fg07372*	Abhydrolase_2	5	5	4	4	4	4	4	4	4	4	4
FGSG_03358	*Fg03358*	Abhydrolase_2	4	4	4	4	4	4	4	4	4	4	4
FGSG_09154	*Fg09154*	Abhydrolase_2	4	4	4	4	4	4	4	4	4	4	4
FGSG_09256	*Fg09256*	Abhydrolase_2	4	4	4	4	4	4	4	4	4	4	4
FGSG_08571	*Fg08571*	Abhydrolase_1	4	4	4	4	4	4	4	4	4	4	4
FGSG_03532	*TRI8*	LIP	4	4	4	4	4	4	4	4	2	4	4

FGSG_03486	*Fg03486*	LIP	4	4	4	4	4	4	4	4	4	4	4
FGSG_03846	*Fg03846*	LIP	4	4	4	4	4	4	4	4	4	4	4
FGSG_03911	*Fg03911*	Phospholip_A2_3	4	4	4	4	4	4	4	4	4	4	4
FGSG_01571	*Fg01571*	Abhydrolase_3	4	4	4	4	4	4	4	4	4	4	4
FGSG_01603	*LIP1*	Coesterase	4	4	4	4	4	4	4	4	4	4	2
FGSG_01711	*Fg01711*	Coesterase	4	4	4	4	4	4	4	4	4	4	4
FGSG_02133	*FAEC2*	Tannase	4	4	4	4	4	4	4	4	4	4	4
FGSG_02944	*Fg02944*	Coesterase	4	4	4	4	4	4	4	4	4	4	4
FGSG_02987	*FAEC3*	Tannase	4	4	4	4	4	4	4	4	4	4	4
FGSG_03217	*Fg03217*	Tannase	4	4	4	4	4	4	4	4	4	4	4
FGSG_03243	*Fg03243*	Coesterase	4	4	4	4	4	4	4	4	4	4	4
FGSG_03601	*Fg03601*	Coesterase	4	4	4	4	4	4	4	4	4	4	4
FGSG_03687	*Fg03687*	Coesterase	4	4	4	4	4	4	4	4	4	4	4
FGSG_03738	*Fg03738*	Abhydrolase_3	4	4	4	4	4	4	4	4	4	4	4
FGSG_04657	*Fg04657*	Abhydrolase_3	4	4	4	4	4	4	4	4	4	4	4
FGSG_05597	*Fg05597*	Abhydro_lipase	4	4	4	4	4	4	4	4	4	4	4
FGSG_05935	*Fg05935*	Coesterase	4	4	4	4	4	4	4	4	4	4	4
FGSG_06437	*Fg06437*	Coesterase	4	4	4	4	4	4	4	4	4	4	4
FGSG_06807	*Fg06807*	DUF676	4	4	4	4	4	4	4	4	4	4	4
FGSG_08192	*Fg08192*	Abhydrolase_3	4	4	4	4	4	4	4	4	4	4	4
FGSG_08746	*Fg08746*	Abhydrolase_3	4	4	4	4	4	4	4	4	4	4	4
FGSG_09122	*Fg09122*	Coesterase	4	4	4	4	4	4	4	4	4	4	4
FGSG_09181	*Fg09181*	Coesterase	4	4	4	4	4	4	4	4	4	4	4
FGSG_09264	*Fg09264*	Abhydrolase_3	4	4	4	4	4	4	4	4	4	4	4
FGSG_10020	*Fg10020*	PGAP1	4	4	4	4	4	4	4	4	4	4	4
FGSG_10308	*Fg10308*	DUF676	4	4	4	4	4	4	4	4	4	4	4
FGSG_10713	*Fg10713*	Coesterase	4	4	4	4	4	4	4	4	4	4	4

FGSG_11555	*Fg11555*	Abhydrolase_3	4	4	4	4	4	4	4	4	4	4	4
FGSG_11562	*Fg11562*	Tannase	4	4	4	4	4	4	4	4	4	4	4
FGSG_12548	*FAEC1*	Tannase	4	4	4	4	4	4	4	4	4	4	4
FGSG_12678	*Fg12678*	DUF676	4	3	3	3	4	4	4	4	4	4	4
FGSG_13493	*Fg13493*	DUF676	4	4	4	4	4	4	4	4	4	4	4
FGSG_13655	*Fg13655*	Coesterase	4	4	4	4	4	4	4	4	4	4	4
FGSG_13982	*Fg13982*	Tannase	4	4	4	4	4	4	4	4	4	4	4
FGSG_07080	*Fg07080*	Coesterase	4	4	4	4	4	4	4	4	4	4	4
FGSG_03209	*FGL4*	Coesterase	4	4	4	4	4	4	4	4	4	4	4
FGSG_09099	*Fg09099*	Coesterase	4	4	4	4	4	4	4	4	4	4	4
FGSG_03095	*Fg03095*	Coesterase	4	4	4	4	4	4	4	4	4	4	4

Stress responses

Locus ID	Gene name	Stress responses												
		H_2O_2	Menadione	NaCl	KCl	Sorbitol	$FeSO_4$	pH4	pH11	Congo Red	Sodium dodecyl sulfate	Iprodione	Fludioxonil	Benomyl	Tebuconazole
FGSG_05906	*FGL1*	4^k	4^k	4^k	4^k	4^k	4^k	4^k	4^k	4^k	4^k	4^k	4^k	4^k	4^k
FGSG_08945	*Fg08945*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_04818	*FGL3*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_01240	*FGL2*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_02082	*Fg02082*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_12119	*Fg12119*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_02360	*Fg02360*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_03612	*Fg03612*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_03530	*Fg03530*	4	4	4	4	4	4	4	4	4	4	4	4	4	4

FGSG_11578	*Fg11578*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_11229	*Fg11229*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_09358	*Fg09358*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_04848	*Fg04848*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_13219	*Fg13219*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_11112	*Fg11112*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_00968	*Fg00968*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_07261	*Fg07261*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_04677	*Fg04677*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_03129	*Fg03129*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_01223	*Fg01223*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_11280	*Fg11280*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_02634	*Fg02634*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_00520	*Fg00520*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_08139	*Fg08139*	4	4	4	4	4	4	4	4	4	2	4	4	4	4
FGSG_07555	*FgTGL4*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_11174	*Fg11174*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_11429	*Fg11429*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_05297	*Fg05297*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_06645	*Fg06645*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_00208	*Fg00208*	4	4	4	4	4	4	4	4	4	2	4	4	4	4
FGSG_03062	*Fg03062*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_08150	*Fg08150*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_10693	*Fg10693*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_02823	*Fg02823*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_03875	*Fg03875*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_01969	*Fg01969*	4	4	4	4	4	4	4	4	4	4	4	4	4	4

FGSG_02073	*Fg02073*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_09917	*FgPLD1*	6	4	4	4	4	6	6	2	6	2	4	4	4	4
FGSG_06175	*FgPLD3*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_01973	*FgPLD2*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_09004	*Fg09004*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_06404	*Fg06404*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_07372	*Fg07372*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_03358	*Fg03358*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_09154	*Fg09154*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_09256	*Fg09256*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_08571	*Fg08571*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_03532	*TRI8*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_03486	*Fg03486*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_03846	*Fg03846*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_03911	*Fg03911*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_01571	*Fg01571*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_01603	*LIP1*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_01711	*Fg01711*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_02133	*FAEC2*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_02944	*Fg02944*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_02987	*FAEC3*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_03217	*Fg03217*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_03243	*Fg03243*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_03601	*Fg03601*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_03687	*Fg03687*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_03738	*Fg03738*	4	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_04657	*Fg04657*	4	4	4	4	4	4	4	4	4	4	4	4	4	4

FGSG_05597	*Fg05597*	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_05935	*Fg05935*	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_06437	*Fg06437*	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_06807	*Fg06807*	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_08192	*Fg08192*	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_08746	*Fg08746*	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_09122	*Fg09122*	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_09181	*Fg09181*	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_09264	*Fg09264*	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_10020	*Fg10020*	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_10308	*Fg10308*	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_10713	*Fg10713*	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_11555	*Fg11555*	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_11562	*Fg11562*	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_12548	*FAEC1*	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_12678	*Fg12678*	4	4	2	2	2	4	4	4	4	4	4	4	4
FGSG_13493	*Fg13493*	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_13655	*Fg13655*	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_13982	*Fg13982*	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_07080	*Fg07080*	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_03209	*FGL4*	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_09099	*Fg09099*	4	4	4	4	4	4	4	4	4	4	4	4	4
FGSG_03095	*Fg03095*	4	4	4	4	4	4	4	4	4	4	4	4	4

[a] Percentage of average radial growth on complete media (CM) of each mutant compared to wild-type strain was scored (5, more than wild-type strain; 4, 91-100%; 3, 61-90%; 2, 31-60 %; 1, 0-30 %; 0, 0 %).

[b] Percentage of average radial growth on minimal media (MM) of each mutant compared to wild-type strain was scored (5, more than wild-type strain; 4, 91-100%; 3, 61-90%; 2, 31-60 %; 1, 0-30 %; 0, 0 %).

[c] Number of perithecium (NP) was scored from "0" to "5" (1, less than 25% of wild-type strain; 2, 25-50% less; 3, 50-75% less; 4, 75-100% less; 5, more than wild-type strain; 0, no perithecium).

[d] Perithecia maturation (PM) was determined by size compared to wild-type strain (4, normal; 3, 1-2 day delayed; 2, 3-7 days delayed; 1, more than 7 days delayed maturation).

[e] Ascospore formation (AF) represents the existence and morphology of ascospores (4, normal ascospores; 2, abnormal shape of ascospore; 0, no observable ascospores).

[f] Ascospore discharge (AD) was determined by discharged number of ascospores compared to wild-type strain (4, normal; 2, reduced; 0, no discharge).

[g] Conidiation represents conidia production of each mutant compared to wild-type strain (4, normal; 2, reduced; 0, no visible conidia production).

[h] Toxin production (ZEA, zearalenone; DON, deoxynivalenol) was quantified compared to wild-type strain (6, overproduction; 4, similar to wild-type strain; 2, reduced production; 0, no detectable production).

[i] Virulence on wheat heads was scored (4, normal; 0, markedly reduced virulence compared to wild-type strain).

[j] Lipase activity of each mutant was determined by fluorescence emitted on the rhodamine B plate containing olive oil compared to wild-type strain (6, increased; 4, normal; 2, reduced; 0, no detectable activity).

[k] Radial growth of mutants on each stress-inducing media was evaluated compared to growth on mock complete media (CM) (2, susceptible; 4, normal; 6, resistant).

II. Vegetative growth, conidiation, and sexual development

We observed vegetative growth and colony morphology of the mutants on complete medium (CM) and minimal medium (MM). *fg08139, fg05297, fg00208, fgpld1,* and *fg12678* showed reduced mycelial growth, especially on MM, and interestingly, the growth rate of *fg07372* was markedly enhanced compared to the wild-type on both CM and MM (Figure 2A and 2B).

To assess conidiation, we counted the number of conidia and observed conidial morphology of the mutants in carboxymethyl cellulose medium (CMC). Only *fgpld1* showed reduced conidiation by approximately 65% and produced abnormal conidia with shorter size as described in the previous study (Ding et al., 2017) (Figure 2C and 2D).

With respect to sexual development, *fg08139, fg05297, fgpld1,* and *fg12678*, which showed reduced mycelial growth, were also defective in perithecial maturation. *fg08139, fg05297* and *fg12678* produced a number of smaller immature perithecia than the wild-type but all mutants had no defects in the morphology of ascospores and ascospore discharge (Figure 3A). The deletion of *fgpld1* completely abolished perithecial formation as shown in the previous study (Ding et al., 2017).

III. Stress response, mycotoxin biosynthesis, and virulence

We evaluated the responses of the mutants under various stress conditions, including oxidative stress, osmotic stress, cell wall stress, pH stress, and several

fungicide treatments. A few mutants had altered sensitivity or resistance to the stress agents (Table 3). Among them, *fg12678* exhibited strong sensitivity to osmotic stress induced by NaCl and KCl (Figure 3B).

When assayed for mycotoxin production, the amounts of zearalenone (ZEA) and deoxynivalenol (DON) were enhanced in *fgpld1* (Table 3). For virulence, only *fgpld1* showed defects in the virulence as previously reported (Ding et al., 2017).

These phenotypic results indicate that some lipases and esterases are involved in the important biological processes, but most play redundant roles in *F. graminearum*, so that the loss of a single gene does not lead to significant phenotypic defects.

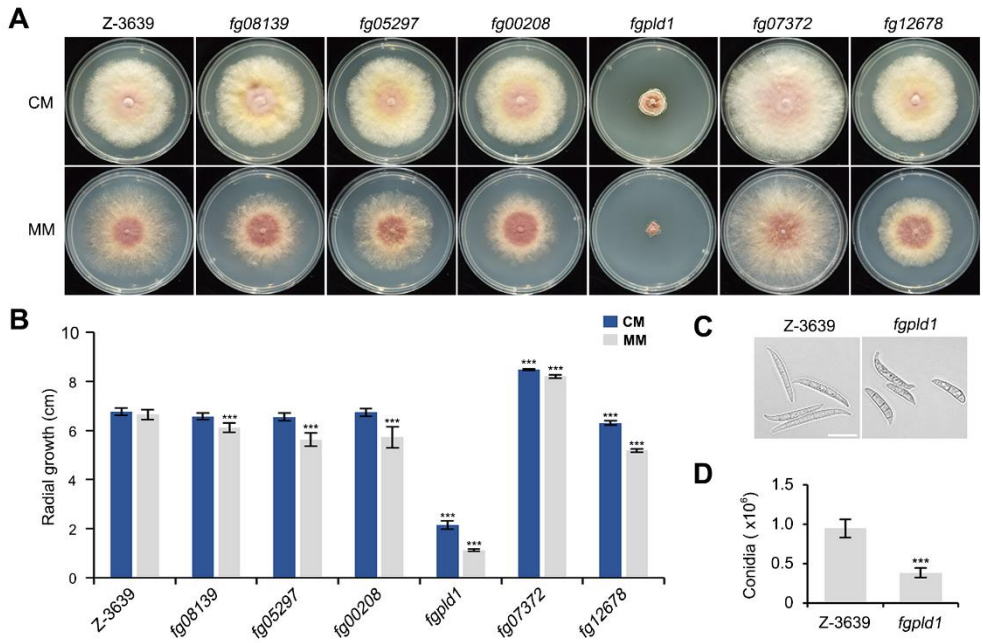


Figure 2. Vegetative growth and conidiation of lipase mutants. (A) The mycelial growth on complete medium (CM) and minimal medium (MM). The strains were imaged 4 days after inoculation. (B) Radial growth on CM and MM. Radial growth was measured 4 days after inoculation. Significant differences (***P < 0.001) compared to the wild-type are indicated by asterisks. (C) Conidial morphology. The photographs were taken 3 days after conidial induction in carboxymethyl cellulose medium (CMC). Scale bar = 20 μm. (D) Conidial production. The number of conidia was measured 5 days after inoculation in CMC.

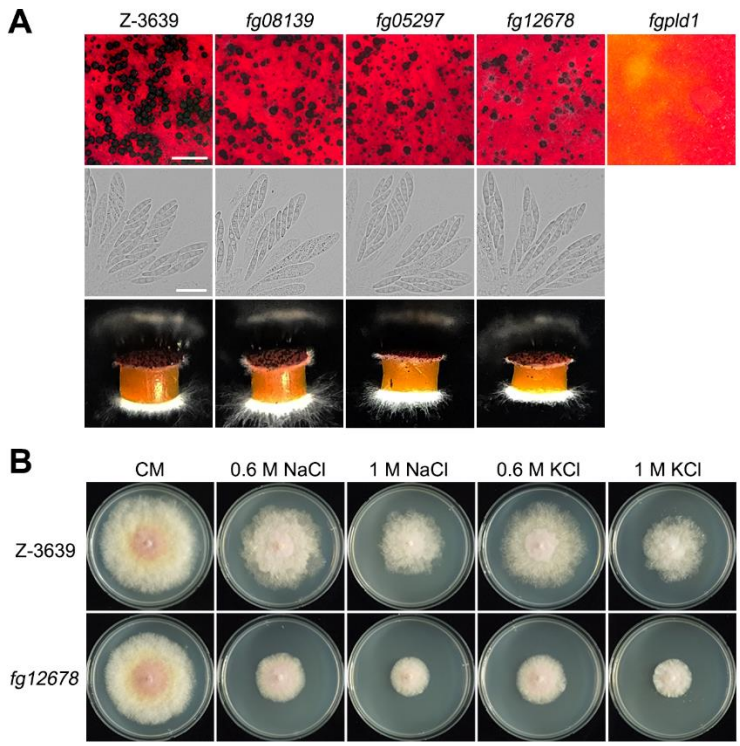


Figure 3. Sexual development and stress response. (A) Perithecium formation, ascospore formation, and forcible ascospore discharge. The perithecia and the asci were imaged 8 days after sexual induction on carrot agar. Scale bar = 1000 μm (top panel, for dissecting microscopic pictures), 20 μm (middle panel for bright field (BF) images). White cloudy materials (bottom panel) show discharged ascospores. The discharged ascospores were imaged 48 h after initiation of the assay. (B) Osmotic stress sensitivity. Strains were incubated on CM and CM supplemented with osmotic stress agents (0.6 M NaCl, 1 M NaCl, 0.6 M KCl, 1 M KCl). The photographs were taken 5 days after inoculation.

IV. FgLip1 and Fgl1 act as core extracellular lipases in *F. graminearum*

Olive oil is primarily composed of triglycerides (~99%) and secondarily other lipid sources (Boskou et al., 2006). To evaluate the lipase activity, we inoculated the mutants on MM supplemented with olive oil as a sole carbon source, which was used as a lipase-inducing medium for overall experiments. The fluorescence formed by the interaction of rhodamine B and free fatty acids was observed 2 days after inoculation under ultraviolet (UV) light. Contrary to expectations, no mutants exhibited reduced fluorescence except for the deletion mutant of *FgLIP1*, which was previously reported to encode a secreted lipase (Feng et al., 2005) (Figure 4A). The interesting thing was that the deletion of *FGL1*, which led to significantly reduced lipase activity in the previous study (Voigt et al., 2005), made no difference to the fluorescence. We hypothesized that FgLip1 might primarily act on the hydrolysis of triglycerides, which compose the majority of olive oil, while Fgl1 might have different substrate preferences.

In order to precisely analyze the lipase activities of *fglip1* and *fgl1,* we took two approaches. First, we constructed a *fglip1 fgl1* double mutant and the complemented strains of the mutants. *fglip1 fgl1* exhibited almost no fluorescence while weak fluorescence was still detected in *fglip1* (Figure 4A). The fluorescence was restored in the *FgLIP1* complemented strain. Second, we cultured the strains in the lipase-inducing medium, and the lipase activities of the culture supernatants were measured at various time points using para-nitrophenyl palmitate (pNPP) as a

substrate (Figure 4B). The amount of para-nitrophenol released by enzymatic hydrolysis of pNPP was measured spectrophotometrically (Winkler and Stuckmann, 1979). The lipase activity of the wild-type increased over time after induction and *fglip1* had consistently reduced activity compared with the wild-type. *fgl1* showed reduced lipase activity within the first 8 h, but almost recovered its activity at 12 h after inoculation (Figure 4B). The lipase activity of *fglip1 fgl1* was not or barely detected at overall time points, indicating that the extracellular lipase activity of *F. graminearum* completely depends on FgLip1 and Fgl1. To figure out if recovery of the lipase activity in *fgl1* at 12 h was due to upregulation of *FgLIP1*, we analyzed the relative transcript level of *FgLIP1* at 2, 4, 8, 12, and 24 h after inoculation in the lipase-inducing medium via quantitative reverse transcription (qRT)-PCR assays (Figure 4C). The wild-type and *fgl1* showed similar expression patterns of *FgLIP1*, which implies that a mechanism other than transcriptional regulation exists to regulate lipase activity.

Previous studies showed that *F. graminearum* accumulates lipid droplets when cultured in a rich medium and degrades the stored lipid droplets in carbon starvation conditions (Nguyen et al., 2011; Josefsen et al., 2012). We hypothesized that extracellular lipase activity of the strains might also affect the distribution of the intracellular lipid droplets in the starvation or olive oil-supplemented conditions. We observed the distribution of intracellular lipid droplets by Nile red staining to investigate lipid droplet utilization and accumulation of the mutants. All strains grew normally and accumulated lipid droplets in the mycelia when sucrose was given. In the starvation condition, the lipid droplets significantly decreased in all strains

(Figure 5A). When olive oil was supplemented as a sole carbon source, the lipid droplets decreased after 6 h cultivation (Figure 5B). However, after 12 h, the wild-type recovered the intracellular lipid droplets by normally utilizing the olive oil as a carbon source. *fglip1* and *fgl1* showed a delay in lipid droplet recovery but started to accumulate the lipid droplets 24 h after inoculation. *fglip1 fgl1* failed to recover the intracellular lipid droplets after 24 h, which corresponds to the barely detected lipase activity in Figure 4B.

These results suggest that the extracellular lipase activity in *F. graminearum* depends entirely on FgLip1 and Fgl1, and the absence of both *FgLIP1* and *FGL1* almost completely blocks the extracellular lipase activity of *F. graminearum*.

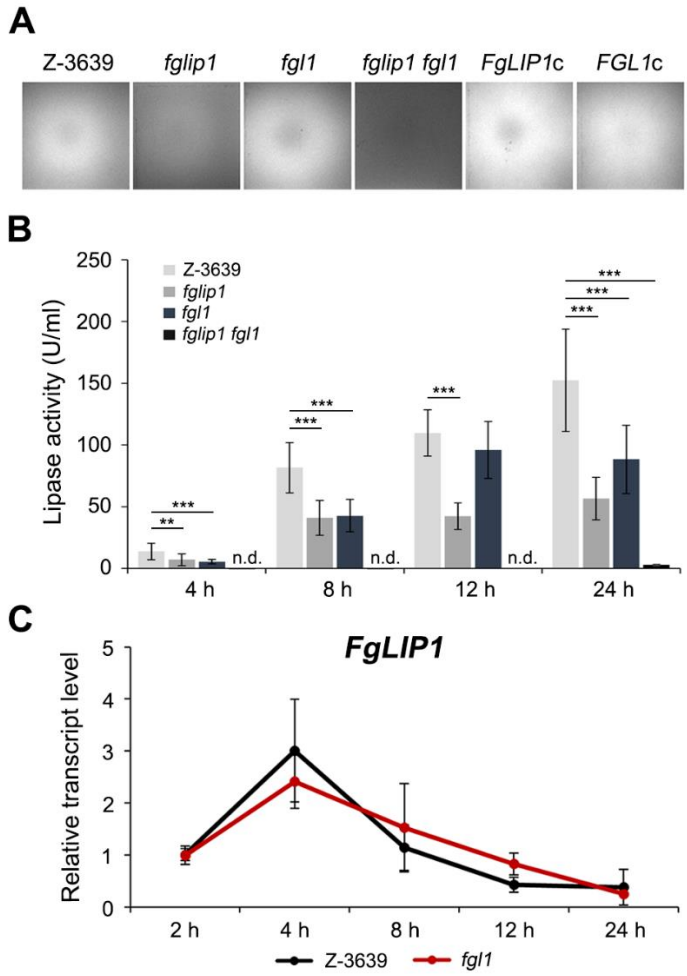

A
Z-3639
fglip1
fgl1
fglip1 fgl1
FgLIP1c
FGL1c
B
Lipase activity (U/ml)
Z-3639
fglip1
fgl1
fglip1 fgl1
n.d.
4 h
8 h
12 h
24 h
C
FgLIP1
Relative transcript level
2 h
4 h
8 h
12 h
24 h
Z-3639
fgl1

Figure 4. Lipase activity of *fglip1* and *fgl1*. (A) Lipase activity of *fglip1, fgl1, fglip1 fgl1* and the complemented strains. Each strain was inoculated on MM supplemented with 1 % olive oil and 0.0005 % rhodamine B. Images were captured 2 days after inoculation under UV light. (B) Lipase activity of *fglip1, fgl1*, and *fglip1 fgl1*. The culture supernatant of each strain was harvested at indicated time points after inoculation in the lipase-inducing medium. Lipase activity was measured spectrophotometrically using para-nitrophenyl palmitate (pNPP) as a substrate. An asterisk indicates a significant difference (**P < 0.005, ***P < 0.001) between the wild-type and the deletion mutant at each time point. n.d., not detected. (C) Fold changes of *FgLIP1* in *fgl1*. Total RNA was extracted after 2, 4, 8, 12, 24 h cultivation in the lipase-inducing medium. The transcript abundances were estimated via qRT-PCR.

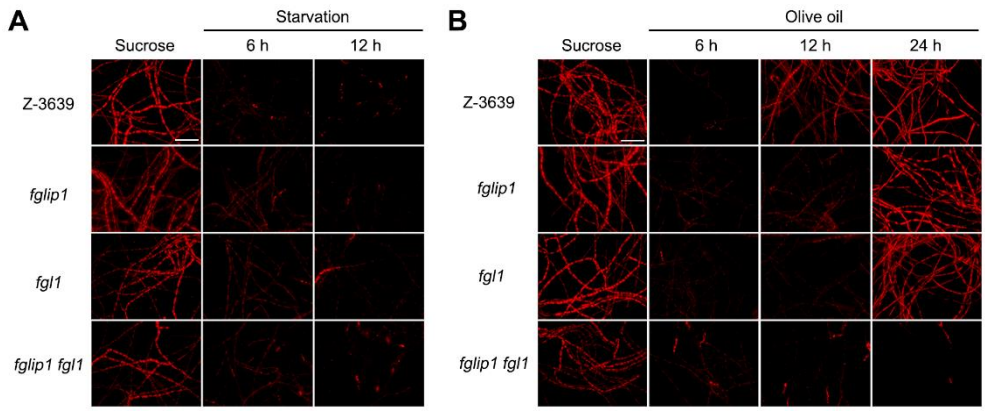


Figure 5. Visualization of intracellular lipid droplets in lipase mutants. (A and B) The mycelia were cultured in MM, MM without carbon source (A) and MM supplemented with 1 % olive oil as a sole carbon source (B) for indicated time points. The mycelia were stained with Nile red solution to observe intracellular lipid droplets. Scale bar = 50 μm.

V. The enzymatic characteristics of FgLip1 and Fgl1

To further investigate the enzymatic characteristics of FgLip1 and Fgl1, we analyzed the triacylglycerol lipase activity by detecting the free glycerol liberated from triglycerides. Glycerol is the final product of triglyceride hydrolysis and is generated after the degradation of monoglycerides. Interestingly, we found that *fgl1* had a severe defect in its ability to liberate free glycerol from triglycerides (Figure 6A). The triacylglycerol lipase activities of *fglip1* and *fgl1* were restored in the complemented strains (Figure 7),. Since *fgl1* showed no visible defect in olive oil degradation on the rhodamine B plate (Figure 4A), we hypothesized that *fgl1* might have a defect in the hydrolysis of diglycerides or monoglycerides and have a preference for triglyceride.

We incubated 40 unit (U) of culture supernatants of the wild-type, *fglip1*, and *fgl1* with triolein and analyzed the hydrolysis products; oleic acids, monoolein, 1,2-diolein, 1,3-diolein, and triolein, via thin-layer chromatography (TLC) (Figure 6B). We observed that culture supernatants of all strains generated similar amounts of oleic acid, but monoolein was largely accumulated in *fgl1* compared to the wild-type and *fglip1*. We subsequently analyzed the amount of free glycerol and found that the liberated glycerol was significantly lower in *fgl1* than in the wild-type (Figure 6C). Our results support the proposal that Fgl1 might play a key role in the later stages of triolein hydrolysis. Since *fglip1* had no defect in the full hydrolysis of triolein, we estimated that although degradation of triolein was slow, the later hydrolysis processes proceed very quickly by Fgl1. We further analyzed the amount of the oleic acid liberated from the triolein (Figure 6D). Supporting our proposal, in

fglip1, which is expected to have a low triolein degrading capacity, the production of oleic acid was slow at the beginning while it was quickly generated in *fgl1*, which is expected to have a high preference for triolein. Taken together, the results indicate that FgLip1 has a substrate preference for triglyceride, and Fgl1 has a substrate preference for diglycerides or monoglycerides, which can act significantly when *F. graminearum* faces various lipid sources in the environment.

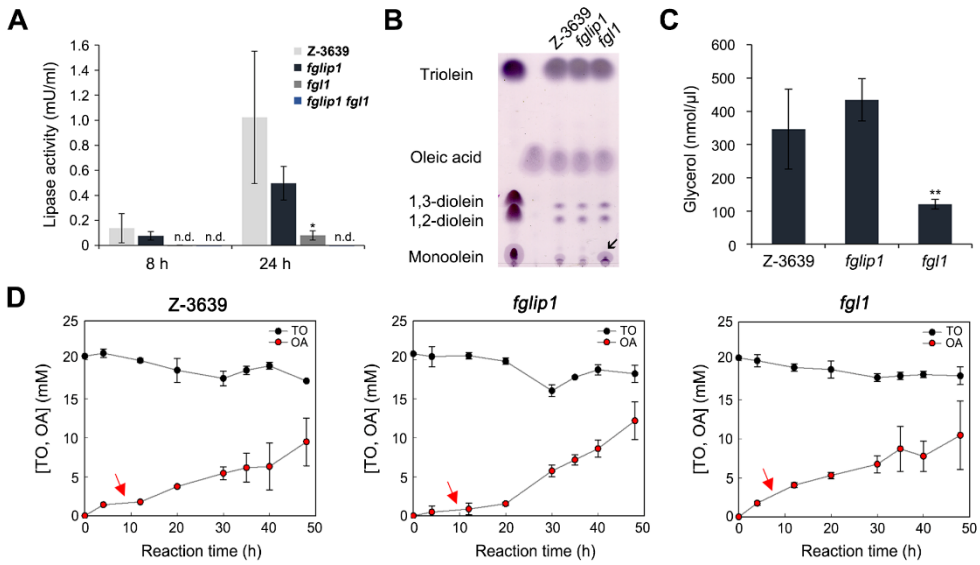

A
Lipase activity (mU/ml)
Z-3639
fglip1
fgl1
fglip1 fgl1
n.d.
n.d.
*
n.d.
8 h
24 h
B
Z-3639
fglip1
fgl1
Triolein
Oleic acid
1,3-diolein
1,2-diolein
Monoolein
C
Glycerol (nmol/µl)
**
Z-3639
fglip1
fgl1
D
Z-3639
fglip1
fgl1
TO
OA
[TO, OA] (mM)
Reaction time (h)

Figure 6. Enzymatic characteristics of FgLip1 and Fgl1. (A) Triacylglycerol lipase activity**.** The strains were incubated in the lipase-inducing medium for indicated time points. The culture supernatants were harvested and used for the analysis of triacylglycerol lipase activity. An asterisk indicates a significant difference (*$P < 0.05$) between the wild-type and the deletion mutant at each time point. n.d., not detected. (B) Thin-layer chromatography (TLC) of hydrolysis products of triolein. 40 U of culture supernatants were reacted with 10 μl of triolein at 37 °C for 20 h on a rotary shaker. Total lipids were extracted from the reaction mixture and compared with reference standards on the TLC plates; oleic acids, monoolein, 1,2-diolein, 1,3-diolein, and triolein. The arrow indicates the accumulated monoolein. (C) Glycerol assay. The amount of glycerol in the reaction mixture was measured. An asterisk indicates a significant difference (**$P < 0.005$) compared to the wild-type. (D) Quantification of triolein and oleic acids. 50 U of culture supernatants were reacted with 40 mM triolein dissolved in isooctane at 37 °C with vigorous mixing. Total lipids were extracted at 0, 4, 12, 20, 30, 35, 40, 48 h after reaction, and a portion of the lipid extracts was used to analyze the amount of triolein and oleic acid via high-performance liquid chromatography (HPLC). The arrow indicates the amount of oleic acid produced at the beginning of the reaction. TO, triolein; OA, oleic acid.

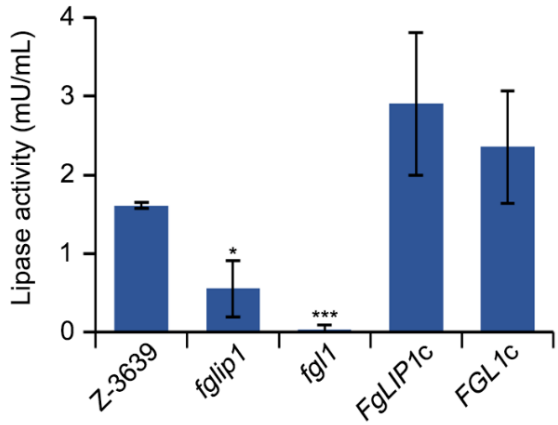


Figure 7. Triacylglycerol lipase activity of *fglip1*, *fgl1*, and complemented strains. Each strain was cultured for 12 h in the lipase-inducing medium and the culture supernatant was used for the analysis of triacylglycerol lipase activity. An asterisk indicates a significant difference (*P<0.01, ***P < 0.001) compared to the wild-type.

VI. Identification of transcription factors involved in lipase activity

To identify the regulatory mechanisms of lipase activity in *F. graminearum*, we screened the lipase activity of 1729 strains corresponding to 657 transcription factor (TF) mutants (Son et al., 2011b) on MM containing olive oil and rhodamine B. Twelve TF mutants exhibited altered fluorescence compared to the wild-type (Figure 8), and the lipase activities were measured using pNPP as a substrate (Figure 9A). Among them, *myt3*, *fgsas3*, and *gzzc258*, which had the lowest lipase activity, and *gzzc066* with the highest lipase activity, were selected for further experiments. A putative Myb-like transcription factor Myt3 and a histone acetyltransferase FgSas3 have been characterized previously (Kim et al., 2014; Kong et al., 2018). Gzzc258 was reported to affect the lipase activity in *F. graminearum* (Le, 2011), and Gzzc066 encodes an ortholog of the transcriptional activator of gluconeogenesis Ert1 in yeast (Turcotte et al., 2009; Gasmi et al., 2014).

We hypothesized that selected TFs might regulate the transcription of *FgLIP1* or *FGL1* in the lipase-inducing condition. The relative transcript levels of *FgLIP1* and *FGL1* in the TF mutants were analyzed 12 h after induction by qRT-PCR (Figure 9B). *FgLIP1* and *FGL1* were markedly downregulated in *myt3*, *fgsas3*, and *gzzc258*, and the expression of *FgLIP1* was highly induced in *gzzc066* in comparison with the wild-type.

We then overexpressed *FgLIP1* or *FGL1* in *myt3*, *fgsas3*, and *gzzc258*, and conversely, deleted these genes in *gzzc066* (Figure 9C and 9D). Overexpression of *FgLIP1* or *FGL1* in *myt3*, *fgsas3*, and *gzzc258* rescued the defects in the lipase

activity. *gzzc066 fglip1* double mutant and *gzzc066 fglip1 fgl1* triple mutant exhibited almost no fluorescence on the rhodamine B plate, while we could not observe a visible difference between *gzzc066* and the *gzzc066 fgl1* double mutant. The altered lipase activity of the selected TF mutants was restored in the complemented strains (Figure 10) and the constitutive expression of *FgLIP1* and *FGL1* in the overexpressing strains was validated via qRT-PCR (Figure 11A). These data indicate that several transcription factors, including *MYT3, FgSAS3, GzZC258*, and *GzZC066,* co-regulates the lipase activity of *F. graminearum* by regulating *FgLIP1* and *FGL1*.

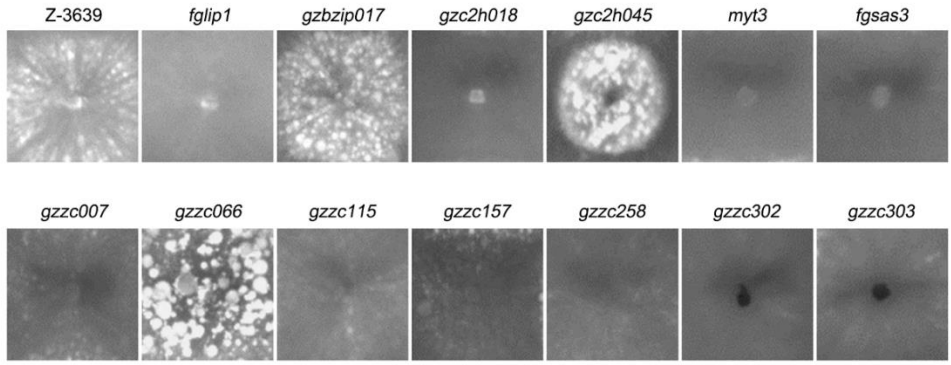


Figure 8. Lipase activity screening of TF mutants. Each TF mutant was inoculated on MM containing 1 % olive oil and 0.0005 % rhodamine B. The pictures were taken 2 days after inoculation under UV light.

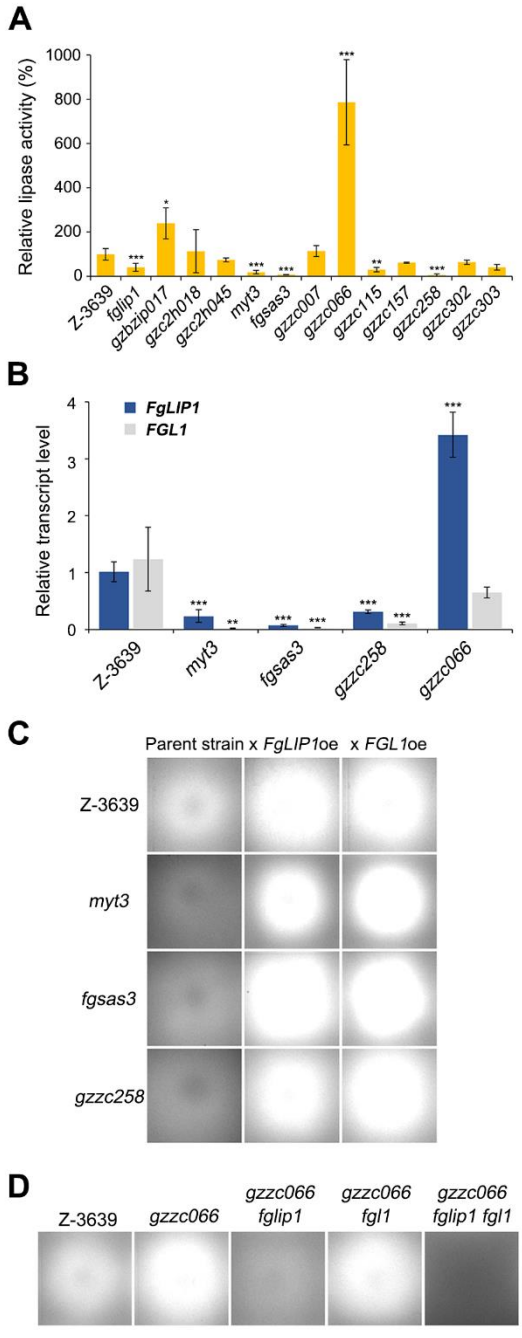

A
Relative lipase activity (%)
1000
800
600
400
200
0
Z-3639
fglip1
gzbzip017
gzc2h018
gzc2h045
myt3
fgsas3
gzzc007
gzzc066
gzzc115
gzzc157
gzzc258
gzzc302
gzzc303
B
FgLIP1
FGL1
Relative transcript level
4
3
2
1
0
Z-3639
myt3
fgsas3
gzzc258
gzzc066
C
Parent strain x FgLIP1oe x FGL1oe
Z-3639
myt3
fgsas3
gzzc258
D
Z-3639
gzzc066
gzzc066 fglip1
gzzc066 fgl1
gzzc066 fglip1 fgl1

Figure 9. Characterization of transcription factors (TFs) involved in the lipase activity. (A) Lipase activity of primarily selected twelve TF mutants**.** The culture supernatant of each strain was harvested 12 h after inoculation in lipase-inducing medium. Lipase activity was measured spectrophotometrically using pNPP as a substrate. Significant differences (*P<0.01, **P < 0.005, ***P < 0.001) compared to the wild-type are indicated by asterisks. (B) Relative transcript levels of *FgLIP1* and *FGL1* in four TF mutants. Total RNA was extracted 12 h after inoculation in lipase-inducing medium. The relative transcript levels were analyzed by qRT-PCR. An asterisk indicates a significant difference (*P<0.01, **P < 0.005, ***P < 0.001) in the relative transcript level of each gene compared to the wild-type. (C and D) Lipase activity of TF mutants carrying an *FgLIP1* or *FGL1* overexpression (C) or deletion (D). The strains were inoculated on MM containing 1 % olive oil and 0.0005 % rhodamine B. The pictures were taken 2 days after inoculation under UV light.

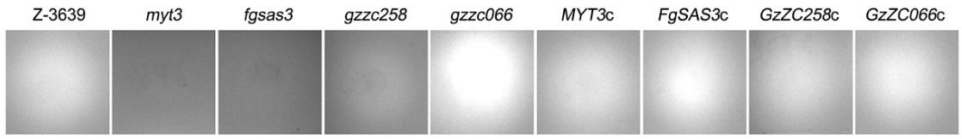


Figure 10. Lipase activity of four TF mutants and the complemented strains. Each strain was inoculated on MM containing 1% olive oil and 0.0005% rhodamine B. The pictures were taken 2 days after inoculation under UV light.

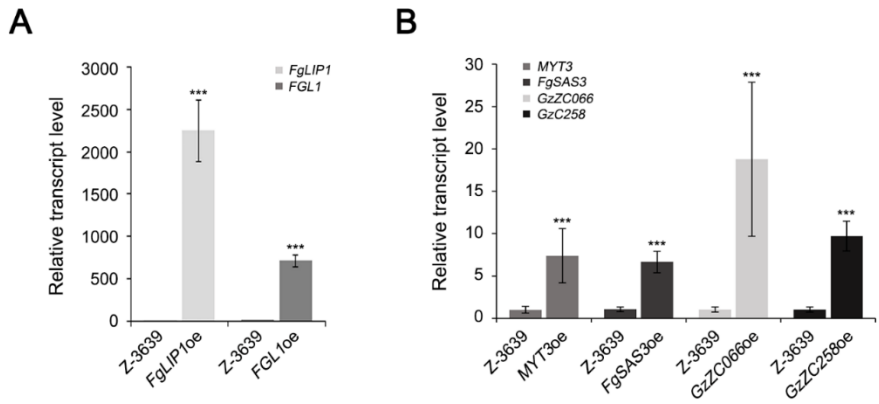


Figure 11. Overexpression of *FgLIP1, FGL1,* and four TF genes. (A and B) Overexpression of *FgLIP1, FGL1* (A), and *MYT3, FgSAS3, GzZC258,* and *GzZC066* (B) were confirmed via qRT-PCR. Total RNA of each strain was extracted 24 h after cultivation in CM, and the relative transcript level was quantified. Significant differences (***P < 0.001) in the relative transcript level of each gene in comparison with the wild-type are indicated with asterisks. The relative transcript abundances of the indicated gene in Z-3639 was arbitrarily set to 1.

VII. Gzzc258 regulates lipase activity in a *FgLIP1*- and *FGL1*-dependent manner

To identify a key lipase regulator among the selected TFs, we measured the lipase activity of the overexpressing strain of each TF gene at 12 h after induction. The constitutive expression of each gene was validated by qRT-PCR (Figure 11B). The lipase activity was induced to about 10-fold of the wild-type in the *GzZC258* overexpression mutant while the overexpression of other genes made no measurable difference, indicating that Gzzc258 is a key lipase regulator in *F. graminearum* (Figure 12A).

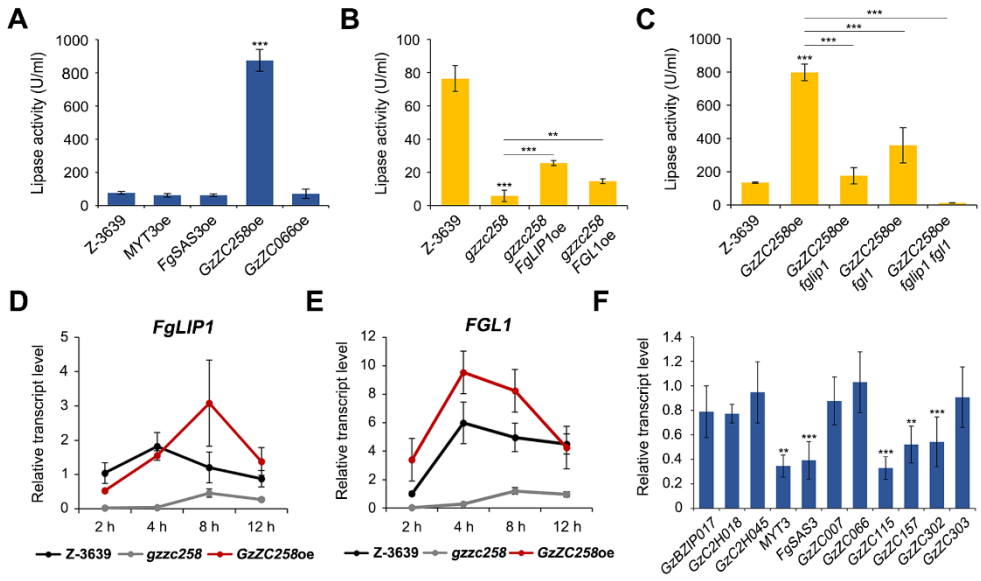
We subsequently focused on the regulatory role of Gzzc258 in the transcription of *FgLIP1* and *FGL1*. When *FgLIP1* or *FGL1* was overexpressed in *gzzc258,* the lipase activity was partially restored to about 4.5-fold and 2.5-fold of *gzzc258,* respectively (Figure 12B). When *FgLIP1* or *FGL1* was deleted in the *GzZC258* overexpression mutant, the lipase activity was largely decreased compared to the overexpressing strain of *GzZC258* (Figure 12C). In particular, when *FgLIP1* and *FGL1* were both deleted in the *GzZC258* overexpression mutant, the lipase activity was barely detectable.

We additionally analyzed the transcript levels of *FgLIP1* and *FGL1* in *gzzc258* and the *GzZC258* overexpression mutant at 2, 4, 8, and 12 h after inoculation in the lipase-inducing medium (Figure 12D and 12E). The transcript levels of *FgLIP1* and *FGL1* were barely detected in *gzzc258* and recovered slightly after 8 h. In contrast, the *GzZC258* overexpressing strain had significantly higher transcript levels of *FgLIP1* and *FGL1* than the wild-type. Taken together, the results

demonstrate that Gzzc258 is a key lipase regulator which regulates lipase activity in a *FgLIP1*- and *FGL1*-dependent manner.

Gzzc258 encodes 889 amino acids and contains the $Zn(II)_2Cys_6$ DNA-binding domain (PF00172, IPR001138) and the fungal-specific transcription factor domain (PF04082, IPR007219) found in a number of fungal transcription factors including XlnR, which regulates the extracellular cellulolytic and xylanolytic genes (Hasper et al., 2004) (Figure 13A). The nuclear localization signal, $_{57}$GCRRRKIKC$_{65}$, was predicted in Gzzc258 by NucPred (Brameier et al., 2007), suggesting that Gzzc258 might act as a nuclear transcription factor in *F. graminearum*. We further analyzed the transcript levels of twelve primarily selected TF genes in *gzzc258*. We found that *MYT3, FgSAS3, GzZC115, GzZC157*, and *GzZC302* were markedly downregulated in *gzzc258* in the lipase-inducing condition (Figure 12F). Our results indicate that Gzzc258 is a master lipase regulator in *F. graminearum*, affecting complex genetic networks involved in lipase activity. Based on the lipase activity of the TF mutants (Fig. 6A) and the altered transcript levels of the TF genes (Figure 12F), we suggest a genetic network model regarding the lipase activity in *F. graminearum* (Figure 13B).

When the intracellular lipid droplets of *myt3, fgsas3, gzzc258*, and *gzzc066* were visualized by Nile red staining, we observed that the lipid droplet recovery was delayed in *gzzc258*, as in *fgl1* and *fglip1* (Figure 14). Interestingly, *myt3* and *fgsas3* were defective in degrading intracellular lipid droplets in the starvation condition, implying the involvement of Myt3 and FgSas3 in overall nutrient utilization.


A
Lipase activity (U/ml)
1000
800
600
400
200
0
***
Z-3639
MYT3oe
FgSAS3oe
GzZC258oe
GzZC066oe
B
Lipase activity (U/ml)
100
80
60
40
20
0
**
***
***
Z-3639
gzzc258
gzzc258 FgLIP1oe
gzzc258 FGL1oe
C
Lipase activity (U/ml)
1000
800
600
400
200
0
***
***
***
***
Z-3639
GzZC258oe
GzZC258oe fglip1
GzZC258oe fgl1
GzZC258oe fglip1 fgl1
D
FgLIP1
Relative transcript level
5
4
3
2
1
0
2 h
4 h
8 h
12 h
Z-3639
gzzc258
GzZC258oe
E
FGL1
Relative transcript level
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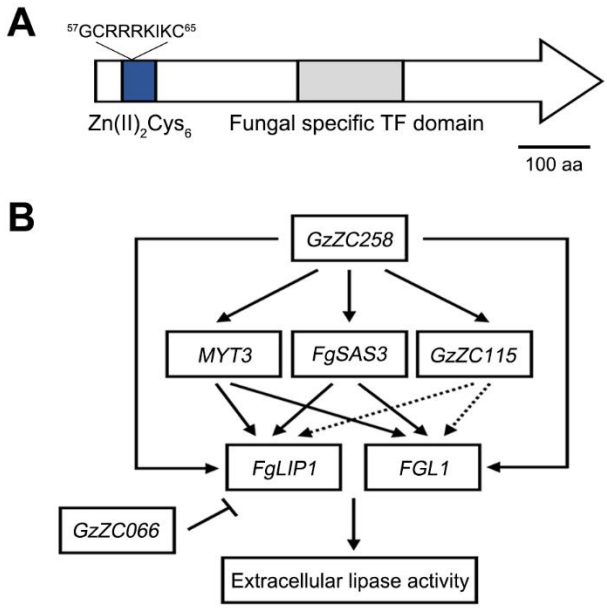
10
8
6
4
2
0
2 h
4 h
8 h
12 h
Z-3639
gzzc258
GzZC258oe
F
Relative transcript level
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
**
***
***
**
***
GzBZIP017
GzC2H018
GzC2H045
MYT3
FgSAS3
GzZC007
GzZC066
GzZC115
GzZC157
GzZC302
GzZC303

Figure 12. Gzzc258 as a key lipase regulator in *F. graminearum*. (A-C) Lipase activity of overexpressing strains of four TF mutants (A), *gzzc258* carrying an *FgLIP1* or *FGL1* overexpression (B), and *GzZC258*oe carrying an *FgLIP1* or *FGL1* deletion (C). The strains were incubated for 12 h in the lipase-inducing medium and the culture supernatant was used for the analysis of lipase activity using pNPP as a substrate. An asterisk indicates a significant difference (**P < 0.005, ***P < 0.001) compared to the wild-type (A), gzzc258 (B), and *GzZC258*oe (C). (D and E) Transcript abundances of *FgLIP1* (D) and *FGL1* (E) in *GzZC258* deletion and overexpression mutant. Total RNA was extracted 2, 4, 8, 12 h after inoculation in the lipase-inducing medium and the relative transcript levels were quantified via qRT-PCR. The relative transcript abundances of the indicated gene at 2 h after inoculation in Z-3639 was arbitrarily set to 1. (F) The relative transcript level of TF genes in *gzzc258*. Total RNA was extracted 2 h after cultivation in the lipase-inducing medium and the transcript abundances were quantified via qRT-PCR. An asterisk indicates a significant difference (**P < 0.005, ***P < 0.001) in comparison with the wild-type. The expression of the indicated genes in Z-3639 was arbitrarily set to 1.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Fungal specific TF domain
100 aa
B
GzZC258
MYT3
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FgLIP1
FGL1
GzZC066
Extracellular lipase activity
C
Z-3639
gzzc258
GzZC258c
D
Z-3639
gzzc258
Lipase activity (U/mg)
500
400
300
200
100
0
***
***
***
0
1
3
5
Days after sexual induction

Figure 13. A schematic figure of lipase regulatory mechanism in *F. graminearum,* and the lipase activity in the sexual development stages. (A) Domain structure of Gzzc258. (B) Proposed genetic network of *FgLIP1, FGL1,* and TF genes. (C) Perithecium formation of *gzzc258* and complemented strains. The photographs were taken 8 days after sexual induction on carrot agar. Scale bar = 500 μm. (D) Lipase activity of *gzzc258* in sexual development stages. Whole cell extracts were prepared from carrot agar cultures 0, 1, 3, and 5 days after sexual induction and used for the lipase activity analysis using pNPP as a substrate. An asterisk indicates a significant difference (***P < 0.001) between the wild-type and *gzzc258* at each time period.

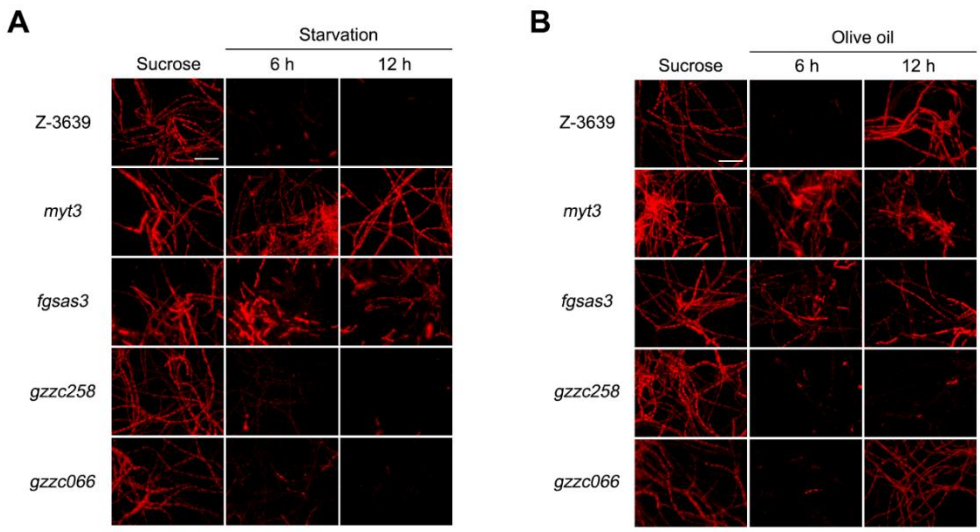

Figure 14. Visualization of intracellular lipid droplets in *F. graminearum* strains. (A and B) The mycelia were cultured in MM, MM without carbon source (A) and MM supplemented with 1 % olive oil as a sole carbon source (B) for indicated time points. The mycelia were stained with Nile red solution. Scale bar = 50 μm.

IX. Lipase activity during the sexual development stages

F. graminearum accumulates the lipid bodies in the early perithecial formation and lipid oxidation processes occur in the later stages of sexual development (Guenther et al., 2009; Sikhakolli et al., 2012). We observed the sexual reproduction of the selected TF mutants to find the relationship between lipase activity and sexual development. *myt3* and *fgsas3* failed to produce any perithecia, and *gzzc258* only developed immature and tiny perithecial structures after sexual induction. *gzzc066* was not able to discharge ascospores at all. (Figure 13C and Figure 15). We subsequently analyzed the lipase activities of *gzzc258* and the wild-type at 0, 1, 3, and 5 days after sexual induction (Figure 13D). The lipase activity of the wild-type rapidly increased after sexual induction and finally reached about 7-fold of the vegetative stages. *gzzc258* showed delayed enzymatic induction, and the lipase activity only increased to about 3-fold of the vegetative stages. Our results suggest that lipase activity is induced in the sexual development stages and is required for perithecial maturation.

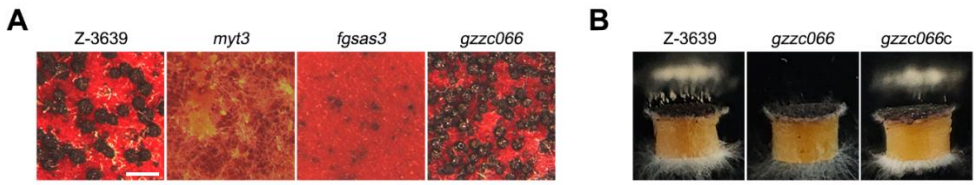

Figure 15. Sexual development of *F. graminearum* strains. (A) Perithecium formation. The perithecia were imaged 8 days after sexual induction. Scale bar = 500 μm. (B) Forcible ascospore discharge. White cloudy materials represent discharged ascospores. The pictures were taken 48 h after initiation of the assay.

DISCUSSION

We identified a total of 86 putative lipase-encoding genes with conserved lipase or esterase domains and analyzed the phenotypic changes of 86 putative lipase deletion mutants in various developmental processes. Seven mutants, *fg08139, fg05297, fg00208, fgpld1, tri8, fg07372,* and *fg12678,* showed defects in the analyzed phenotypes (Figure 2 and 3, Table 3), especially in vegetative growth and sexual development. Except for *tri8* which only showed a defect in the DON production (McCormick and Alexander, 2002), all genes but *Fg12678* belonged to phospholipase families. These results indicate that most lipases play overlapping roles in developmental processes, and therefore a single gene disruption does not lead to significant phenotypic defects. Research on intracellular lipases is commonly conducted via the generation of double or triple mutants in yeast (Athenstaedt and Daum, 2005; Yazawa et al., 2012). We found that three ortholog genes of *Saccharomyces cerevisiae* Tgl3, a main enzyme responsible for intracellular lipase activity, exist in *F. graminearum* with a significant match (Expect < $1e^{-30}$) (Athenstaedt and Daum, 2003). Based on this, we expect that a single lipase gene would play a dispensable role in *F. graminearum,* but a double or triple mutant would lead to defects in several biological processes.

None of the mutants including *fgl1* showed defects in virulence on wheat spikelets in our laboratory conditions, except for *fgpld1*. Previous studies also have demonstrated the dispensable roles of fungal lipases in virulence in *M. oryzae, F.*

oxysporum, and *B. cinerea* (Reis et al., 2005; Wang et al., 2007; Bravo-Ruiz et al., 2013). We expect that functional redundancy of the lipase genes exists in the virulence of *F. graminearum*, or the majority of lipase genes contribute to nutritional uptake from the plant tissue, not to fungal virulence.

FgLip1 and Fgl1 are previously reported extracellular lipases in *F. graminearum*, which are highly induced during the infection stages and in the presence of lipid sources such as olive oil and wheat germ oil (Feng et al., 2005; Voigt et al., 2005). FgLip1 and Fgl1 both harbors the signal peptides in their N-terminus of 15 amino acid residues and 20 amino acid residues, respectively, suggesting that they function as secretory lipases. We investigated the extracellular lipase activities of the putative lipase mutants (Figure 4). Only *fglip1* had defects in the decomposition of the olive oil on the plate, indicating that the majority of extracellular triglyceride lipase activity of *F. graminearum* depends on FgLip1. When we quantified the lipase activities of the strains using pNPP as a substrate, a single deletion of *FgLIP1* or *FGL1* showed significant defects compared with the wild-type. We further demonstrated in Figure 5 that these lipases hydrolyze extracellular olive oil, allowing the cells to utilize the extracellular lipid sources. When olive oil was supplemented as a sole carbon source, the wild-type initially used the internal lipids as carbon sources, but the intracellular lipid droplets began to accumulate again as extracellular lipases decompose the external olive oil. However, when *FgLIP1* or *FGL1* was deleted, the mutant lacked the ability to decompose external olive oil, so cells continued to use internal lipids, resulting in defects in the internal lipid droplets recovery. This result allowed us to visually

demonstrate how *F. graminearum* uses lipids inside and outside of the cell when the carbon sources are scarce or the external lipid sources are present. Despite several extracellular lipases other than FgLip1 and Fgl1 had been characterized in *F. graminearum* (Nguyen et al., 2010), we conclude that FgLip1 and Fgl1 might constitute most of the lipase activity in *F. graminearum* since *fglip1 fgl1* had no pNPP hydrolysis activity in our study. However, other results can be obtained if the lipases are induced with other lipid sources, or if the activity is analyzed for other substrates such as pNP-butyrate.

fgl1 had a severe defect in generating glycerol from triglycerides, which means a defect in the full hydrolysis of triglycerides (Figure 6A). Based on our results, we could hypothesize that (i) FgLip1, which is expected to account for most of the activity in *fgl1*, would primarily act on the hydrolysis of triglycerides and have lower activity on the later substrates, and (ii) Fgl1 would have a higher preference for the later substrates than FgLip1. Our TLC analysis shows that monoglycerides are accumulated in *fgl1*, which supports our proposal (Figure 6B). To analyze detailed enzymatic characteristics of the lipases, further research is needed by reacting the purified enzymes with various substrates.

Although we demonstrated that FgLip1 and Fgl1 are required for the extracellular lipase activity in *F. graminearum*, we could not find any defects in the assessed phenotypes. Plant pathogenic fungi encounter various environments in their life cycles. Previous studies suggest that plant pathogenic fungi face nutrient deprivation during their proliferation in the plant tissue (Coleman et al., 1997; Solomon et al., 2003). After infection of the wheat florets, *F. graminearum* colonizes

the wheat stems and produces the specified hyphal coils, termed perithecium initials, for overwintering (Guenther and Trail, 2005). For successful colonization, nutrient uptake from the plant tissue is required via secretion of various degrading enzymes (Ospina-Giraldo et al., 2003; Gibson et al., 2011; Kubicek et al., 2014). A previous study revealed the induced expression of lipases, including FgLip1 and Fgl1, during wheat colonization (Guenther et al., 2009), so we expect extracellular lipases in *F. graminearum* might contribute to fungal survival outside of laboratory conditions.

We identified three transcription factors, Myt3, Gzzc258, and Gzzc066, and one histone acetyltransferase, FgSas3, involved in the lipase activity in *F. graminearum*. Previously, Ctf1α/β in *Fusarium* species, which are homologous to FarA and FarB in *A. nidulans*, have been characterized as a cutinase and lipase regulator in several fungi (Li et al., 2002; Rocha et al., 2008; Le, 2011; Garrido et al., 2012; Bravo-Ruiz et al., 2013; Wang et al., 2020). Among the primarily selected twelve TFs, Gzzc115 and Gzzc258 had sequence similarity up to 84% and 19% with Ctf1α of *Fusarium solani*, respectively. We found that *myt3*, *fgsas3*, *gzzc258*, and *gzzc066* showed altered transcript levels of *FgLIP1* and *FGL1*, and the overexpression or deletion of *FgLIP1* or *FGL1* directly recovered their lipase activities (Figure 9B and C). These results suggest that the altered lipase activities of the TF mutants were not due to defects in secretion or post-translational modification, but to the transcriptional defects of *FgLIP1* and *FGL1*. Among the selected TFs, we designated Gzzc258 as a key lipase regulator since the overexpression of *GzZC258* led to enhanced lipase activity and increased transcription of *FgLIP1* and *FGL1* (Figure 12). We found that multiple TF genes involved in lipase activity, including

MYT3 and *FgSAS3*, were downregulated in *gzzc258* in the lipase-inducing conditions, suggesting a complex genetic network of the lipase regulatory genes.

In this study, we demonstrated that *F. graminearum* greatly increases its lipase activity during its sexual developmental stages, while *gzzc258* only produced immature perithecial initials with lower induction of the lipase activity (Figure 13C and D). Since *fglip1*, *fgl1*, and even *fglip1 fgl1* successfully developed mature perithecia (data not shown), we conclude that intracellular, rather than extracellular, lipases might act for the cellular lipid accumulation and degradation during sexual reproduction stages.

In conclusion, we constructed genome-wide deletion mutants of putative lipases in *F. graminearum* and provided the phenotypic database of the mutants. Our study shows that *F. graminearum* possesses abundant lipases that work for cellular growth and differentiation. We identified several TFs that co-regulate the expression of the extracellular lipases, FgLip1 and Fgl1, and designated Gzzc258 as a key lipase regulator. We revealed the relationship of lipase activity and sexual development in *F. graminearum*, suggesting the potential roles for lipases in various cellular mechanisms. Our study provides extensive genetic sources regarding fungal lipases and reveals the complex mechanisms regulating extracellular lipase activity in plant pathogenic fungi.

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CHAPTER II

Development of a versatile copper-responsive gene expression system in the plant pathogenic fungus *Fusarium graminearum*

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ABSTRACT

Fusarium graminearum has been widely used as a model organism to study various biological processes of plant pathogenic fungi because of its amenability to genetic manipulation. Gene deletion and overexpression/constitutive expression of target genes are tools widely used to investigate the molecular mechanism underlying fungal development and virulence. However, for fine-tuning gene expression and studying essential genes, a conditional gene expression system is necessary that enables repression or induction of gene expression by modifying external conditions. Until now, only a few conditional expression systems have been developed in plant pathogenic fungi. This study proposes a versatile conditional gene expression system in *F. graminearum* using the promoter of a copper-responsive gene, *F. graminearum* copper-responsive 1 (*FCR1*). Transcript levels of *FCR1* were found to be greatly affected by copper availability conditions. Moreover, the promoter (P_{FCR1}), 1 kb upstream of the *FCR1* open reading frame, was sufficient to confer copper-dependent expression of a green fluorescent protein gene and *FgENA5*. We also demonstrated the applicability of this conditional gene expression system to an essential gene study by replacing the promoter of *FgIRE1*, an essential gene of *F. graminearum*. This enabled the generation of *FgIRE1* suppression mutants, which allowed functional characterization of the essential gene. This study reported the first conditional gene expression system in *F. graminearum* using both repression and induction. This system would be a convenient way to precisely control gene expression and will be used to determine the biological functions of various genes, including essential ones.

INTRODUCTION

Investigating the molecular mechanisms underlying various biological phenomena first requires examining the molecular functions of the related genes. The development of basic molecular techniques, such as gene knockout and overexpression, has accelerated molecular genetic research; however, few methods are available to study essential genes or fine-tune gene expression in non-model organisms. A conditional gene expression system can overcome this limitation by allowing repression or induction of gene expression simply by modifying the culture conditions. This technique is a convenient molecular tool and furthermore, has a potential for industrial applications (Mach and Zeilinger, 2003).

Fusarium graminearum is an important plant pathogenic fungus that causes Fusarium head blight on wheat and barley, as well as ear rot on maize (Leslie and Summerell, 2006). *F. graminearum* infections result in severe yield losses worldwide (Goswami and Kistler, 2004), and contaminate grains with harmful mycotoxins such as trichothecenes and zearalenone (Desjardins, 2006). Genetic manipulations via highly efficient homologous recombination have led to this fungus being used as a model for large-scale, in-depth molecular genetic research of plant pathogenic fungi (Son et al., 2011; Wang et al., 2011; Yun et al., 2015; Jiang et al., 2020). However, only one conditional gene expression system is currently available in *F. graminearum*. This system is based on the P_{ZEAR} promoter which is activated by zearalenone or the estrogenic compound β-estradiol (Lee et al., 2010; Lee et al., 2011a) and has been successfully applied to analyze the function of essential genes

in this fungus (Lee et al., 2011b; Bui et al., 2016; Tang et al., 2018; Liu et al., 2019; Nguyen et al., 2020). The P_{ZEAR} system has limitations that it cannot be utilized in studies of zearalenone biosynthesis. Moreover, only one-directional control (induction) is available when supplementing with zearalenone or β-estradiol. Therefore, there is the need to develop more versatile conditional gene expression systems in *F. graminearum*.

Several conditional gene expression systems have been developed in fungi. A doxycycline-dependent Tet-on/Tet-off system was established in *Aspergillus fumigatus*, *Aspergillus niger*, and *Candida albicans* (Park and Morschhäuser, 2005; Vogt et al., 2005; Meyer et al., 2011). In *Saccharomyces cerevisiae* and *Cryptococcus neoformans,* the *GAL7* promoter has been widely used to activate or repress gene expression using galactose and glucose, respectively (Johnston, 1987; Wickes and Edman, 1995). The *thiA* promoter is repressible by thiamine in *Aspergillus oryzae* (Shoji et al., 2005). In *Aspergillus nidulans* and *A. fumigatus, alcA* promoter is induced by glycerol, ethanol, or threonine, and is repressed during growth on glucose (Waring et al., 1989; Felenbok et al., 2001; Romero et al., 2003).

In this study, we focused on the copper-regulated promoters that have been used in several fungal species. Copper is an essential trace element that acts as a cofactor in many enzymes, but it can be detrimental to cells if accumulated in excess (Kim et al., 2008). Living organisms protect themselves from copper toxicity by maintaining cellular copper homeostasis. In *F. graminearum*, the P-type ATPase transporter FgCrpA and transcription factor FgAceA play a role in copper tolerance (Liu et al., 2020). Previous studies of *Schizosaccharomyces pombe and C.*

neoformans revealed that the promoter of the high-affinity copper transporter could drive strong copper-dependent regulation of target genes (Bellemare et al., 2001; Ory et al., 2004). Similarly, conditional gene expression systems using copper-regulated promoters have been proposed in several model fungal species (Gebhart et al., 2006; Willyerd et al., 2009; Lamb et al., 2013).

This study aimed to develop a new conditional gene expression system in *F. graminearum* based on a native copper-responsive promoter. We successfully identified *F. graminearum* copper-responsive 1 (*FCR1*) and demonstrated that the *FCR1* promoter (P_{FCR1}) could drive gene expression in a copper-dependent manner. We further assessed the general applicability of this promoter for studying essential genes and fine-tuning gene expression. This study provides an easy and convenient method to generate conditional mutants. This should help determine the biological functions of the genes underlying fungal development, virulence, and mycotoxin production, which had previously proven difficult to study.

MATERIALS AND METHODS

I. Strains and culture conditions

The *F. graminearum* wild-type strain Z-3639 (Bowden and Leslie, 1999) and all mutants derived from the wild-type (Table 1) were stored as mycelial suspensions in 20% glycerol solution at -80 °C. Production of HK146 and HK147 (*Fgena5* deletion mutant and *FgENA5* overexpressing strain) is described elsewhere (Son et al., 2015). Culture medium was prepared as described in the *Fusarium* laboratory manual (Leslie and Summerell, 2006). The growth temperature was set at 25°C unless otherwise indicated.

II. Nucleic acid manipulations and PCR primers

For genomic DNA isolation, each strain was cultured in 5 ml of CM for 3 days in a rotary shaker at 200 rpm, and genomic DNA was extracted according to the *Fusarium* laboratory manual (Leslie and Summerell, 2006). Total RNA was extracted from mycelia ground in liquid nitrogen using the Easy-Spin Total RNA Extraction Kit (Intron Biotech, Seongnam, Republic of Korea). Restriction endonuclease digestion and agarose gel electrophoresis were performed following standard protocols (Sambrook and Russell, 2001). Southern blot hybridization was performed with the North2South Biotin Random Prime Labeling Kit and the North2South Chemiluminescent Hybridization and Detection Kit (Thermo Scientific, USA). The PCR primers used in this study (Table 2) were synthesized by an oligonucleotide synthesis facility (Bionics, Seoul, Republic of Korea).

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

Strain	Genotype	Source or reference
Z-3639	*Fusarium graminearum* wild-type	Bowden & Leslie, 1999
HK146	Δ*Fgena5::GEN*	Son *et al.*, 2015
HK147	*FgENA5::GEN-*$P_{EF1\alpha}$*-FgENA5*	Son *et al.*, 2015
P_{FCR1}*-GFP*	P_{FCR1}*-GFP-HYG*	This study
P_{FCR1}*-FgENA5*	*FgENA5::HYG-*P_{FCR1}*-FgENA5*	This study
P_{FCR1}*-FgIRE1*	*FgIRE1::HYG-*P_{FCR1}*-FgENA5*	This study

Table 2. Primers used in this study.

Purpose	Name	Sequence
qRT-PCR	00773-RT-F	TCAATGGCAAAGAAGGCAACAC
	00773-RT-R	TAGCCAACAGCATGACAAAGTAGG
	07059-RT-F	TGTTACTGAATCGACTCCCATCCT
	07059-RT-R	GCCAAGCGAAACAGAAACCAT
	06061-RT-F	GAGCATCACCTCTCCAGCAGTTTA
	06061-RT-R	GACCTTCCAAACCCTCCAAATC
	UBH-RT-F	GTTCTCGAGGCCAGCAAAAAGTCA
	UBH-RT-R	CGAATCGCCGTTAGGGGTGTCTG
***FgIRE1* deletion**	IRE1-5F	GGGGGTAGCAATAAGCGAATCC
	IRE1-5N	AGAATTGGGTCTTGTCTTGGATGC
	IRE1-5R	gcacaggtacacttgtttagagGTTGGTGAATGCGGAGTCGG
	IRE1-3F	ccttcaatatcatcttctgtcgGGACGGCATTGCATTGATTGTA
	IRE1-3N	AGAATAGCGTCGGGTTTTACTGGA
	IRE1-3R	ACCGCGAGACAAACTTCCAATG
***HYG*-P_{FCR1}-*GFP* fusion**	Hyg-F	GAGCAAAAACAGGAAGGCAAAATG
	Hyg-R	TTGTTAACTGGTTCCCGGTCG
	GFP-F-Pfcr1	ggcatgtcagaaactgccaccATGGTGAGCAAGGGCGAGG
	GFP-R	TTGCGGGACTCTAATCATAAAAAC
	Pfcr1-F-HYG	cgaccgggaaccagttaacaaGTGGAACGTGGTGGAAGAGTGAC
	Pfcr1-R1	GGTGGCAGTTTCTGACATGCC
	Hyg-F1	GGCTTGGCTGGAGCTAGTGGAGG
	GFP-R1	GCAAGACCGGCAACAGGATTC
	Pfcr1-R	GTCGAGGAAAGGTAGCAGCGTAGA
Promoter fusion of *FgENA5*	ENA5-5F	GGCAAAATTGTGGAGGTGGTTA
	ENA5-5N	ATGATGAAGAGAAACAGCACGAGC
	ENA5-5R-Pfcr1	cctccactagctccagccaagccGTGTAACCCATGAGGAACCAACTT
	ENA5-3F-Pfcr1	ggcatgtcagaaactgccaccCAAAATGAGTCAGAACCCTACAGAA
	ENA5-3N-Pfcr1	CCACGAGCATGCCACTTACC
	ENA5-3R-Pfcr1	GCAAAGTTGTCGTCGGTTAGGA
Promoter fusion of *FgIRE1*	IRE1-5F	GGGGGTAGCAATAAGCGAATCC
	IRE1-5N	AGAATTGGGTCTTGTCTTGGATGC
	IRE1-5R-Pfcr1	cctccactagctccagccaagccTGAATGAAGGGTTGGAATGGAATA
	IRE1-3F-Pfcr1	ggcatgtcagaaactgccaccTAACCTTGGCCCAAAATGCTG
	IRE1-3N-Pfcr1	ATTACTGCGCATTCGGTCTTCTG
	IRE1-3R-Pfcr1	AGGTGTCGACGGATATTTTTCTGG

III. Characterization of *FCR1*

To identify putative copper-responsive genes in *F. graminearum*, BLASTp was performed to compare the amino acid sequences of *S. pombe* Ctr4 (GenBank Accession No. NP_587968), *C. neoformans* Ctr4 (XP_775793) and *N. crassa* TCU-1 (XP_964373) to the *F. graminearum* genome database (https://fungidb.org/). Genes that showed a significant match (E value < 1e-10) were considered putative copper-responsive genes. The phylogenetic tree (Figure 1) was constructed using ClustalW and the MEGA-X program, with 1,000 bootstrap replicates performed by the neighbor-joining method (Kumar et al., 2018).

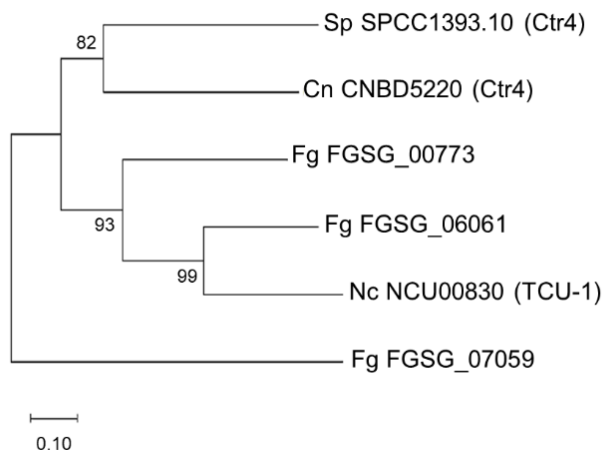

Figure 1. Phylogenetic tree of putative copper-responsive proteins in *F. graminearum* and other representative fungal species. The MEGA-X program was used to perform ClustalW alignment using the neighbor-joining method with 1000 bootstrap replicates. Sp, *Schizosaccharomyces pombe*; Cn, *Cryptococcus neoformans*; Fg, *Fusarium graminearum*; Nc, *Neurospora crassa*

IV. Genetic manipulations and fungal transformations

The double-joint (DJ) PCR method (Yu et al., 2004) was employed to construct the fusion PCR products required for targeted gene deletion and promoter replacement. Fungal transformation was performed as previously described via homologous recombination (Son et al., 2011).

To generate the P_{FCR1}-*GFP* strain, hygromycin resistance gene cassette (*HYG*) and *GFP* were amplified from the pIGPAPA plasmid (Horwitz et al., 1999) using the primers HYG-F/HYG-R and GFP-F-Pfcr1/GFP-R. The -1,009 to -1 bp region upstream of the *FCR1* translational site was amplified from the wild-type strain using the primers Pfcr1-F-HYG/Pfcr1-R1. Three fragments were fused by the DJ PCR method, and the resulting PCR product was used as a template to produce the final construct, *HYG*-P_{FCR1}-*GFP,* with the primers HYG-F1 and GFP-R1. Subsequently, the *HYG*-P_{FCR1}-*GFP* construct was cloned into the pGEM-T Easy vector following the manufacturer's instructions for the pGEM-T and pGEM–T Easy Vector Systems Kit (Promega, Madison, WI, USA), using *E. coli* DH10B. Plasmid DNA was extracted with the DNA-spin Plasmid DNA Purification Kit (Intron Biotech) and used to transform the *F. graminearum* wild-type protoplasts.

For the promoter replacement of *FgENA5* with P_{FCR1}, *HYG* was amplified from the pIGPAPA plasmid (Horwitz et al., 1999) using the primers HYG-F/HYG-R, and P_{FCR1} was amplified from the wild-type strain with the primers Pfcr1-F-HYG/Pfcr1-R. The two fragments were fused by the double joint (DJ) PCR method (Yu et al., 2004), and the *HYG*-P_{FCR1} construct was amplified with the primers HYG-F1 and Pfcr1-R1. The 5′ and 3′ flanking regions of *FgENA5* were amplified from the

wild-type strain using the primers ENA5-5F/ENA5-5R-Pfcr1 and ENA5-3F-Pfcr1/ENA5-3R-Pfcr1, respectively. After fusion PCR of the resulting three fragments, the final PCR construct was obtained with nested primers. The final PCR products were used to transform fungal wild-type protoplasts. The P_{FCR1}-*FgIRE1* strains were generated using the same strategy.

To generate *FgIRE1* deletion mutants, the 5' and 3' flanking regions of *FgIRE1* and a geneticin resistance cassette (*GEN*) were amplified from the wild-type strain and pII99, respectively. The three amplicons were fused by DJ PCR, and the third round of PCR was performed using nested primers. The resulting amplicons were transformed into the wild-type strain.

V. Microscopic observation

Microscopic observation was performed with a Leica DM6 B microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Leica DMC6200 camera using the fluorescent filter L5 (part no. 11504166). Conidial suspensions of the P_{FCR1}-*GFP* strain were inoculated in CM at 2×10^5 conidia per milliliter, and mycelia were harvested 24 h after incubation on a rotary shaker (200 rpm). The mycelia were observed under GFP light 8 and 12 h after reinoculation in CM and CM supplemented with $CuSO_4$ (10 or 50 μM) or BCS (25 or 50 μM).

VI. qRT-PCR

Total RNA was extracted with an Easy-Spin Total RNA Extraction Kit (Intron Biotech). First strand cDNA was synthesized using SuperScript III reverse

transcriptase (Invitrogen, Carlsbad, CA, USA). 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 the corresponding primers (Table 2). The endogenous housekeeping gene ubiquitin C-terminal hydrolase (*UBH*) was used as a reference gene for normalization. The PCR was repeated three times with two biological replicates. The relative transcript levels of target genes were calculated as previously described (Livak and Schmittgen, 2001).

To compare copper-dependent expression of FGSG_00773, FGSG_06061 and FGSG_07059, conidial suspensions of the wild-type were inoculated in CM at 5×10^4 conidia per milliliter. Mycelia were harvested 36 h after incubation on a rotary shaker (200 rpm), and recultured in CM and CM supplemented with 200 μM $CuSO_4$ or 300 μM BCS. Total RNA was extracted after 1, 4, 8 and 12 h. Copper concentration-dependent relative transcript levels of *FCR1* were examined using wild-type mycelia prepared by the same strategy. Total RNA was extracted 12 h after reinoculation in CM and CM supplemented with $CuSO_4$ (10–200 μM) or BCS (25–300 μM). For analysis of the expression pattern of *FCR1* over time, wild-type mycelia were prepared using the same approach and recultured for 1, 2, 4, 8, 12, 24 and 48 h after the addition of 10 μM $CuSO_4$ or 25 μM BCS. Total RNA was isolated, and the relative transcript levels were analyzed by qRT-PCR.

RESULTS AND DISCUSSION

I. Identification of a copper-responsive gene in *F. graminearum*

S. pombe ctr4^{+} (GenBank Accession No. NP_587968), *C. neoformans CTR4* (XP_775793), and *N. crassa tcu-1* (XP_964373) are representative copper-responsive genes that encode high-affinity copper transporters (Labbé et al., 1999; Bellemare et al., 2001; Ory et al., 2004; Lamb et al., 2013). The expression of these genes is regulated by copper availability, and they have previously been used for conditional gene expression systems. Based on amino acid sequence identity with *S. pombe* Ctr4, *C. neoformans* Ctr4, and *N. crassa* TCU-1, we identified three candidates for copper-responsive genes in *F. graminearum*; FGSG_00773, FGSG_06061, and FGSG_07059. These genes have not been functionally characterized in *F. graminearum*.

To examine the responses of candidate genes to copper conditions, we investigated their relative transcript levels at 1, 4, 8 and 12 h after the addition of 200 μM $CuSO_4$ and 300 μM of the copper chelator bathocuproinedisulfonic acid (BCS) in complete medium (CM) via quantitative reverse transcription (qRT)-PCR assays (Figure 2). All three genes showed reduced transcript levels under $CuSO_4$-supplemented conditions (copper-sufficient conditions) and increased transcript levels when BCS was added (copper-deficient conditions). The highest levels of repression and induction were observed 12 h after the addition of $CuSO_4$ and BCS. Under copper-deficient conditions, FGSG_00773 showed a higher induction effect

(by about 8-fold) than those of FGSG_06061 and FGSG_07059 (about 5- and 4-fold, respectively). With sufficient copper, the transcript levels of FGSG_06061 decreased rapidly after 1 h, while the transcript levels of FGSG_07059 and FGSG_00773 started to decrease after 4 h. At 12 h after inoculation, FGSG_00773 showed a maximum inhibitory effect (0.03-fold) while the transcript levels of FGSG_06061 and FGSG_07059 were 0.07- and 0.2-fold, respectively. Taken together these results identified gene FGSG_00773 as the most responsive to copper availability conditions. We designated this gene *F. graminearum* copper-responsive 1 (*FCR1*) and selected it for further characterization.

To start mapping the promoter region of *FCR1*, we compared the sequences upstream of the open reading frame (ORF) of *FCR1* and its putative orthologs in *S. pombe* and *C. neoformans*. The upstream regions of copper transporter genes usually contain a *cis*-acting copper-signaling element (CuSE), which is similar to the metal regulatory element (MRE) in copper metalloregulatory transcription factors in *S. cerevisiae* and *Candida glabrata* (Koch and Thiele, 1996; Beaudoin and Labbé, 2001). The CuSE is represented as the consensus sequence 5'-D(T/A)DDHGCTGD-3' (D = A, G or T; H = A, C or T). GCTG and a T/A located four nucleotides upstream of the first G are the core sequences of CuSE. While the *CTR4* promoter regions of *S. pombe and C. neoformans* contained CuSE, we could not find any CuSE within 2 kb upstream of *FCR1* ORF. Instead, we identified the sequence 5'-ATATCGCTGC-3', which shows substantial similarity to CuSE, at position -874. In this sequence, 9/10 nucleotides of CuSE are present, including the core GCTC and T/A sequences. We predict that *FCR1* might be regulated in a copper-dependent

manner through a different pathway or mechanism than previously identified copper-responsive genes.

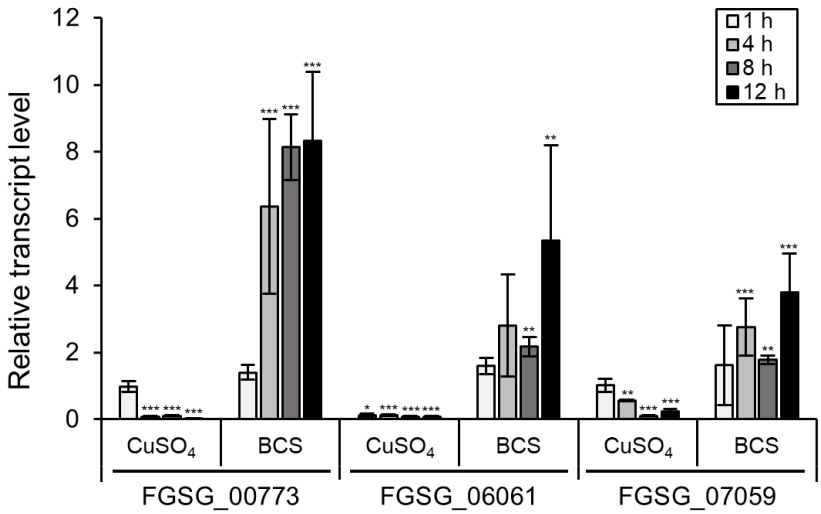

Figure 2. Identification of copper-responsive genes in *F. graminearum*. Relative transcript levels of putative copper-responsive genes in copper-enriched and copper-deprived conditions. Fold-change values were analyzed by qRT-PCR. Total RNA was extracted from the wild-type strain grown for 1, 4, 8 and 12 h in CM and CM supplemented with 200 μM $CuSO_4$ or 300 μM BCS. The relative transcript level of each gene in CM at each hour was arbitrarily set to 1 and omitted from the graph. Then, relative transcript abundances of each gene in the presence of $CuSO_4$ and BCS at each hour was analyzed based on that value. Bars represent the mean ± SDs of two biological and three technical replications, and significant differences (*$P < 0.01$, **$P < 0.005$, ***$P < 0.001$) are indicated with an asterisk.

II. The optimal concentration of $CuSO_4$ and BCS for copper-responsive expression of *FCR1*

The relative transcript levels of *FCR1* were analyzed via qRT-PCR under various copper concentrations to confirm that *FCR1* expression depends on the copper concentration. The results showed that all tested concentrations of $CuSO_4$ (10, 25, 50, 100, 150, 200 μM) and BCS (25, 50, 100, 150, 200, 300 μM) effectively repressed and induced the expression of *FCR1*, respectively (Figure 3A). We further investigated whether $CuSO_4$ and BCS could induce phenotypic alterations in this fungus, to distinguish between the effects of the reagents themselves and those of altered gene expression. The *F. graminearum* wild-type strain Z-3639 did not show significant defects in vegetative growth (Figure 4), conidiation or sexual reproduction under treatment conditions of 10 μM $CuSO_4$ or 25 μM BCS (data not shown). Given that minimum experimental concentrations of $CuSO_4$ (10 μM) and BCS (25 μM) worked well in controlling *FCR1* expression without inducing phenotypic alterations in *F. graminearum*, we used them for further experiments.

To examine the expression pattern of *FCR1* over time under established working concentrations, we analyzed the transcript levels of *FCR1* at various time points (Figure 3B). The repressive effect of adding 10 μM $CuSO_4$ started 12 h after inoculation and was maintained until at least 48 h. The inducing effect of adding 25 μM BCS reached a maximum 8 h after inoculation and gradually decreased thereafter. Similarly, a previous study of *F. graminearum* showed that the transcript levels of the zearalenone-inducible gene (*ZEAR*) decreased over time, even in the presence of a high concentration of extracellular zearalenone (Lee et al., 2010).

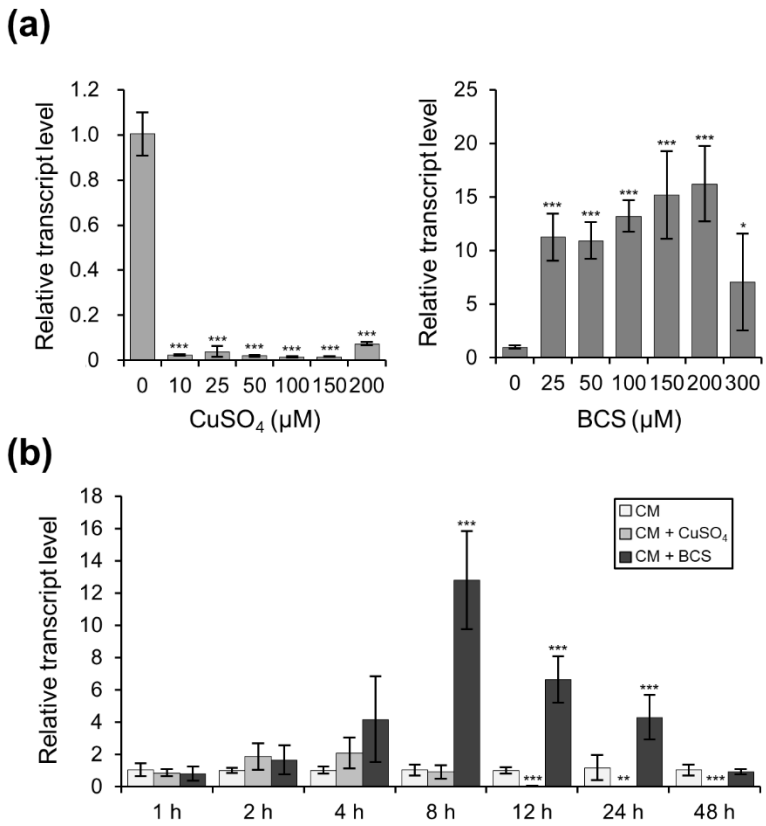

Figure 3. Relative transcript levels of *FCR1* under various copper concentrations and time points. (a) Copper concentration-dependent relative transcript levels of *FCR1*. Total RNA was extracted from the wild-type strain 12 h after inoculation in CM and CM supplemented with $CuSO_4$ (10–200 μM) or BCS (25–300 μM), and transcript levels were quantified by qRT-PCR. (b) Relative transcript levels of *FCR1* at various time points. Total RNA was isolated from the wild-type strain cultured for the indicated time points after the addition of 10 μM $CuSO_4$ and 25 μM BCS. Fold change values were analyzed by qRT-PCR. Bars represent the mean ± SDs of two biological and three technical replications, and significant differences (*$P < 0.01$, **$P < 0.005$, ***$P < 0.001$) are indicated with an asterisk.

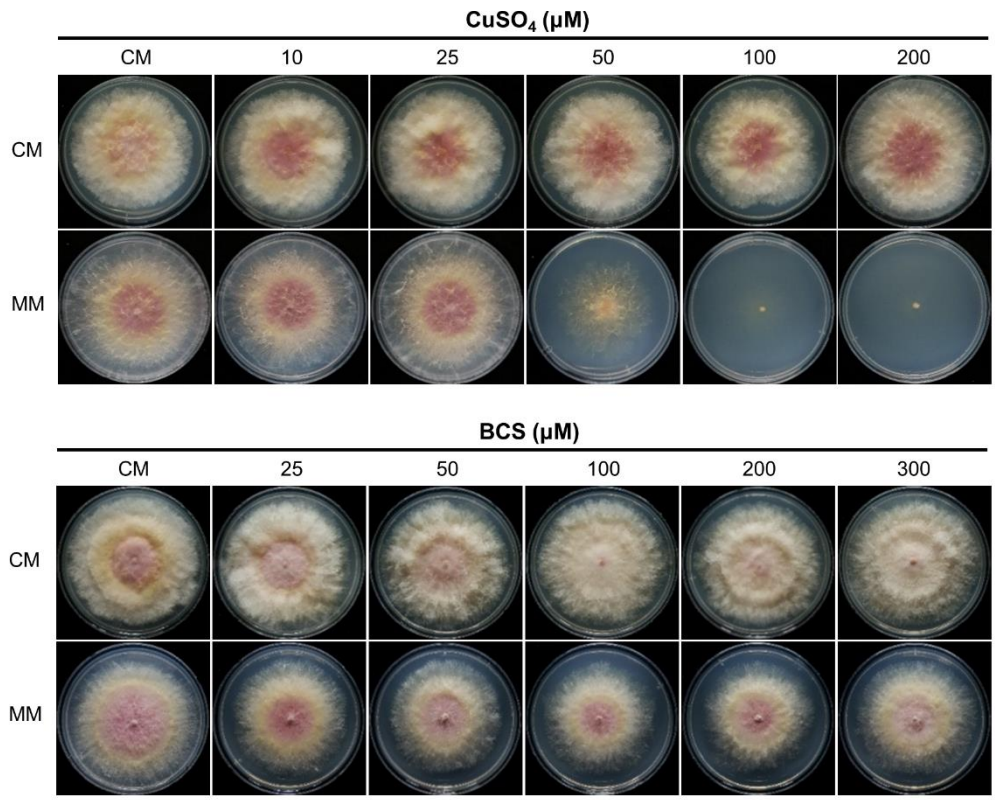

Figure 4. Mycelial growth with various copper and BCS concentrations. The wild-type strain was inoculated on CM and CM supplemented with $CuSO_4$ (10–200 μM) or BCS (25–300 μM). The pictures were taken 5 days after inoculation.

III. P_{FCR1} can drive heterologous gene expression in a copper-dependent manner

To confirm that the promoter of *FCR1* can regulate the expression of other genes in a copper-dependent manner, we used the green fluorescent protein (*GFP)* gene and *FgENA5* gene. The -1,009 to -1 bp upstream region of *FCR1* (P_{FCR1}) was fused to the *GFP* gene, and the P_{FCR1}-*GFP* cassette was randomly integrated into the genome of the *F. graminearum* Z-3639 strain. The GFP fluorescence pattern of the P_{FCR1}-*GFP* strain (Figure 5) was consistent with the expression pattern of *FCR1* analyzed by qRT-PCR (Figure 3B). At 8 h after inoculation, GFP fluorescence began to increase in the presence of BCS, although there was no change in the $CuSO_4$-supplemented conditions. At 12 h after inoculation, GFP fluorescence was dramatically reduced under copper-enriched conditions and increased under copper-deprived conditions. We observed stronger GFP fluorescence at higher BCS concentrations at both time points, but differences at higher $CuSO_4$ concentrations were not visually evident. These results clearly showed that P_{FCR1} could both repress and induce gene expression depending on the external copper conditions.

To validate the applicability of P_{FCR1} to *F. graminearum* functional genetics, we replaced the promoter of *FgENA5*, a cation stress-related gene, with P_{FCR1} (Figure 6A). In a previous study, *Fgena5* deletion mutants were found to be very sensitive to lithium-derived cation stress whereas overexpression of *FgENA5* increased lithium tolerance (Son et al., 2015). We expected P_{FCR1}-*FgENA5* strains to exhibit a similar phenotype to the *Fgena5* deletion mutant (HK146) under copper-supplemented conditions, and a similar phenotype to the *FgENA5* overexpression

mutant (HK147) in the presence of BCS. When copper was added under lithium-derived cation stress (0.1 M LiCl), the growth of P_{FCR1}-*FgENA5* strains was significantly reduced, although not to the same extent as the *Fgena5* deletion mutant (Figure 7). Under copper-deficient conditions, P_{FCR1}-*FgENA5* strains showed almost as much lithium tolerance as the *FgENA5* overexpression mutant. Although 10 μM $CuSO_4$ and 25 μM BCS were successful for copper-dependent gene regulation, higher concentrations of $CuSO_4$ (50 μM) and BCS (50 μM) led to a higher level of repression and induction. These results indicate that the conditional gene expression system using P_{FCR1} can be applied to functional genetic studies of target genes, particularly via concentration-dependent fine-tuning of gene expression.

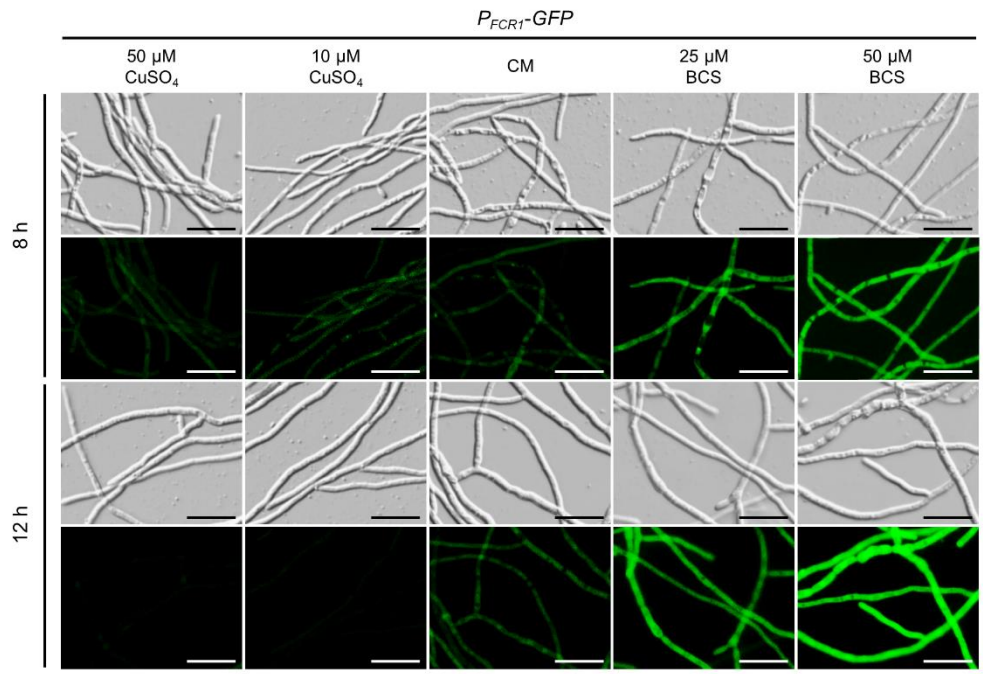

Figure 5. GFP expression of the *P_{FCR1}-GFP* mutant. GFP fluorescence was observed 8 and 12 h after inoculation in CM and CM supplemented with $CuSO_4$ (10 or 50 µM) or BCS (25 or 50 µM). Scale bars represent 25 µm.

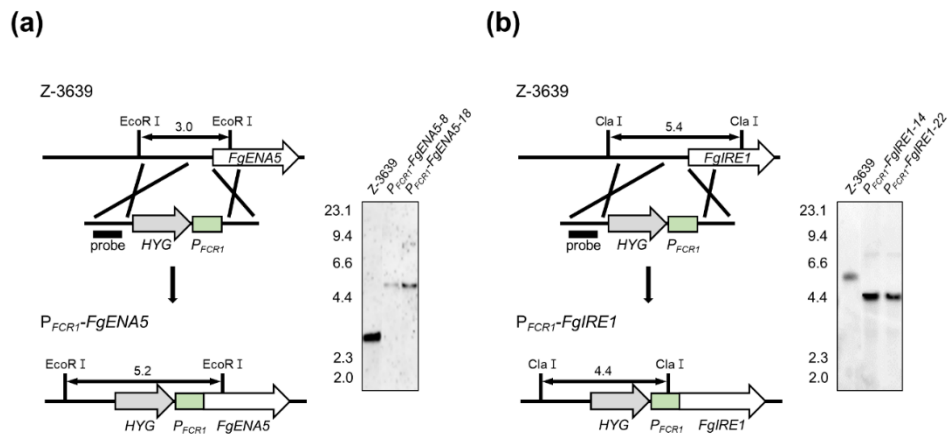

Figure 6. Promoter replacement. Schematic illustrating the strategy for promoter replacement of *FgENA5* (a) and *FgIRE1* (b) (left panel). Southern blot analyses confirming genetic manipulations (right panel). Lane 1, wild-type Z-3639; lanes 2 and 3, promoter-replaced mutants. Sizes of the DNA standards (kb) are indicated to the left of the blots.

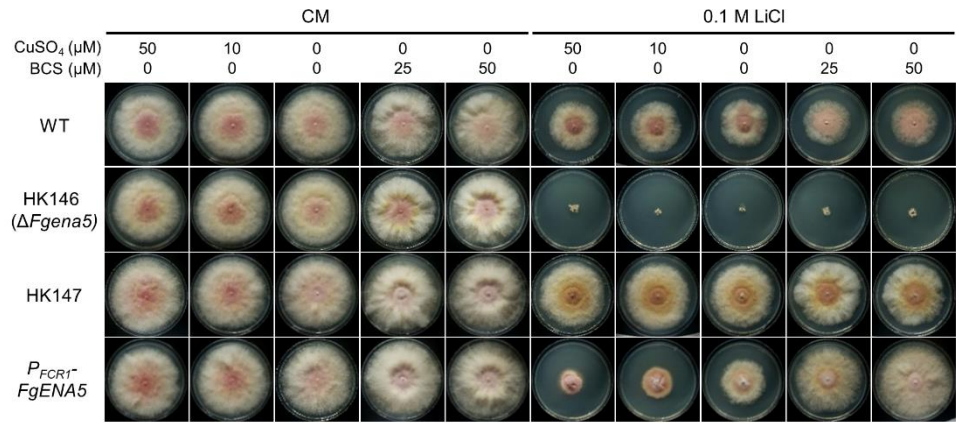

Figure 7. Cation sensitivity of *F. graminearum* strains. The strains were inoculated on CM and CM supplemented with 0.1 M LiCl, and mycelial growth was observed under the indicated $CuSO_4$ and BCS concentrations. The pictures were taken 5 days after inoculation. WT, wild-type strain Z-3639; HK146, Δ*Fgena5*; HK147, *FgENA5* overexpression strain.

IV. Functional analysis of the *F. graminearum* essential gene *FgIRE1* using P_{FCR1}

The functional analysis of essential genes is difficult as it is not possible to apply the key tool of gene deletion. In fact, inability to obtain deletion mutants is an indication that the target gene might be essential. Ire1 is known to function as an endoplasmic reticulum (ER) stress sensor kinase in many fungal species. There is evidence that *IRE1* might be an essential gene in several fungi. For instance, no *ireA* (*IRE1* ortholog) deletion mutants were obtained in either *A. oryzae* (Tanaka et al., 2015) or *A. niger* (Mulder and Nikolaev, 2009; Carvalho et al., 2010). Consistent with this, the ortholog of *IRE1* (locus ID: FGSG_00775, *FgIRE1*) is known to be essential in *F. graminearum* (Wang et al., 2011). In this study, repeated trials to obtain a deletion mutant of *FgIRE1* were also unsuccessful. We concluded that *FgIRE1* is essential for basic growth and other physiological functions of *F. graminearum,* and generated conditional suppression mutants of *FgIRE1* by replacing its native promoter with P_{FCR1} (Figure 6B). P_{FCR1}-*FgIRE1* strains showed reduced vegetative growth on CM under copper-supplemented conditions, confirming that *FgIRE1* is an essential gene directly involved in survival (Figure 8).

IRE1 disruption mutants have shown high sensitivity to ER stress in many fungal species (Cheon et al., 2011; Miyazaki et al., 2013; Krishnan and Askew, 2014; Fan et al., 2015). We expected P_{FCR1}-*FgIRE1* strains to be more sensitive to ER stress under copper-enriched conditions and less sensitive under copper-depleted conditions. When copper was added under ER stress, induced by tunicamycin (TM), P_{FCR1}-*FgIRE1* strains showed severe growth inhibition as the TM concentration

increased (Figure 8A). When 5 μg/ml TM was applied, the P_{FCR1}-*FgIRE1* strains hardly grew under copper-enriched conditions. In the presence of BCS, P_{FCR1}-*FgIRE1* strains recovered tolerance to ER stress to a level similar to the wild-type.

Previous studies have demonstrated that heat stress and the heat shock response are closely related to the ER stress response (Liu and Chang, 2008; Liu et al., 2012). The *C. neoformans ire1* deletion mutant showed extreme thermosensitivity compared to the wild-type (Cheon et al., 2011; Jung et al., 2016). To analyze the heat stress response of *F. graminearum* strains, we observed the growth of the P_{FCR1}-*FgIRE1* strains at 25°C and 30°C under copper- and BCS-supplemented conditions (Figure 8B). At 30°C, compared to 25°C, P_{FCR1}-*FgIRE1* strains showed markedly reduced growth under copper-enriched conditions, although the wild-type did not show any growth defect in response to heat stress. When BCS was added, P_{FCR1}-*FgIRE1* strains recovered heat tolerance similar to the wild-type. These results demonstrate that *FgIRE1* was successfully suppressed by adding copper, and reduced expression of *FgIRE1* led to higher sensitivity to ER and heat stress. We expect that in-depth study of *FgIRE1* and other essential genes will be possible using P_{FCR1}, which enables both suppression and overexpression of target genes depending on the copper concentration. In conclusion, we found that the transcription of *FCR1* is regulated in response to external copper concentrations, and that copper-dependent regulation of the target gene is possible by replacing the native promoter with P_{FCR1}. This study is the first to develop a conditional gene expression system that enables both repression and induction in *F. graminearum*. The P_{FCR1} system is generally applicable for studying essential genes and would be a valuable

tool to generate suppression or overexpression mutants in a fast and convenient way. We expect precise control of gene expression to facilitate extensive functional genetic studies of *F. graminearum*.

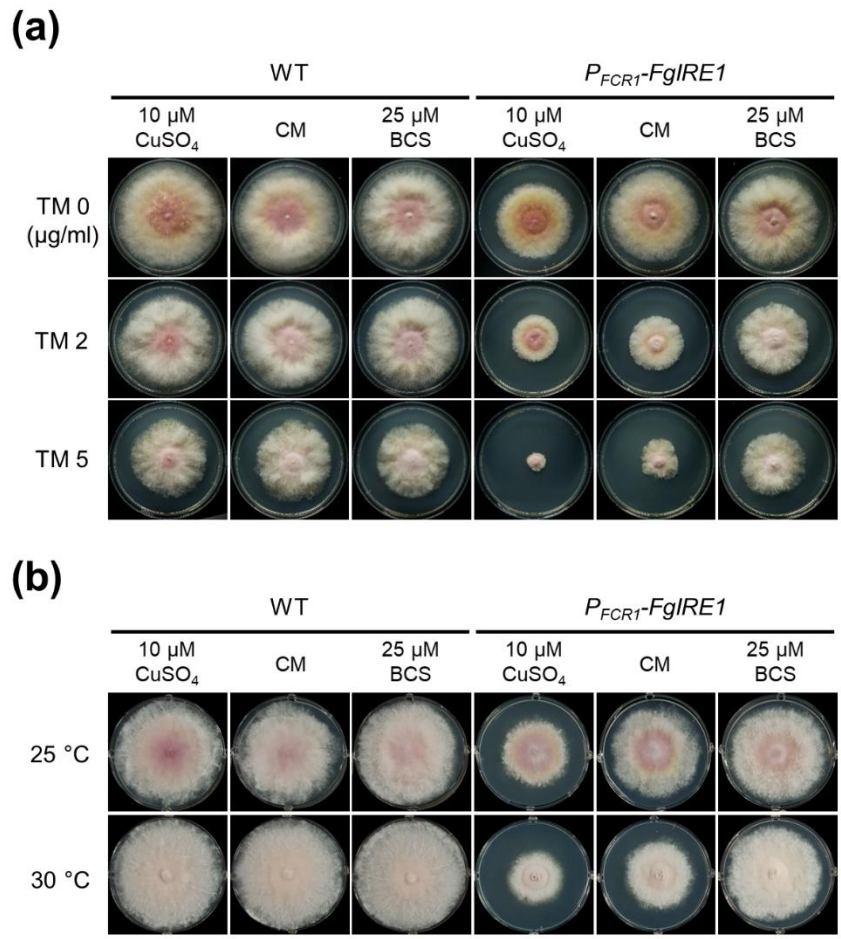

Figure 8. ER stress and heat stress sensitivity of *P_{FCR1}-FgIRE1*. (a) ER stress sensitivity. The strains were inoculated on CM and CM supplemented with ER stress agents (0.02 μg/ml TM, 0.05 μg/ml TM). Mycelial growth was observed under 10 μM $CuSO_4$ and 25 μM BCS treatment conditions. The pictures were taken 5 days after inoculation. (b) Heat stress sensitivity. Strains were incubated at 25°C and 30°C on CM and CM supplemented with 10 μM $CuSO_4$ and 25 μM BCS. The pictures were taken 3 days after inoculation.

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CHAPTER III

Identification of essential genes for the establishment of spray-induced gene silencing (SIGS)-based disease control in *Fusarium graminearum*

This chapter is in preparation for publication.

ABSTRACT

The plant pathogenic fungus *Fusarium graminearum* causes Fusarium head blight (FHB) in major cereal crops such as wheat, barley, and rice, resulting in significant economic losses. As resistance to chemical fungicides continues to increase in *F. graminearum*, there is a growing need to develop novel disease control strategies. To discover essential genes that could serve as new disease control targets, we selected essential gene candidates that had failed to be deleted in previous studies. Eleven genes were confirmed to be essential by constructing conditional promoter replacement mutants, and their growth was significantly reduced under repressing conditions. Two genes were identified as essential as they could not be deleted through a clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9)-mediated deletion strategy. We synthesized double-stranded RNAs (dsRNAs) targeting these essential genes and analyzed their protective effects in plants using a spray-induced gene silencing (SIGS) method. When dsRNAs targeting *Fg10360, Fg13150,* and *Fg06123* were applied to detached barley leaves prior to fungal inoculation, disease lesions were greatly reduced. Our findings provide evidence of the potential of essential genes identified by a SIGS method to be effective targets for the control of fungal diseases.

INTRODUCTION

Fusarium graminearum is a plant pathogenic fungus that causes diseases in cereal crops such as wheat, barley, rice, and maize worldwide (1). Fusarium head blight (FHB) inflicts damage to major food crops and has become a more severe thereat as the global population increases, not only reducing yields, but also posing a risk to food and feed safety by accumulating mycotoxins such as deoxynivalenol (DON) and zearalenone (ZEA) on the infected grains (2). The fungicides most extensively used to control FHB and fungal plant diseases are sterol demethylation inhibitors (DMIs) targeting the fungal cytochrome P450 lanosterol C-14α-demethylase (CYP51) to inhibit fungal ergosterol biosynthesis (3). However, prolonged use of DMI fungicides since the 1970s has led to development of reduced sensitivity in several plant pathogenic fungi, including *F. graminearum* (4-6), which highlights the need to identify new antifungal targets and to establish sustainable crop protection methods.

Essential genes, which are crucial for viability of the organism, have been primary targets for commercial antifungal drugs since their inhibition can result in the complete prevention of pathogen growth. For example, DMI fungicides (e.g., tebuconazole, propiconazole, and prothioconazole) targets the product of the essential gene *CYP51*/*ERG11*, and the double deletion of *FgCYP51A* and *FgCYP51B* is lethal in *F. graminearum* (7). Echinocandins (e.g., caspofungin, anidulafungin, and micafungin) target the essential cell wall synthesis enzyme Fks1, 1,3- β-D-glucan synthase, in *Aspergillus, Candida,* and *Cryptococcus* species (8, 9). Due to the

importance of essential genes as major targets of antifungal agents, large-scale studies have been conducted to identify essential genes in the model eukaryotic microorganism *Saccharomyces cerevisiae* and in human fungal pathogens such as *Candida albicans*, *Cryptococcus neoformans,* and *Aspergillus fumigatus* (10-13).

Common methods to identify essential genes include gene disruption to determine the feasibility of generating deletion mutants (14), parasexual genetics to assess the viability of haploid progeny (13), heterokaryon rescue to examine the viability of uninucleate spores (15), and conditional promoter replacement (CPR) to investigate growth defects under gene-repressing conditions (10, 12). With respect to plant pathogenic fungi, including *F. graminearum*, several studies have used a CPR strategy to investigate essential genes (16, 17), but large-scale forward or reverse genetics-based identification of essential genes has not been achieved. Moreover, although many essential genes have been identified as potential antifungal targets in fungal pathogens, there has been limited validation of their effectiveness.

Recently, RNA interference (RNAi) processes have garnered significant interest as sustainable and environmentally friendly means of plant protection. RNAi is a highly conserved biological mechanism within the eukaryotic kingdom through which double-stranded RNAs (dsRNAs) recognize mRNA molecules in a sequence-specific manner, leading to suppression of translation or degradation of the targeted mRNA. The use of RNAi-based plant disease control has been shown to effectively suppress essential genes or virulence-related genes of pests and pathogens (18-20). Furthermore, previous studies have revealed that RNAi is a cross-kingdom phenomenon, in which small RNA (sRNA) molecules move between host plants and

pathogens to silence genes, suggesting the potential to utilize host RNAi machinery for plant protection (21).

Host-induced gene silencing (HIGS) was initially used to control plant diseases by generating transgenic plants that produce dsRNAs to trigger gene silencing in pests and pathogens. However, regulatory issues surrounding genetically modified (GM) plants and the difficulty of breeding experiments have led to the development of an alternative approach, spray-induced gene silencing (SIGS). Briefly, SIGS involves spraying dsRNAs onto plant surfaces, similar to the application of conventional pesticides by farmers. Previous studies have shown that external application of dsRNAs targeting virulence genes (e.g., *BcDCL1* and *BcDCL2*) and essential genes (e.g., *BcCYP51*, *BcCHS1*, and *BcEF2*) significantly reduces disease lesions caused by *Botrytis cinerea*, which causes grey mold diseases in crops such as strawberries and tomatoes (20, 22). Similarly, in *F. graminearum*, spray application of *FgCYP51*-dsRNAs provided strong plant protection on barley leaves (19).

Despite promising results for RNAi-based plant disease control, the limited availability of target sources has posed a challenge. Here, we used a reverse genetics and CPR strategy to identify 13 essential genes in *F. graminearum* that could serve as potential antifungal targets. Among these, application of a SIGS method identified *Fg10360, Fg13150,* and *Fg06123* as effective targets to control fungal diseases in plants.

MATERIALS AND METHODS

I. Strains and culture conditions

The *F. graminearum* wild-type strain Z-3639 (23) was used as a host strain for transformation experiments. All strains used in this study were stored as mycelial suspensions in 20% glycerol solution at −80 °C. Culture media were prepared as described in the *Fusarium* laboratory manual (1). Conidial production of *F. graminearum* strains was induced in carboxymethyl cellulose (CMC) medium (24). To estimate the growth of conditional promoter replacement (CPR) mutants under repressing or inducing conditions, P_{ZEAR}-CPR mutants were inoculated on complete medium (CM; repressing conditions) or CM supplemented with 30 μM β-estradiol (inducing conditions), and P_{FCR1}-CPR mutants were inoculated on CM supplemented with 50 μM $CuSO_4$ (repressing conditions) or 25 μM bathocuproinedisulfonic acid (BCS; inducing conditions).

II. Nucleic acid manipulations, Southern blotting and PCR

Fungal genomic DNA was extracted from freeze-dried mycelial powder as previously described (1). Southern blot hybridization was performed using a North2South™ Biotin Random Prime Labeling Kit and North2South™ Chemiluminescent Hybridization and Detection Kit (Thermo Fisher Scientific, Waltham, MA, USA). The PCR primers (Table 1) were synthesized by an oligonucleotide synthesis facility (Bioneer, Daejeon, Republic of Korea).

Table 1. Primers used in this study.

Primers used to generate deletion mutants

Gene name	Oligo name	Sequence (5' to 3')
Fg07504	07504-5F	CCGATATTGCCATTGGAACAGAT
	07504-5R	gcacaggtacacttgtttagagGAGGTGGAGATGGAGAGGAAGAA
	07504-3F	ccttcaatatcatcttctgtcgTCCAGCATTGACGGACCATCTA
	07504-3R	CGTCCCATCTTCATCGCCTAC
	07504-5N	CTGGAGGAGGGATACGACGACA
	07504-3N	ATTTGCGGACAGGGGATAGTTG
	07504 with 5F	GAGCAGGAACAGCAACAGGAACT
	07504-ORF-F	GAAAAGTTTCAAGCGGACAGAGC
	07504-ORF-R	ATTGCTATCACGACCGCTTTGTT
Fg07909	07909-5F	CGAGCGTGAGCTTAGAATGAGATG
	07909-5R	gcacaggtacacttgtttagagTTATCCGGAGTTGTGAAAGGTTGA
	07909-3F	ccttcaatatcatcttctgtcgATGCGCAGTGGTTAGAGATGTTCA
	07909-3R	CAACCCCAAGCTGCACTACTAAGG
	07909-5N	GAAAGCTCCTCCACGACCACATA
	07909-3N	CAATGCAGGGGCCGTCTCTA
	07909 with 5F	GAGTTGTGTTTGCATCGTCAGGAG
	07909-ORF-F	GCTTGAGTCGAACCCAGATTACA
	07909-ORF-R	AAAGGCTTGCAGGCGTGATT
Fg08845	08845-5F	CGTCTCATCTGCTCGTCACTCCT
	08845-5R	gcacaggtacacttgtttagagAGACCAGAGACCAGAGACCCACTT
	08845-3F	ccttcaatatcatcttctgtcgGCGCGGACTCTACCTCACAATA
	08845-3R	CTGGCTTTTTACGCTTGCTTTCTT
	08845-5N	GGAGAGGGAGAGGGTAGAGGATGA
	08845-3N	TGCTGAACGATGAATGTGATGTCT
	08845 with 5F	CCGGGTCAACTCGATAAAGGAT
	08845-ORF-F	GAGGAGTCAGACGAGGAGGAGGAT
	08845-ORF-R	CCCACGAACAAGAAGACCGAGTA
Fg09629	09629-5F	TCCTACACGAGCAGCACCAATG
	09629-5R	gcacaggtacacttgtttagagAAACATGCGGGAACCTTACCA
	09629-3F	ccttcaatatcatcttctgtcgTCGGCTCCCCCACCACTC
	09629-3R	TACCTGGCATTCGCATCCTAAC
	09629-5N	TCGGACAGGGATCTCAAGTTCTC
	09629-3N	CATGAATGGGATCGTGTAGTTGGA
	09629 with 5F	CTCATGGACTTCCTCGGCTGTT
	09629-ORF-F	GCGACACCGACTTCCGTAAAA

	09629-ORF-R	CTCCGTACTCTGTCTTCACCTTGG
Fg10360	10360-5F	CACAAATGCCTGAGAAACGAGAGA
	10360-5R	gcacaggtacacttgtttagagTCGGGGGTGATGGAGGAAT
	10360-3F	ccttcaatatcatcttctgtcgCCATACCTCAACGGAACCAACAG
	10360-3R	ACGGCTCTGGCTGGGTCAA
	10360-5N	GGTCAGGCCGTCTATTCCACTTC
	10360-3N	CACGTGGAGTTTATCAGCGGAGTA
	10360 with 5F	AGCTGCTCGGCGACATCATTAT
	10360-ORF-F	ACTTCATACACGATGGGGGATACC
	10360-ORF-R	ATGGCCAAAGAGTGCTGCGA
Fg08474	08474-5F	GGATGCTGTAGTTGGGAGGAATG
	08474-5R	gcacaggtacacttgtttagagCAGCGGAATGCAGAGTATGTATGG
	08474-3F	ccttcaatatcatcttctgtcgAGGAGCGAAATGTTATGAGGTGC
	08474-3R	TCTTCCCCTCCTTCGTCATAACTT
	08474-5N	GTTCCATCGGGCTGTCGTATC
	08474-3N	ACGCAGGAGCAGAAGCAGACC
	08474 with 5F	CTGCGATGGGTGAGGTGAATG
	08474-ORF-F	CACAACTGGCTAGACTCGCTGGTA
	08474-ORF-R	TCAAGCCCTTACACAAAACCTCAA
Fg00374	00374-5F	GGCCTGCCAATGGATGAATAGT
	00374-5R	gcacaggtacacttgtttagagCGGGAAGAAGAATAAGCGTAGAAA
	00374-3F	ccttcaatatcatcttctgtcgTGGGTAATGGGTTAGTGGAGCA
	00374-3R	GAGCCTTTGACCCCTACTGACAC
	00374-5N	GCAACAGGTCCAGTAGGCAAGAA
	00374-3N	TAAGCAGATGGGCAGCGTTGT
	00374 with 5F	AACACTTTCTTCATGGTCGGGTAA
	00374-ORF-F	ACTGACGAGCCCGATTATTATGAG
	00374-ORF-R	CCTTCTGGTTGGCGAGTCTTTC
Fg13150	13150-5F	GAAATGATAACGAGCCCCACACT
	13150-5R	gcacaggtacacttgtttagagGTCTGGGGCGAATTGAACTGA
	13150-3F	ccttcaatatcatcttctgtcgGTGTGCTAGCATTATGTGGACTCG
	13150-3R	GCGCCTTGGCACCTATGTATCT
	13150-5N	GGCAAGAAGAATGCGACAACG
	13150-3N	ACATGGCGCGGTAGTTTTTACAC
	13150 with 5F	GGTAGTGGCCAATGCGATGTAGT
	13150-ORF-F	TTCAGCTTCTGGTGGTGCCC
	13150-ORF-R	TGGTGTTTGTCGTTGCTGGATAAG
Fg06123	06123-5F	ATCTCACGGCCCATCCATTG
	06123-5R	gcacaggtacacttgtttagagCATCGGCCGTTTCGTCAGC
	06123-3F	ccttcaatatcatcttctgtcgGATGATTTTGTGCGGATGACTGG

	06123-3R	TCGACCACCGAAGAAAGCAGTTA
	06123-5N	CCATCTCAGTCACCCAACGCTTA
	06123-3N	TCGGGCAAAGACATCCAACAAT
	06123 with 5F	GCGCTGGTAGGCCTCTCAAGTA
	06123-ORF-F	CACGAGGATGCGGTTTACGATAC
	06123-ORF-R	CCATCGAGGCGCACATAACA
Fg05512	05512-5F	CTTACCATCCCAGCCCATACCTC
	05512-5R	gcacaggtacacttgtttagagAATCCTCTTCCACCCTGTCATCC
	05512-3F	ccttcaatatcatcttctgtcgGCGATCGATCTTGGTGTTTGTCT
	05512-3R	GGTGACGAGCTGCCTTACAACTAC
	05512-5N	GGATGGGGGTCTTTTCGTCG
	05512-3N	CAAACGATCAGGAACAGGCATT
	05512 with 5F	CAAGTTAGCACGCCGAAAAAGATA
	05512-ORF-F	CTACAAAATCCGATGCTGCTGCTA
	05512-ORF-R	GAGACCATTTATCGTGCTGCTGAA
Fg01290	01290-5F	CGTGTTGGCAGCGGCAGTA
	01290-5R	gcacaggtacacttgtttagagGGGATGCCCAATTTTCCTCAGT
	01290-3F	ccttcaatatcatcttctgtcgTTGACGGACGGGATGAAACG
	01290-3R	TGTAGCGTCAGCGGCGTAAA
	01290-5N	TTCTAGGCAGAGGGCAGTGGTATT
	01290-3N	GGTTGCTTAGCACGCTTGGAGT
	01290 with 5F	ACTCGTCGGACTTCACCACTCTG
	01290-ORF-F	AGGGGGATACAGGGTGACGG
	01290-ORF-R	TTCAACCAGCTTCTTCACCAAAAC
Fg02622	02622-5F	ATCCGGTTGGTTGAGGAATACG
	02622-5R	gcacaggtacacttgtttagagTCGAGACTGAGGGGCTGATTTT
	02622-3F	ccttcaatatcatcttctgtcgAGCAAGACAACACGCCTAACGAT
	02622-3R	CTACGATCAATCACAACCCACTCC
	02622-5N	ACCGTTCCTAGGCCAAGACACT
	02622-3N	GATGAATGGTATGAACGGGACAAA
	02622 with 5F	TCATCTCCATCTTCGTCCAGTGC
	02622-ORF-F	CCCGATGACATAATGGACACAGAC
	02622-ORF-R	TTGAGGACCTGAGACTTGGCTGTA
Fg08655	08655-5F	CTTTGCTTGTTCGCGTTTTTGT
	08655-5R	gcacaggtacacttgtttagagAGAAAGCTGGTGATGATGTTGTGG
	08655-3F	ccttcaatatcatcttctgtcgGGCTGGCGTTGTTGATACTGTC
	08655-3R	CCAGCAAAGACTCCAATAGACTCG
	08655-5N	GATGGTTGCGGAGGAGTAGAGTTT
	08655-3N	CCACCACTGCCTCCAACAAC
	08655 with 5F	CGGCGGTGTACTTGAGAATGAC

	08655-ORF-F	CGTTTCCTTTCTCCCCTTTCCTA
	08655-ORF-R	CAGCAGCGCCATCGTCAGA
Fg10017	10017-5F	GTGGCCATGATGAACCTAACGA
	10017-5R	gcacaggtacacttgtttagagAGCTCCTTCGTCACTCACCCTC
	10017-3F	ccttcaatatcatcttctgtcgGAAGTGATGGTCAATTTCAGTGGC
	10017-3R	CGGCTGGATGAGGAAGAAGAGTT
	10017-5N	TCCAATGATACCCGATACGCTAAC
	10017-3N	GTTCACAACACAATCCCTCCACTAT
	10017 with 5F	CAGCAGCATTTGGGTTTGAGAC
	10017-ORF-F	CAACAGGAGATGGCCAAGAAGACT
	10017-ORF-R	GAAATAGGGGTGTCTCGGTAGTCG
Fg04221	04221-5F	CATCCTCTACGCCTTTGTGACG
	04221-5R	gcacaggtacacttgtttagagGCTGCAACGCTGTGGGAAGT
	04221-3F	ccttcaatatcatcttctgtcgGACAAATCGGAGTTCTTCACAGGA
	04221-3R	CGCACCCAACAGTCAAAATACAC
	04221-5N	CCATCGAGGGGACTTTGTTAGG
	04221-3N	GAAGGCCAAGCAAGCGTCTCT
	04221 with 5F	CCGCACCATTTCTTCGTTACATA
	04221-ORF-F	AAATGGTGCGGCGACTGTTAT
	04221-ORF-R	CAGTATCCGCAAGTTCCGAGTG
Fg10458	10458-5F	ACTCGTTGTCTTCTTTGGGGTTGT
	10458-5R	gcacaggtacacttgtttagagTGTTGATCGGGGGACTAGTTCTTA
	10458-3F	ccttcaatatcatcttctgtcgATTCAATGCCCAAAGCGGTC
	10458-3R	GTGGTTGCGTATGACAAAATGGTT
	10458-5N	GTGCGGTCCTGGCTTTCATTAG
	10458-3N	ATGCTGGGCGTGAGACAAATG
	10458 with 5F	TTTGGCCCACATCTTGACTTTTG
	10458-ORF-F	GCGACAGATGCGAGAAAAATGA
	10458-ORF-R	GGGTTGCGTAGCGTCGTGTAG
Fg11551	11551-5F	TCGCGAGACTCAAGACCAACAA
	11551-5R	gcacaggtacacttgtttagagGTCATGGTTCAGGCTGCTTGC
	11551-3F	ccttcaatatcatcttctgtcgATTGGTCTGCCGGTTTATTTTGTT
	11551-3R	CACGATTGGCGCAGGATTC
	11551-5N	CGGTCGCATCGTCTGGTCTC
	11551-3N	ATCATCTCGCGCACAAATAAGTCC
	11551 with 5F	GCGACGTGAGTTGGTGTAGCA
	11551-ORF-F	GAGACGAAATGCTCCAACTGTGTTA
	11551-ORF-R	GGGTTGCACTTCCTGTATCGTCA
Fg11356	11356-5F	GCAGAGCGCAACCCCATAGT
	11356-5R	gcacaggtacacttgtttagagGCTTCGGCGCAGTAGACAATG

	11356-3F	ccttcaatatcatcttctgtcgCTATCTCTGGCACAACCGCACTT
	11356-3R	CATCAGAATCTCGCCGTTGCTA
	11356-5N	CTCCGATGGCAAGAATAGCGTTAC
	11356-3N	AGCTGAAGCTGTTGCCGTAGTT
	11356 with 5F	ATGGCGAAACAAGTCAAAGTATGG
	11356-ORF-F	TGCGTTAAGCCATACCGAGTCA
	11356-ORF-R	CTTCTGCGGGAATATCACCACTTT
Fg07924	07924-5F	GTACGTACGGCTGGAAAAGTCG
	07924-5R	gcacaggtacacttgtttagagGTGGGCGGAGCTGGAGTAAG
	07924-3F	ccttcaatatcatcttctgtcgTATGATATCGAGGCGTTGGTGTG
	07924-3R	CGCCAACACAATTTCCAAGTC
	07924-5N	AGGAGACAGCCAGGACACGGT
	07924-3N	CAATGTACGAGATGCCAGAGGAAA
	07924 with 5F	GCAAGTGGGTGGATGTAAGTGAAG
	07924-ORF-F	GCAGCAGCAGTGTTTGAGTCCTT
	07924-ORF-R	GCACAGGCTGGTTCTTGATTACAT
Fg08064	08064-5F	AGCCATTCTTCTCAGCGACAAAC
	08064-5R	gcacaggtacacttgtttagagCGCTATAGGGTGGTCAGAGAGGA
	08064-3F	ccttcaatatcatcttctgtcgACATTTGGGGTTGGCATCAGTAT
	08064-3R	TTTGGTGTGGTGAGGAGCGTA
	08064-5N	TGGTTTTGGTAGTCTTTTGAGGCT
	08064-3N	GAGAAAGGATGGCAAAAGTGATGA
	08064 with 5F	GTTGGTGTGATCGGTAGTGGTTG
	08064-ORF-F	AACTCCTCAATTGCGACCGAAC
	08064-ORF-R	TGCATGCAGTGTTTTCGTCGTAG
Fg09064	09064-5F	ATGTTAGCTGTTGTTCACCGTTCC
	09064-5R	gcacaggtacacttgtttagagGATGGGGAATGGCAGTTGATAA
	09064-3F	ccttcaatatcatcttctgtcgGTATTGACTGCCGTGTTCTGGATT
	09064-3R	AGTTGTGGGTAGGGCTTTAGGAGA
	09064-5N	CGAATCCCGTACTTGTCAGCATC
	09064-3N	ACCATCAGCACCAACAATCAAGTC
	09064 with 5F	TCTCTGCCCTTGCTCTGGTTCT
	09064-ORF-F	CTGGGGCTACATAGTCGGGAATC
	09064-ORF-R	CACTCCAGCTTGAACATCCGTAAA
Fg07609	07609-5F	CCGGCAAGACCAGTGATGATT
	07609-5R	gcacaggtacacttgtttagagGATGGGTTGGACTTGGGACAG
	07609-3F	ccttcaatatcatcttctgtcgAGCAGGCGTATGGGACTATGTAAC
	07609-3R	TAACCAGCCATCACGAAACAATC
	07609-5N	CAATTTGTAAGGGGCAGCGTGT
	07609-3N	TGCCGTACTCCGCCTGTGTT

	07609 with 5F	GTAACGGGTGGTAAGCTCCTGAC
	07609-ORF-F	TCCGCCTTCCACGCTACCTTA
	07609-ORF-R	GCCTCTTGCTTTTCGATTCATTCT
Fg04862	04862-5F	CAGCGAGGATAGTAGCGAAGGTT
	04862-5R	gcacaggtacacttgtttagagAAGATGAGCGAACAAGCGAATG
	04862-3F	ccttcaatatcatcttctgtcgGAAGAGGCATATTGACCGAACCA
	04862-3R	CCGATTTGCAGAGATGGAGGAG
	04862-5N	GGGGAACCTTGCAGAGTCACA
	04862-3N	AAGCGGAATAGGCAACAGGATAA
	04862 with 5F	TGGCAGCAAATTAGGGCAGAC
	04862-ORF-F	TGGTCGTACTTGTGATGGCTATGA
	04862-ORF-R	TTCTCATCTTTCAGTCTCCCATCA
Fg00800	00800-5F	CGCATATTCACGGCATTGT
	00800-5R	gcacaggtacacttgtttagagCGAGTCACGGCAACGAGAATG
	00800-3F	ccttcaatatcatcttctgtcgACCCGTGGGAATTTGTTTTGTG
	00800-3R	TAAAGATCGCCAAATCGTCATAGC
	00800-5N	GCTGATTAGAGCCAATAGCGAGTG
	00800-3N	TGGGCTCGCATACAGAATAGGTG
	00800 with 5F	CGAGGACGCATTTGACAGGTG
	00800-ORF-F	TAATGCGCCACCCAAAAACTC
	00800-ORF-R	ACGTCTCCGCATCTCTGTTTCAA
	GEN-For	CGACAGAAGATGATATTGAAGG
	GEN-Rev	CTCTAAACAAGTGTACCTGTGC
	GEN-G3	GGGAAGGGACTGGCTGCTATTG
	GEN-G2	GCAATATCACGGGTAGCCAACG
	GEN with 5F	GTTGCCTAGTGAATGCTCCGTAACA

Primers used to generate CPR mutants

Gene name	Oligo name	Sequence (5' to 3')
Fg09629	09629-PZ-5F	=09629-5F
	09629-PZ-5R	tccactagctccagccaagccATCAAGACCCCTTAAAATGTTTGA
	09629-PZ-3F	gagagaacgaaagtaaccatgATGTCCGACTCAAAGAAAGAAGGC
	09629-PZ-3R	TGAGGGGTTAAGGAGTGCTGAGT
	09629-PZ-5N	=09629-5N
	09629-PZ-3N	CGGCGATCCAAAGTGAGTACTAGT
Fg10360	10360-PZ-5F	=10360-5F
	10360-PZ-5R	tccactagctccagccaagccGATGGATGGTTGATCGGTTCG
	10360-PZ-3F	gagagaacgaaagtaaccatgGCCATGAGTGTCGCATTCG

	10360-PZ-3R	GAATAAACGCCGTGCCAAAAAC
	10360-PZ-5N	=10360-5N
	10360-PZ-3N	CGAGTCAATGAACCCCGTGTAAA
Fg08474	08474-PF-5F	=08474-5F
	08474-PF-5R	cctccactagctccagccaagccTCGAACTCAGTACTCGTCGGAAAG
	08474-PF-3F	ggcatgtcagaaactgccaccATGGTCCCACCACCTCGAGC
	08474-PF-3R	ACCCCCTCGTTGATATTGACTTTC
	08474-PF-5N	=08474-5N
	08474-PF-3N	GCTTGGATCTTGTTGGGAGGTTT
Fg00374	00374-PZ-5F	ACAGCTCTTAGACCAACCGAACG
	00374-PZ-5R	tccactagctccagccaagccAAAACTGCAGTGGTGGGGTGAT
	00374-PZ-3F	gagagaacgaaagtaaccatgATGGCCGATATCGATGATTATCTC
	00374-PZ-3R	CCTTCTGGTTGGCGAGTCTTTC
	00374-PZ-5N	GGTCTTATCGAAAATGTCCACTCCA
	00374-PZ-3N	CATATTGGGCTCGTCGTTCTCTG
Fg13150	13150-PZ-5F	GGCTGACCGCATTGTTCCTT
	13150-PZ-5R	tccactagctccagccaagccCCATACATAAACTGTAGCCATTCCA
	13150-PZ-3F	gagagaacgaaagtaaccatgATGGCGTCAGAGCTTGAAGGC
	13150-PZ-3R	TGGTGTTTGTCGTTGCTGGATAAG
	13150-PZ-5N	GAAATGATAACGAGCCCCACACT
	13150-PZ-3N	AAACATCGGGATTCGTGAGCC
Fg06123	06123-PF-5F	=06123-5F
	06123-PF-5R	cctccactagctccagccaagccCTAAAGGCAATTCCCATCCGTG
	06123-PF-3F	ggcatgtcagaaactgccaccTCAGCACCTTGAACTCCCTCGT
	06123-PF-3R	CCCAACGAATGACAGGACCAC
	06123-PF-5N	=06123-5N
	06123-PF-3N	ACAACAACACAGCCGTCTCTCCT
Fg05512	05512-PZ-5F	=05512-5F
	05512-PZ-5R	tccactagctccagccaagccGTGCTAAAATTGTCCAACCGCTG
	05512-PZ-3F	gagagaacgaaagtaaccatgATGACTGAGGTCGGAGCCGC
	05512-PZ-3R	TATGGGCGCTCCGATTTGAC
	05512-PZ-5N	=05512-5N
	05512-PZ-3N	CATGCTCCGGACTCGTATCTGTAG
Fg01290	01290-PZ-5F	=01290-5F
	01290-PZ-5R	tccactagctccagccaagccGGATGCCCAATTTTCCTCAGTAA
	01290-PZ-3F	gagagaacgaaagtaaccatgGACCGAGACCGAAAATGCTCC
	01290-PZ-3R	GGCACGCTCTTAAACCATACACTC
	01290-PZ-5N	=01290-5N
	01290-PZ-3N	CAGTGCACAATCCAGATACAAAGGT

Fg02622	02622-PZ-5F	=02622-5F
	02622-PZ-5R	tccactagctccagccaagccGTGCTAAAATTGTCCAACCGCTG
	02622-PZ-3F	gagagaacgaaagtaaccatgATGAGGTACGTCCTTTCACTAAACATC
	02622-PZ-3R	CTCATCCTCCTCGGGCTCTACTG
	02622-PZ-5N	=02622-5N
	02622-PZ-3N	TGCGATAGATTCCGGTCACTTCA
Fg08655	08655-PZ-5F	=08655-5F
	08655-PZ-5R	tccactagctccagccaagccGCGACTGGCTTGTGATTTCTGTG
	08655-PZ-3F	gagagaacgaaagtaaccatgATGCCTAAGAACAAGGGAAAGGTAA
	08655-PZ-3R	TCAGCTGCAAACCTCAAGAGAAG
	08655-PZ-5N	=08655-5N
	08655-PZ-3N	GCTTTTGAGACGAATGCGATGAT
Fg10017	10017-PF-5F	=10017-5F
	10017-PF-5R	cctccactagctccagccaagccGCCGTGGTATAGATTGTCAGAGGT
	10017-PF-3F	ggcatgtcagaaactgccaccATGACTTCGTCCATTGCTGGC
	10017-PF-3R	ATCGCCACTTCGCAACACAAC
	10017-PF-5N	TGGAGAGGTGGACGGTTATGCT
	10017-PF-3N	CGGGGTCGAATTGTATGGAGTA
Fg04221	04221-PZ-5F	=04221-5F
	04221-PZ-5R	tccactagctccagccaagccTACCTTAGTCGATGGGGAAAAATG
	04221-PZ-3F	gagagaacgaaagtaaccatgATGTCGGGCCGCAAGCTG
	04221-PZ-3R	ATCTTGGCTGCTTCCTGTATCTGA
	04221-PZ-5N	=04221-5N
	04221-PZ-3N	TAGTTGCCGGCTGTGGTTTCTC
Fg07924	07924-PZ-5F	=07924-5F
	07924-PZ-5R	tccactagctccagccaagccCGGTTGTTGGAGGTGGTTGAG
	07924-PZ-3F	gagagaacgaaagtaaccatgATGTCCGACTCACCTCCCCC
	07924-PZ-3R	GCATTGTGGGATTGCATTAGTAGC
	07924-PZ-5N	=07924-5N
	07924-PZ-3N	TTTTTCATCCCCGACAGTGCTAC
	HYG-F1	GGCTTGGCTGGAGCTAGTGGAGG
	ZEAR-R2	CATGGTTACTTTCGTTCTCTCTGGTC
	FCR1-R	GGTGGCAGTTTCTGACATGCC
	HYG-H3	CGTTATGTTTATCGGCACTTTGC
	HYG-H2	GCTGCTCCATACAAGCCAACC

Primers used to generate deletion mutants using Cas9 RNP

Gene name	Oligo name	Sequence (5' to 3')

PKS12	PKS12-CAS9-5F	GCTGCGAATGATAATCCACGACT
	PKS12-CAS9-5R	gcacaggtacacttgtttagagACGTGATTCAGAGTTGGGATTAGC
	PKS12-CAS9-3F	ccttcaatatcatcttctgtcgCACGATGGAGAGGGCTGTTTG
	PKS12-CAS9-3R	TCATCAGCTTTCACTCCGTCACAT
	PKS12-CAS9-5N	GGGCAGTGCCTGCTGAGTCTT
	PKS12-CAS9-3N	ACCCAACAACCCATCAATCTCAGT
Fg04274	04274-CAS9-5F	TACGATGCGCTACGGTGTTCA
	04274-CAS9-5R	gcacaggtacacttgtttagagTCCATTCGACCCCACCAACTT
	04274-CAS9-3F	ccttcaatatcatcttctgtcgAGGGCAACCCCACCTGACTAA
	04274-CAS9-3R	TTGGCGTTGGAAGCACATTTG
	04274-CAS9-5N	CGCCACGGTATTATGACTGACG
	04274-CAS9-3N	TCTCATCCCTCCATCATCAGTCG
Fg00374	00374-CAS9-5F	ACCCCAGCCCTCACCTTCAA
	00374-CAS9-5R	gcacaggtacacttgtttagagGAAGGCGAGGTACGGTGACAG
	00374-CAS9-3F	ccttcaatatcatcttctgtcgCGAAAAATTCAGCAAGCAAAAAGT
	00374-CAS9-3R	AGCTAGCGCGATAAAACCTCAAC
	00374-CAS9-5N	CCATCCCATACTTGCATCTCCTG
	00374-CAS9-3N	TGACCCCTACTGACACCCGATAC
Fg05512	05512-CAS9-5F	GATCGTGCGGTCTAGGCTGTAAT
	05512-CAS9-5R	gcacaggtacacttgtttagagTAGGGCGATCAATAACAAACAGTG
	05512-CAS9-3F	ccttcaatatcatcttctgtcgGGGTCGAGGTTTGAAGGCATAAG
	05512-CAS9-3R	GCTTGATGGTTCTGATTCCCTATG
	05512-CAS9-5N	CCAGATCGGCATCCTGTTTGT
	05512-CAS9-3N	GTGCTGCTAGATGGGTCGCTGT

Primers used to synthesize dsRNA

Gene name	Oligo name	Sequence (5' to 3')
Fg13150	13150_dsR_F	taatacgactcactatagggTCACCAACGCCACGGAAGAC
	13150_dsR_R	taatacgactcactatagggTCGTCTTCGTCCTCTTCCTCTTCA
Fg10360	10360_dsR_F	taatacgactcactatagggGACACGAGCGCGAGGTTCA
	10360_dsR_R	taatacgactcactatagggGCAGCCGCTTGACCTCCTC
Fg06123	06123_dsR_F	taatacgactcactatagggGGCTGTGTTGTTGTACCCTCTTCA
	06123_dsR_R	taatacgactcactatagggTGGCTGCTGGGCGAATGA
GFP	GFP_dsR_F	taatacgactcactatagggATGGTGAGCAAGGGCGAGG
	GFP_dsR_R	taatacgactcactatagggACGCCGTAGGTGAAGGTGGTC
FgCYP51A	CYP51A_dsR_F	taatacgactcactatagggACTCTCCGCGTCCACTCTTCC
	CYP51A_dsR_R	taatacgactcactatagggCGGGCTCTTCGTTCCTTTAGAC
FgCYP51B	CYP51B_dsR_F	taatacgactcactatagggCCACATGATGATTGCCCTCCTTAT
	CYP51B_dsR_R	taatacgactcactatagggCGCGCATGATGGAGTGGATA

FgCYP51C	CYP51C_dsR_F	taatacgactcactatagggCTCTCAACGGCCAAGTCATCAA
	CYP51C_dsR_R	taatacgactcactatagggCCGTAATCCACTGTTTGACCATCT
Fg00374	00374_dsR_F	taatacgactcactatagggCAAGCCGCATTAAGACTGAGCA
	00374_dsR_R	taatacgactcactatagggAACACTTTCTTCATGGTCGGGTAA
Fg01290	01290_dsR_F	taatacgactcactatagggACGATCATCTCTGGAAAGGACGAC
	01290_dsR_R	taatacgactcactatagggCTCAGTCCATCAGCGGCAAGA
Fg02622	02622_dsR_F	taatacgactcactatagggCGTTGTCCTTTTGCACTATCCTGA
	02622_dsR_R	taatacgactcactatagggGACCTCTTGACTGGCAACTCTG
Fg04221	04221_dsR_F	taatacgactcactatagggTTATTGGGCGAGAGTATGCGAGA
	04221_dsR_R	taatacgactcactatagggTGTTCGATAAGCTTGCGGGGATTC
Fg05512	05512_dsR_F	taatacgactcactatagggAGTCCAAGAAGGGCGAAGAAAAGT
	05512_dsR_R	taatacgactcactatagggAGACCATTTATCGTGCTGCTGAA
Fg07924	07924_dsR_F	taatacgactcactatagggCAGCAGTGTTTGAGTCCTTGTCT
	07924_dsR_R	taatacgactcactatagggAAAGGATGCAACAAGGGAACAGA
Fg08474	08474_dsR_F	taatacgactcactatagggTCAAAGCGAATCTGGAAAGTCAAT
	08474_dsR_R	taatacgactcactatagggCGTTTCCATCCCTGCCGTTA
Fg08655	08655_dsR_F	taatacgactcactatagggCGTGGAAAGATGCGAAAGAAGG
	08655_dsR_R	taatacgactcactatagggCAGCAGCGCCATCGTCAGA
Fg09629	09629_dsR_F	taatacgactcactatagggGCCGTGGAGCTGGTCAATA
	09629_dsR_R	taatacgactcactatagggTCTGTCTTCACCTTGGTTGCCTC
Fg10017	10017_dsR_F	taatacgactcactatagggAAAGCCCGGCCGAAATCT
	10017_dsR_R	taatacgactcactatagggCCGGAACACGAGACCAAAGATT

III. Genetic manipulations and fungal transformations

The fusion PCR products used for gene deletion and promoter replacement were constructed using the double-joint (DJ) PCR method (25). To generate deletion mutants, the 5' and 3' flanking regions of the target genes were amplified from the genomic DNA of the wild-type *F. graminearum*, and the geneticin resistance cassette (*GEN*) was amplified from the pII99 plasmid (26). The three amplicons were fused using a DJ PCR method, and the final products were obtained using the nested primers. The resulting PCR products were transformed into the fungal wild-type protoplasts. The transformants were screened using diagnostic PCR, and the mutants

were confirmed by Southern blot analysis with a flanking region probe (Figure 1). For transformants showing bands with unexpected sizes in the Southern blot analyses, we additionally confirmed the knockout mutants using a probe located in the open reading frame (ORF) region.

To generate P_{ZEAR}-CPR mutants, the hygromycin resistance gene cassette (*HYG*)- P_{ZEAR} was amplified from the P_{ZEAR}-*FgHSP90* strain (HK226) (16), and the 5' flanking regions and partial ORF regions of the target genes were amplified from genomic DNA of the wild-type strain. The primers were designed to exclude ~200−1000 bp upstream of the gene translational site to remove the native promoter. After fusion PCR of the resulting three amplicons, the third round of PCR was performed using the nested primers. The final constructs were used to transform the fungal wild-type strain. To generate P_{FCR1}-CPR mutants, an *HYG*-P_{FCR1} construct was amplified from the *HYG*-P_{FCR1}-*GFP* vector constructed in our previous study (27). The rest of the process was performed using the same strategy. All CPR mutants were confirmed by Southern blot analyses using a flanking region probe (Figure 2). Transformation experiments for *F. graminearum* were performed as described in the previous study (28).

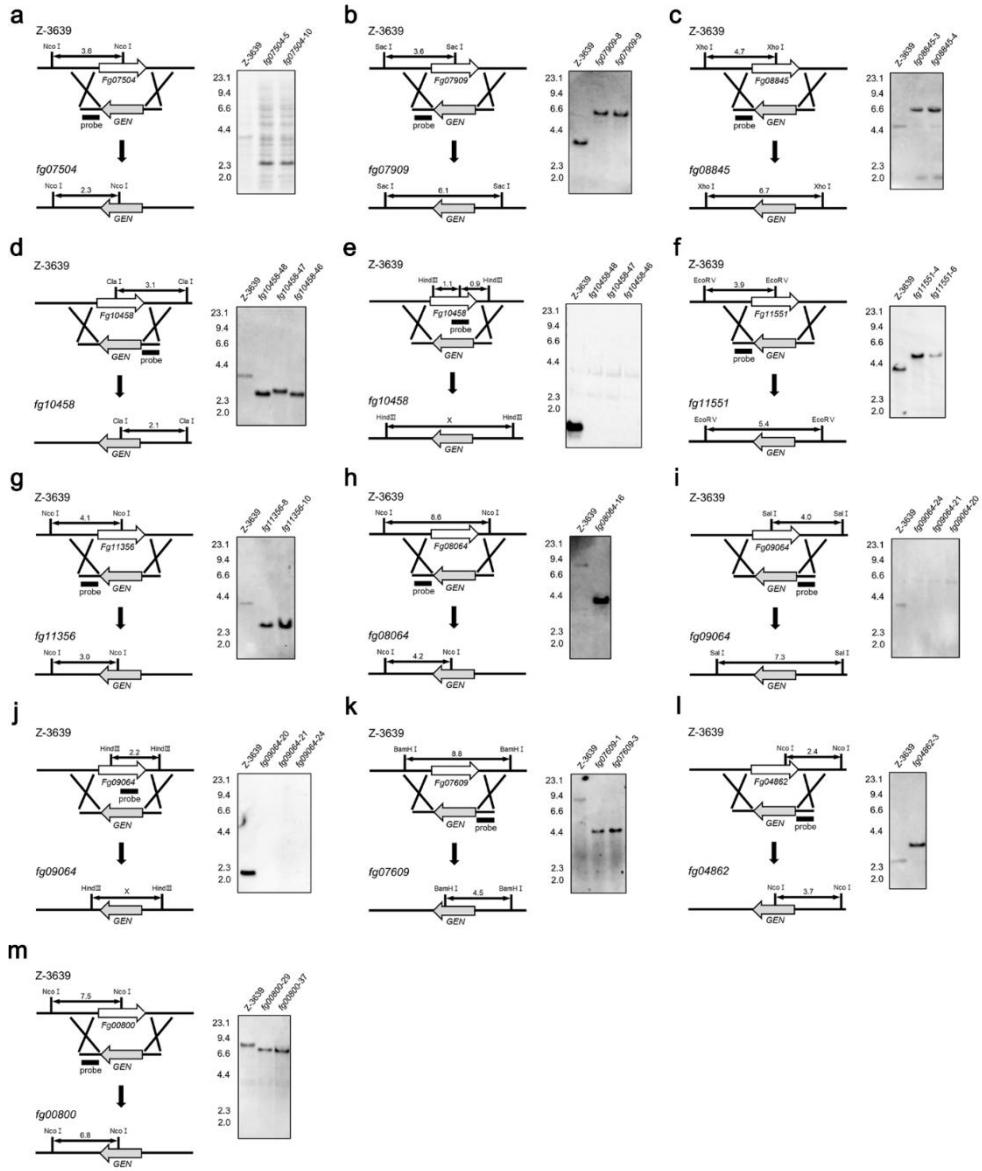


Figure 1. Southern blot analyses of deletion mutants. (a−m) Schematic illustration of the gene deletion strategy (left panel) and Southern blot analyses confirming genetic manipulations (right panel). Sizes of the DNA standards (kb) are indicated to the left of the blots.

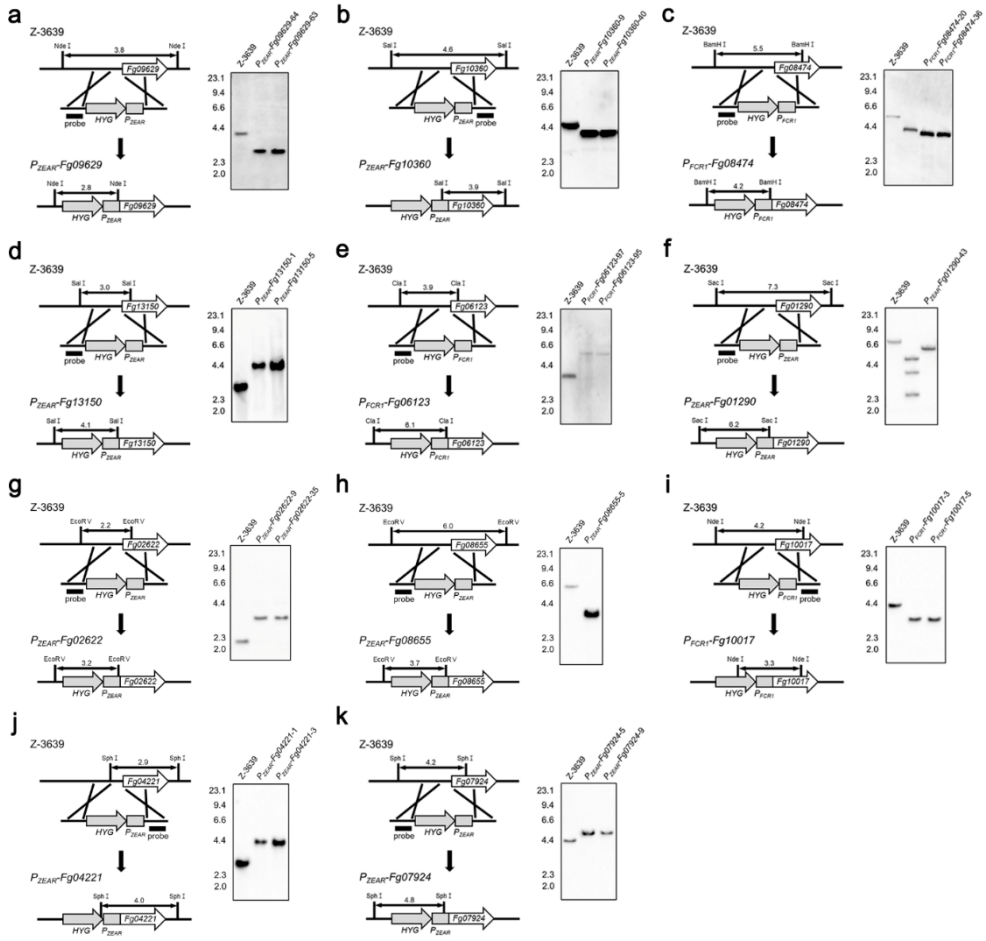


Figure 2. Southern blot analyses of conditional promoter replacement (CPR) mutants. (a−k) Schematic overview of the promoter replacement strategy (left panel) and Southern blot analyses confirming genetic manipulations (right panel). Sizes of the DNA standards (kb) are indicated to the left of the blots.

IV. Single guide RNA (sgRNA) design and Cas9 complex ribonucleoprotein (RNP)-mediated gene deletion

The clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9)-mediated gene deletion was performed as described in the previous study (29). In brief, single guide RNAs (sgRNAs) with an $(N)_{20}$NGG sequence were designed within the ORF region of the target genes based on a web-based tool (CHOPCHOP) (30). Synthesized sgRNAs were purchased from Macrogen (Seoul, Republic of Korea). The sgRNAs used in this study are listed in Table 2. To generate the Cas9 RNP complex, 10 μL of reaction mixture containing 0.1 ng TrueCut™ Cas9 Protein v2 (Thermo Fisher Scientific), 160 ng sgRNA, and 1× Cas9 nuclease reaction buffer was incubated at 37℃ for 30 min. The resulting Cas9 RNP complex was mixed with the final fusion PCR products and transformed into the 150 μL fungal wild-type protoplasts (5×10^5).

Table 2. SgRNAs used in this study.

Target gene	Sequence	GC content (%)	Off-targets
PKS12	CGTAAGACATGATCTCAGAGCGG	45	0
Fg04274	GATTTCTTTAGGCTGGCTGGCGG	50	0
Fg00374	CGACGAGCCCAATATGAGTGCGG	55	0
Fg05512	ATTTTGACGAACTGGCTCAGGGG	50	0

V. BLASTMatrix analyses

All sequence information of the essential gene candidates used for used in the BLASTMatrix analyses was retrieved from the online database Comparative Fungal Genomics Platform (CFGP, http://cfgp.riceblast.snu.ac.kr) (31). The BLASTMatrix tool in CFGP was used to identify homologous proteins across taxa using "[2008] All genome datasets" and "Whole genome sequences in CFGP" as reference datasets.

VI. BLAST similarity search of essential genes in *F. graminearum* for plot generation

The amino acid sequences of 13 *F. graminearum* essential genes were compared with the *S. cerevisiae* genome on *Saccharomyces* Genome Database (https://www.yeastgenome.org), the *Botrytis cinerea* genome on FungiDB (https://fungidb.org/fungidb/app), the human genome on GenBank (https://www.ncbi.nlm.nih.gov/gene/), and the Chinese spring wheat genome, IWGSC RefSeq v2.1, on GrainGenes (https://wheat.pw.usda.gov/GG3/). The e-values of the best hits were converted into the corresponding negative logarithm. The value of 200 was set as the maximum, and 0 was set as the minimum value including the genes with no BLAST match.

VII. Design and *in vitro* synthesis of dsRNA

The small interfering RNA (siRNA)-Finder (si-Fi) software (default

parameters) (32) was used to predict sequences with likely efficient RNAi of essential genes identified in *F. graminearum*. The sequence for dsRNA synthesis was selected at about 250 bp, and NCBI BLASTN was performed to confirm that none of the sequences were preserved in plants or animals. The dsRNAs were synthesized using a MEGAscript™ RNAi Kit (Thermo Fisher Scientific) following the manufacturer's protocol. Primers with the T7 promoter sequence at the 5′ end (Table 1) were synthesized at an oligonucleotide synthesis facility (Bioneer) and used for dsRNA amplification. A pIGPAPA template was used to synthesize *GFP*-dsRNA (33).

VIII. External dsRNA application to barley leaves

Second leaves of three-week-old barley were detached, transferred to square Petri dishes (120×120×17 mm), and wounded at a length of about 7 mm. Each plate contained three detached leaves, and 600 ng of dsRNA in 500 μL of nuclease-free water was sprayed per plate. After drying the sprayed dsRNA suspension for 1 h, 15 μL of *F. graminearum* conidial suspension containing 2×10^4 conidia/mL in sterile water was drop-inoculated onto the leaves. The plates were incubated at 25℃ for five days.

RESULTS AND DISCUSSION

I. Identification of essential gene candidates in *F. graminearum*

Previous studies in yeast and human fungal pathogens such as *Candida albicans*, *Cryptococcus neoformans,* and *Aspergillus fumigatus* have identified essential genes either through a forward-genetic approach by generating random insertional mutants or through a reverse-genetic approach based on identification of genes sharing homology to known essential genes in *Saccharomyces cerevisiae* (10-13). The forward-genetic approach requires screening a large number of mutants generated across the entire genome, which can be a labor-intensive process. On the other hand, the main limitation of the reverse-genetic approach is that it cannot facilitate the identification of novel genes. In this study, we employed a reverse-genetic approach to identify essential genes based on genes that had failed to be deleted in previous studies of *F. graminearum* and aimed to discover both known and novel essential genes. In *F. graminearum*, several large-scale genetic research studies have been conducted by constructing gene-knockout mutant libraries on transcription factors (TFs) (34), kinases (35), phosphatases (36), cytochrome P450 (CYPs) (37), peroxidases (38), G-protein coupled receptors (GPCRs) (39, 40), and orphan proteins (41). To identify essential genes in *F. graminearum*, we manually searched for the genes that had not been deleted in previous studies and selected them as essential gene candidates (34-37) (Figure 3).

The potential for off-target RNAi effects in non-target organisms is a major

issue when applying RNAi as a crop protection strategy. Since off-target silencing commonly occurs in genes with homologous target sequences, the ideal target of RNAi lacks orthologs in both mammals and the host plants. To narrow down the essential gene candidates, we analyzed homologous proteins of essential gene candidates in various eukaryotic species in the chromista, fungi, metazoa, and viridiplantae kingdoms using BLASTMatrix software in the Comparative Fungal Genomics Platform (CFGP, http://cfgp.riceblast.snu.ac.kr) (Figure 3) (31). The similarity was visualized in color based on the e-value, which represents the degree of conservation of each gene. Most candidate genes in the phosphatase and kinase groups were highly conserved across taxa in eukaryotic species. The genes in the transcription factor (TF) and cytochrome P450 (CYP) groups had much lower degree of conservation in animals and plants than kinases and phosphatases. Since many genes in the CYP group have unclear functions and share similarities to *S. cerevisiae ERG11*, the known target of DMI fungicides, we selected the TF group for further essential gene confirmation.

We first attempted to generate deletion mutants for essential gene candidates in the TF group using homologous recombination and the split-marker method. The resulting geneticin-resistant transformants were screened using diagnostic PCR, and the mutants were confirmed by Southern blot analysis (Figure 1). We successfully generated knockout mutants for 11 genes but failed to obtain deletion mutants for another 13 genes after screening more than 96 transformants from at least two independent trials.

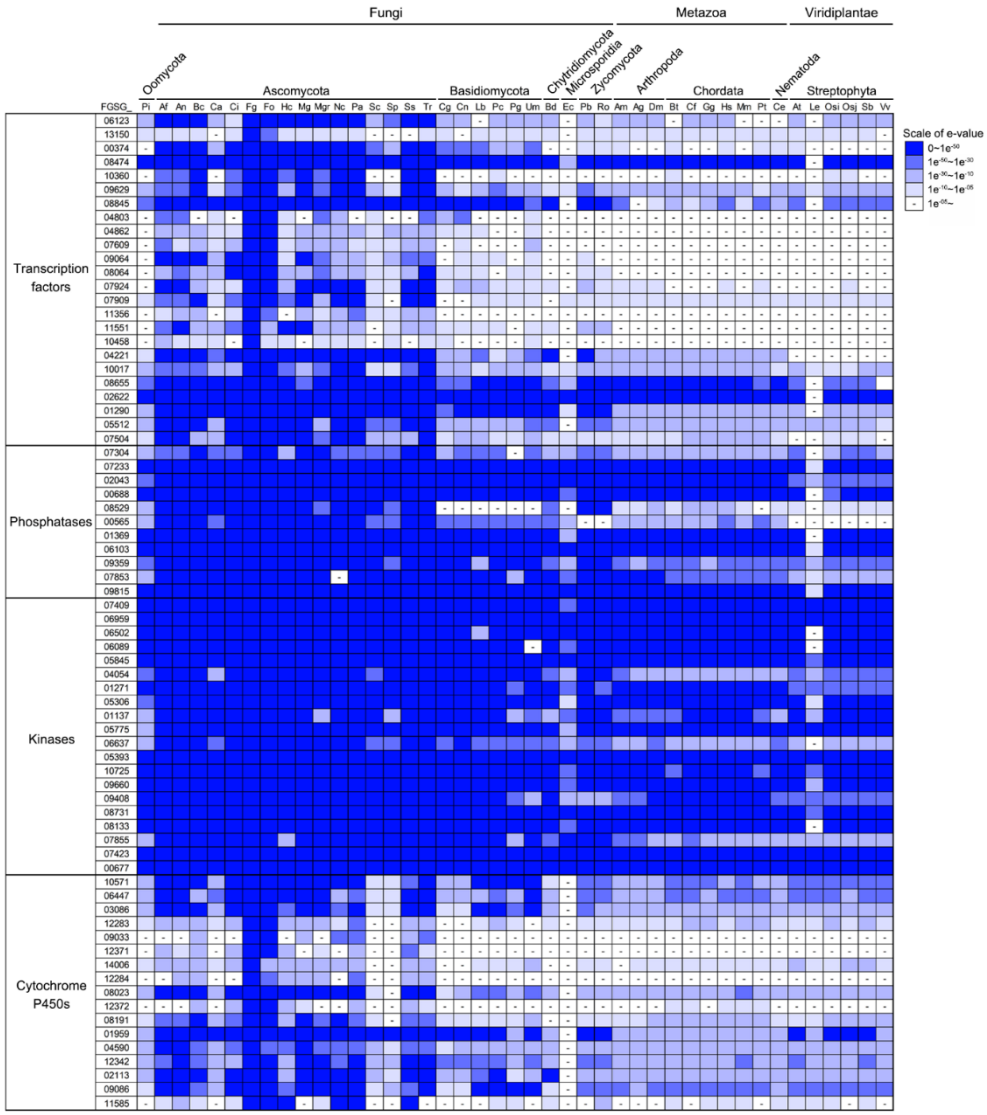

Fungi
Metazoa
Viridiplantae
Oomycota
Ascomycota
Basidiomycota
Chytridiomycota
Microsporidia
Zygomycota
Arthropoda
Chordata
Nematoda
Streptophyta
FGSG_
Pi Af An Bc Ca Ci Fg Fo Hc Mg Mgr Nc Pa Sc Sp Ss Tr Cg Cn Lb Pc Pg Um Bd Ec Pb Ro Am Ag Dm Bt Cf Gg Hs Mm Pt Ce At Le Osi Osj Sb Vv
Transcription factors
Phosphatases
Kinases
Cytochrome P450s
Scale of e-value
0~1e-50
1e-50~1e-30
1e-30~1e-10
1e-10~1e-05
1e-05~

Figure 3. BLASTMatrix of essential gene candidates. Amino acid sequences of essential gene candidates were matched with genome databases for multiple species across taxa using BLASTMatrix software in the Comparative Fungal Genomics Platform (CFGP, http://cfgp.riceblast.snu.ac.kr). Matches with the genomes of 43 representative species were presented. Pi, *Phytophthora infestans*; Af, *Aspergillus fumigatus*; An, *Aspergillus nidulans*; Bc, *Botrytis cinerea*; Ca, *Candida albicans*; Ci, *Coccidioides immitis*; Fg, *Fusarium graminearum*; Fo, *Fusarium oxysporum*; Hc, *Histoplasma capsulatum*; Mg, *Magnaporthe grisea*; Mgr, *Mycosphaerella graminicola*; Nc, *Neurospora crassa*; Pa, *Podospora anserina*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Ss, *Sclerotinia sclerotiorum*; Tr, *Trichoderma reesei*; Cg, *Cryptococcus gattii*; Cn, *Cryptococcus neoformans*; Lb, *Laccaria bicolor*; Pc, *Phanerochaete chrysosporium*; Pg, *Puccinia graminis*; Um, *Ustilago maydis*; Bd, *Batrachochytrium dendrobatidis*; Ec, *Encephalitozoon cuniculi*; Pb, *Phycomyces blakesleeanus*; Ro, *Rhizopus oryzae*; Am, *Apis mellifera*; Ag, *Anopheles gambiae*; Dm, Drosophila melanogaster; Bt, *Bos taurus*; Cf, *Canis familiaris*; Gg, *Gallus gallus*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Pt, *Pan troglodytes*; Ce, *Caenorhabditis elegans*; At, *Arabidopsis thaliana*; Le, *Lycopersicon esculentum*; Osi, *Oryza sativa* (indica); Osj, *Oryza sativa* (japonica); Sb, *Sorghum bicolor*; Vv, *Vitis vinifera*.

II. Validation of essential genes using a conditional promoter and CRISPR/Cas9-mediated deletion strategy

The conditional promoter replacement (CPR) strategy is a powerful tool for identification of essential genes (10-12). To confirm the essentiality of 13 identified candidate genes, we generated CPR mutants using two conditional promoters developed in *F. graminearum*, the zearalenone-inducible *ZEAR* promoter (P_{ZEAR}) and copper-regulatable *FCR1* promoter (P_{FCR1}) (27, 42). The native promoter of each gene was replaced with P_{ZEAR} or P_{FCR1} by homologous recombination and using the split-marker method as described in Figure 4a, and the phenotype was analyzed under repressing or inducing conditions. The conditional mutants were first generated using P_{ZEAR} and then secondarily produced using P_{FCR1} to address the genes for which we failed to generate growth-defective P_{ZEAR}-CPR mutants under repressing conditions. A previous study of *C. albicans* reported that a tetracycline (Tet)-regulatable promoter was insufficient to repress the essential gene *ERG26* (43), which may also explain our inability to generate growth-defective CPR mutants for several genes.

The P_{ZEAR} promoter is activated by zearalenone or the estrogenic compound β-estradiol. We obtained P_{ZEAR}-CPR mutants for eight genes, *Fg09629, Fg10360, Fg13150, Fg01290, Fg02622, Fg08655, Fg04221*, and *Fg07924*, that displayed severe growth defects when cultured on complete medium (CM) in the absence of β-estradiol. The vegetative growth of the mutants was restored when 30 μM β-estradiol was added (Figure 4b). Among the five remaining genes, P_{FCR1}-CPR mutants were constructed for *Fg08474, Fg06123,* and *Fg10017*; repeated trials to

generate growth-defective suppression mutants for *Fg00374* and *Fg05512* were unsuccessful. When copper was added, the P_{FCR1}-CPR mutants of *Fg08474, Fg06123,* and *Fg10017* exhibited growth inhibition, while the addition of the copper chelator bathocuproinedisulfonic acid (BCS) restored mycelial growth (Figure 4c). All CPR mutants were confirmed by vegetative growth under repressing or inducing conditions and Southern blot analyses (Figure 2).

Since we could not obtain growth-defective CPR mutants for *Fg00374* and *Fg05512,* we applied a CRISPR-Cas9-mediated deletion strategy to validate the essentiality of *Fg00374* and *Fg05512*. In *F. graminearum,* a previous study reported that genetic manipulation using a preassembled Cas9 RNP greatly enhances the deletion efficiency of *PKS12* and *Fg04274* (29). We attempted to disrupt *Fg00374* and *Fg05512* using Cas9 RNP and screened at least 40 transformants from two independent trials, using *PKS12* and *Fg04274* as controls. The use of Cas9 RNP significantly increased the number of transformants and the deletion efficiency of *PKS12* from 44 to 182 and from 56.8 % to 86.3 %, respectively, and those of *Fg04274* from 76 to 189 and from 73.6 % to 93.6 % (Figure 5). Similarly, the number of transformants for disrupting *Fg00374* and *Fg05512* increased from 40 to 92 and 72 to 167, respectively, when using Cas9 RNP, but diagnostic PCR revealed that all transformants maintained the wild-type allele. These results suggest that *Fg00374* and *Fg05512* are essential genes whose absence leads to lethal phenotype in *F. graminearum*.

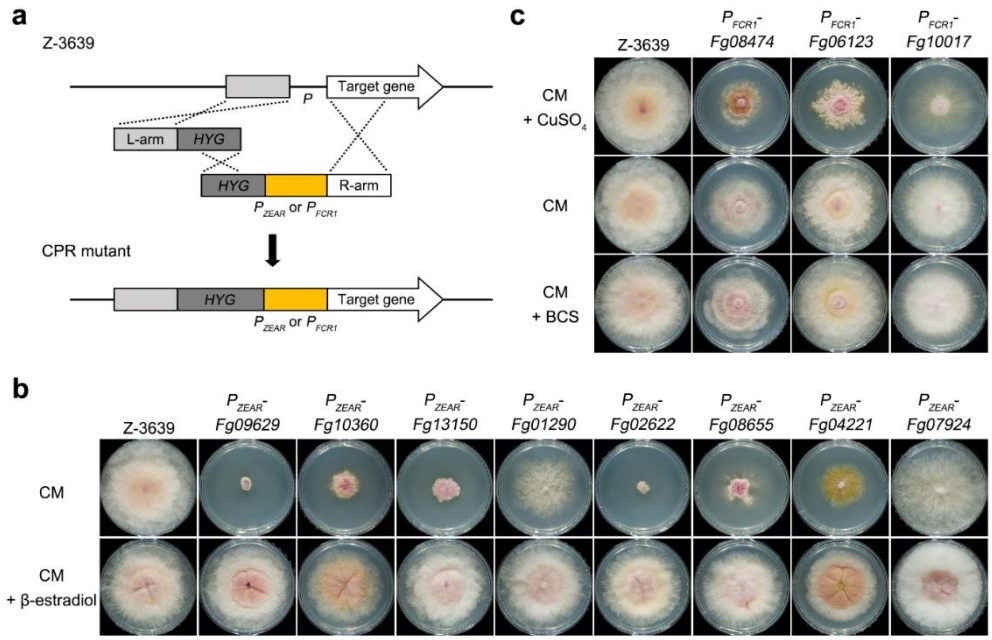
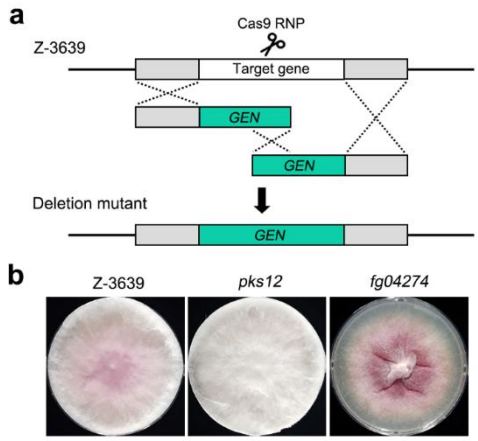


Figure 4. Conditional mutants for essential genes in *F. graminearum*. (a) Schematic representation for the conditional promoter replacement (CPR) strategy. The native promoter (*P*) of the target gene was replaced with P_{ZEAR} or P_{FCR1} using promoter replacement cassettes containing homologous flanking sequences (L-arm and R-arm). (b, c) Mycelial growth of CPR mutants under repressing and inducing conditions. P_{ZEAR}–CPR mutants (b) were inoculated on complete medium (CM; repressing conditions) and CM supplemented with 30 μM β-estradiol (inducing conditions). P_{FCR1}–CPR mutants (c) were inoculated on CM or CM supplemented with 50 μM $CuSO_4$ (repressing conditions) or 25 μM BCS (inducing conditions). The photographs were taken four days after inoculation.



c

Target gene	Cas9 RNP	Total no. of transformants	Deletion efficiency (%)
PKS12	-	44	56.8
PKS12	+	182	86.3
Fg04274	-	76	73.6
Fg04274	+	189	93.6
Fg00374	-	40	0
Fg00374	+	92	0
Fg05512	-	72	0
Fg05512	+	167	0

Figure 5. Generation of *F. graminearum* deletion mutants using Cas9 RNP. (a) Schematic representation of the CRISPR/Cas9-mediated deletion strategy. (b) Colony morphology of Z-3639 (*F. graminearum* wild-type strain), *pks12* and *fg04274*. The strains were incubated for four days on complete medium (CM). (c) Cas9 RNP-mediated deletion efficiency of *PKS12*, *Fg04274*, *Fg00374* and *Fg05512* genes. The number of transformants was counted from two independent trials.

III. Characterization of essential genes in *F. graminearum*

Here, we identified 13 essential genes required for viability in *F. graminearum* (Table 3). The nearest homolog of each gene in *S. cerevisiae* was identified using BLASTp analysis in *Saccharomyces* Genome Database (https://www.yeastgenome.org). Among 13 genes, eight were previously demonstrated to be essential in *S. cerevisiae* or *Schizosaccharomyces pombe* and comprised essential biological processes of RNA processing (*SNU23*), cell cycle control (*CDC39*), transcription (*TFC3*), glycosylation (*SEC59*), histone acetylation *(EAF1*), mitochondrial ribosomal function (*RML2*), DNA replication (*MCM2*), and translation (*TIF11*). We searched for the closest homologs of these yeast genes in *F. graminearum*, and the bidirectional best hits (BBH) revealed that all these gene pairs are more similar to each other than to any other genes in the respective genome.

The five remaining essential genes had homologs that are not essential in *S. cerevisiae* or *S. pombe.* The *Fg00374* gene is homologous to yeast *SRC1*, an inner nuclear membrane protein involved in chromatin segregation during mitosis, which has a paralog *HEH2* that arose from whole-genome duplication. While a single deletion of each gene is viable in *S. cerevisiae*, the *src1 heh2* double mutant is lethal or displays a growth defect. In *Aspergillus nidulans*, the heterokaryon rescue method has shown *src1* to be an essential gene (44).

The *Fg04221* gene is homologous to *S. cerevisiae PEP7*, which is involved in vesicle-mediated vacuolar protein sorting. A previous study (45) showed that *S. cerevisiae PEP7* is nonessential in the S288c reference strain but essential in the Σ

1278b strain in which the genome is nearly identical to that of the S288c strain. While *vac1*, also denoted as *pep7*, was successfully deleted in *A. nidulans* showing severe growth defects, all attempts to delete NRRL3_05482 (Yeast *PEP7* ortholog) failed in *A. niger* (46, 47). These findings suggest that the gene essentiality of *PEP7* varies among fungi (from yeast to filamentous) even among evolutionally close strains.

Each of *Fg10360*, *Fg10017*, and *Fg07924* are most similar to yeast *NRG2*, *HCM1*, and *ZNF1*, respectively, but the BLAST e-values and sequence identity percentages show that these gene pairs have a low degree of similarity. Since the yeast *NRG2*, *HCM1*, and *ZNF1* genes each have much closer homologs in *F. graminearum*, these three essential genes might play as yet unknown important functions in *F. graminearum*. The *Fg10360* gene is homologous to *BcCND13*, which is upregulated following the addition of the drug cyclosporin A (CsA) (48), suggesting its involvement in the stress responses or calcineurin-dependent pathways. The *Fg10017* gene shares weak homology with four forkhead transcription factors in the yeast, *HCM1*, *FKH2*, *FHL1*, and *FKH1*, but possesses the conserved Fhl1 domain, the transcriptional regulator of ribosomal protein (RP) genes. In *S. cerevisiae*, the loss of *FHL1* leads to extreme growth defects and its interaction partner *IFH1* is an essential gene. A previous study of *Magnaporthe oryzae* has reported the failure to obtain the deletion mutant of *MoFOX2*, the homolog of *Fg10017* (49). The *Fg07924* gene shares weak homology with several respiration- and carbon metabolism-related genes including *ZNF1* in *S. cerevisiae*, indicating its biological functions in carbon source metabolism.

Table 3. *S. cerevisiae* homologs of *F. graminearum* essential genes.

Locus	*S. cerevisiae* homolog[a]	Description in *S. cerevisiae*	Probability (e-value)	Similarity (%)	BBH[b]	Essential in *S.cerevisiae*/ *S. pombe*[c]	Essential in other species[d]
FGSG_09629	Snu23	Component of the U4/U6.U5 snRNP complex	8e-04	27	Yes	Yes/No	
FGSG_10360	Nrg2	Negative regulator of glucose-controlled genes	3e-03	16	No	No/No	
FGSG_08474	Cdc39	Subunit of the CCR4-NOT1 core complex	2e-168	24	Yes	Yes/Yes	
FGSG_00374	Src1	Chromosome linkage inner nuclear membrane protein	1e-25	21	Yes	No/No	*A. nidulans* (44)
FGSG_13150	Tfc3	Subunit of RNA polymerase III transcription initiation factor complex	5e-05	15	Yes	Yes/Yes	
FGSG_06123	Sec59	Dolichol kinase	5e-13	18	Yes	Yes/Yes	
FGSG_05512	Eaf1	Component of the NuA4 histone acetyltransferase complex	3e-12	17	Yes	No/Yes	
FGSG_01290	Rml2	Mitochondrial ribosomal protein of the large subunit (L2)	5e-110	46	Yes	No/Yes	
FGSG_02622	Mcm2	Protein involved in DNA replication	0.0	56	Yes	Yes/Yes	
FGSG_08655	Tif11	Translation initiation factor eIF1A	4e-53	63	Yes	Yes/Yes	
FGSG_10017	Hcm1	Forkhead transcription factor	5e-07	11	No	No/No	*M. oryzae* (49)
FGSG_04221	Pep7	Adaptor protein involved in vesicle-mediated vacuolar protein sorting	1e-45	25	Yes	No/No	*A. niger* (47)
FGSG_07924	Znf1	Zinc cluster transcription factor that regulates respiratory growth	5e-07	15	No	No/No	

[a] The standard name of the nearest homolog in *S. cerevisiae* is given.

[b] BBH, Bidirectional best hit.

[c] The essentiality of the nearest homolog in the *S. cerevisiae* and *S. pombe* genomes is given based on the *Saccharomyces* Genome Database (https://www.yeastgenome.org) and PomBase (https://www.pombase.org).

[d] For genes whose *S. cerevisiae* or *S. pombe* homologs are not essential, the previous studies suggesting the essentiality of the nearest homolog in the indicated fungal species are given.

IV. Prioritizing antifungal targets using BLAST analysis

The essential genes that are not conserved in host plants or mammal species but are found in other plant pathogenic fungi are considered the best antifungal targets that can be used to control a wide range of pathogenic fungi with fewer side effects. To prioritize antifungal targets of the essential genes identified in this study, a BLAST similarity search was performed using the protein sets for *S. cerevisiae,* ascomycete plant pathogenic fungus *Botrytis cinerea*, wheat, and humans. The e-value of the best hits was converted to the corresponding negative log value and plots displaying the gene similarity were generated as previously conducted in *C. albicans* and *C. neoformans* (10, 13) (Figure 6). The lower is the value, the lower is the similarity of the compared species. Figure 6a shows the BLAST results of 13 essential proteins in *F. graminearum* against the *S. cerevisiae* and the wheat proteins, and Figure 6b shows the BLAST results against the *B. cinerea* and the human proteins. All genes were highly conserved in *B. cinerea* compared to other species, with BLAST e-values less than 1e−50. The essential genes located at the bottom of the plots, which indicate low conservation in wheat (Figure 6a) or humans (Figure 6b) (BLAST e-values > 1e−10), are marked in black on the plots. Seven out of the 13 essential genes were absent in the wheat genome, and five genes were absent in the human genome. Therefore, *Fg10360, Fg00374, Fg13150, Fg10017,* and *Fg07924* in *F. graminearum* were potentially suitable targets for antifungal agents. However, some known antifungal targets, such as *ERG11*, are highly conserved in both humans and wheat, but can still be suitable targets if the agent and/or formulation are designed to be selectively toxic to pathogenic fungi.

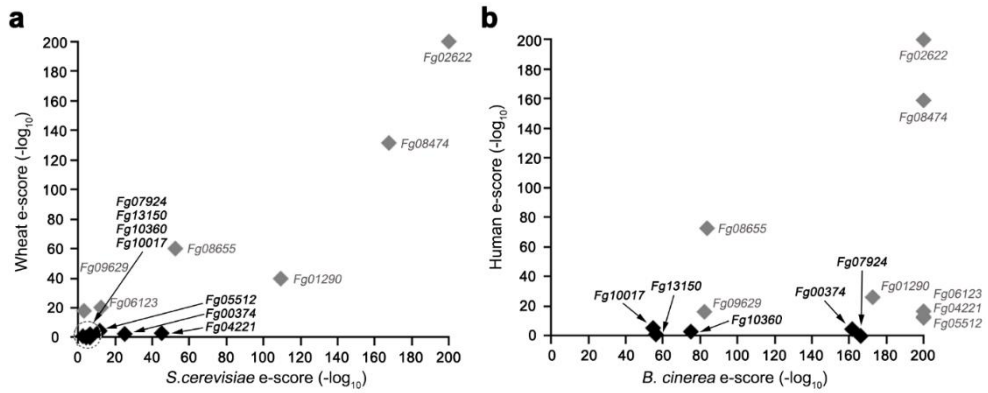


Figure 6. BLAST similarity search comparing *F. graminearum* essential genes with *S. cerevisiae*, wheat (a), *B. cinerea,* and human genomes (b). The amino acid sequences of essential genes in *F. graminearum* were compared with the protein sets of the indicated species. The degree of similarity was expressed as the e-value of the best hits converted into the corresponding negative logarithm. The maximum value was set as 200 and the minimum value was set as 0, which included genes with no BLAST matches within the species assessed. The genes displaying a low conservation in wheat or humans (BLAST e-value > 1e−10) are marked in black on the plots.

V. SIGS-mediated control of *F. graminearum* infections on detached barley leaves

To demonstrate that the identified essential genes could be potential targets of antifungal agents, we conducted RNAi experiments. About 250 bp of dsRNAs was designed using the siRNA-Finder (si-Fi) software (32) to identify efficient siRNA sequences for RNAi. Although prioritization of essential genes as antifungal targets was conducted as shown in Figure 6, we performed RNAi experiments for all 13 genes to identify those that can be efficiently inhibited. The prepared dsRNA solutions were sprayed onto the surface of detached barley leaves 1 h before *F. graminearum* inoculation. A *GFP*-dsRNA was used as a negative control, and a mixture of *CYP51A*-dsRNA, *CYP51B*-dsRNA, and *CYP51C*-dsRNA, which were previously reported to be effective (19), was used as a positive control and was named *CYP51*-dsRNA. Five days after inoculation, *Fg10360*-dsRNA-, *Fg13150*-dsRNA-, and *Fg06123*-dsRNA-sprayed barley leaves exhibited substantially smaller lesions compared to the water- or *GFP*-dsRNA-treated leaves (Figure 7). Of these, *Fg10360* and *Fg13150* appeared to be more attractive targets for antifungal agents given they were poorly conserved in wheat and human genomes (Figure 6). We observed weak inhibitory effects when targeting other essential genes with dsRNAs. Previous studies have shown that the inhibitory effect of dsRNAs can vary depending on the specific sequences used (50). Therefore, using dsRNAs with different sequences or designing dsRNAs that can target multiple essential genes could potentially result in more effective outcomes.

In conclusion, in this study, we identified 13 essential genes in *F. graminearum*, including genes previously characterized in yeast and other fungal species, and also uncharacterized genes using a CPR strategy and CRISPR-Cas9-mediated deletion method. Our RNAi experiments showed that silencing of *Fg10360*, *Fg13150*, and *Fg06123* by external dsRNA application led to reductions in fungal disease progression, suggesting that they could be new targets for RNAi-based disease control. This research enhances our understanding of essential biological processes in filamentous ascomycete fungi, and we anticipate that this research will accelerate the development of dsRNA-based antifungal agents targeting essential genes in pathogenic fungi.

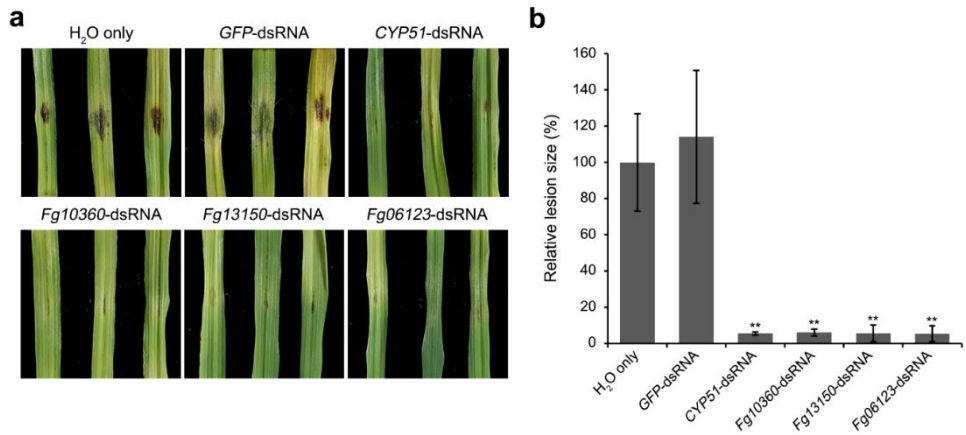


Figure 7. RNAi-based control of *F. graminearum* infections by applying *Fg10360*-dsRNA, *Fg13150*-dsRNA, and *Fg06123*-dsRNA to barley leaves. (a) Lesions developed on detached barley leaves 5 d after inoculation. Detached barley leaves were sprayed with 600 ng of dsRNA 1 h before *F. graminearum* inoculation. Sterile water (mock), *GFP*-dsRNA (negative control), and *CYP51*-dsRNA (positive control) were used as controls. (b) The size of the lesions was measured using imageJ software and expressed relative to the control treatment with sterile water (mock). Error bars represent mean values ± SD from three replicates. Asterisks indicate statistical significance (**P< 0.01, students t-test).

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유전자 억제 기반 붉은곰팡이병 방제를 위한 필수유전자 발굴

김 시 은

초 록

붉은곰팡이(*Fusarium graminearum*)는 전 세계적으로 밀과 보리 등 주요 곡물 작물에서 붉은곰팡이병을 유발하는 중요한 식물병원균이다. 붉은곰팡이병 방제를 위해 사용 가능한 내성 작물 품종이 부족하고, 화학적 항진균제에 대한 감수성이 적은 붉은곰팡이 균주가 등장함에 따라, 보다 지속 가능한 병 방제 전략을 확립하고 새로운 병 제어 타겟을 찾는 것이 필요하다. Spray-induced gene silencing (SIGS)는 병원균의 병원성 유전자 또는 필수 유전자를 저해하는 이중가닥 RNA (double-stranded RNA, dsRNA)을 식물체 외부에서 처리하여 식물병을 방제하는 병 방제 전략이다. 본 연구에서는 붉은곰팡이에서 지질분해효소 유전자와 필수 유전자를 동정한 후 그들의 기능을 조사하고, SIGS 접근법을 통해 해당 유전자의 항진균 약제 대상으로서의 잠재력을 평가하였다.

지질분해효소는 tri-, di-, and monoglycerides를 유리지방산과 글리세롤로 분해하는 효소이다. 식물체를 감염하는 과정에서 식물병원균은 왁스와 지질 고분자로 이루어진 식물 표피층을 분해하기 위해 외분비

효소의 일종으로 지질분해효소를 분비한다. 본 연구에서는 총 86개의 지질분해효소 유전자 후보군을 동정하였고, 이들 각각을 붉은곰팡이 유전체에서 삭제하였다. 86개의 지질분해효소 유전자 후보군에 대해 포괄적인 기능적 분석을 수행한 결과, 대부분의 지질분해효소 삭제변이체는 병원성을 포함한 대부분의 표현형에서 야생형 균주와 동일한 형질을 나타내었는데, 이는 붉은곰팡이 내에서 지질분해효소가 상당히 중복된 기능을 수행함을 의미한다. 이들 지질분해효소 중 FgLip1과 Fgl1은 세포외의 지질 분해에 필수적인 외분비 지질분해효소로 확인되었다. 또한 본 연구에서는 *FgLIP1*과 *FGL1*의 전사 조절에 중요한 역할을 하는 세 개의 전사 조절 인자(transcription factor, TF)와 1개의 히스톤 아세틸전이효소(histone acetyltransferase)를 밝혀내었다. 특히 Gzzc258은 붉은곰팡이에서 가장 핵심이 되는 지질분해효소 조절인자로서, 붉은곰팡이의 유성생식 발달 단계에서 지질분해효소 유도에 주요한 역할을 하는 것으로 확인되었다.

생물의 생존에 필수적인 필수 유전자는 항진균제의 제어 대상으로 사용되었을 때 병원균의 성장을 완전하게 억제할 수 있기 때문에, 상용화된 항진균제의 주된 타깃으로 여겨진다. 본 연구에서는 붉은곰팡이의 필수 유전자를 동정하기 위해, 기존 연구에서 삭제변이체 제작에 실패했다고 보고된 필수 유전자 후보군을 선별하였다. 여러 독립적인 형질전환 실험을 수행한 결과, 이들 필수 유전자 후보군 중 13개 유전자에 대해서 삭제변이체를 얻는 데 실패하였다. 해당 유전자의 필수성을 검증

하기 위해, 조건부 발현 프로모터를 이용하여 조건부 발현 균주를 제작하였고, 유전자의 발현이 저해되는 조건에서의 균주 생장을 관찰하였다. Zearalenone 또는 에스트로겐계 물질인 β-estradiol에 의해 유전자 발현이 유도되는 P_{ZEAR} 시스템이 이미 확립되어 있었지만, 일부 필수 유전자 후보군에는 적용이 불가능했기 때문에, 해당 과정을 위해 붉은곰팡이의 구리 반응성 프로모터(*F. graminearum* copper-responsive 1(*FCR1*) promoter (P_{FCR1}))를 사용한 조건부 유전자 발현 시스템을 새롭게 개발하였다. P_{FCR1}은 녹색 형광 단백질 유전자와 *FgENA5* 유전자의 발현을 구리 의존적으로 효과적으로 조절하였다. 또한 P_{FCR1}을 이용해 붉은곰팡이의 필수 유전자인 *FgIRE1*의 조건부 발현 균주를 제작 하여, 해당 필수 유전자의 기능을 분석할 수 있었다.

P_{ZEAR} 또는 P_{FCR1}을 사용한 조건부 발현 프로모터 치환 전략을 통해 총 11개 필수 유전자가 확정되었다. 또한 CRISPR/Cas9 방법을 통해서 삭제변이체를 제작하는 데 실패한 2개 유전자를 추가적으로 필수 유전자로 확정하였다. 확정한 필수 유전자를 제어하는 dsRNA를 식물체 외부에 처리하였을 때 실제로 병원균에 대한 보호 효과가 나타나는지 검증하기 위해, dsRNA를 보리 잎에 처리한 후 붉은곰팡이를 접종하여 병징을 관찰하였다. *Fg10360*-dsRNA, *Fg13150*-dsRNA, 그리고 *Fg06123*-dsRNA를 처리한 보리 잎에서는 물 혹은 *GFP*-dsRNA를 처리한 보리 잎에 비해 병반이 상당히 감소하였는데, 이는 이들 필수 유전자가 RNA 간섭(RNA interference, RNAi)을 기반으로 하는 병 방제 기

법의 유망한 타깃이 될 수 있음을 시사한다. 따라서 본 연구는 식물병원균에서 RNA 간섭을 기반으로 하는 항진균제 타깃에 대한 자원을 제공하며, 또한 곰팡이의 지질분해효소에 대한 종합적인 데이터베이스와 유전자 도구로서 사용될 수 있는 조건부 발현 프로모터 시스템을 제시한다.

주요어 : 붉은곰팡이, 필수유전자, 지질분해효소, 조건부 발현 프로모터, RNA 간섭

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