FUNCTIONAL CHARACTERIZATION OF CANDIDATE CO-FACTOR GENES INVOLVED IN A-TO-I MRNA EDITING IN *FUSARIUM GRAMINEARUM*

by

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Dedicated to Dzung Tien Vu, Tung Thanh Ngo, Brett Clark, Karie Clark, and Chancelor Clark.

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ABSTRACT

Adenosine-to-Inosine (A-to-I) mRNA editing is a post-transcriptional modification of specific sites within the mRNA that has only recently been observed in filamentous fungi. In the wheat scab fungus *Fusarium graminearum*, this phenomenon has shown to be facilitated by FgTad2 and FgTad3, homologs of Adenine Deaminase Acting on tRNA (ADAT). Interestingly, these two proteins are constitutively expressed in all different life stages, in contrast to only the sexual stage-specific nature of A-to-I mRNA editing in *F. graminearum*. To understand the molecular mechanisms regulating this process, six candidate co-factor genes were identified which interact with FgTad2 and/or FgTad3, specifically during sexual reproduction. Deletion mutants of four candidate co-factor genes were successfully generated. All four mutants displayed normal asexual development of *F. graminearum*, but four mutants also altered sexual function. Those four mutant led to formation of morphologically normal perithecia and ascospores, but the perithecia failed to discharge ascospores. More interestingly, in *FGSG_10943* deletion mutant, most of these ascospores germinated precociously within the perithecium. I also observed, that among the candidate co-factor genes which are specifically expressed during sexual reproduction, *FGSG_10943* was significantly upregulated during the later stage of sexual development. This gene is restricted in nature to only a few orders of fungi in the class Sordariomycetes that form dark pigmented ascocarps, particularly Hypocreales and Glomerellales. Taken together, these results indicate that the four candidate co-factor genes are dispensable for vegetative growth of the fungus and involved in ascospore discharge. *FGSG_10943* appears to be involved in autoinhibition of ascospores inside the perithecia and interact with FgTad2 during sexual reproduction to mediate A-to-I mRNA editing in *F. graminearum*.

CHAPTER 1. INTRODUCTION

1.1 The wheat scab fungus Fusarium *graminearum*

1.1.1 The disease: Fusarium Head Blight

One of the most destructive cereal diseases world-wide that are caused by fungi and occur under a wide range of environmental conditions is Fusarium Head Blight (FHB), which is caused by several *Fusarium species*. Over the past few decades, there have been several FHB epidemics resulted in billions of dollars loss in the wheat and barley production industry in North America (Turkington *et al.*, 2014; Canadian Grain Commission, 2018). In Europe, the expansion of wheat farming area to more wet and rainy European regions has aggravated the risk of FHB incidence (Giancaspro *et al.*, 2016). The first FHB disease symptom includes premature bleaching of head tissue on wheat, and appearance of a pink to reddish mold on corn silk 3-6 days after infection (Schmale and Bergstrom, 2003). Later in the season, small black fruiting bodies may occur on the infected wheat spikelet. FHB results in reduction of yield and also results in devaluation of the quality of wheat flour, barley malt, and feeding value of small grains. More importantly, it poses a threat to human and animal health since fungal infection of wheat florets contaminates grains with a mycotoxin called deoxynivalenol (DON) (Jansen et al., 2005). When DON is accumulated in harvested grains, its consumption led to vomiting and feed refusal by livestock, and has been associated with human sickness (Goswami and Kistler, 2004).

1.1.2 The pathogen: Fusarium graminearum

Among all phytopathogens, fungi are the most common agents of diseases on wheat and barley (Zhou, 2011). Fusarium is a genus of filamentous fungi, belonging to the phylum Ascomycetes, that are pervasive in terrestrial ecosystems and infect a wide range of plants including wheat, corn and barley. Several important species are *Fusarium graminearum* (teleomorph *Gibberella zeae*), *Fusarium culmorum* and *Fusarium avenaceum* (teleomorph *Gibberella avenacea*) (Buerstmayr *et al.*, 2009). Individual species of this genus may be the most common causal agent depending on certain grain hosts, cultivation areas, or specific environmental conditions. Among all Fusarium species that cause scab, *Fusarium graminearum* is the most

dominant causal agent in the United States, Canada, several European countries and China (McMullen *et al.*, 1997; Kant *et al.*, 2017). At early stages of infection, *F. graminearum* is a biotrophic fungus which grow intercellularly, expanding through the stem tissues. At later stages, the fungus grows intracellularly and quickly colonizes host tissue leading to necrosis of plant tissues.

F. graminearum has both sexual and asexual stages, with sexual development being a critical point of the FHB disease cycle. The sexual stage is initiated by the formation of binucleate hyphae that lead to the establishment of fruiting bodies and perithecia. In the laboratory, it takes 2 weeks for *F. graminearum* to complete its life cycle (Cavinder *et al.,* 2012) In the field, perithecia are the overwintering structure which arise from those hyphae that grow on infected crop residues (Figure 1).

F. graminearum has genes required for both mating types (Mat1-1 and Mat1-2) present in its haploid genome, making it a homothallic fungus (Trail, 2009). Thus, it does not require a compatible partner to form fruiting bodies. This advantage gives *F. graminearum* the ability to produce homozygous offspring of an individual trait without crossing. Within a fertilized perithecium, many tubulous sacs (asci), each containing eight ascospores are formed following meiosis. Within one week after fertilization, mature perithecia eject ascospores through a small slit in perithecial apex (Figure 2A) (Trail and Common, 2000).

Ascospore discharge is one of the most important functions of perithecia since the fungus relies on its ability to forcibly eject ascospores into the air to initiate host colonization. Ascospores are shot from the ascus by turgor pressure inside the ascus (Hallen and Trail, 2008). Alternatively, conidiation, or sporulation in the asexual stage is a secondary reproductive cycle for massive generation of conidia (Ohara *et al.*, 2004). These asexually produced, sickle-shaped structures born on sporodochia on the surface of infected plants or crop residue may lead to primary or secondary infection.

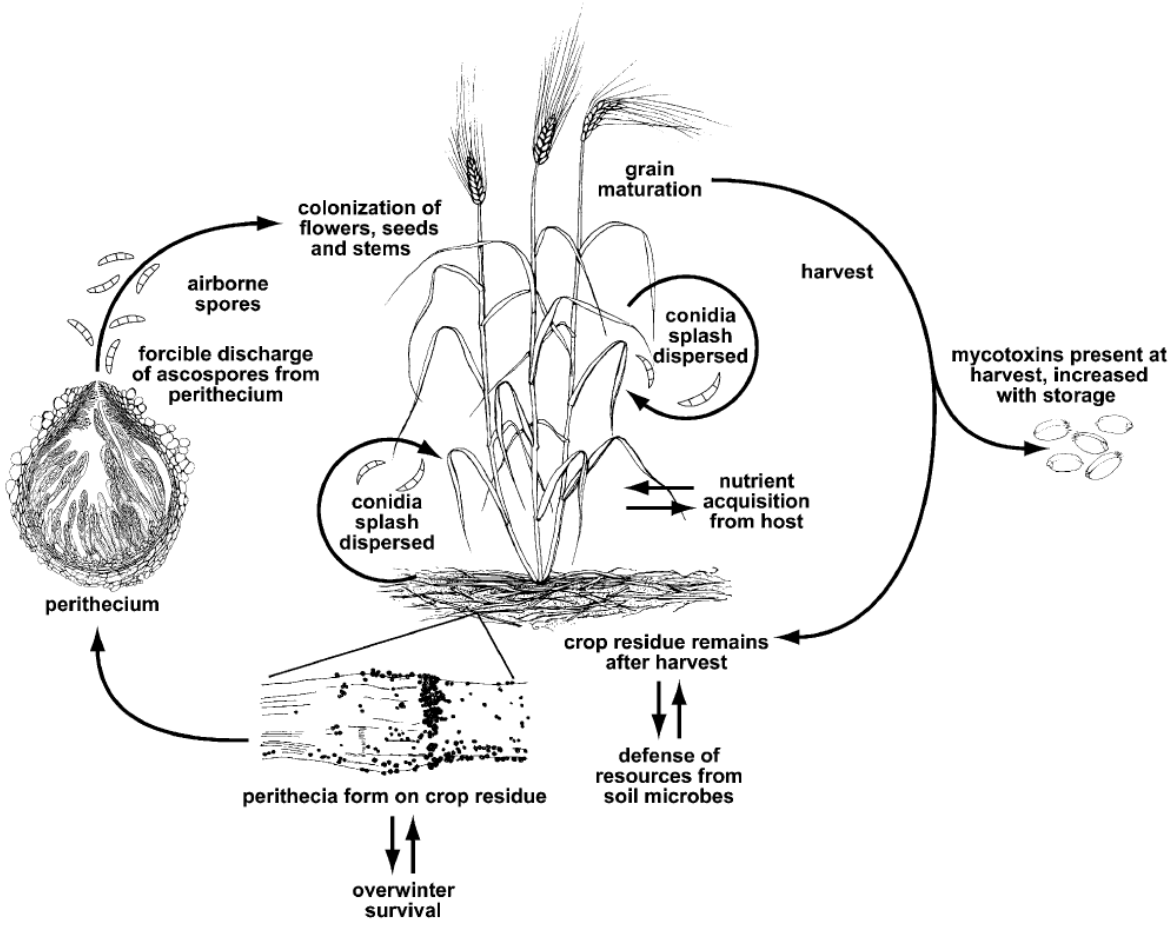


Figure 1. Generalized life cycle of *Fusarium graminearum* (Trail, 2009). Details of specific aspects of the cycle are discussed in the text.

Ascospores, the primary inoculum of FHB disease, are sexually reproduced inside perithecia and can germinate within six hours upon landing on susceptible parts of the host plant (Bayer and Verreet, 2004. Single-celled, uninucleate initials are the first form during the ascospore development process (Figure 2B). Following nucleus division, a septum is synchronously formed in the middle of a spore, leading to a two-celled spore with uninucleate cells (Headrick, 1988). Four-celled spores, each with uninucleate cells, are fully developed ascospores and have been considered the norm in Fusarium. Interestingly, ascospores generally do not germinate inside perithecia; in fact, they germinate when shot into the air and land on a plant surface or a growth medium under a wet and warm environment (Manstretta *et al.*, 2016).

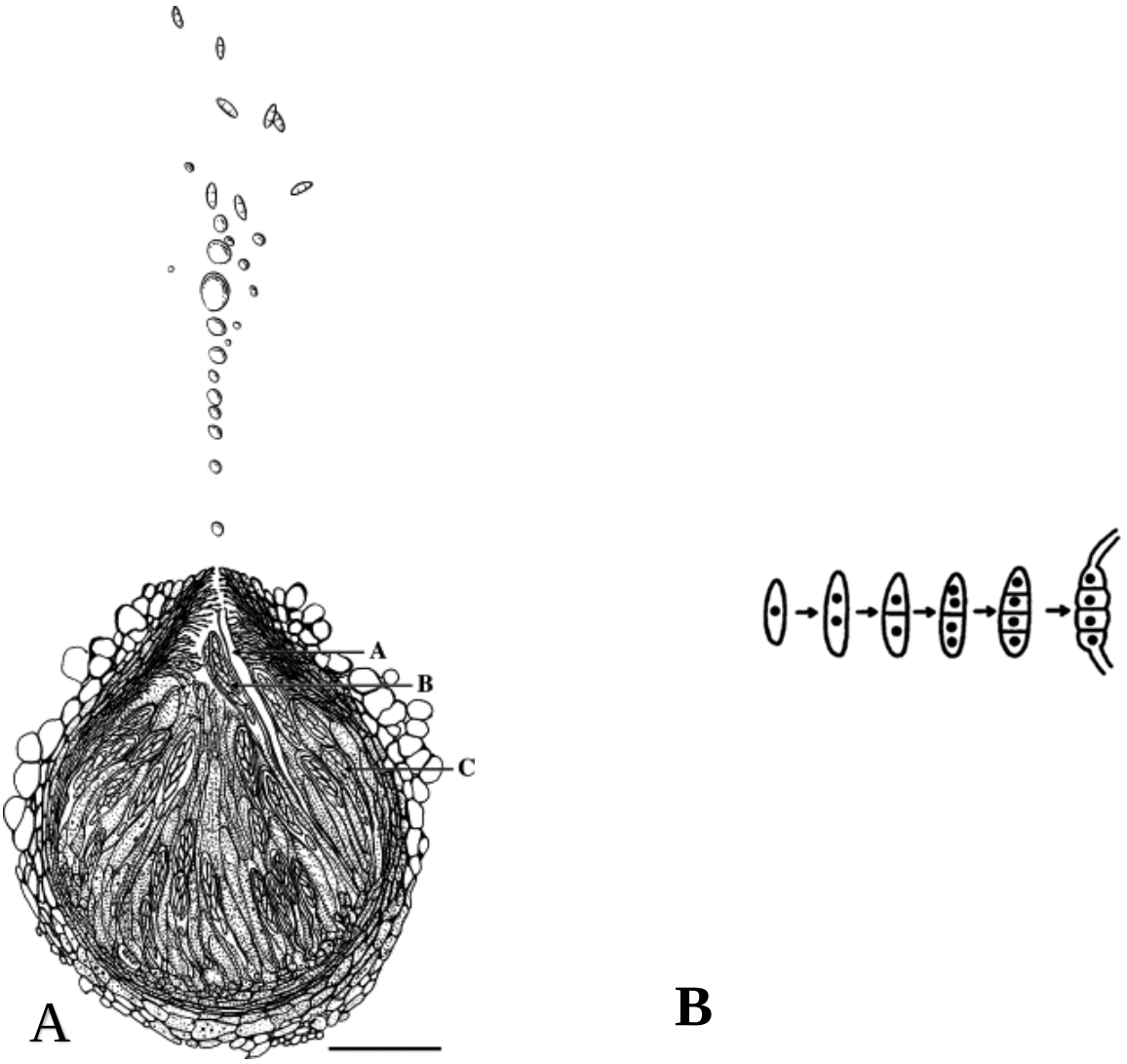


Figure 2. Main biological structures of *Fusarium graminearum* during sexual development. A. Longitudinal section through a mature perithecium *(Trail et al., 2005).* An ascus retracting following ejection of spores and fluid (smaller A); a mature ascus, extending upward to the end of the ostiolar canal to discharge (smaller B); and a mature, un-extended ascus (small C). Bar = 50 μm; B. Typical sequence of nuclear and morphological conditions during ascospore ontogeny. Mature ascospores consist of four cells, which germinate only when being shot out of the perithecia and land on the host surface.

1.2 RNA editing

The central dogma of molecular biology was simply understood as "DNA makes RNA, and RNA makes protein", and once viewed as a solid 1:1 parallel co-linearity of a gene's DNA sequence, its RNA copy and products. However, several biological processes at the transcription and translation stage alter the transcripts and amino acid sequences, making those sequences differ

from that predicted from the genome template, and represent a form of genetic recoding. One of these processes is RNA editing, a post-transcriptional modification of specific sites within the RNA nucleotide sequence (Gott and Emeson, 2000). The term RNA editing was first proposed in 1986 when the insertion or deletion of uridine (U) nucleotides into specific positions of the mitochondrial cox2 mRNA precursors of trypanosome species to restore their open reading frames (ORFs) was discovered (Rob Benne *et al.*, 1986). Since its discovery, RNA editing has proven to be evolutionarily conserved in all domains of life including eukaryotes, prokaryotes, archaea, and viruses. To date, more than 160 distinct types of RNA nucleotide modifications with the potential to impact the function and stability of RNA molecules have been reported (Boccaletto *et al.*, 2018).

A-to-I mRNA editing is the predominant form of RNA editing in metazoans. The growing number of RNA editing events detected in numerous species reveals the significance of this molecular process. Indeed, RNA editing regulates several cellular processes in insects and humans (Keegan *et al.*, 2011), and regulates conserved toxin-antitoxin systems in bacteria (Bar Yaacov *et al.*, 2017). Even though the occurrence of RNA-edited sites has been well characterized in metazoans (Pinto *et al.*, 2014; Grice *et al.*, 2015), the identification and characterization of RNA editing in fungi has been under-investigated. In fact, RNA editing of nuclear protein-coding sequences was thought to be absent in fungi. The mechanism of RNA editing in fungi without the presence of Adenine deaminase acting on RNA (ADARs) remains unclear, and how RNA editing has emerged is still under debate. In this literature review, RNA modification and the current knowledge and findings of RNA editing in fungi will be discussed.

1.2.1 A-to-I mRNA editing

A journalist can edit a manuscript by inserting, deleting, or changing a word; similarly, RNA nucleotide sequences can be edited with reference to their genome template by insertion, deletion, or base deamination. RNA editing by deamination occurs mostly in messenger RNA (mRNAs) and transfer RNA (tRNAs) (Maas and Rich, 2000). If such base changes occur in the anticodon of tRNAs, the codon-recognition efficiency of tRNAs can be altered during translation (Gerber and Keller, 2001). On the other hand, base modifications that appear in coding regions of mRNA can alter the amino-acid specificity of codons, leading to the synthesis of protein isoforms that were not predicted from the genome template. Thus, mRNA editing is of great interest since it can change the flow of genetic information, leading to genetic recoding.

Generally, there are three main forms of mRNA editing: U insertion or deletion, C-to-U editing, and A-to-I editing. U insertion or deletion happens mostly in mitochondrial transcripts, while C-to-U and A-to-I editing can also be found in the nuclear transcripts of humans and other animals (Sloan, 2017; Eisenberg and Levanon, 2018). C-to-U editing in metazoans is catalyzed by the cytidine deaminase family of "Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like" (APOBEC), or the activation-induced deaminase AID/APOBEC family (Knisbacher *et al.*, 2016). In humans, this editing event occurs when APOBEC1 edits a specific cytosine in the long apolipoprotein B (*Apob*) pre-mRNA which recodes a CAA codon to a stop codon (Teng *et al.*, 1993, Navaratnam *et al.*, 1993). All enzymes belonging to the AID/APOBEC family (excluding APOBEC2 and APOBEC4) have specific cytosine deaminase capacity on single-stranded DNA or RNA. To date, C-to-U RNA editing has only been observed in the mitochondria, plastids and nuclear mRNAs of humans and animals, whereas no identification of C-to-U editing in the nuclear mRNAs of fungi has been reported.

A-to-I editing is the most abundant modification of mRNA in metazoans and is mediated by the adenine deaminase family of (ADARs) (Nishikura, 2010). ADARs are present only in metazoans, and members of this enzyme family possess an adenosine deaminase domain and several double-stranded RNA (dsRNA)-binding motifs (Nishikura 2016). A-to-I mRNA editing occurs when ADARs bind to dsRNA to deaminate the adenine base in adenosine nucleotides by hydrolytic deamination (Sommer *et al.*, 1991) (Fig. 3a). The nonstandard hypoxanthine base of inosine (I) pairs with cytosine and is subsequently read by cellular machineries as a guanosine (G) in translation (Basilio *et al.*, 1962). Thus, A-to-I editing on mRNAs is likely to substitute amino acids during translation, alter splice sites during RNA processing, and recode protein sequences (Zinshteyn and Nishikura, 2009)

In humans, ADAR1 is responsible for millions of RNA editing sites mainly in the non-coding repeat regions, whereas ADAR2 mediates the editing of coding regions (Bazak *et al.*, 2014). Even though protein recoding is rare in humans, thousands of A-to-I editing sites in the protein-coding regions of octopus have been reported (Albertin *et al.*, 2015). More interestingly, more than 57,000 editing sites in the coding regions of transcripts were modified by A-to-I RNA editing, leading to the recoding of several proteins in the nervous system of squids (Liscovitch-Brauer *et al.*, 2017). These studies show the great potential of A-to-I RNA editing to extensively alter the proteome in eukaryotic organisms.

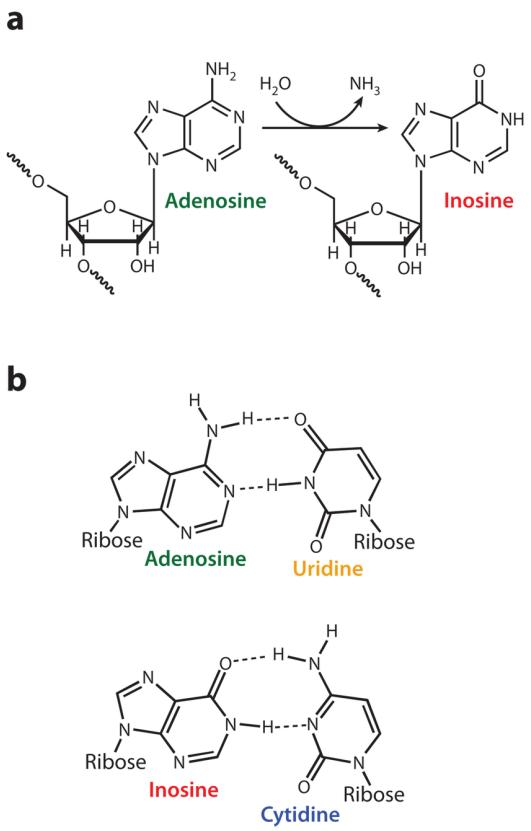


Figure 3. Deamination of adenosine to inosine by ADAR (Nishikura, 2009). (a) A hydrolytic deamination reaction converts adenosine to inosine. (b) In contrast to adenosine that pairs with uridine, inosineand guanosine pair with cytidine in a Watson-Crick-bonding configuration.

1.2.2 A-to-I tRNA editing

RNA editing is critical for tRNA to be fully functional and there are significantly more modified nucleotides in tRNAs than in mRNAs. Generally, post-transcriptional modifications of tRNA have two important roles: 1) contributing to structural integrity and 2) facilitating interactions with molecular co-factors (Piñeyro *et al.* 2014; Torres *et al.* 2014).

In tRNA, there are three main positions of Inosine, a nonstandard nucleoside present in tRNAs in both eukaryotes and prokaryotes at position 34, the first nucleotide of the anticodon loop (wobble-position); position 37 (after the anticodon); and position 57 (at the TΨC-loop) (Fig. 4). A-to-I editing in tRNA is mediated by the members of the Adenosine deaminases acting on tRNA (ADAT) family (Torres et al., 2014). The first member of this enzyme family to be crystalized was the tRNA-specific adenosine deaminase (tadA), which mediates the deamination of Adenine at position 34 of the $tRNA^{Arg}$ in *E.coli* (Wolf *et al.*, 2002). TAD2 and TAD3 (two homologs of

ADAT2 and ADAT3 in yeast), are two subunits of a protein complex involved in a two-step process that deaminates A34 in eukaryotes (Torres *et al.*, 2014). In contrast, the deamination of Adenine at position 37 in the anticodon loop is mainly catalyzed by Tad2 whereas Tad3 plays a minimal role (Gerber *et al.*, 1998; Torres *et al.*, 2014). The enzymes responsible for the deamination of Adenine at position 57 in archaea remains unknown.

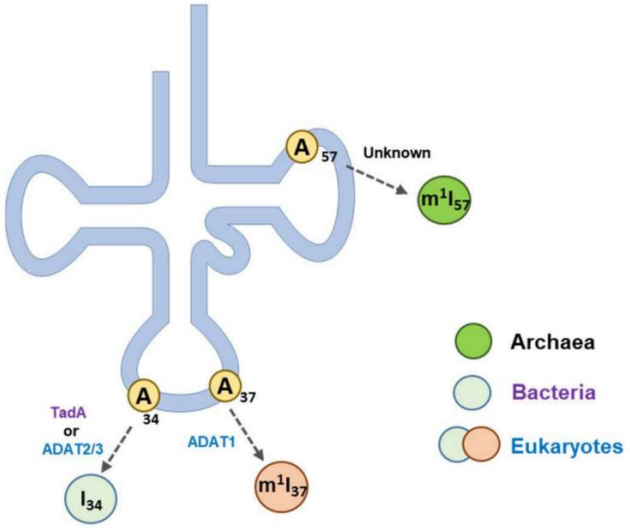


Figure 4. A-to-I editing in tRNAs (Srinivasan *et al.*, 2021). Adenosines at positions 34, 37, and 57 of tRNAs can be modified to inosines.

1.2.3 Current knowledge of A-to-I mRNA editing in filamentous fungi

For many years, A-to-I editing was presumed to be absent in the fungal kingdom and to rarely occur in nuclear protein-coding transcripts. Several reasons underlaid these presumptions including: 1) the enzymes with both adenine deaminase domain and dsRNA binding domain, or the adenine deaminase family of ADARs which mediate A-to-I editing in metazoans are not encoded in fungi (Nishikura, 2010); 2) mRNA editing was not observed in the fungal model species *Saccharomyces cerevisiae;* and 3) the specific developmental stage in which RNA editing occurs in ascomycetes has been understudied. The A-to-I editing of mRNA in fungi; however, was discovered in 2016. To be specific, thousands of A-to-I editing sites were detected by genome-wide analyses in several filamentous ascomycetes that belong to the Sordariomycetes and the Pezizomycetes classes (Liu *et al.*, 2016, 2017; Teichert *et al.*, 2017). These discoveries suggest that different mechanisms for A-to-I RNA editing are present in filamentous fungi and independent

of ADARs. Despite the abundance of RNA editing events in filamentous fungi, the mechanism of A-to-I RNA editing in this kingdom remains poorly understood.

Interestingly, the number of RNA editing sites increases in correlation with the development of perithecia in *F. graminearum, N. crassa, S. macrospora,* and *P. confluens* (Liu *et al.,* 2016, 2017; Teichert et al., 2017). These findings suggest that the A-to-I mRNA editing in fungi happens specifically during the sexual stage. Despite the abundance of A-to-I editing events in several fungal species, no editing events were reported in *S. cerevisiae* and *S. pombe*. The shared characteristic of A-to-I mRNA editing events in different fungi species suggest that editing mechanisms are conserved in those fungi belonging to the higher classes of filamentous ascomycetes.

Several of those filamentous fungi possess three ADATs genes homologous to TAD1, TAD2, and TAD3 in yeast (Liu et al., 2016, 2017; Teichert et al., 2017). The *Fgtad1* deletion mutant does not have any defects in normal development, while deletions of *TAD2* and *TAD3* are lethal (Sun et al., 2021 – New Phytologist). Using the Repeat-Induced Point mutation (RIP) approach, our lab has identified RIP mutations in FgTAD2 and FgTAD3 that specifically affect RNA editing and sexual reproduction, but have no effect on vegetative growth. Furthermore, our lab showed that affinity purified FgTad2 proteins have A-to-I RNA editing activities in in vitro editing assays with mRNA substrates (Bian et al., unpublished). These genetic and biochemical data indicate that FgTad2 and FgTad3 are responsible for A-to-I mRNA editing in *F. graminearum*.

Table 1: Occurrence of fungal and bacterial RNA editing of nuclear protein-coding transcript in the chronological order of the discoveries.

Date	Species	Types of RNA editing	Number of editing sites	Affecting	Original discoveries
2014	*Ganoderma lucidum*	C-to-U, A-to-G, G-to-A, and U-to-C conversions.	8,906	mRNA	Abundant and selective RNA-editing events in the medicinal mushroom *Ganoderma lucidum* (Zhu *et al.*, 2014)
2016	*Fusarium graminearum*	A-to-I (G)	26,056	mRNA	Genome-wide A-to-I RNA editing in fungi independent of ADAR enzymes (Liu *at al.*, 2016)
2017	*Neurospora crassa*	A-to-I (G)	40,677	mRNA	A-to-I RNA editing is developmentally regulated and generally adaptive for sexual reproduction in Neurospora crassa (Liu *et al.*, 2017)
2017	*Pyronema confluens; Sordaria macrospora*	A-to-I	2,592 for *P.confluens;* 5,900 for *S.macrospora*	mRNA	RNA editing during sexual development occurs in distantly related filamentous ascomycetes (Teichert *et al.*, 2017)
2017	*Escherichia coli*	A-to-I	15	tRNA	RNA editing in bacteria recodes multiple proteins and regulates an evolutionarily conserved toxin–antitoxin system (Bar-Yaacov *et al.*, 2017)

Although *FgTAD2* and *FgTAD3* have been shown to be responsible for RNA editing, both are constitutively expressed in all life stages of *F. graminearum,* but RNA editing specifically occurs during sexual reproduction. Furthermore, FgTad2 and FgTad3 proteins lack a dsRNA binding domain or RNA recognition motif (RRM). Therefore, I hypothesized that FgTad2 and FgTad3 interact with stage-specific cofactors during sexual reproduction to edit mRNA. To test this hypothesis, we used the affinity purification approach to identify proteins interacting with FgTad2 and/or FgTad3 specifically in perithecia (Bian et al., unpublished). The goal of this MS thesis study is to functionally characterize genes encoding putative FgTad2-interacting proteins identified by MS-MS analysis.

CHAPTER 2. MATERIALS AND METHODS

2.1 Candidate genes bioinformatics

The genomes and predicted amino acid sequences of the candidate genes from *F. graminearum* were downloaded from Ensembl Genomes (Howe et al., 2019). To identify their homologs, the amino acid sequence of each gene was used as the query for a BlastP search of the non-redundant protein sequence database at NCBI. Approximately 20 hits had query cover above 70% (E-value cut off 5e-5) and were chosen for phylogenetic analysis. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018) using the Neighbor-Joining method (Saitou and Nei, 1987) and computed with the Poisson correction method (Zuckerkandl and Pauling, 1965) to generate multiple sequence alignment and a phylogenetic tree.

2.2 Fungal strains and culture conditions

The *F. graminearum* strain PH-1 (wild-type, WT) and all the transformants generated in this research were stored long-term in 20% glycerol at -80°C and maintained as working stock on V8 (Campbell) agar plates at 25°C. For conidiation, mycelia on 4-day-old V8 plates were collected, blended in a commercial blender for 30 seconds, then introduced into 100ml CMC medium (15g Carboxymethylcellulose, 1g NH_4NO_3, 1g KH_2PO_4, 0.5g $MgSO_4$ x $7H_2O$, 1g yeast extract in 1L double distilled water, autoclaved) in a 250ml flask. For isolation of genomic DNA and isolation of protoplasts, the conidia harvested from 3-day old CMC medium was introduced into 100ml YEPD medium (3g yeast extract, 10g peptone, and 20g dextrose in 1L double distilled water, autoclaved), incubated at 28°C for 12 hours with continuous shaking at 175 rpm.

2.3 Fungal genomic DNA extraction

Mycelia from overnight YEPD cultures were collected using two layers of Miracloth in a Nalgene 100mm funnel. Genomic DNA extraction was performed by grinding mycelia tissue in liquid nitrogen and transfer to a 1.5 ml microcentrifuge tube. Then 500 µl of preheated CTAB extraction buffer were added to the dried tissue powder, then vortexed for 20 seconds.

The mixture of tissue powder and CTAB was kept at 65°C for 10 min to promote separation of polysaccharides during isolation of DNA. A solution of 1ml chloroform: isoamyl alcohol, mixed

at a ratio of 24:1, was added to the tube, mixed thoroughly and centrifuged for 5 min at 16,000 g at room temperature to remove insoluble matter. The aqueous phase was carefully transferred into a clean 1.5ml microcentrifuge tube, followed by the addition of 2 µl of RNase A, then incubated at 37°C for 30 min. To precipitate DNA, 600 µl of ice-cold isopropanol were added to the tubes, mixed thoroughly, kept at -20°C for at least 2 hours, and centrifuged for 5 min at 16,000 g. The supernatant was discarded. After being washed once with ice-cold 70% isopropanol, the pellet was air-dried to remove ethanol completely, and resuspended in 30 µl double distilled water. Finally, concentration of genomic DNA was quantified using the NanoDrop-1000 spectrophotometer at 260 nm and stored at -20°C.

2.4 Generation of single-gene knockout mutants

2.4.1 Generation of gene knockout construct

Gene replacement was generated using the split-marker approach (Goswami, 2012). The linear Hygromycin marker gene (*hph* gene) was used as a selectable marker for transformants. The upstream and downstream flanking regions of the target gene were replaced with the *hph* gene by homologous recombination. Six gene-specific primers were designed on chromosomal DNA sequence for each sexual-stage-specific candidate gene from *F. graminearum* Two linear PCR products for each gene were obtained from the homologous recombination at two different locations; between the flanking regions of *F. graminearum* genomic DNA (200 ng/µl), and within the overlapping regions of the *hph* gene (Fig. 4). Primers for each gene of interest were designed using Primer-BLAST (Ye *et al.*, 2012). Primer concentrations were reduced to 50 nM instead of the recommended 100 nM to achieve cleaner results. Homologous arms containing complementary sequences to the selectable marker primers were added to the 5' end of 2R and 3F primers for the overlap of the flanking sequence and *hph* sequence. In the first round PCR, *F. graminearum* genomic DNA (100 ng/µl) was used as the template, primer 1F and 2R were used to amplify the 5' flanking sequence, while primer 3F and 4R were used to amplify the 3' flanking sequence. Each flanking sequence is 700-800 bp long. HYGT plasmid, primer pair HYG-F/ HY-R, primer pair YG-F/ HYG-R were used to amplify two-thirds of the *hph* gene.

The primers used to amplify the flanking sequences for each gene are shown in Table 2. In the fusion PCR, the upstream flanking sequence and the first two-thirds of the marker gene

generated from PCR round 1 were used as the template (50ng/ 1000bp). Primer pair 1F/ HY-R was used to amplify construct I for transformation; for construct II, the downstream flanking sequence and the second two-thirds of marker gene were used as the template, and primer pair 4R/YG-F were used. The PCR products were purified using a gel extraction kit from QIAgen prior to fusion PCR.

Gene replacement constructs for the candidate genes were generated using the PCR fusion method following standard PCR conditions as suggested by the manufacturer with a few modifications. GoTaq DNA Polymerase from Promega was used. In the first round PCR, cycling reactions were 2 min at 95°C followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 2 min at 72°C. Finally, an extension cycle of 5 min at 72°C was added. In the fusion PCR, there were 2 rounds: the first round only contained DNA template, i.e.,no primer added, and had 15 cycles of 30 s at 94°C, 30 s at 55°C, and 2 min at 72°C. The second round of fusion PCR included primers added to the reaction, with 35 cycles of 30 s at 94°C, 30 s at 55°C, and 2 min at 72°C. The resulting PCR products were either purified or used directly for protoplast transformation of *F. graminearum*.

Table 2. List of templates and primer sequences used to generate knockout mutant

DNA template	**Primers**	**Sequence (5' – 3')**
Genomic DNA from the transformant's mycelia	H850	ATGTTGGCGACCTCGTATTGG
	H852	TTCCTCCCTTTATTTCAGATTCAA
FGSG_10943 deletion transformant	10943/5F	TAGTATCTGCGTCTCTTCCAACAG
	10943/6R	CCGAGCAAGCATACGCATCAACAC
	10943/7F	CGCCAACTCCCAGTCTATCCAA
	10943/8R	GAAACATATTCCAGCCTGCCCCA
FGSG_07774 deletion transformant	07774/5F	CCTATCCAGACTACCAACTCTGG
	07774/6R	GGAAGACGAAGGTGATGTTGGTG
	07774/7F	CTGAGGACCCCTGGTTTCTTCCAA
	07774/8R	GCGACAACTGGAGATTATACTCGG

Table 2 continued

	04494/5F	*CCGGAACCACTTCTACAGTCACTT*
FGSG_04494 deletion transformant	04494/6R	ACATCCCCTTCCTTCTCTTCC
	04494/7F	CATTCATGGAAGGCTACGACGACC
	04494/8R	TTAAAGGCACGCACCGATACCA
FGSG_10589 deletion transformant	10589/5F	ATCGCTCAGCCCCAGGAATAT
	10589/6R	GCTGTTGGAAGGATTTGGCCCGTA
	10589/7F	ATCACCAACGGCCGAGAAGAGTAG
	10589/8R	TTCTGACACTCCGTATTTGTGC
FGSG_02267 deletion transformant	02267/5F	AAGTGAGAGATACCCAGTTGCTGAC
	02267/6R	CCTTGAAGTGAGGGTTAAAGTC
	02267/7F	CTTCAATGTCAAAGCTCCTGGGCC
	02267/8R	AATACACCCTGTCGTCCTGGGTA
FGSG_059222 deletion transformant	05922/5F	AAGCCAAACCCAAAACATGTCC
	05922/6R	ACGGGTTGAAGGAGCTCAGT
	05922/7F	GAGGAACAGAGTCAGTATTAGGC
	05922/8R	GATTCTGGGTTGCGAAATATGCC
	10943/1F	CGACACCAGTCACCACTAACTC
	10943/2R	AATGCTCCTTCAATATCATCTTCTGTCA GAACCAACAGTCTCTGATACAC
	10943/3F	CGTCCGCAATGTGTTATTAAGTCGACAC GCAGAACAGTAAACGACGGATG
	10943/4R	GGAGTCCGGTTATGATAGCAGCAT
	07774/1F	TACCGTCTAGCTCCACTGACCAA
	07774/2R	AATGCTCCTTCAATATCATCTTCTGTAGG GAAGGTCATAGCGGAAG
	07774/3F	CGTCCGCAATGTGTTATTAAGTCGACCGT TGATCGCCATGGCTCATTGGA
	07774/4R	GTTATTTCCACACATCAGGGCG

Table 2 continued

	04494/1F	*AATCGCGGCATCTAATAGGGGTCT*
	04494/2R	AATGCTCCTTCAATATCATCTTCTGTCAG ACGGAGGGAGCTTCTAACA
	04494/3F	CGTCCGCAATGTGTTATTAAGTCGACGGT AGTTCTTAGGTACTGGACC
	04494/4R	ATCGCATCCGTCACAATGTCTGC
	10589/1F	CGTAGTAAACGATGATGAGAGACC
	10589/2R	AATGCTCCTTCAATATCATCTTCTGTAGG CCGCTTGTTACCAGTTCATCC
	10589/3F	CGTCCGCAATGTGTTATTAAGTCGACTCA CGTAATTCGCTCTCCAAGAG
	10589/4R	TCGACGTTCCGTATCTCCACTAA
	02667/1F	AGGACACGACACAAGGTCTCACAA
	02667/2R	AATGCTCCTTCAATATCATCTTCTGTCATT GGTATCTGCATTGGAGCCG
	02667/3F	CGTCCGCAATGTGTTATTAAGTCGACAAT GAGGTGCGGATACTGCTGAT
	02667/4R	TCGGAAGCTCGGATGCCAATTGAG
	05922/1F	AATGCGTAGAACCGCGTAGAACCA
	05922/2R	AATGCTCCTTCAATATCATCTTCTGTACAG GCCGAAGAACCAGTCTTG
	05922/3F	CGTCCGCAATGTGTTATTAAGTCGACGCGA TGAATCACGGAAGAGAA
	05922/4R	TGTGCCTTATAGCAGCGTACC
	HYG/ F	GGCTTGGCTGGAGCTAGTGGAGGTCAA
	HY/ R	GTATTGACCGATTCCTTGCGGTCCGAA
	YG/ F	GATGTAGGAGGGCGTGGATATGTCCT
	HYG/ R	AACCCGCGGTCGGCATCTACTCTATTC

Note: Underlined sequences indicates homologous sequences required for recombination.

2.4.2 Isolation of *F. graminearum* protoplasts and PEG-mediated transformation

F. graminearum strain PH-1 protoplasts were isolated and transformed as described by Turgeon et al. (2010). Briefly, mycelia in YEPD liquid medium were harvested and digested in protoplasting enzyme solution (0.25 g digesting enzyme from Trichoderma, 0.05 g Kitalase, 10 ml 1.2M KCl) for 1.5 – 2 hours. The protoplasts were filtered through three layers of Miracloth, washed twice with 1.2M KCl by centrifugation at 4000 g for 5 min at 4°C. Protoplasts were resuspended in STC (200 g sucrose, 7.35 g $CaCl_2$, 10 mM Tris-HCl) to the final concentration of 10^8 protoplasts/ ml. 7% DMSO were added to preserve the protoplasts in a -80°C freezer. For PEG-mediated transformation, 10 µl of each fragment from the fusion PCR were added to 200 µl protoplast suspension and mixed thoroughly. After incubation for 20 min, 1.25 ml PTC (40% PEG in STC) were added and the mixture was incubated at room temperature for 20 min to facilitate transformation.

Subsequently, 5 ml of TB3 liquid medium (3 g yeast extract, 3 g casamino acids and 20% sucrose in 1L double distilled water) were added to the tube. The transformation tubes were shaken at 100 rpm for 16 hours at room temperature, then supplemented with 50°C TB3 agar medium, Ampicillin (50 µg/ml), and hygromycin (150 µg/ml). The next day, the plate was overlayed with 10 ml of warm TB3 agar supplemented with 200 µg/ml Hygromycin. After 3 days, transformants were transferred onto V8 plates.

2.4.3 Identification of single gene knock-out mutants

Transformants that formed a single colony on V8 agar supplemented with 250mg/ L hygromycin B (Calbiochem, La Jolla, CA, U.S.A.) were isolated and confirmed by PCR using genomic DNA. Four PCR reactions were utilized along with several combinations of primers as listed in Table 1. See Fig.4 for primer locations. Table 2 shows the specific sequence of primers that were designed for confirming this purpose.

Table 3. List of primer pairs used for confirmation of successful transformation

Primer combination	Amplicon
H852/ H850	Internal region of *hph* gene
7F/ HY-R	5' end of the mutant gene
8R/ YG-F	3' end of the mutant gene
5F/ 6R	Negative screen.

2.5 Assays for growth and conidiation defects

For comparative growth rate and colony morphology, the diameter of each colony formed by mycelia on plastic petri dishes (Fisher Scientific, 100 mm x 15 mm) containing CM (Correll et al., 1987) and 5×YEG (Zheng et al., 2000) agar media were measured after incubation at room temperature for 4 days. To compare conidia production rate and morphology, four 5-mm^2 diameter agar blocks were blended in a commercial blender for 20 seconds with 100 ml CMC liquid medium in a 250 ml flask, continuously shaken at 175 rpm at room temperature for 5 days. Conidia production rate was determined with a hemacytometer.

2.6 Assays for perithecia formation and ascospore discharge

Five-day-old mycelium growing on the surface of carrot agar (400 g carrot, 1 L water, 15 g agar) were scraped away with a small, sterile spatula. Self-crossed dishes were incubated at room temperature under fluorescent lights to induce perithecium formation. Ascospores, asci, cirrhi and perithecia were observed for 2-3 weeks after inoculation. At 6 days-post-fertilization (dpf), a 1-cm semicircle of carrot agar were cut out and placed on a glass microscope slide inside a moisture chamber. Ascospores that are shot out of the perithecia were visual examination by naked eye after 2 days.

2.7 Plant infection assay

Virulence was determined by degree of discoloration of corn silks. Fresh corn silks from inside the husks of young corn ears were collected for assays. . , The V8 agar containing mycelia was cut into 4 mm^3 blocks to inoculate the lower end of 3-4 fresh corn silks inside a moist chamber

at room temperature. Double distilled water was supplied every 2 days to maintain the moisture. After 6 days, the discoloration of corn silk was scored.

2.8 Complementation of the *FGSG_10943* deletion mutants

Complementation constructs were generated using the yeast gap repair approach (Zhou et al., 2011). Briefly, a fragment containing the entire *FGSG_10943* gene with its native promoter and 3' untranslated region were amplified with primers 10943-CM/F and 10943-CM/R (Table 3) and transformed into yeast competent cells with pFL2 vector. After 2 days, yeast transformants were isolated and screened for true transformants using XXYYY method. Yeast DNA of true transformants was extracted and cloned into the vector pFL2. Subsequently, the resulting complementation construct was transformed into protoplasts of the *FGSG_10943* deletion mutant as described in section 2.3.2. The 10943-c transformants were confirmed by PCR.Move Table 3 to this position after it is first mentioned.

2.9 Generation of the of the Δ10943-GFP fusion construct

The yeast gap repair approach was used to generate Green Fluorescence Protein (GFP) fusion construct as described above. Specifically, primers 10943-GFP/F and 10943-GFP/R were designed to amplify the region containing entire *FGSG_10943*, RP27 promoter and GFP sequence (Table 4). Q5 High fidelity polymerase (New England Biolabs) was used with the following PCR conditions: 98°C for 30 seconds, followed by 34 cycles at 98°C for 10 seconds, 65°C for 30 seconds and 72°C for 2.5 minutes.Finally, 2 minutes of final extension at 72°C were added. The resulting PCR products were analyzed by gel electrophoresis and the amplicons were purified using QIAgen gel extraction kit.

The purified amplicon was mixed with pFL2 vector and transformed to the *E. coli* strain DH5α by electroporation at 1800 V and spread to LB plates containing 50µg/ml ampicillin. The *E.coli* transformants harboring the pFL2 plasmid containing the Green Fluorescence construct were verified by colony PCR, and sequences of recombinant plasmids were identified by DNA sequencing. Valid fusion constructs were transformed into protoplasts of a *FGSG_10943* deletion mutant.

Table 4**.** List of primers used to generate complementation and GFP fusion constructs

Primer name	Sequence
10943-CM/ F	CGACTCACTATAGGGCGAATTGGGTACTCAAATTGG GGACCACAAGCGGTTCACTA
10943-CM/ R	AACATATTCCAGCCTGCCCC
10943-GFP/F	CAGATCTTGGCTTTCGTAGGAACCCAATCTTCAATGT CAACTCGGAACGGAAGTG
10943-GFP/R	CACCACCCCGGTGAACAGCTCCTCGCCCTTGCTCAC CCCTAGAAACCCACCATCGAAC

Note: The underlined sequences are specific for the FGSG_10943 gene.

CHAPTER 3. RESULTS AND DISCUSSION

3.1 Bioinformatic analysis of *Fusarium graminearum* candidate genes

Considering that A-to-I RNA is a stage-specific process occurring during sexual development in filamentous fungi (Liu et al., 2016), I first investigated genes that are highly expressed during the sexual stage of *F. graminearum.* The six previously identified *F. graminearum* genes *(*Cuomo *et al.*, 2007) chosen for this project were FGSG_10943 (GenBank accession no. EYB28934), FGSG_07774 (GenBank accession no. XP_011327591), FGSG_04494 (GenBank accession no. XP_011321099), FGSG_10589 (GenBank accession no. XP_011319592), FGSG_02667 (GenBank accession no. XP_011318617.1) and FGSG_05922 (GenBank accession no. XP_011324532.1). All these genes are predicted to interact with TAD2 and/or TAD3 based on the MS-MS analysis from Yang Li.

Table 5**.** Sequence information of *F. graminearum's* A-to-I mRNA editing candidate co-factors.

Gene_ID	BlastP Annotation	Length/AA	Pfam domain	Pulldown components
FGSG_10943	Hypothetical protein	630	N/A	FgTad2
FGSG_07774	Hypothetical protein	196	N/A	FgTad2 and FgTad3
FGSG_04494	Hypothetical protein	461	N/A	FgTad2 and FgTad3
FGSG_10589	Hypothetical protein	94	N/A	FgTad2 and FgTad3
FGSG_02667	Hypothetical protein	605	DUF2235	FgTad2
FGSG_05922	Hypothetical protein	122	N/A	FgTad2

The A-to-I editing candidate co-factors are highly expressed during the sexual stage of *F. graminearum.* Results from qRT-PCR with RNA isolated from 12h YEPD cultures and 8 dpf perithecia (Liu et al, 2016) showed that the transcripts in 4 of them were abundant in perithecia, but rare in macroconidia, vegetative hyphae, and infected plant tissues (Fig. 2). These results indicate that *FGSG_10943, FGSG_04494, FGSG_10589,* and *FGSG_02667* were specifically expressed during the sexual development of *F. graminearum.*

More interestingly, based on data from the strand-specific RNA-seq using RNA extracted from *F. graminearum* mating culture collected at 1 to 8 days post fertilization (dpf), Accession No.

PRJNA384311 showed the expression level of *FGSG_10943* and *FGSG_04494* was increased during perithecia development. To be specific, their transcripts were almost negligible at the early stage of sexual development, but started to escalate at 3dpf (Fig. 3). The abundance of transcripts of these two genes kept increasing and reached their peak at 5 and 6 dpf. For *FGSG_04494*, in comparison with 2dpf young perithecia, expression was significantly upregulated by 1,400 fold at 5dpf. The timing of up-regulated expression of *FGSG_10943* and *FGSG_04494* suggests they might be involved in ascus and ascospore development in perithecia. Thus, because of their stage-specific expression and potential interaction with FgTad2 and/ or FgTad3 from the LC-MS/MS analysis, my candidate genes may play important roles during sexual development and A-to-I RNA editing in *F. graminearum.* This last sentence is not clear.

Most of the candidate genes encode proteins unique to ascocarp-forming ascomycetes. Except for *FSGS_02667*, which has homologs in *Candida albicans*, other candidate genes lack distinct homologs in budding and fission yeasts and in other Taphrinomycotina and Saccharomycotina species (Fig.1A, 1B, and 1C). None of my candidate genes encode proteins that are conserved in *Neurospora crassa*. *FGSG_10943, FGSG_07774* and *FGSG_05922* are restricted to only a few orders of fungi in the class of Sordariomycetes that form dark pigmented ascocarps, particularly Hypocreales and Glomerellales. To search for possible genes that might not be predicted by automated annotation, the amino acid sequence of each candidate gene was used as queries to search against the genome sequences of *N. crassa*, *A. nidulans, S. cerevisiae, S. pombe* by TBlastN; however, no homolog was detected. Thus, none of the candidate genes has any putative conserved domain that can be detected by NCBI CDD tools.

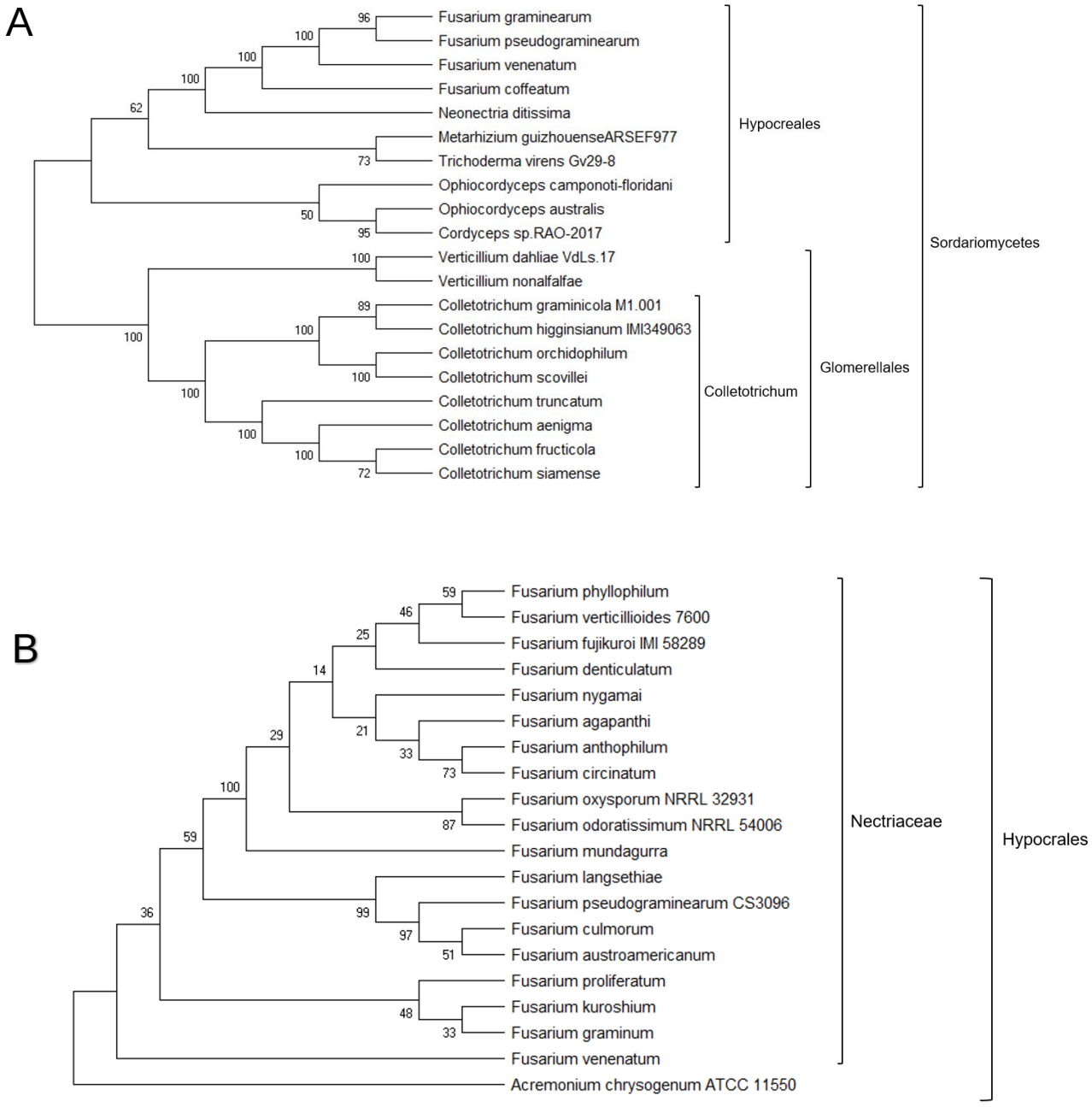
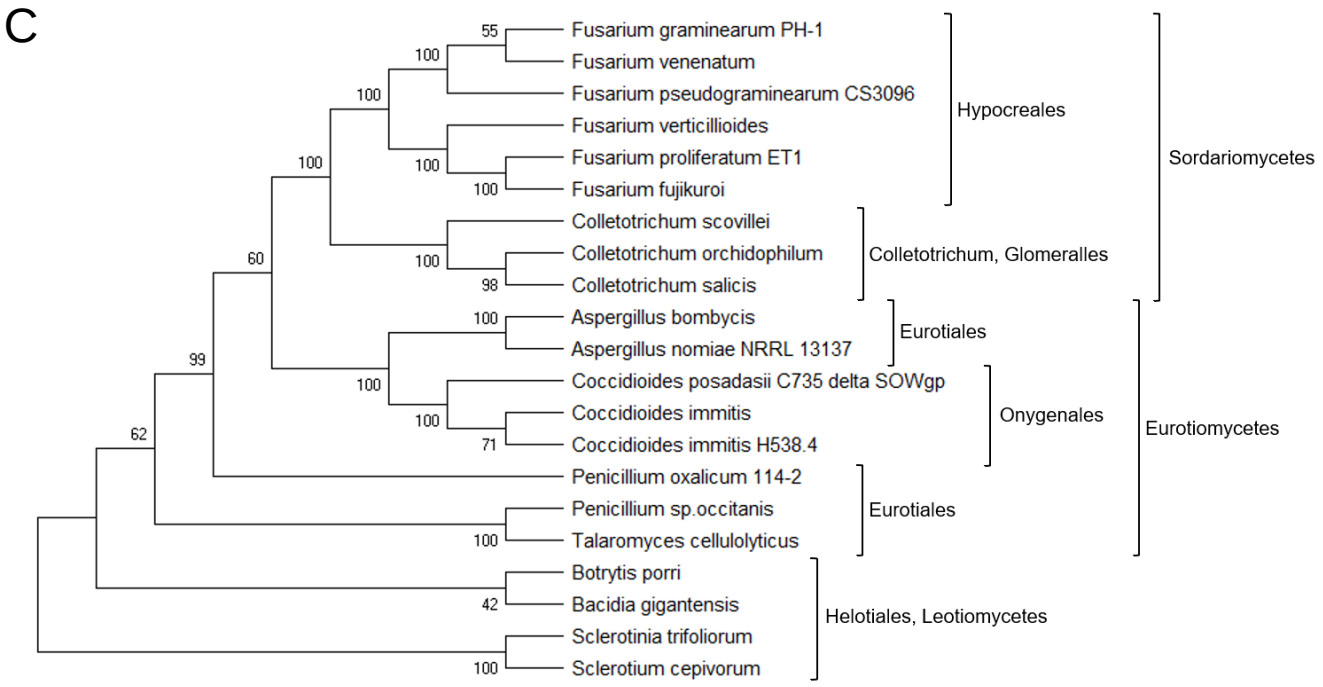
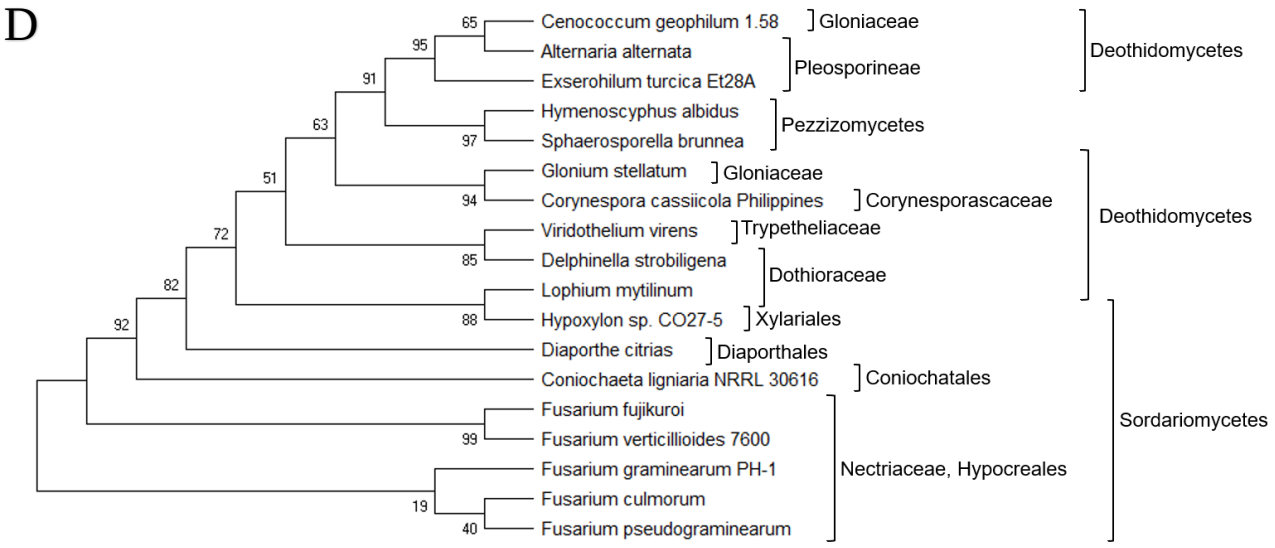


Figure 5. Phylogenetic distribution of candidate genes in kingdom Fungi. A, Phylogentic distribution of FGSG_10943. B, Phylogenetic distribution of FGSG_07774. C, Phylogenetic distribution of FGSG_04494. D, Phylogenetic distribution of FGSG_10589. E, Phylogenetic distibution of FGSG_02667. F, Phylogenetic distribution of FGSG_05922. The tree was constructed from the ClustalX-aligned sequences using a MEGA version X program and the Neighbor-Joining method. The numbers in nodes indicate bootstrap percentages (1000 replicates). Pairwise distances between 20 orthologs were analyzed in p-distance methodology by MEGA X.

Figure 5 continued





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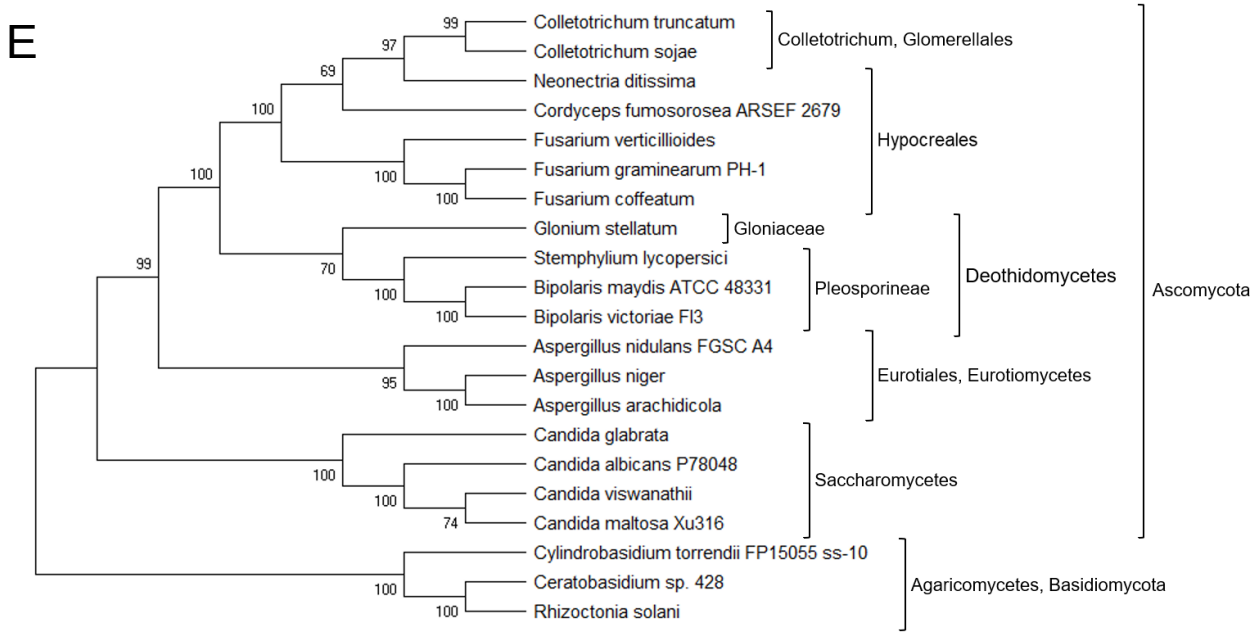

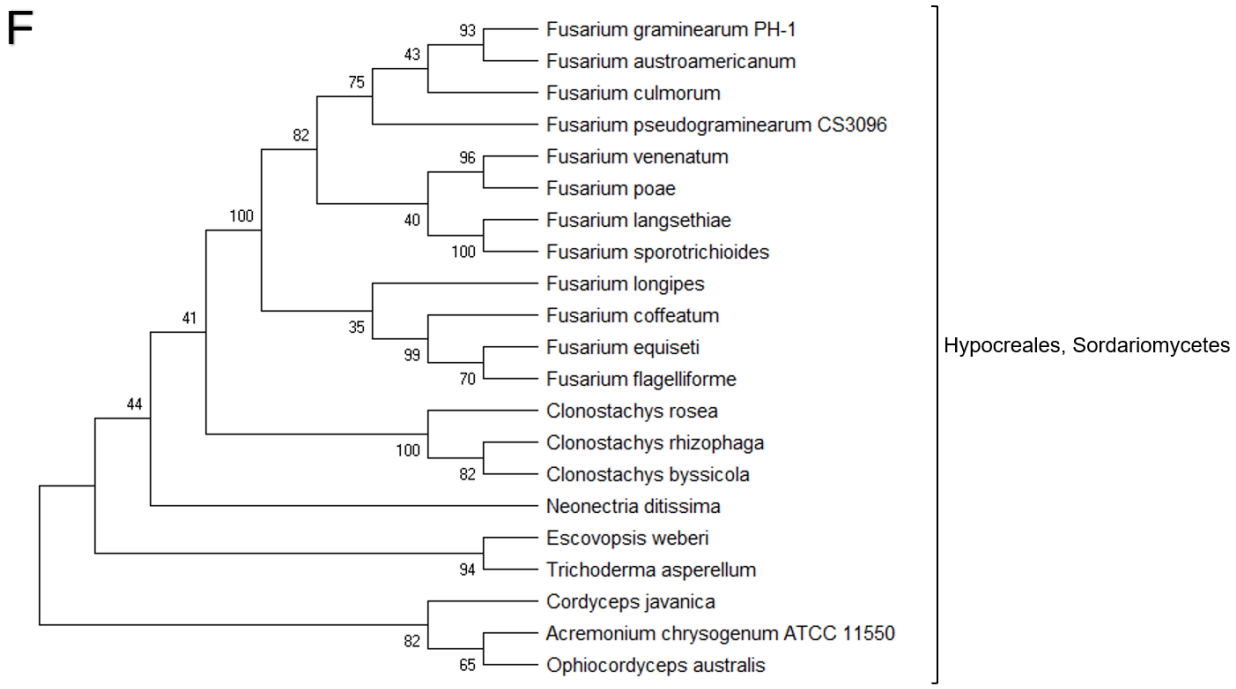

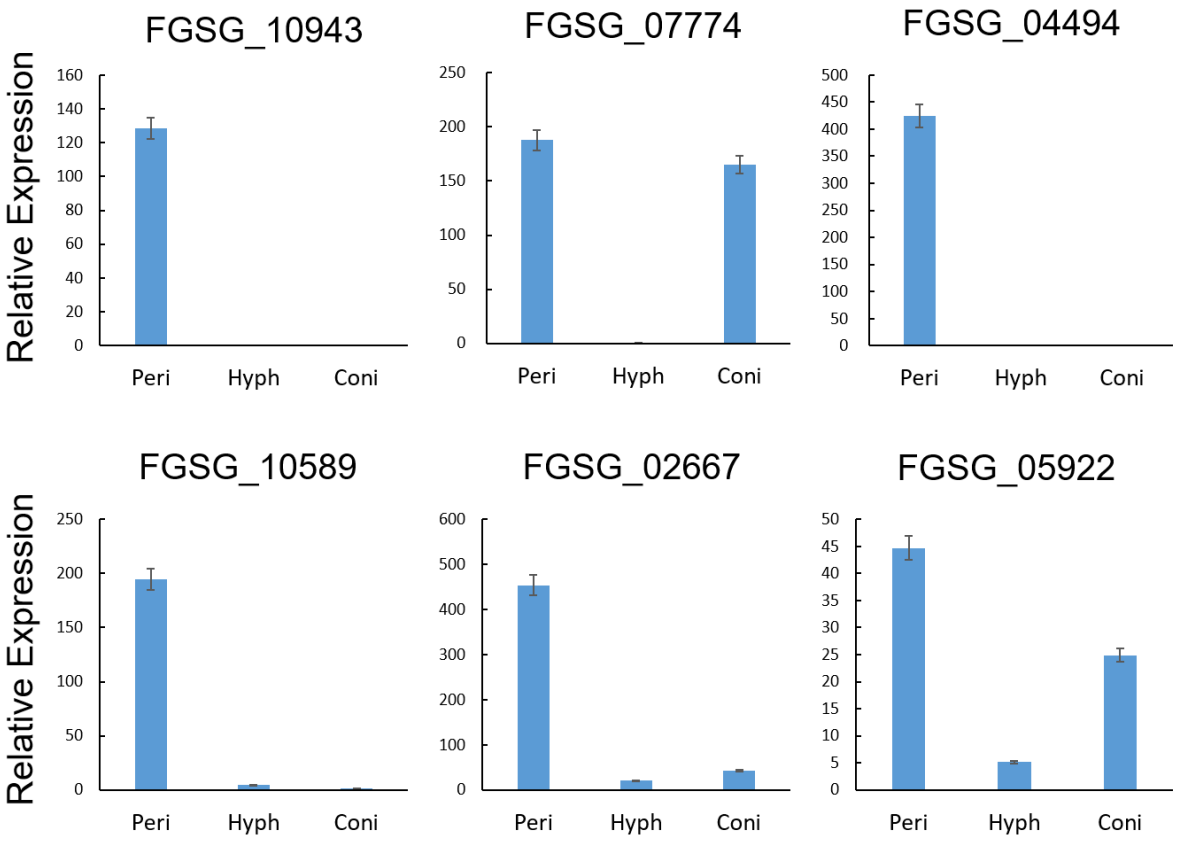

Figure 6. Relative expression level of candidate genes based on published RNA-seq data. The expression level of candidate genes was assayed by qRT-PCR with RNA isolated from 3 days CMC cultures (Coni), 12h YEPD cultures (Hyph), and 8 dpf perithecia (Peri). Bars indicate standard errors of the mean from three independent replicates.

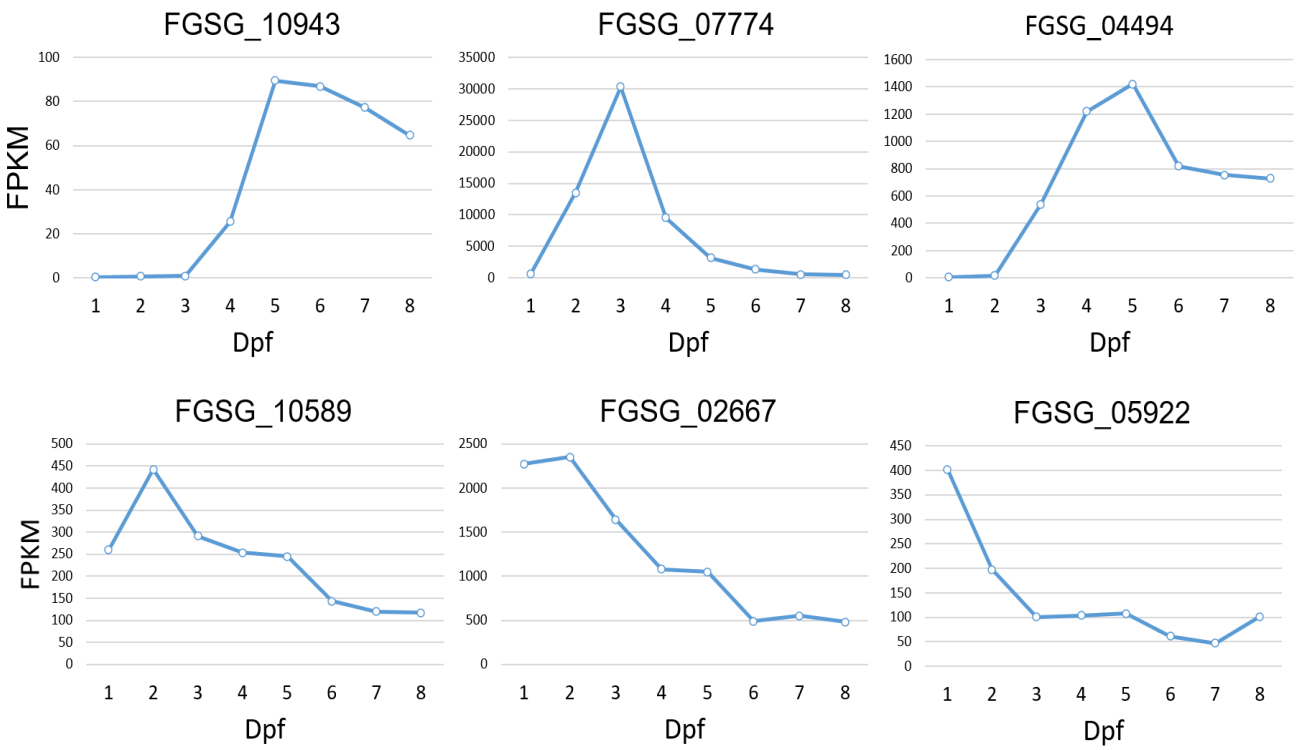

Figure 7. The abundance of candidate genes transcripts in different sexual stages based on published RNA-seq data of mating cultures collected at 1–2 dpf and perithecia sampled at 3–8 dpf. FPKM: Fragments Per Kilobase of exon per Million fragments mapped.

3.2 Targeted deletion of candidate genes and their complementation using the split-marker approach

To determine the function of candidate genes in *F. graminearum*, the split-marker approach was applied using the hygromycin phosphotransferase (hph) cassette to generate knockout transformants (Fig. 4). Two gene replacement constructs for each targeted gene were generated and transformed into the protoplasts of the *F. graminearum* wild-type strain PH-1 (Cuomo *et al.*, 2007). Ten to twenty transformants per transformation for each gene were first screened with negative primer pair 5F-6R. After identifying transformants which cannot be amplified with 5F-6R, the putative deletion mutants of each gene were identified and further confirmed by PCR using three different positive primer pairs. The lack of 5F-6R band and the existence of H850-H852 band indicate the marker was inserted into the genome and replaced the targeted gene. Visible bands at the right size when amplified with 7F-HY and YG-8R indicate the full length of *hph* replaced the

target sequence. Transformants that lack the internal region of the targeted gene's ORF plus show a band at the right size when amplified with H850-H852, 7F-HY and YG-8R were used as the true mutants. We have successfully knocked out six candidate genes, and there were four candidate genes that shows phenotype in deletion mutants, which are: *FGSG_10943*, FGSG_07774, FGSG_04494, and FGSG_05922.

After phenotyping for sexual stage, the *Δ10943* mutant had more significant defect during sexual reproduction than did other mutants; therefore, it was complemented by introducing *FGSG_10943* into the Δ10943 strain with Native promoter and 3' UTR. The presence of the inserted *FGSG_10943* gene was confirmed by PCR using the same approach.

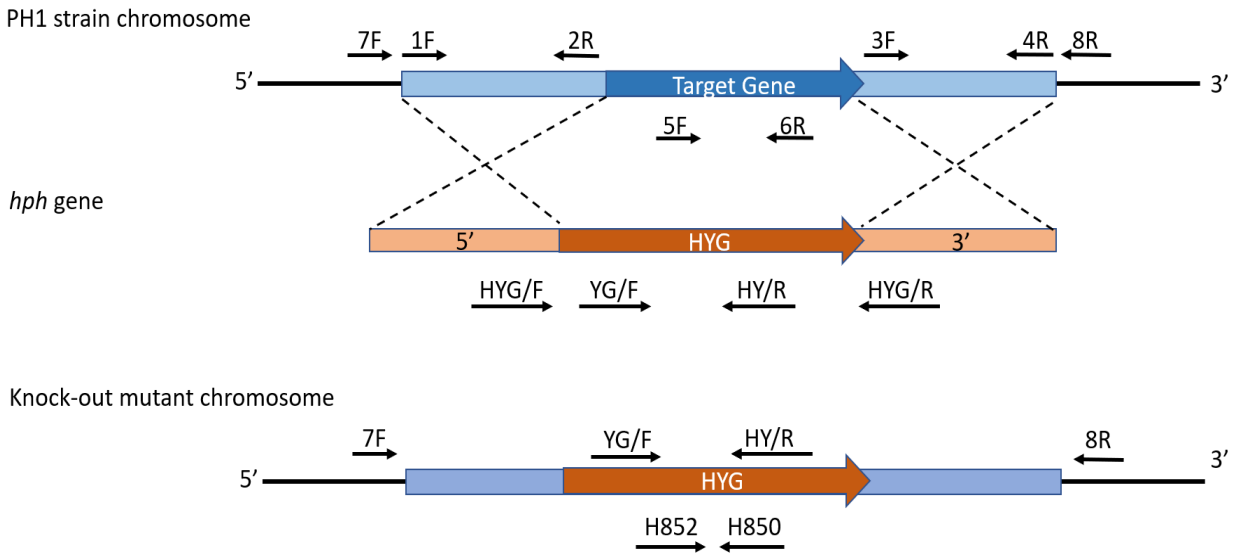

Figure 8. Diagrammatic representation of target gene deletion strategy and primers' locations to generate knockout mutant. Primer pairs 1F/2R and 3F/4R were used for amplification of upstream and downstream flanking sequences of the PH1 strain chromosome. Primer HYG-F/HY-R and YG-F/HYG-R were used to generate amplicons from *hph* gene. Blue and orange arrows indicate the orientation of the target genes and hph genes, respectively. Homologous recombination is denoted by dash lines. See Table 2 for primer sequences.

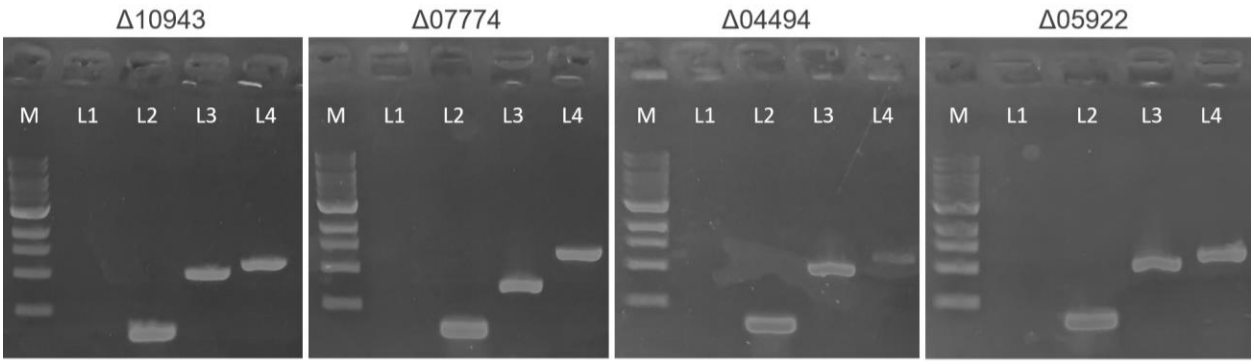

Figure 9. PCR assays for validation of the deletion of candidate genes. Deletion of the labelled candidate gene in the mutant strains was confirmed by the lack of PCR products amplified with primers 5F and 6R (L1). The insertion of *hph* gene was verified by the amplification with primers H855F and H856R (L2). PCR products amplified by primer pairs 7F/H855R (L3) and H856F/8R (L4) showed the existence of homologous recombination at the upstream and downstream flanking sequences of the labelled candidate gene, respectively, in the mutant strain selected for detailed analysis. All primers were described in Table 2.

3.3 Candidate genes are dispensable for vegetative growth, conidiation, and virulence

All four mutants had no obvious defect in colony morphology formed on CM agar plates (Fig. 8A) and produced typical conidium with 4 to 6 septa (Fig. 8B) in liquid CMC medium. The conidia production was also normal (Fig. 8C) and there were no defects in conidia germination in YEPD as well as hyphal tip morphology on agar slab (data not shown). In comparison with the wild-type strain PH-1, my candidate genes were normal in both asexual-stage development and virulence (Fig. 7 and Fig. 8). These results showed that, consistent with stage-specific expression during sexual reproduction, *FGSG_10943*, *FGSG_07774*, *FGSG_04494* and *FGSG_05922* are not needed for hyphae growth, conidiation, and virulence.

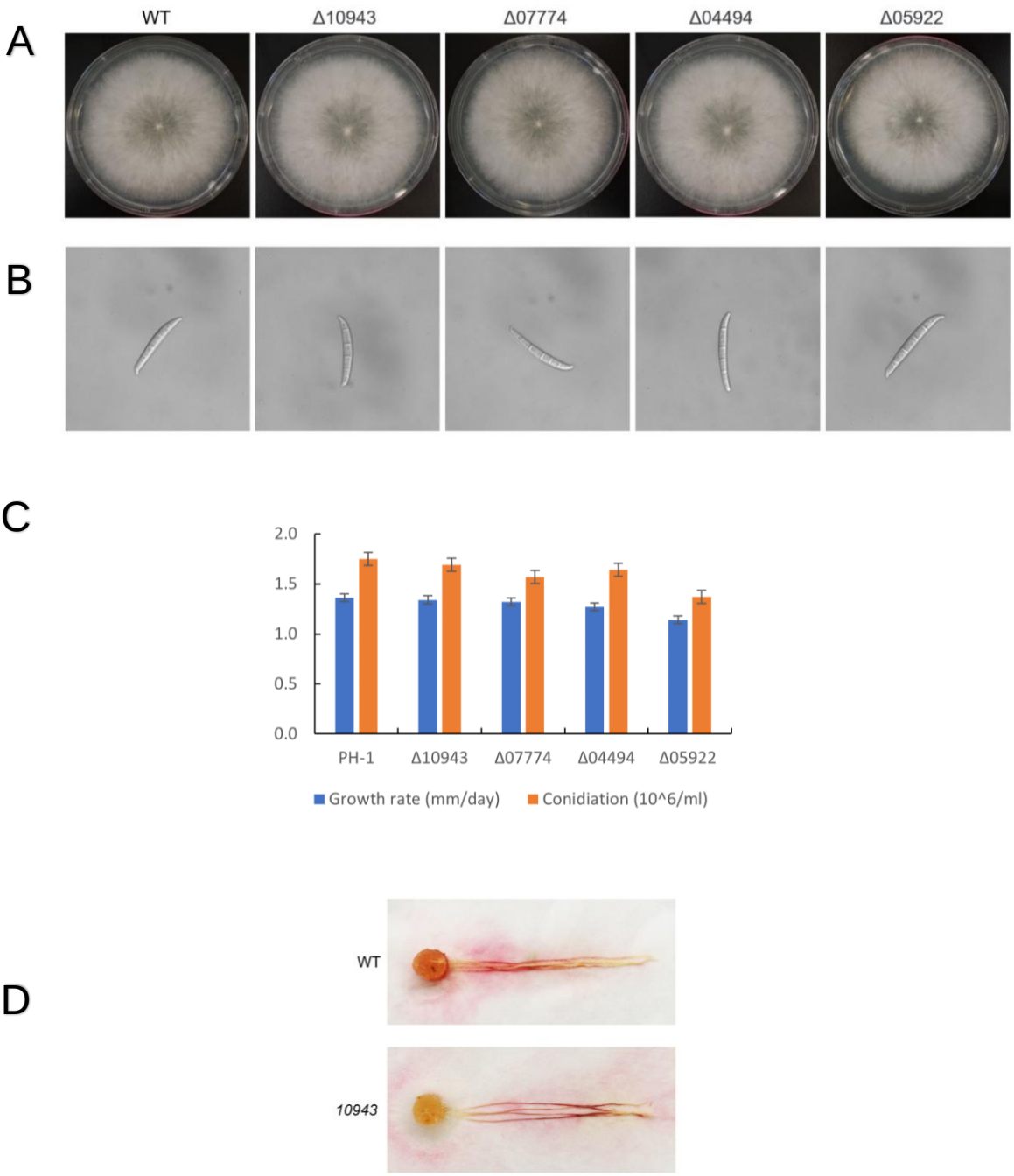

Figure 10. Morphology of the Wild-type PH-1 (WT) and *Δ10943, Δ07774, Δ04494* and *Δ05922* mutants. **A,** Colony morphology formed on CM agar plates. Photographs were taken after 3 days of incubation. **B,** Conidia morphology produced in liquid CMC culture after incubation for 3 days, shaken at room temperature. **C,** The vegetative growth rate and conidum production of *F. graminearum* strains. The means and standard errors were calculated from at least three independent experiments for vegetative growth assay, and two independent experiments for conidiation assay. **D,** Infection assay with the *Δ10943* mutant and Wild-type PH1. Corn silks were inoculated with culture blocks of the same set of strains and assessed at 6 dpi.

3.4 Ascospore discharge is defected in four deletion mutants

As a homothallic fungus, *F. graminearum* is capable of sexual reproduction regardless of the presence of an opposite sex partner. Thus, after self-fertilization, this fungus will produce abundant fruiting bodies called perithecia containing ascospores inside. The *FGSG_10943*, *FGSG_07774*, *FGSG_04494* and *FGSG_05922* deletion mutant (namely *Δ10943*, *Δ07774*, *Δ04494*, and *Δ05922* was as prolific as the wild-type strain, producing copious dark-pigmented perithecia on mating agar plates at 7 days after self-fertilization. Nevertheless, mutant perithecia do not have ascospore cirrhi on their surface (Fig. 9A), even after 2 weeks of incubation under fluorescent light, indicating that the perithecia failed to eject ascospores in the *Δ10943*, *Δ07774*, *Δ04494*, and *Δ05922* mutant.

To further verify this observation, I inspected ascospore discharge as previously described (Cavinder *et al*., 2012). Under the same conditions, We observed that while a plentiful number of ascospores were forcibly shot from wild-type perithecia after incubation for 16 h, ascospore discharge was not detected in the *Δ10943*, *Δ07774*, *Δ04494*, and *Δ05922* mutant under naked eye (Fig. 9B). Thus, it appears that these candidate genes might involve in ascospore ejection from perithecia in *F. graminearum*.

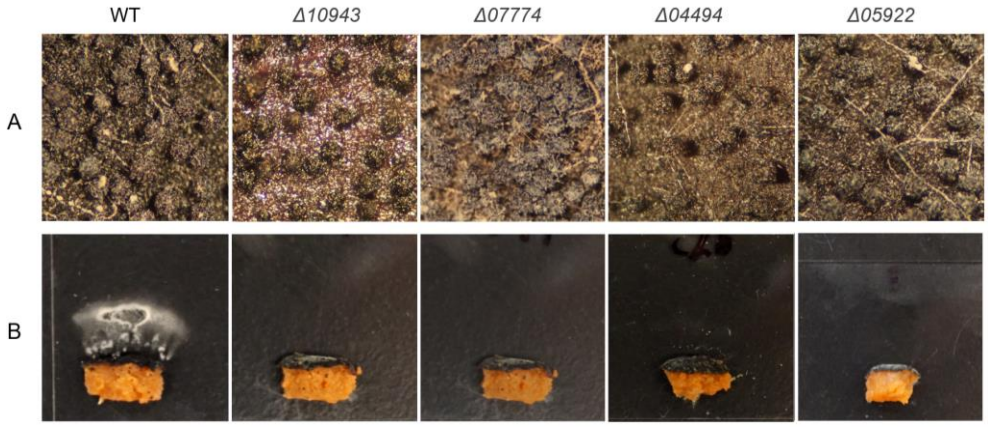

Figure 11. The *Δ10943*, *Δ07774*, *Δ04494*, and *Δ05922* mutant was defective in ascospore discharge.

3.5 Ascospores germinated inside perithecia during late stage of sexual development

Even though cirrhi were rare on the surface of perithecia, abundant slender ascospores containing two or four cells each were produced inside the perithecia of the *Δ10943* mutant within 2 weeks after inoculation. The wild type's perithecia contained rosettes of asci of similar sizes with eight ascospores contained in each ascus, while the *Δ10943* mutant had some asci that contain fewer than eight ascospores. Moreover, while ascospores inside perithecia produced by wild-type strain PH-1 did not germinate, mutant ascospores had germinated inside perithecia by 12 dpf under the same condition (Fig. 10C). More interesting, only one end of mutant ascospores germinated with germ tubes, while the other end did not germinate inside perithecia (Fig. 10D). More importantly, germination was not observed with ascospores that were still inside intact asci of the mutant.

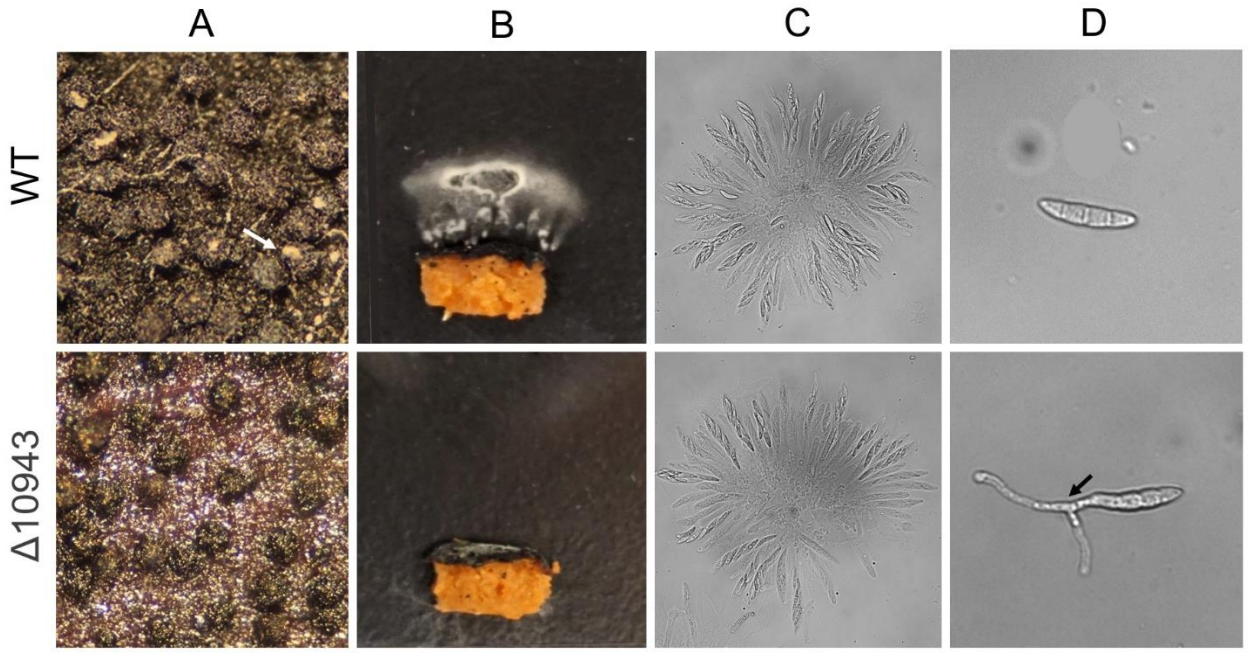

Figure 12. The *Δ10943* mutant was defective in late sexual-stage development. **A,** Perithecia of the wild-type PH-1 (WT) and *Δ10943* mutant were examined at 8 dpf. Cirrhi were only detected in the wild-type strain. Arrows point to cirrhi. **B,** Ascospore discharge from perithecia was examined on 5 mm^3 agar blocks from mating plates with perithecia of the same set of strains. Whitish accumulation appears as evidence for ascospore-discharge capability of perithecia after incubation for 24 h. **C,** Morphology of asci rosette from the same set of strains. **D,** Ascospore inside perithecia from labeled strain at 12 dpf. Arrows mark the germ tube in ascospore in the mutant.

3.6 The Δ10943-3'UTR complemented Δ10943 mutant

Typically, the 3'-untranslated region (3'-UTR) and terminator sequence (described as the 3'-end sequence) are not critical for the recovery of a gene's function in complementation assays with *F. graminearum* (Liu *et al*., 2015; Zhang *et al*., 2017). Certainly, in hygromycin phosphotransferase, a widely used genetic marker, its cassette for transformation selection does not contain the 3′-end sequence (Carroll *et al*., 1994; Catlett *et al*., 2003). Thus, the 10943-GFP fusion construct that had RP27 promoter, but lacked the 3′-UTR, was first amplified and transformed in to the *Δ10943* protoplasts. The resulting *Δ10943* /10943 -GFP transformants remained defective in late sexual development. They were unable to discharge ascospores and produced germinating ascospores, showing unsuccessful complementation of *Δ10943* using this GFP fusion construct. Since the fusion with GFP may disrupt the gene's function, a 10943-fragment containing its native promoter, open reading frame (ORF), and 3′-UTR (lacking the terminator) were constructed and then transformed into the *Δ10943* mutant. All the resulting *Δ10943*/10943WT-GFP transformants were normal in hyphal growth and conidiation. The perithecia formed by the *Δ10943*/10943-3'UTR transformants formed some ascospore cirrhi and there were 8 ascospores per asci (Fig, 11), indicating the *FGSG_10943* deletion mutant is complemented by the insertion of Δ10943-3'UTR construct.

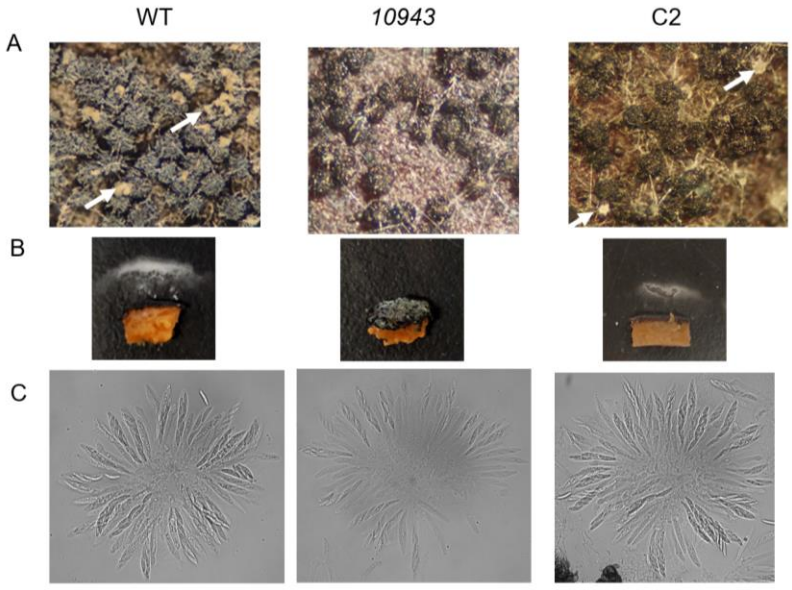

Figure 13. The complemented strain (C2) partially complemented the defect of *Δ10943*-deletion mutant during sexual reproduction. The arrows in WT and C2 point toward cirrhi.

CHAPTER 4. CONCLUSION

All six selected candidate genes were identified by analyzing the LC-MS/MS results, and each is specifically up-regulated during sexual development in *F. graminearum.* All of them were successfully knocked out using targeted gene deletion by the split-marker approach, and each single deletion mutant was confirmed by PCR. Deletion mutants of four candidate genes had no effect on vegetative morphology and growth but have defect in ascospore discharge. One of them, the *FGSG_10943* deletion mutant has ascospores germinating inside perithecia at later stages of sexual development. My or the results suggest that these selected sexual-stage specific candidate genes: *FGSG_10943, FGSG_07774, FGSG_04494* and *FGSG_05922* are dispensable for the vegetative stages but involved in the ascospore discharge of *F. graminearum,* and *FGSG_10943* is not essential but contributed to the sexual development and A-to-I mRNA editing in *F. graminearum.*

Sexual reproduction serves a crucial role in the life cycle of *F. graminearum*. When checking the sexual reproduction of the deletion mutants, deletion of *FGSG_10943* resulted in defects in ascospore germination and release during this important stage. Since *FGSG_10943* deletion mutants were not able to discharge ascospores, and this gene is not well conserved among many families in Sordariomycetes, it is possible that FGSG_10943 is functionally related to the physical ejection of ascospores from asci and its orthologs may have evolved only in some orders of Sordariomycetes, including Hypocreales and Glomerellales. This defect is similar to that of the *FgAMD1, FgKIN1, gea1* mutants (Cao *et al.*, 2017, Luo *et al.*, 2014, Son *et al.*, 2013). Although its exact function is not clear, *FGSG_10943* appears to have an important role in ascospore discharge, and auto-inhibition of ascospore germination inside perithecia in later development stages. It would be intriguing to further confirm the interaction with FgTad2 and FgTad3 during development of sexual reproduction by Co-immunoprecipitation and Yeast two-hybrid assay. It will also be interesting to learn if other genes display expression pattern changes in mutant perithecia by RNA-seq analysis.

Normally, ascospores inside perithecia of *F. graminearum* do not germinate until they are released and land on plant surfaces or growth media. Nevertheless, it was observed that the *FGSG_10943* deletion mutant ascospores germinated from one end and created bundles of ascospores with long germ tubes tangled together inside 2-week-old perithecia. This defect is

similar to the *fgkin1* and *fgamd1* mutants, in which ascospore germination after the breakdown of the ascus wall might be responsible for its defect in cirrhus formation and ascospore discharge. The mechanism behind auto-inhibition of ascospore germination inside perithecia of Sordariomycetes, however, has not yet been elucidated. Further, the broader biological and ecological significance of this trait are not yet fully understood.

In summary, I have (1) identified candidate co-factor genes that can interact with FgTad2 and FgTad3 to mediate A-to-I mRNA editing and (2) provided experimentally useful deletion mutant strains of candidate genes, and (3) determined these candidate genes may have important roles during sexual reproduction in *F. graminerum*. The questions that remain are: (1) does the protein encoded by *FGSG_10943* interact with FgTad2 and TAD3 during Co-IP assay and Yeast Two-hybrid, (2) if there is an interaction does this result in changes in A-to-I mRNA editing events, and in turn (3) would there be defect in the sexual development stages of *F. graminearum*. Answering some of these questions would provide insight into the relationship and importance of FgTad2 and FGTad3 and their interacting co-factors to the A-to-I mRNA editing in filamentous fungi.

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