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A Novel Transcriptional Factor Important for Pathogenesis and Ascosporogenesis in *Fusarium graminearum*

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Fusarium head blight or scab caused by *Fusarium graminearum* is an important disease of wheat and barley. The pathogen not only causes severe yield losses but also contaminates infested grains with mycotoxins. In a previous study, we identified several pathogenicity mutants by random insertional mutagenesis. One of these mutants was disrupted in the *ZIF1* gene, which encodes a b-ZIP transcription factor unique to filamentous ascomycetes. The Δ*zif1* mutant generated by gene replacement was significantly reduced in deoxynivalenol (DON) production and virulence on flowering wheat heads. It was defective in spreading from inoculated florets to the rachis and other spikelets. Deletion of the *ZIF1* ortholog *MoZIF1* in the rice blast fungus also caused reductions in virulence and in invasive growth. In addition, the Δ*zif1* mutant is defective in sexual reproduction. Although it had normal male fertility, when selfed or mated as the female in outcrosses, the Δ*zif1* mutant produced small, pigmented perithecia that were sterile (lack of asci and ascospores), suggesting a female-specific role for *ZIF1* during fertilization or ascus development. Similar female-specific defects in sexual reproduction were observed in the Δ*Mozif1* mutant. When mated as the female, the Δ*Mozif1* perithecia failed to develop long necks and asci or ascospores. The *ZIF1* gene is well conserved in filamentous ascomycetes, particularly in the b-ZIP domain, which is essential for its function. Expression of *ZIF1* in *Magnaporthe oryzae* complemented the defects of the Δ*Mozif1* mutant. These results indicate that this b-ZIP transcription factor is functionally conserved in these two fungal pathogens for plant infection and sexual reproduction.

Fusarium graminearum (teleomorph *Gibberella zeae*) infects wheat, barley, maize, and other crop plants (Bai and Shaner 2004; Goswami and Kistler 2004). In North America, it is the predominant species causing scab or head blight of wheat and barley. In addition to yield losses caused by this pathogen, infested cereals often are contaminated with deoxynivalenol (DON) and other toxins that are harmful to human and animals (Walter et al. 2010). *F. graminearum* overwinters in plant debris and infects susceptible floral tissues with ascospores as the primary inoculum (Schmale et al. 2005; Trail et al. 2002,

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2005). Infection of wheat heads is initiated when ascospores are deposited on or inside flowering spikelets, which can occur from the beginning of anthesis to the dough stage of kernel development. The fungus then spreads from the infected floret to the rachis and causes severe damage under conducive environmental conditions.

The first pathogenicity factor characterized in *F. graminearum* is the trichodiene synthase gene, *TRI5*, which is essential for DON production and spreading within colonized wheat heads (Bai et al. 2002; Proctor et al. 1995). In the past two decades, the trichothecene biosynthesis pathway has been extensively studied in *Fusarium* spp. In *F. graminearum*, most genes encoding regulatory or transporter proteins and enzymes for metabolic steps in DON biosynthesis have been well characterized, including *TRI3, TRI4, TRI5, TRI6, TRI7, TRI10,* and *TRI101* (Alexander et al. 2009; Desjardins, 2003; Seong et al. 2009). *TRI6* and *TRI10* are pathway-specific transcriptional regulators of the *TRI* clusters. Although it is not clear what plant factors stimulate the fungus to synthesize trichothecenes, there are studies on environmental conditions and chemical compounds that induce DON production in axenic cultures (Audenaert et al. 2010; Gardiner et al. 2009ab).

In the past few years, a variety of genetic and genomic resources have been accumulated for *F. graminearum*, including 10× coverage of the genome sequence (Cuomo et al. 2007) and a whole-genome Affymetrix GeneChip (Guldener et al. 2006). The availability of these resources has significantly advanced or benefited genetic studies on molecular mechanisms regulating plant infection and fungus–plant interactions in this important pathogen (Goswami and Kistler 2004; Trail 2009; Walter et al. 2010). To date, genes with various biological or biochemical functions have been shown to be important for plant infection and spreading in *F. graminearum*, such as the Cid1 type C cyclin (Zhou et al. 2010), FgPtc1 type 2C protein phosphatase (Jiang et al. 2010), chitin synthases (Kim et al. 2009), and topoisomerase (Baldwin et al. 2010). Components of and genes regulated by conserved signaling pathways are among the best-studied pathogenicity factors in *F. graminearum* (Hou et al. 2002; Jenczmionka and Schafer 2005; Jenczmionka et al. 2003; Urban et al. 2003; Yu et al. 2008). A number of genes related to primary metabolism, including *CBL1, MSY1, HMR1, NPS6,* and *CPS1*, also have been shown to be required for virulence (Lee et al. 2009a and b; Oide et al. 2006; Seong et al. 2005, 2006).

In a previous study, we used the restriction enzyme-mediated integration (REMI) insertional mutagenesis approach to generate mutants with reduced or lost virulence (Seong et al. 2005). One of these mutants was disrupted in the *ZIF1* gene

that encodes a putative b-ZIP transcription factor unique to filamentous ascomycetes. In this study, we generated and characterized the Δ*zif1* mutant. Like REMI mutant M7, the Δ*zif1* mutant was significantly reduced in DON production and virulence on flowering wheat heads. It also displayed female-specific defects in sexual reproduction. Similar defects in plant infection and female fertility were observed in the *Magnaporthe oryzae* mutant deleted from the *ZIF1* ortholog. Zif1 is well conserved in the class Sordariomycetes but none of its orthologs have been characterized in other fungi. Results from this study indicate that *ZIF1* may be conserved in fungal pathogens for female fertility and invasive growth after penetration or initial colonization.

RESULTS

The *ZIF1* gene is disrupted in REMI mutant M7.

REMI mutant M7 was generated in a previous study by transforming the *Eco*RI-digested plasmid vector pKY37 into the wild-type strain PH-1 (Seong et al. 2005). It had reduced virulence on flowering wheat heads. The conventional plasmid rescue approach was used to identify the flanking sequence of the integrated pKY37. Plasmid pMS7 containing a 3.8-kb genomic fragment (Fig. 1A) was isolated and sequenced. Sequencing analysis revealed that the transforming vector pKY37 was inserted in the *Eco*RI site located in the predicted gene FGSG_01555.3 (Fig. 1A). This gene, named *ZIF1* (for bZIP transcription factor 1) in this study, encodes a 568-amino acid protein with a basic-leucine zipper (bZIP) domain. It has no distinct homolog in the budding or fission yeast but is conserved in filamentous ascomycetes (Supplementary Fig. 1). However, none of the *ZIF1* orthologs have been functionally characterized in other fungi. Except for the bZIP domain, there is no other known domain or motif in the Zif1 protein. Nevertheless, the glutamine-rich regions adjacent to the bZIP domain are conserved in different fungi and may function in mediating protein–protein interactions and transcriptional activation (Escher et al. 2000).

Deletion of the *ZIF1* gene has no effect on conidiation or conidium morphology.

Because the disrupted *ZIF1* in mutant M7 may be not a null allele, we generated the *ZIF1* gene replacement construct (Fig. 1A) by the split-marker approach (Catlett et al. 2003). After transforming protoplasts of PH-1, putative Δ*zif1* mutants were identified by polymerase chain reaction (PCR) and confirmed by Southern blot hybridization (Fig. 1B). When hybridized with a *ZIF1* fragment amplified with primers BTN-1F and BTN-2R, the wild-type and ectopic transformants had the 6.3-kb *Pst*I band. The Δ*zif1* mutant ZP10 had no hybridization signals (Fig. 1B). Similar to REMI mutant M7, ZP10 had a slight reduction in the growth rate (Table 1) but it had no defects in conidiation, conidium morphology, and germination.

The Δ*zif1* mutant has reduced virulence.

In infection assays with flowering wheat heads, the Δ*zif1* mutant usually only caused typical scab symptoms on the inoculated florets 14 days postinoculation (dpi). Symptoms rarely spread to nearby spikelets on the same wheat heads (Fig. 2A). The average disease index of the Δ*zif1* mutant was approximately 1 (Table 1). Under the same conditions, the wild type and the ectopic transformant spread from the inoculated florets to nearby spikelets. The disease index for the wild-type and ectopic transformant was >6 (Table 1), indicating that the Δ*zif1* mutant was significantly reduced in virulence on wheat.

Because *F. graminearum* also is a corn pathogen, we assayed the virulence of mutants M7 and ZP10 on corn stalks and silks.

In plants inoculated with the wild type and an ectopic transformant, areas of stalk rot often extended from the inoculation site to or close to the nodes above and below 14 dpi (Fig. 2B). In contrast, Δ*zif1* mutants caused only limited stalk rot in inoculated corn piths (Fig. 2B). Lack of extensive spreading in corn pith tissues indicates that *ZIF1* is a virulence factor for stalk rot. Mutants M7 and ZP10 also were significantly reduced in virulence in corn silk infection assays (data not shown).

Similar defects of mutants M7 and ZP10 in growth and plant infection suggested that disruption or deletion of the *ZIF1* gene was responsible for the observed phenotypes. To further confirm this observation, we reintroduced the full-length *ZIF1* gene into protoplasts of ZP10. Bcom9 (Table 2) was one of the resulting geneticin-resistant transformants that contained a single copy of the transforming complementation construct (data not shown). Bcom9 had the wild-type growth rate and virulence (Table 1; Fig. 2A), indicating that the reintroduced *ZIF1* allele complemented the Δ*zif1* mutant. We also generated the *ZIF1*^ΔbZIP allele deleted from the entire b-ZIP domain (amino acids 276 to 339) and transformed it into ZP10. Several transformants, including YP6 and YP8, were confirmed by Southern analysis to contain the transforming *ZIF1*^ΔbZIP construct. However, these *ZIF1*^ΔbZIP transformants had phenotypes similar to that of the original Δ*zif1* mutant, indicating that the b-ZIP domain is essential for *ZIF1* function.

Ascus and ascospore formation are blocked in the Δ*zif1* mutant.

We assayed mating and ascospore production in the Δ*zif1* mutant because ascospores are the primary inoculum in *F. graminearum*. At 7 days after fertilization, the Δ*zif1* mutant formed smaller and fewer perithecia than the wild type on carrot agar plates (Fig. 3A). When examined under a microscope, abundant asci and ascospores were visible in perithecia formed by the wild type but not in Δ*zif1* mutant perithecia (Fig. 3A).
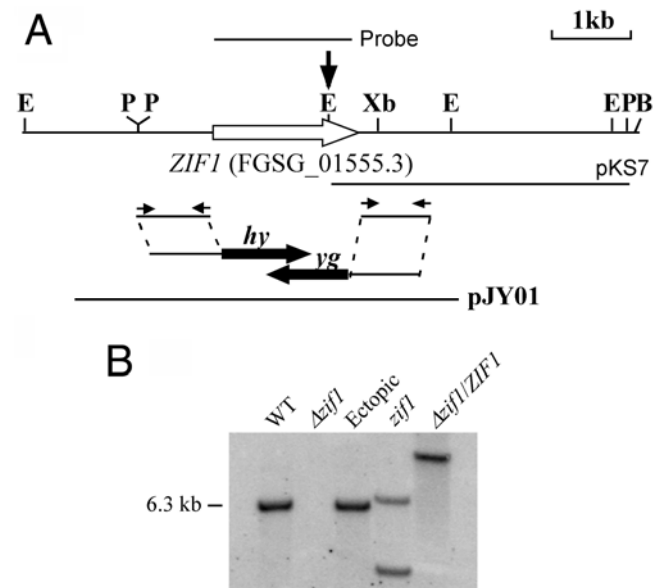

Fig. 1. *ZIF1* gene and generation of the Δ*zif1* mutant. **A,** Schematic drawing of the *ZIF1* locus. The insertion site of transforming vector in the *ZIF1* gene was marked with a vertical black arrow. Plasmid pKS7 was recovered by plasmid rescue from mutant M7. Plasmid pJY01 was used for complementation assays. Smaller arrows represented primers used for generating the Δ*zif1* mutant. B, *Bam*HI; E, *Eco*RI; P, *Pst*I; Xb, *Xba*I. **B,** Southern blot analysis of *Pst*I-digested genomic DNA of the wild type (WT) PH-1, Δ*zif1* mutant ZP10, ectopic transformant ZP4, REMI mutant M7 (*zif1*), and complemented transformant Bcom9. The probe was amplified with primers bZF1F and bZF2R.

When matured, the wild-type perithecia oozed out ascospores and formed cirrhi at the ostiole (Fig. 3B). Perithecia produced by the Δ*zif1* mutant failed to enlarge and form cirrhi even after incubation for 21 days or longer (Fig. 3B).

When examined by transmission electronic microscopy, ascospores and asci were visible in the perithecia formed by PH-1 7 days after fertilization. In contrast, no ascospores were observed in perithecia of the Δ*zif1* mutant (Fig. 3C). These data indicate that, although it is dispensable for protoperithecium or perithecium formation, *ZIF1* must play a critical role in the late events of ascocarp development and formation of asci and ascospores in selfing crosses (Δ*zif1* × Δ*zif1*).

ZIF1 is dispensable for male fertility in outcrosses.

Although it is a homothallic fungus, *F. graminearum* also can outcross. When the *zif1* mutant ZP10 (*hyg*R) was crossed with a *nit1* mutant 11622 that is resistant to potassium chlorate (Hou et al. 2002), mature perithecia with cirrhi were formed on the 11622 side of the confrontation zone 2 to 3 weeks after fertilization. By screening through cirrhi from 50 perithecia, we identified 2 perithecia that produced recombinant *nit1 hyg*R progeny resistant to both potassium chlorate and hygromycin at 150 μg/ml. From these two outcrossing perithecia, we isolated 35 hygromycin-resistant progeny. All of them displayed characteristic phenotypes of the Δ*zif1* mutant, including reduced virulence and defects in sexual reproduction (data not shown). When grown on potassium chlorate-amended complete medium (CM), 18 progeny failed to grow and, therefore, were determined to be *NIT1*, whereas 17 progeny grew on the medium and, therefore, were determined to be *nit1*. These data indicated a 1:1 segregation at the *NIT1* locus. Therefore, we conclude that the observed defects of the Δ*zif1* mutant but not *nit1* co-segregated with the *hph* marker. Because the Δ*zif1* mutant was sterile in selfing crosses, the fertility of the *ZIF1* ♀ × Δ*zif1* ♂ cross indicates that *ZIF1* is dispensable for male fertility but plays a critical role in female fertility.

DON production is reduced in the Δ*zif1* mutant.

Because of its importance in *F. graminearum* pathogenesis, DON production was measured in diseased wheat kernels from inoculated wheat florets 14 dpi. On average, kernels from wild-type-inoculated heads contained DON at 500 ppm, whereas
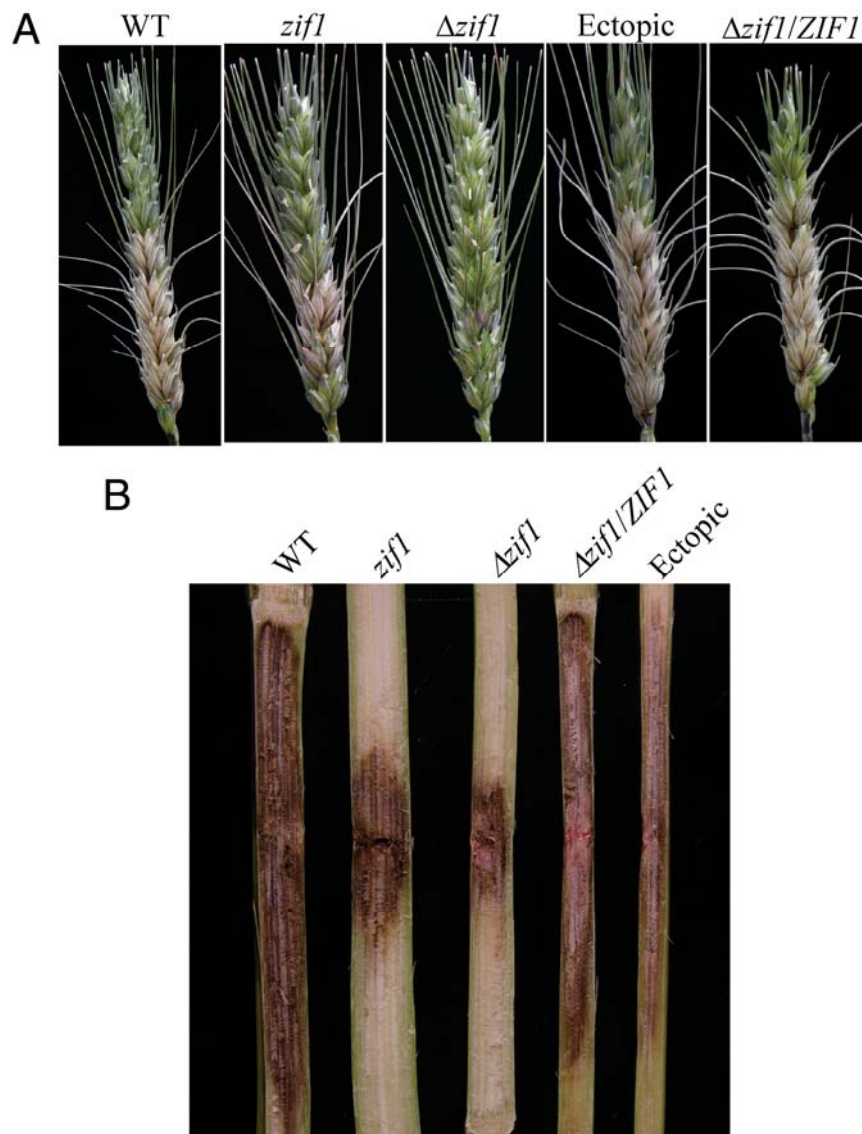

Fig. 2. Wheat head and corn stalk rot infection assays. **A,** Flowering wheat heads were inoculated with conidia from the wild type (WT) PH-1, REMI mutant M7 (*zif1*), Δ*zif1* mutant ZP10, ectopic transformant ZP4, and complemented strain Bcom9. Typical wheat heads were photographed 14 days postinoculation (dpi). **B,** Corn stalks were inoculated with toothpicks carrying conidia from PH-1, ZP10, ZP4, and Bcom9. Infected corn stalks were split longitudinally at the inoculation sites and examined 14 dpi.

kernels from Δ*zif1* mutant-inoculated heads contained fourfold less DON (Table 1). The complemented transformant Bcom9 produced similar amount of DON in infested wheat kernels (Table 1).

We then assayed DON production in axenic cultures as described (Gardiner et al. 2009a). In agmatine cultures, the Δ*zif1* mutant was reduced more than 15-fold in DON production compared with the wild type (Table 1). DON production was increased in the complemented strain Bcom9 (Table 1), suggesting the involvement of *ZIF1* in regulating trichothecene synthesis. To further confirm this observation, we assayed the expression of several *TRI* genes (Kimura et al. 2007) in agmatine cultures. In the Δ*zif1* mutant, the expression level of *TRI5* and *TRI6* was reduced more than sixfold (Fig. 4A). *TRI5* encodes the key enzyme for DON synthesis and *TRI6* encodes a major transcriptional regulator of DON synthesis (Seong et al. 2009). For the other *TRI* genes assayed, *TRI4* and *TRI12* had no obvious changes but *TRI8* and *TRI14* also had reduced expression levels in the Δ*zif1* mutant (Fig. 4A). Interestingly, *TRI3* expression was increased approximately twofold in the Δ*zif1* mutant. These data confirmed that deletion of *ZIF1* reduced the expression of the key structural and regulatory genes of DON synthesis. However, unlike the *TRI*-cluster-specific transcription factor Tri6 (Seong et al. 2009), Zif1 appears to have different regulatory effects on various *TRI* genes.

Expression of the *ZIF1* gene.

The *ZIF1*-eGFP (enhanced green fluorescent protein) fusion construct under its native promoter control was transformed into the Δ*zif1* mutant ZP10. The resulting transformant NP18 was normal in growth, sexual reproduction, and virulence (data not shown), indicating that the P_{ZIF1}-*ZIF1*-EGFP construct complemented the defects of the Δ*zif1* mutant. However, we failed to observe GFP signals in vegetative hyphae, conidia, and young perithecia. Zif1-eGFP fusion proteins may be relatively unstable and only transiently localized to the nucleus.

We then assayed *ZIF1* expression by quantitative reverse-transcription (qRT)-PCR assays with RNA isolated from different culture conditions or plant infection stages. The expression level of *ZIF1* was relatively low in freshly harvested conidia and young germlings (10 h) but increased in mature vegetative hyphae (Fig. 4B). The highest expression level was in developing perithecia (5 days postfertilization). These results were consistent with the defects of Δ*zif1* mutant in ascospore production. According to microarray data deposited in the PLEXdb database, *ZIF1* also is upregulated during barley infection (Boddu et al. 2006), indicating that it may be involved in plant infection.

ZIF1 is important for spreading to the rachis.

To visualize fungal growth in infected plant tissues, we transformed the RP27-GFP construct pRM7 (Mehrabi et al. 2008) into protoplasts of PH-1 and ZP10. The resulting transformants, RM7PH1 and RM7zp10 (Table 1), had strong GFP signals in the cytoplasm of conidia and vegetative hyphae. In wheat heads inoculated with RM7PH1, extensive fungal growth was visible in the rachis tissues next to the inoculated site 5 dpi (Fig. 5). Under the same conditions, hyphal growth of the Δ*zif1* mutant failed to spread from the inoculated floret to the rachis. GFP signals were only observed at the site (likely the scission zone) where the inoculated floret was removed (Fig. 5). These data indicate that the Δ*zif1* mutant colonized the inoculated floret but its spread to the rachis and other spikelets was restricted, which may explain the reduced virulence of the Δ*zif1* mutant (Table 1).

The Δ*zif1* mutant was hypersensitive to reactive oxygen species.

When assayed for growth on CM, the Δ*zif1* mutant had no obvious changes in sensitivities to 1 M sorbitol, 0.7% NaCl,

Table 1. Growth, conidiation, deoxynivalenol (DON) production, and virulence of *Fusarium graminearum* strains

| Strain | Growth rate (mm/day)[a] | Conidiation (×10⁵ spores/ml) | DON (ppm) | | Disease index[d] |
			Infected kernels[b]	Axenic cultures[c]	
PH-1	11.5 ± 0.5	42.1 ± 0.9	505.0 ± 249.3	2,410.1 ± 100.0	6.8 ± 1.2
ZP10	10.5 ± 0.0	39.5 ± 3.9	117.0 ± 68.4	137.7 ± 61.4	0.6 ± 0.2
Bcom9	12.0 ± 0.5	26.3 ± 3.2	530.0 ± 86.1	1,026.8 ± 233.3	5.9 ± 1.9
M7	9.5 ± 0.5	62.2 ± 4.6	205.3 ± 46.1	483.8 ± 123.8	1.6 ± 1.4

[a] Average and standard deviation of daily extension in colony radius during 5 days of growth on V8 agar in Φ15-cm petri plates at 25°C.
[b] DON production in infected wheat kernels harvested from inoculated wheat heads 14 days postinoculation. Mean and standard deviation were calculated from four independent infection assays.
[c] DON production in the defined medium with 0.2% agmatine.
[d] Mean and standard deviation of diseased spikelets per wheat head.

Table 2. *Fusarium graminearum* and *Magnaporthe oryzae* strains used in this study

Strain	Brief description	Reference
F. graminearum		
PH-1	Wild-type	Trail et al. 2003
11622	*nit1* mutant of PH-1	Hou et al. 2002
M7	REMI mutant disrupted in the ZIF1 gene	Seong et al. 2005
ZP10	Δ*zif1* gene replacement mutant	This study
ZP4	An ectopic knockout mutant	This study
Bcom9	Complemented transformant of ZP10 (Δ*zif1/ZIF1*)	This study
NP18	Transformant of ZP10 expressing P_{ZIF1}-*ZIF1*-eGFP	This study
YP6	Transformant of ZP10 expressing the *ZIF1*^ΔbZIP allele	This study
YP8	Transformant of ZP10 expressing the *ZIF1*^ΔbZIP allele	This study
RM7PH1	Transformant of PH-1 expressing P_{RP27}-eGFP (pRM7)	This study
RM7zp10	Transformant of ZP10 expressing P_{RP27}-eGFP (pRM7)	This study
M. oryzae		
Guy11	Wild-type (MAT1-2, avr-Pita)	Chao and Ellingboe 1991
70-15	Wild-type (MAT1-1, AVR-Pita)	Chao and Ellingboe 1991
bzip24	ΔMozif1 mutant of 70-15 (MAT1-1)	This study
MFC1	Transformant of bzip24 expressing P_{ZIF1}-*ZIF1*-eGFP	This study

Congo red at 200 μg/ml, or 0.01% sodium dodecyl sulfate (SDS), indicating that *ZIF1* is not involved in response to hyperosmotic and cell wall stresses. However, deletion of *ZIF1* resulted in increased sensitivity to H_2O_2. In the presence of 0.08% H_2O_2, the Δ*zif1* mutant had no visible growth but the wild type and complemented transformants still formed colonies (Supplementary Fig. 2).

The *ZIF1* ortholog is important for virulence in *M. oryzae*.

To determine whether the function of *ZIF1* in plant infection is conserved in other fungi, we generated the gene replacement mutant of MGG_03288.6, which was orthologous to *ZIF1* (named *MoZIF1* in this study). The Δ*Mozif1* mutant (Supplementary Fig. 3) was normal in vegetative growth, conidiation, and appressorium formation (data not shown). However, its virulence was reduced in infection assays with rice seedlings (Fig. 6A). When assayed for appressorial penetration with onion epidermal cells, the Δ*Mozif1* mutant had reduced growth of infectious hyphae compared with the wild type (Fig. 6B). These results indicate that *MoZIF1* is important for invasive growth and lesion development in *M. oryzae*.
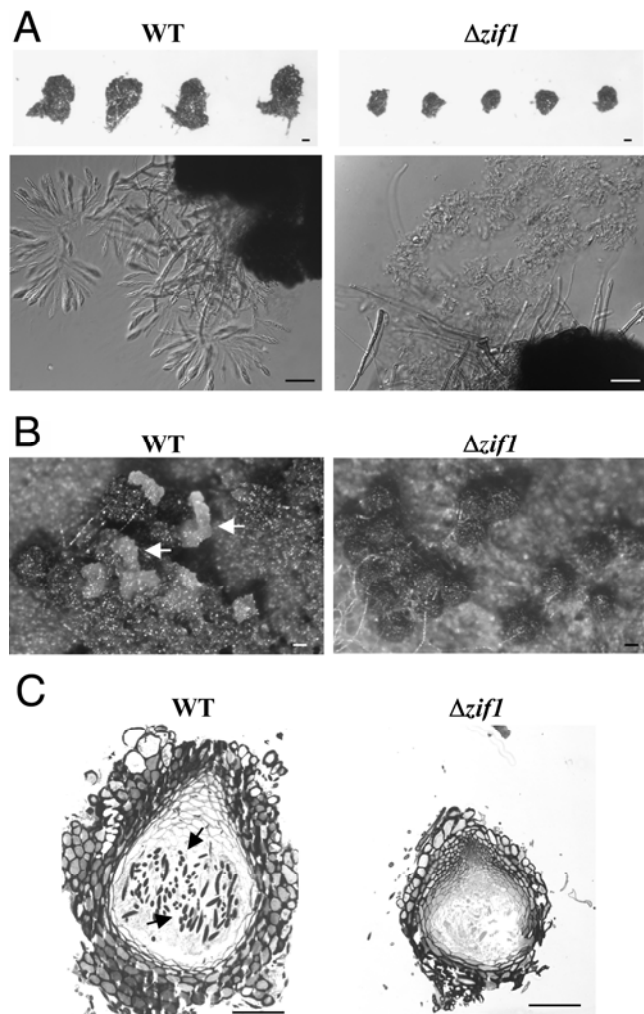

Fig. 3. The Δ*zif1* mutant was defective in sexual reproduction. **A,** Representative perithecia of the wild type (WT) PH-1 and Δ*zif1* mutant (ZP10) were examined under a dissecting (upper panels) or compound (lower panels) microscope 10 days after fertilization. Fascicles of asci were observed in PH-1 but not in ZP10. **B,** Ascospores oozed from mature perithecia as cirrhi (marked with arrows) in the WT but not mutant perithecia 14 days after fertilization. **C,** Perithecia of PH-1 and ZP10 were examined by transmission electron microscopy. Arrows point to asci and ascospores. Bar = 50 μm.

MoZIF1 also is essential for female fertility.

In *M. oryzae*, perithecia are normally embedded within the substrate but develop long beaks that protrude from the surface of the substrate and through which ascospores are released.
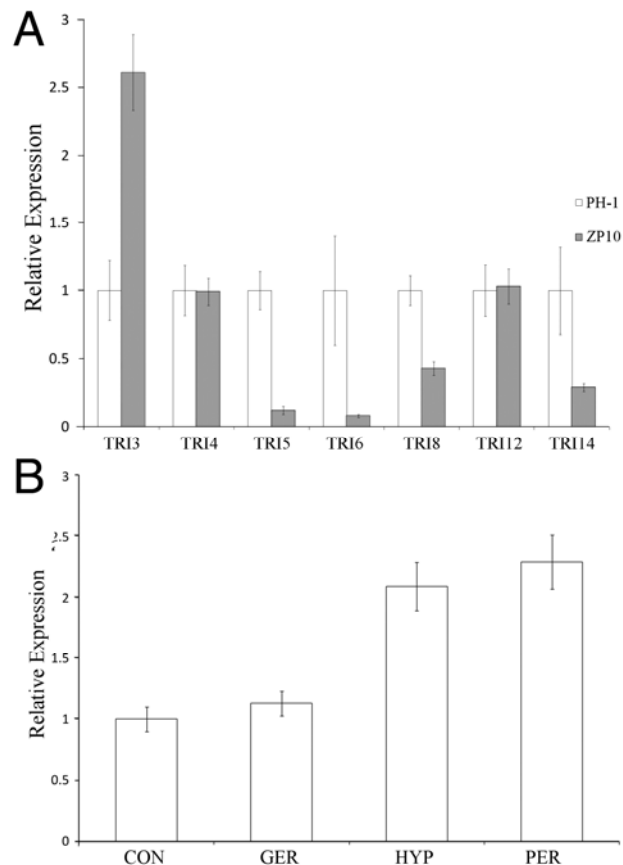

Fig. 4. Quantitative reverse-transcription polymerase chain reaction analyses of the *TRI* and *ZIF1* genes. **A,** Expression levels of selected *TRI* genes in the wild type (WT) and Δ*zif1* mutant. RNA was isolated from agmatine cultures. For each gene, its relative expression in the WT was normalized to the value 1. **B,** Expression of *ZIF1* in freshly harvested conidia (5-day-old CMC cultures), germlings (10 h in complete medium [CM]), vegetative hyphae (36 h CM cultures), and developing perithecia (5 days after fertilization).
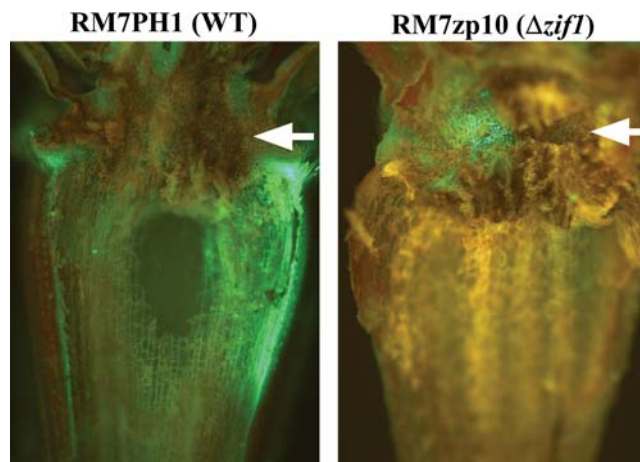

Fig. 5. Rachises from wheat heads inoculated with strains RM7PH1 (wild type [WT]) and RM7ZP10 (Δ*zif1*) were dissected and examined under an epifluorescence microscope 5 days postinoculation. Arrows marked the site where the inoculated spikelet was removed. Hyphal growth represented as green fluorescent protein signals was visible in rachis tissues from wheat heads inoculated with RM7PH1 but not RM7zip10.

When the ∆Mozif1 mutant (*MAT1-1*) was crossed with the wild-type strain Guy11 (*MAT1-2*), mature perithecia with long beaks protruding from the surface were formed on the Guy11 side of the confrontation zone after incubation for 3 to 4 weeks (Fig. 7A). Ascospore progeny were isolated from these perithecia (Guy11 as the female), suggesting that *MoZIF1* is dispensable for male fertility. However, no perithecia with long necks were observed on the mutant side of the confrontation zone (Fig. 7A). After removing aerial hyphae, immature perithecia were observed in these areas in which the ∆Mozif1 mutant was mated as the female. These perithecia were pigmented but failed to produce long necks (Fig. 7A). Perithecia of the ∆Mozif1 mutant also were smaller than those of the wild type (Fig. 7B), and sterile (Fig. 7C). Therefore, *MoZIF1* is required for late stages of perithecium development and the production of asci and ascospores (Fig. 7B).

Complementation of the ∆Mozif1 mutant with *ZIF1*.

To further test whether *ZIF1* and *MoZIF1* share similar biological functions, we transformed the P_{ZIF1}-*ZIF1*-eGFP fusion into the ∆Mozif1 mutant. Transformant MFC1 (Table 2) was identified by PCR with primers RGF and NGR to contain the *ZIF1*-eGFP construct. In infection assays with rice seedlings, it had increased virulence compared with the ∆Mozif1 mutant. The number of blast lesions formed by MFC1 was similar to that of the wild type (Fig. 6), indicating that the *F. graminearum ZIF1* gene functionally complemented the defects of the *M. oryzae* ∆Mozif1 mutant in plant infection. However, transformant MFC1, like *F. graminearum* NP18 (∆zif1/*ZIF1*-eGFP), lacked detectable GFP signals in conidia, vegetative hyphae, appressoria, and infectious hyphae (data not shown).

Genes regulated by *ZIF1* in liquid cultures.

The *F. graminearum* whole-genome GeneChip (Guldener et al. 2006) was used for microarray analysis. In comparison with the wild type, 214 and 328 genes were down- and upregulated, respectively, more than fivefold in the ∆zif1 mutant (Supplementary Tables 1 and 2). Among the downregulated genes, more than 60% encode hypothetical proteins with unknown biological functions (Supplementary Fig. 4). The second largest Munich Information Center for Protein Sequences (MIPS) category (Mewes et al. 2002) is related to metabolism (20.5%). Of these genes, 20 (9.56%) are involved in secondary metabolism (MIPS subcategory 01.20). Many genes belonging to MIPS category 20 (cellular transport, 12.4%) and 32 (defense and virulence, 7.65%) also were downregulated in the ∆zif1 mutant. Some of them may be involved in plant infection. Among the upregulated genes, approximately 57 and 24% encode hypothetical proteins and proteins involved in metabolism, respectively. In all, 39 genes involved in ribosome biogenesis (subcategory 12.01) have increased expression levels in the ∆zif1 mutant. Only one gene belonging to this subcategory was downregulated.

The gene with the most significantly reduced expression level in mutant ZP10 was FGSG_01767, which is predicted to encode a cytochrome P450 monooxygenase similar to pisatin demethylase of *Nectria haematococca* (George et al. 1998). Its expression also was increased during plant infection (Guldener et al. 2006). If this gene plays a role in detoxifying plant defensive compounds in *F. graminearum*, its downregulation of 230-fold may be related to the reduced virulence of the ∆zif1 mutant. Along with the same line, two other putative cytochrome P450 monooxygenase genes, FGSG_03260 and FGSG_03264, also were downregulated more than fivefold in the mutant. Other significantly downregulated genes with possible functions in plant infection include FGSG_06554 and FGSG_09352, which encode a putative catalase and a membrane protein similar to Pth11 in *M. oryzae* (DeZwaan et al. 1999), respectively.

Interestingly, four of the top six downregulated genes (more than 30-fold reduction) in mutant ZP10 were FGSG_03932 to FGSG_03936. Unfortunately, most of the genes belonging to this putative cluster encode conserved hypothetical proteins, which is not helpful to predict its biological function. Several additional gene clusters were identified among these genes with more than fivefold downregulation in the ∆zif1 mutant (Supplementary Table 3). One of them is the aurofusarin synthesis cluster (Frandsen et al. 2006; Kim et al. 2008), suggesting that *ZIF1* positively regulates aurofusarin biosynthesis in *F. graminearum*. One cluster consists of genes from FGSG_7820 to FGSG_7825. Most of them encode conserved hypothetical proteins unique to filamentous fungi. Some of them may be important for plant infection or sexual reproduction in *F. graminearum*. Interestingly, three downregulated genes, FGSG_01298, FGSG_04894, and FGSG_12978, are homologous to fungal genes involved in self-recognition or vegetative incompatibility.

To verify the microarray data, we selected the following genes for qRT-PCR analysis: FGSG_02087.3 (putative TfdA family oxidoreductase), FGSG_03935 (short-chain alcohol dehydrogenase), FGSG_03936 (succinate semialdehyde dehydrogenase), FGSG_07822 (major facilitator MirA), FGSG_13098 (putative fungal specific transcription factor), FGSG_0393 (Pth11-like receptor), FGSG_01767 (pisatin demethylase), FGSG_03120 (diacylglycerol pyrophosphate phosphatase), FGSG_03710 (NADH-oxidase), FGSG_04587 (WD40-repeat protein), and FGSG_06554 (catalase). All except for FGSG_03710 were confirmed by qRT-PCR to be significantly downregulated in the ∆zif1 mutant (Fig. 8).

DISCUSSION

The *ZIF1* gene disrupted in REMI mutant M7 encodes a relatively large protein (561 amino acids) with only one known functional domain, the b-ZIP domain. Results from BlastP
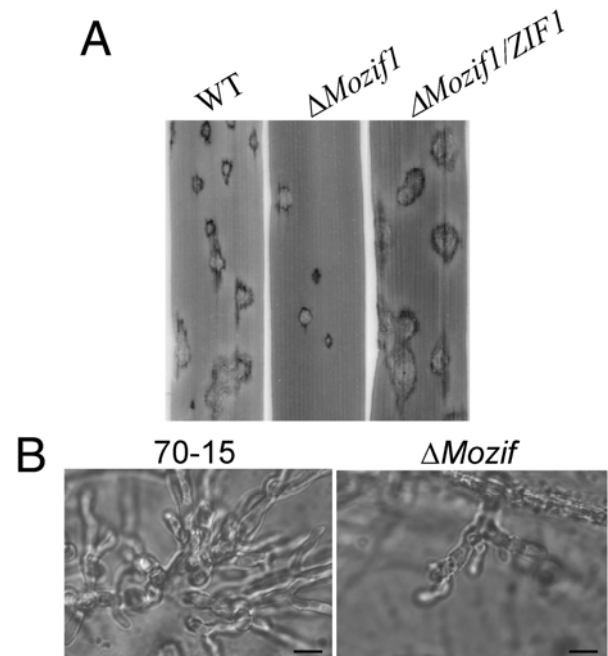

Fig. 6. Infection and penetration assays with the ∆Mozif1 mutant. **A,** Rice leaves sprayed with conidia (1×10^5 conidia/ml) from the wild type (WT) 70-15, ∆Mozif1 mutant, and ∆Mozif1/*ZIF1* complemented transformant MFC1. Typical leaves were photographed 7 days postinoculation. **B,** Penetration assays with onion epidermal cells. In comparison with the WT strain, the ∆Mozif1 mutant had reduced growth of infectious hyphae in penetrated plant cells 48 h postinoculation. Bar = 10 µm.

searches indicate that *ZIF1* is specific to filamentous ascomycetes. Its orthologs are well conserved over the entire protein in the class Sordariomycetes. Surprisingly, one of the maize full-length cDNA clone (GenBank number ACR36092) is highly similar to *ZIF1* (38% identity; e value = 7e^{-70}). Because some fungi, such as *F. verticillioides,* could grow endophytically in corn plants, we used the nucleotide sequence of ACR36092 to search different databases. It has no perfect match with any predicted genes in sequenced fungal genomes but shares the highest similarity (79% identity) with one expressed sequence tag from *Penicillium marneffei* (GenBank number XM_002146056). Therefore, if ACR36092 is from an endophyte present in tissues used for constructing maize cDNA libraries, this fungus is likely a Eurotiomycete that has not been sequenced.

In *F. graminearum*, the disease index of the Δ*zif1* mutant was less than 1, which is consistent with its defects in spreading from inoculated floret to the rachis 5 dpi (Fig. 5). *ZIF1* may play a critical role in invading through the scission zone to the rachis and other spikelets. Because DON is an important virulence factor in *F. graminearum* (Bai et al. 2002), reduced production of DON in the Δ*zif1* mutant may be related to its defects in plant infection. In the foliar pathogen *M. oryzae*, the Δ*Mozif1* mutant penetrated plant cells but had reduced invasive growth, which may be directly responsible for fewer and generally smaller lesions formed by the mutant. Although *F. graminearum* and *M. oryzae* differ in infection mechanisms, the *ZIF1* orthologs are conserved in these two fungi for plant infection processes after the initial colonization. It is possible
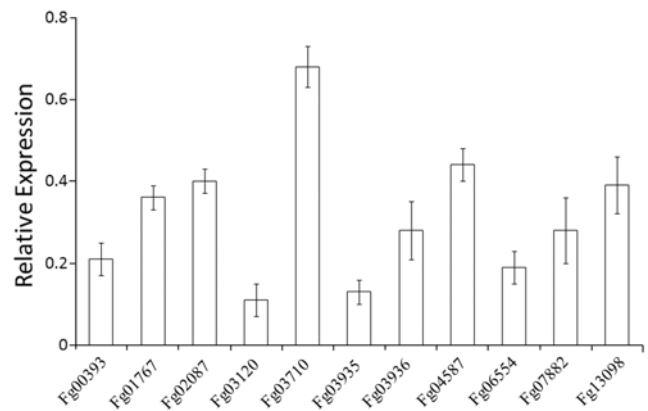

Fig. 8. Quantitative reverse-transcription polymerase chain reaction assays for the expression of selected genes in the Δ*zif1* mutant. After normalizing with *FgACT1*, the relative expression level of each gene in the Δ*zif1* mutant was presented as fold changes in comparison with that of the wild type (normalized to the value 1). Mean and standard deviation were calculated with data from three biological replicates.
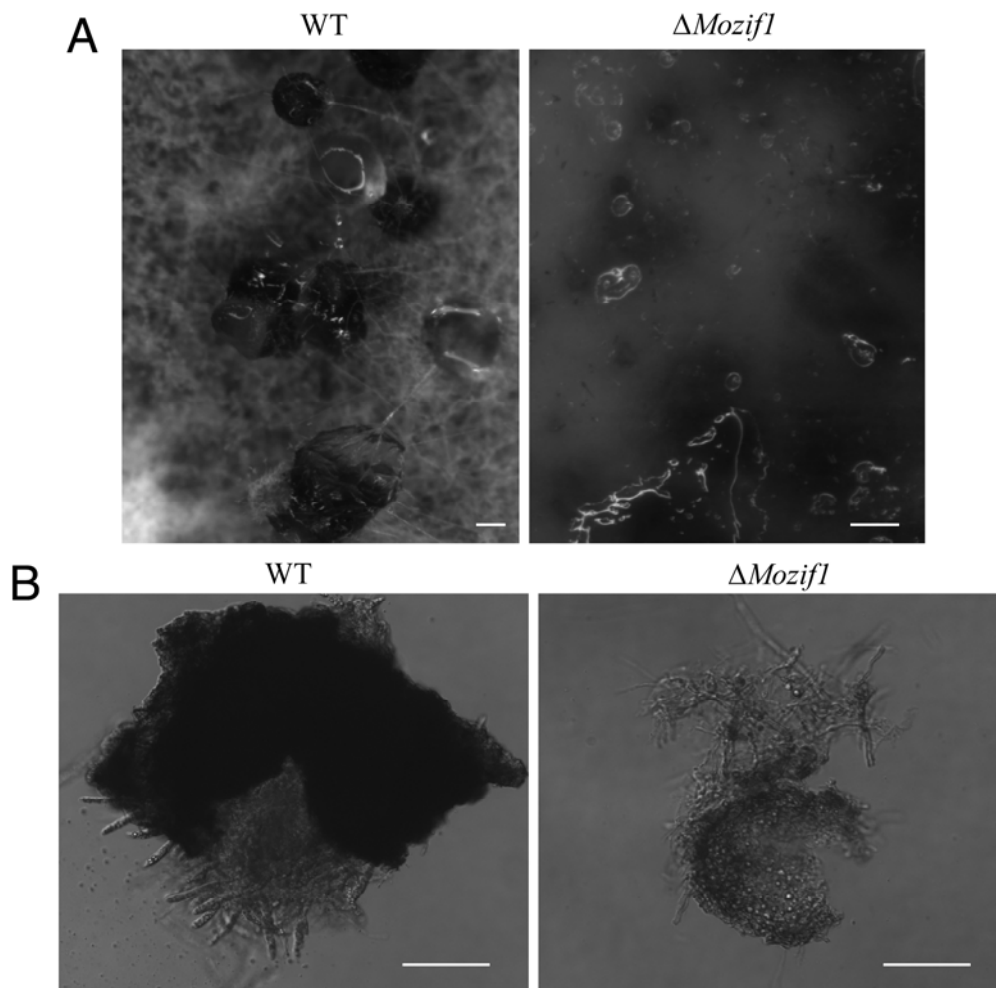

Fig. 7. Defects of the Δ*Mozif1* mutant in sexual reproduction when crossed with the wild-type (WT) strain Guy11. **A,** Close-up view of 4-week-old mating cultures on the Guy11 (WT, left) or Δ*Mozif1* mutant (right) side of the confrontation zone where each strain was mated as the female. Long beaks produced by WT perithecia protruded from the colony surface. For the mutant culture, aerial hyphae were removed to observe smaller perithecia that were pigmented but blocked in the beak formation inside the medium. **B,** Typical perithecia from mating zones where Guy11 or the Δ*Mozif1* mutant was mated as the female. Asci and ascospores were only visible in WT perithecia. Bar = 50 μm.

that the Zif1 transcription factor regulates subsets of genes that are important for fungus–plant interactions during invasive growth or overcoming plant defensive compounds, such as reactive oxygen species (ROS) and phytoalexins. The Δ*zif1* mutant had increased sensitivity to H_2O_2 and a reduced expression level of one putative catalase gene. In yeast, Yap1 is a b-ZIP transcription factor regulating responses to oxidative stress (Delaunay et al. 2000). The Yap1 ortholog FGSG_08800 has not been characterized in *F. graminearum*. In *Ustilago maydis* and *Alternaria alternata*, Yap1 orthologs are important for plant infection (Lin et al. 2009; Molina and Kahmann 2007). However, other transcription factors may be responsible for regulating genes important for detoxifying ROS in planta because the Yap1 ortholog is dispensable for virulence in *Cochliobolus heterostrophus* (Lev et al. 2005).

In infection assays with both wheat heads and corn stalks, the REMI mutant M7 appeared to be more virulent than *zif1* mutant ZP10 (Fig. 2). DON production also was higher in mutant M7 than in ZP10 (Table 1). Because the insertion site of pYK37 was close to the C terminus, the b-ZIP domain and 90% of the amino acid sequence of the Zif1 protein are not affected. It is likely that the disrupted *ZIF1* allele has some residual activities (not a null allele). In the complemented transformant Bcom9, conidiation, virulence, and DON production were increased significantly in comparison with the Δ*zif1* mutant but were not recovered to the wild-type level (Table 1), indicating that a positional effect may affect the expression and function of the ectopically integrated *ZIF1* gene.

In the Δ*zif1* mutant, DON production and the expression of many *TRI* genes were reduced. Microarray analysis revealed that a number of other gene clusters that involved secondary metabolism were downregulated in the Δ*zif1* mutant, including the aurofusarin biosynthetic gene cluster. Therefore, *ZIF1* may be involved in the global regulation of secondary metabolism in *F. graminearum*. One cluster consists of genes from FGSG_7820 to FGSG_7825. Most of them encode conserved hypothetical proteins unique to filamentous fungi. Approximately half of the genes downregulated in the Δ*zif1* mutant have unknown functions. It is possible that some of them are important pathogenicity factors in *F. graminearum*.

ZIF1 also plays a critical role in sexual reproduction. In *F. graminearum*, a homothallic fungus, the Δ*zif11* mutant was sterile on selfing cross plates (Δ*zif1* × Δ*zif1*). However, unlike other female sterile mutants in *F. graminearum*, such as the *gpmk1*, *mgv1*, *FBP1*, and *Gzsyn2* mutants (Han et al. 2007; Hong et al. 2010; Hou et al. 2002; Jenczmionka et al. 2003), the Δ*zif1* mutant still formed small, dark-pigmented perithecia. In *ZIF1* (♀) × Δ*zif1* (♂) outcrosses, fertile perithecia were produced and Δ*zif11* progeny were identified, indicating that *ZIF1* is dispensable for ascospore viability and male fertility. Similar phenomenon was observed in *M. oryzae*, a heterothallic fungus that has weak female fertility or the tendency to lose female fertility under laboratory conditions (Xu et al. 2007; Zeigler 1998). The Δ*Mozif1* mutant was fertile when mated as the male. When mated as the female, the Δ*Mozif1* mutant produced small, sterile perithecia without long necks (Fig. 7). Therefore, unlike other genes known to be related to female fertility in *M. oryzae*, such as *MPS1* and *MGB1* (Nishimura et al. 2003; Xu et al. 1998), *MoZIF1* is dispensable for proto-perithecium formation and initial perithecium development but essential for ascosporogenesis and perithecium maturation.

Our results indicate that the *ZIF1* orthologs play a female-specific role in ascus and ascospore formation in a homothallic and a heterothallic member of order Sordariomycetes. To our knowledge, no other genes with similar functions in sexual reproduction have been reported in filamentous ascomycetes. For the transvection phenomenon in *Neurospora crassa*, the *asm-1* mutant is defective in ascospore development as either the male or female (Aramayo and Metzenberg 1996). It is most likely that *MoZIF1* functions before or during fertilization. Protoperithecia of the Δ*Mozif1* mutant may be defective in fertilization. However, it is also possible that small, pigmented perithecia were developed from mutant protoperithecia after fertilization. *MoZIF1* may be essential for postfertilization events in developing perithecia, such as dikaryotic hyphal growth and crozier formation. Because the mating defects of the Δ*Mozif1* mutant were only observed when it was mated as the female, if *MoZIF1* indeed functions postfertilization then *M. oryzae* must be able to distinguish whether the Δ*Mozif1* mutant was mated as the male or female in the Δ*Mozif1* + *MoZIF1* dikaryotic ascogenous hyphae or Δ*Mozif1 MoZIF1* diploid croziers. In the dikaryon or diploid stage, the function of the *MoZIF1* allele from the female cannot be replaced by the *MoZIF1* allele from the male. In plants and animals, only the maternal or paternal allele of imprinted genes is expressed and functional (Reik and Walter 2001). The female-specific function of *MoZIF1* suggests that it may be regulated by a mechanism similar to genetic imprinting in higher eukaryotes. Therefore, it will be important to determine the regulation and biological function of *ZIF1* and its orthologs in postfertilization events during sexual reproduction in filamentous ascomycetes.

Table 3. Polymerase chain reaction primers used in this study

Primer	Sequence (5′–3′)[a]
BTF1F	TAAAGCGTCGTCATCCTCAG
BTF2R	GACCTCCACTAGCTCCAGCCAAGCCATTGGCGATGCCAGAGAATC
BTF3F	GAATAGAGTAGATGCCGACCGAAAGGGCATACACTAAGCGC
BTF4R	TGGCCAACTAATGGCTCATC
BTN-1F	TTATCGACGCTGCTGTTCTC-
BTN-2R	ACAGGTCCATGGTAAGCAAC
bZP1F	ACTCA*GGTACC*CACCATCCGTAACCGTAAAC
bZP2R	TAGTA*GGTACC*TGAGATGTGTCGTGTAGACC
GFPNAT/F	GGCGAATTGGGTACTCAAATTGGTACCATCCGTAACCGTAAAC
GFPNAT/R	GAACAGCTCCTCGCCCTTGCTCACCAGGTCCATGGTAAGCAAC
GFP27/F	CGTAGGAACCCAATCTTCAAAATGTCGGCTCCTGGCCAGAATAA
GFP27/R	GAACAGCTCCTCGCCCTTGCTCACCAGGTCCATGGTAAGCAAC
ZIF1NGF	CGACTCACTATAGGGCGAATTGGGTACTCAAATTGGGAGCGGTGGACTTGATAA
ZIF1NGR	CACCACCCCGGTGAACAGCTCCTCGCCCTTGCTCACCAGGTCCATGGTAAGCAAC
ZIF1 RGF	CAGATCTTGGCTTTCGTAGGAACCCAATCTTCAAAATGTCGGCTCCTGGCCAGAATAA
BZP1A/R	GAGAATGAAGGTGAGGAGAGCTTCTTGCCCTCCTCGCTA
BZP1B/F	TAGCGAGGAGGGCAAGAAGCTCTCCTCACCTTCATTCTC
BZP1B/R	GAACAGCTCCTCGCCCTTGCTTTACAGGTCCATGGTAAGCAAC

[a] Restriction enzyme sites introduced into the primers are in italic and nucleotide sequences from the *hph* gene are underlined.

MATERIALS AND METHODS

Fungal strains and culture conditions.

The *F. graminearum* wild-type strain PH-1 (NRRL 31084) and transformants generated in this study (Table 2) were routinely maintained on V8 juice agar plates, cultured on carrot agar plates for sexual reproduction, and grown in liquid CMC medium for conidiation (Cappellini and Peterson 1965; Hou et al. 2002; Trail et al. 2003). Protoplast preparation and fungal transformation were performed as described (Hou et al. 2002). CM with hygromycin B at 250 μg/ml or geneticin at 250 μg/ml (Sigma-Aldrich, St. Louis) was used for selection of transformants. The *nit1* mutant 11622 was used to cross with the Δ*zif1* mutant as described (Hou et al. 2002). Ascospore progeny were assayed for growth on CM agar plates with hygromycin B at 250 μg/ml, CM with 1% KClO₃, and minimal medium with 1% NaNO₃ as the sole nitrogen source (Ding et al. 2009; Seong et al. 2006). For testing sensitivities to various stresses, fungal growth was assayed after incubation at 25°C for 3 days on CM plates with 1 M sorbitol, 0.7% NaCl (wt/vol), Congo red at 200 μg/ml, 0.01% SDS (wt/vol), Calcofluor white at 200 μg/ml, or 0.08% H_2O_2.

The *M. oryzae* wild-type and mutant strains were cultured on oatmeal agar plates as described (Park et al. 2006). Transformants were selected on TB3 with hygromycin B at 250 μg/ml or zeocin at 300 μg/ml (Invitrogen, Carlsbad, CA, U.S.A.). Mating and genetic crosses were performed as described (Crawford et al. 1986).

Generation of the Δ*zif1* mutant.

To generate the *ZIF1* gene replacement constructs using the split-marker approach (Catlett et al. 2003), 0.83-kb upstream and 0.85-kb downstream fragments were amplified with primer pairs BTF1F/BTF2R and BTF3F/BTF4R (Table 3), respectively. The *hy* and *yg* fragments of the hygromycin phosphotransferase (*hph*) gene were amplified with primers HYGF, HYR, YGF, and HYGR as described (Mehrabi et al. 2008; Zhou et al. 2010). After transformation, hygromycin-resistant transformants were screened by PCR with primers BTN-1F and BTN-2R (Table 3) and further characterized by Southern blot analysis. For complementation assays, a 3.9-kb fragment containing the *ZIF1* gene was amplified with primers bZP1F and bZP2R (Table 3) and cloned into pHZ100 (Bluhm et al. 2007). The resulting construct pJY01 (Fig. 1A) was transformed into protoplasts of the Δ*zif1* mutant ZP10.

Generation of GFP fusion constructs.

Primers ZIF1NGF and ZIF1NGR (Table 3) were used to amplify the *ZIF1* ORF plus 1.5-kb upstream promoter region. The resulting PCR products were cotransformed into *Saccharomyces cerevisiae* XK1-25 with *Xho*I-digested pFL2 vector, which was generated by replacing the *hph* gene on the vector backbone of pDL2 with the geneticin-resistance marker (Bourett et al. 2002; Bruno et al. 2004). The P_{ZIF1}-*ZIF1*-eGFP fusion construct was identified by PCR with primers ZIP1NF/R and confirmed by sequencing analysis. The same yeast in vivo recombination approach was used to generate the P_{RP27}-*ZIF1*-eGFP construct. Both fusion constructs were transformed into protoplasts of the Δ*zif1* mutant.

Construction of the *ZIF1*[ΔbZIP] allele.

To generate the *ZIF1*[ΔbZIP] allele (deleted from amino acid residues 276 to 339), the *ZIF1* fragments upstream and downstream from the b-ZIP domain were amplified with primer pairs GFPNAT/F-BZPA/R and BZP1B/F-BZP1B/R (Table 3), respectively. The resulting PCR products were co-transformed with *Xho*I-digested pKB04 into yeast strain XK1-25 (Bruno et al.

2004). The *ZIF1*[ΔbZIP] construct recovered from Trp+ yeast transformants was confirmed by sequencing analysis and transformed into mutant ZP10.

The Δ*Mozif1* deletion mutant in *M. oryzae*.

The upstream and downstream flanking sequences of *MgZIF1* were amplified and ligated with the *hph* cassette released from pCX63 (Zhao et al. 2004). With the ligation product as the template, the *MgZIF1* gene replacement construct was amplified with primers bBm and bEI and transformed into the wild-type strain 70-15 as described (Xu and Hamer 1996). Putative *MgZIF1* gene-replacement mutants were screened by PCR and further confirmed by Southern blot analyses. To complement the *M. oryzae* mutant with the *F. graminearum ZIF1* gene, the P_{ZIF1}-*ZIF1*-eGFP fusion construct was transformed into the Δ*Mozif1* mutant. The resulting zeocin-resistant transformants were confirmed by PCR to contain the *ZIF1*-eGFP fusion construct.

Plant infection assays.

Conidia harvested from 5-day-old CMC cultures of *F. graminearum* strains were resuspended to 10^6 conidia/ml in 0.01% (vol/vol) Tween 20. Six-week-old wheat plants of cv. Norm were drop inoculated with conidium suspensions as described (Gale et al. 2002; Kang and Buchenauer 1999). Diseased spikelets in each wheat head were counted 14 dpi to estimate disease indices as described (Gale et al. 2002; Seong et al. 2006). Infection assays with corn silks were performed as described (Seong et al. 2005). For corn stalk infection assays, sterile wooden toothpicks were briefly soaked in conidium suspensions and used to inoculate mature plants of corn cv. Pioneer 2375 as described (Zhou et al. 2010). Stalk rot symptoms were examined 14 dpi.

For *M. oryzae*, conidia harvested from 10-day-old oatmeal cultures were used for appressorium formation, penetration of onion epidermal cells, and rice infection assays as described (Park et al. 2006; Xue et al. 2002; Zhao and Xu 2007). Two-week-old seedlings of rice cv. Nipponbare were used for spray and injection inoculation.

Assay for DON production.

Diseased wheat kernels were harvested from inoculated spikelets 14 dpi and assayed for DON as described (Bluhm et al. 2007). DON production also was assayed with cultures grown in the defined medium with 0.2% agmatine as described (Gardiner et al. 2009a; Zhou et al. 2010).

Microarray analysis.

Conidia harvested from 5-day-old CMC cultures were resuspended in 100 ml of CM to 10^6 conidia/ml. After incubation at 25°C for 16 h, germlings were collected by filtration and used for RNA isolation with the TRIzol reagent (Invitrogen). For both the wild type and the Δ*zif1* mutant, RNA was isolated from three biological replicates. For each sample, 5 μg of total RNA was labeled with the Affymetrix eukaryotic RNA labeling kit (Affymetrix, Santa Clara, CA, U.S.A.). Hybridization and washing of the Fusarium GeneChip (Guldener et al. 2006) were performed at the Purdue Core Genomics Facility with standard Affymetrix procedures. Hybridization signals were scanned with a GeneChip GCS 3000 scanner (Affymetrix). The resulting CEL files were processed with software MAS5.0 (Affymetrix) and further analyzed with GeneSpring GX V7.2 (Agilent Technologies, Santa Clara, CA, U.S.A.).

qRT-PCR analyses.

RNA was isolated with the Trizol reagent (Invitrogen) and used for cDNA synthesis with the StrataScript QPCR cDNA

synthesis kit (Stratagene, La Jolla, CA, U.S.A.) following the instructions provided by the manufacturer. Primers TubQF and TubQR (Bluhm et al. 2007) were used to amplify the β-tubulin gene *TUB2* of *F. graminearum*. PCR was performed with a Stratagene MX3000 system as described (Zhou et al. 2010). After normalizing the expression of tested genes with *TUB2*, relative changes in gene expression levels were calculated by the comparative cycle threshold method (Applied Biosystems, Foster City, CA, U.S.A.). For each gene, qRT-PCR data from three biological replicates were used to calculate the mean and standard deviation.

ACKNOWLEDGMENTS

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AUTHOR-RECOMMENDED INTERNET RESOURCE

PLEXdb database: www.plexdb.org