Transcription Factor RFX1 Is Crucial for Maintenance of Genome Integrity in *Fusarium graminearum*

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The survival of cellular organisms depends on the faithful replication and transmission of DNA. Regulatory factor X (RFX) transcription factors are well conserved in animals and fungi, but their functions are diverse, ranging from the DNA damage response to ciliary gene regulation. We investigated the role of the sole RFX transcription factor, RFX1, in the plant-pathogenic fungus *Fusarium graminearum*. Deletion of *rfx1* resulted in multiple defects in hyphal growth, conidiation, virulence, and sexual development. Deletion mutants of *rfx1* were more sensitive to various types of DNA damage than the wild-type strain. Septum formation was inhibited and micronuclei were produced in the *rfx1* deletion mutants. The results of the neutral comet assay demonstrated that disruption of *rfx1* function caused spontaneous DNA double-strand breaks (DSBs). The transcript levels of genes involved in DNA DSB repair were upregulated in the *rfx1* deletion mutants. DNA DSBs produced micronuclei and delayed septum formation in *F. graminearum*. Green fluorescent protein (GFP)-tagged RFX1 localized in nuclei and exhibited high expression levels in growing hyphae and conidiophores, where nuclear division was actively occurring. RNA-sequencing-based transcriptomic analysis revealed that RFX1 suppressed the expression of many genes, including those required for the repair of DNA damage. Taken together, these findings indicate that the transcriptional repressor RFX1 performs crucial roles during normal cell growth by maintaining genome integrity.

The propagation of cellular organisms depends on the accurate replication and transmission of DNA from one cell to its daughters. However, the DNA of organisms is continually subjected to damage from exogenous and endogenous sources. The DNA damage response has evolved to achieve genome integrity. In response to DNA damage, cells arrest the cell cycle and induce the expression of a set of proteins that facilitate DNA repair through a DNA damage-responsive signal transduction pathway. Components of this pathway have been the subjects of intensive investigation in model organisms (1).

Regulatory factor X1 (RFX1) of *Saccharomyces cerevisiae* (ScRFX1) is a transcription factor (TF) that binds to promoters of target genes involved in DNA repair (2). ScRFX1 recruits the general repressors SSN6 and TUP1 to inhibit the transcription of target genes. In response to DNA damage, MEC1 is activated through an unknown mechanism. Activated MEC1 phosphorylates RAD53 which, in turn, phosphorylates DUN1 kinase. When ScRFX1 is phosphorylated in a DUN1-dependent manner, it loses its DNA-binding capacity. This process leads to the transcriptional activation of target genes, such as ribonucleotide reductases (RNRs). Increased RNR expression facilitates efficient DNA repair because RNRs supply deoxynucleoside triphosphates (dNTPs), which are precursors for DNA repair (3, 4). Therefore, disruption of ScRFX1 function triggers the derepression of RNRs, which makes the cells more resistant to DNA damage (2, 5, 6).

The DNA-binding domain of ScRFX1 shares high sequence similarity with those of the animal RFX TF family (2). Lubelsky et al. reported the functional conservation of RFX from *S. cerevisiae* to humans in response to DNA damage (7). In addition to the DNA damage response, RFX TFs are central regulators in a transcriptional network regulating ciliary gene expression in sensory neurons (8, 9). Loss of RFX function causes the absence of cilia, resulting in severe sensory defects in *Caenorhabditis elegans* (10).

Piasecki et al. suggested that RFX TFs originated early in the unikont lineage (fungi, Amoebozoa, choanoflagellates, and animals) (11). Transcriptional rewiring of many ciliary genes by RFX TFs occurred early in the animal lineage (11, 12). Thus, RFX TFs co-opted control over ciliary gene expression in animals, although their DNA-binding domains were conserved (11).

Functional studies of RFX TFs have been performed in several fungal species. For example, RFX1 in *Penicillium chrysogenum* (PcRFX1) was found to bind to the promoter region of penicillin biosynthetic genes, and knockdown of PcRFX1 reduced penicillin production (13). The *cpcR1* gene of *Acremonium chrysogenum* was initially found to bind promoters of cephalosporin C biosynthesis genes. However, disruption of *cpcR1* did not reduce cephalosporin C production (14, 15). Subsequently, it was revealed that *cpcR1* is required for hyphal fragmentation and, thus, for arthrospore formation (16). In *Schizosaccharomyces pombe*, *sak1* was found to be indispensable for cell viability. Thus, loss of *sak1* caused multiple defects in mitosis and cell morphology (17).

In *Penicillium marneffei*, *rfxA* was found to be essential for cellular division and morphogenesis, particularly during conidiation and yeast growth (18). In contrast to its action in *S. cerevisiae*, the knockdown mutant of *rfxA* was more sensitive to a DNA damage agent in *P. marneffei*. In *Candida albicans*, *rfx2* repressed genes...
related to the repair of DNA damage and regulated hyphal morphogenesis and virulence (5). An rfx2-null mutant was shown to be significantly more resistant to UV irradiation than the wild-type strain. In many fungi, RFX TFs have been shown to be required for normal cell division and morphogenesis; however, in S. cerevisiae, Scrfx1 is dispensable for normal cell growth. Moreover, disruption of RFX TFs causes opposite responses to DNA damage in different fungal species. These findings imply that the functions of RFX TFs are not conserved, despite the presence of a highly conserved DNA-binding domain.

*Fusarium graminearum* is a major cereal pathogen, causing *Fusarium* head blight in wheat, barley, and rice, as well as ear rot and stalk rot in maize. In addition to yield reduction, the fungus contaminates grains with mycotoxins that cause feed refusal and other toxicoses in livestock and pose a threat to food safety (19). *F. graminearum* forms mature perithecia on crop residues and forcibly discharges ascospores, which are the primary inocula (20–23). Conidia produced from sporodochia on infected crops are easily disseminated by rain splash, acting as the secondary inocula (24). Thus, sexual and asexual reproduction of *F. graminearum* are important developmental processes for *Fusarium* head blight (21) and require the elaborate regulation of many genes (25, 26).

Our research goal was to investigate the role of the RFX TF in *F. graminearum* (FgRFX). Therefore, the objectives of this study were (i) to identify the RFX TF in *F. graminearum*, (ii) to characterize its role in morphogenesis and the DNA damage response, and (iii) to identify RFX TF-regulated genes by analyzing the transcriptome. The results of this study suggest that the transcriptional suppressor rfx1 is required for maintenance of genome integrity and normal cell growth in *F. graminearum*.

**MATERIALS AND METHODS**

**Strains and culture conditions.** The *F. graminearum* strains used in this study are listed in Table 1. Standard laboratory methods and culture media for the *Fusarium* species were used (27). Cultures were maintained in complete medium (CM) agar plates. Conidia were produced in carboxymethyl cellulose medium (CMC) or on yeast malt agar (YMA) (28, 29). Unless otherwise stated, the growing temperature of the fungal strains was set at 25°C. All strains were stored as conidia and mycelia in 30% glycerol solution at −80°C.

Conidia were harvested from CMC cultures and transferred to CM containing various DNA-damaging agents at the following concentrations: hydroxyurea (10 mM), methyl methanesulfonate (MMS) (0.1%), and bleomycin (20 μg/ml). Conidia were inoculated on CM agar plates and immediately exposed to UV (254 nm, 480 J/m²). The germination rates of the conidial strains were examined by microscopy 18 h after inoculation, and the percentage of germinated conidia was determined. All statistical analyses were conducted using the R statistical software package (30).

**Nucleic manipulation, primers, and PCR conditions.** Genomic DNA was isolated from lyophilized mycelia as previously described (27). Restriction endonuclease digestion, Southern blotting, and hybridization with 32P-labeled probes were performed in accordance with standard techniques (31). The PCR primers used in this study were synthesized by an oligonucleotide synthesis facility (Bionics, Seoul, South Korea) (see Table S1 in the supplemental material). General PCR procedures were performed in accordance with the manufacturer’s instructions (TaKaRa Bio, Inc., Otsu, Japan).

**Sexual crosses.** For self-fertilization, cultures were grown on carrot agar plates for 5 days. Sexual reproduction was induced by removing aerial mycelia with sterile 2.5% Tween 60 solution (27). For outcrosses, female strains grown on carrot agar plates were fertilized with conidial suspensions from corresponding male strains 5 days after inoculation. The cultures were incubated under a near-UV lamp (365 nm) at 25°C for an additional 7 days.

**Fungal transformation.** For green fluorescent protein (GFP) tagging and complementation, a DNA fragment containing the native promoter and the open-reading frame (ORF) of rfx1 was amplified with RFX1/SF and RFX1/ORF/Rev. The double-joint PCR method (32) was used to fuse the amplicon to the 3′-flanking region of rfx1 and a GFP cassette containing the hygromycin B resistance gene (hyg). Fusion constructs were amplified with nested primers to generate split markers. The resulting constructs were introduced into Δrfx1 protoplasts, using the polyethylene glycol-mediated fungal transformation procedure (33). Transformants were selected under hygromycin B (75 μg/ml).

The ORF of rfx1 was replaced with the GFP cassette to generate strain HK26 (Δrfx1::gfp-hyg). The GFP cassette, 5′-flanking region of rfx1, and 3′-flanking region of rfx1 were fused. The fusion constructs were amplified with nested primers to generate split markers. The resulting constructs were introduced into wild-type protoplasts. rfx1 deletion mutants were selected under hygromycin B (75 μg/ml) and screened for GFP fluorescence in mycelia.

**Fluorescence microscopy.** Differential interference contrast and fluorescence images were captured on an Axio Imager A1 microscope (Carl Zeiss, Oberkochen, Germany) with a charge-coupled-device (CCD) camera. Nuclei of conidia were stained with acriflavin and examined with the 470-nm/525-nm (excitation/emission wavelength) filter set (34). The or-ganelle localizations of fusion proteins RFX1-GFP and histone H1 (hH1)-GFP were imaged with the 470-nm/525-nm filter set. Red fluorescence protein (RFP)-tagged histone H1 was examined with the 560-nm/605-nm filter set. The cell wall was stained with 0.2 μl/ml calcofluor white (Sigma-Aldrich, St. Louis, MO) and observed using the 560-nm/605-nm filter set. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA) and examined with the 365-nm/445-nm filter set (35). Images were analyzed using the AxioVision release 4.7 software package (Carl Zeiss).

**Virulence assays.** The virulence of fungal strains on wheat heads was assessed in the wheat cultivar Eunpamil as previously described (36, 37). Conidia were harvested from CMC cultures and resuspended to 10⁶ spores/ml in 0.01% Tween 20 solution. The center spikelet was drop inoculated with 10 μl of conidial suspension. Inoculated plants were incubated in a humidity chamber at 25°C for 3 days and grown in a greenhouse for an additional 18 days. Infected wheat heads were imaged, and the spikelets with head blight symptoms were counted.

**Toxin analysis.** The conidial suspension was inoculated in defined medium containing 5 mM agmatine (MMA) and incubated for 7 days as previously described (38). Culture filtrates were extracted with an ethyl acetate-methanol mixture (4:1, vol/vol). The extracts were dried (39), and the residues were derivatized with trimethylsilylating reagent [N,O-bis(trimethylsilyl)acetamide (BSA), trimethylchlororosilane (TMCS), and

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

| Strain | Genotype | Reference, source, or parent strains |
|--------|-----------|-------------------------------------|
| Z-3639 | Wild-type | 58                                  |
| Δrfx1  | Δrfx1::gen | 37                                  |
| KH23   | Δrfx1::rfx1-gfp-hyg | This study |
| hH1-GFP | hH1-gfp-hyg | 59                                  |
| Δmat1r | Δmat1::gen | 60                                  |
| mat1g  | Δmat1::hH1-gfp-hyg | 59                                  |
| KH24   | Δrfx1::gen hH1-gfp-hyg | mat1g × Δrfx1 strain |
| mat1r  | Δmat1::hH1-rfp-hyg | 61                                  |
| KH25   | Δrfx1::gfp-hyg hH1-rfp-hyg | mat1r × KH23 |
| KH26   | Δrfx1::gfp-hyg | This study |
| KH27   | Δrfx1::gfp-hyg hH1-rfp-hyg | mat1r × KH26 |
| HK12   | GFP-hyg | 62                                  |
1-(trimethylsilyl)imidazole (TMSI), 3:2:3; Supelco, Bellefonte, PA). The resulting samples were analyzed with a Shimadzu QP-5000 gas chromatograph-mass spectrometer (GC-MS; Shimadzu, Kyoto, Japan) as previously described (40).

Neutral comet assay. Fungal conidia produced in CMC culture were inoculated into 50 ml of YPG liquid medium (3 g yeast extract, 10 g peptone, and 20 g glucose per liter) at 10⁶ spores/ml and grown for 12 h with shaking (33). Mycelia were harvested by filtration and incubated in 35 ml of 1 M NH₄Cl containing Driselase (10 mg/ml) (Sigma-Aldrich) at 30°C to generate protoplasts. For bleomycin treatment of wild-type cells, protoplasts were generated in Driselase solution containing 20 mU/ml bleomycin. Protoplasts were collected by centrifugation 4 h after incubation and resuspended in 1 M NH₄Cl.

Low-gelling-temperature agarose (1%; Sigma-Aldrich) was molten and neutralized with Tris-base at 60°C. The neutralized agarose solution was poured into glass slides and allowed to gel. Slides were submerged in neutral lysis solution (2.5 M NaCl, 100 mM Naacetate, 10 mM Tris, pH 10.0, 25 mM EDTA, 0.5% Triton X-100) and incubated for 30 min at 37°C. The slides were rinsed with deionized water, stained in ethidium bromide solution, rinsed in Tris-acetate-EDTA buffer for 25 min at 0.6 V/cm. The slides were analyzed with the ComET software (TriTek Corp., Sumerduck, VA).

Quantitative real-time (qRT)-PCR. Wild-type and Δrfx1 conidia produced in CMC culture were inoculated into 50 ml of CM. The wild-type culture was incubated for 24 h and the Δrfx1 culture for 32 h with shaking. Mycelia were harvested and subcultured in fresh CM or CM containing 20 mM/ml bleomycin. The cultures were incubated for an additional 2 h. Total RNA was isolated from mycelia that were ground in liquid nitrogen using an Easy-Spin total RNA extraction kit (Intron Bio-tech, Seongnam, South Korea). First-strand cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen).

qRT-PCR was performed with SYBR green supermix (Bio-Rad, Hercules, CA) and a 7500 real-time PCR system (Applied Biosystems, Foster City, CA). An endogenous housekeeping gene, ubiquitin C-terminal hydrolase (ubh; FGSG_01231.3), was used as a reference gene (42). The transcript levels of target genes under different conditions were compared for double-strand break (DSB) detection, in accordance with a standard protocol (41). After overnight lysis, the slides were washed three times in Tris-acetate-EDTA buffer for 30 min each. Electrophoresis was conducted in Tris-acetate-EDTA buffer for 25 min at 0.6 V/cm. The slides were rinsed with deionized water, stained in ethidium bromide solution, rinsed again with deionized water, and dried at room temperature. Dried agarose gels were rehydrated before fluorescence microscopy.

Comet images were captured on an Axio Imager A1 microscope with a CCD camera and the 550-nm/605-nm filter set. Comet images were composed of the comet head and comet tail. The percentage of DNA in the tail was analyzed for individual comet images by using CometScore image analysis software (TriTek Corp., Sumerduck, VA).

RESULTS

Molecular characterization of the rfx1 gene. The Fusarium Comparative Database (Fusarium Comparative Sequencing Project, Broad Institute of Harvard and MIT, http://www.broadinstitute.org) annotated the rfx1 gene (locus FGSG_07420) based on the presence of an RFX DNA-binding domain. We aligned protein sequences from RFX1 homologs using ClustalW in the MEGA 5.2 program and BoxShade. The alignment showed that the RFX DNA-binding domain of FgRFX1 is highly conserved among all characterized RFX proteins (Fig. 1A). Phylogenetic analyses of RFX homologues were performed using a neighbor-joining algorithm, with bootstrap values calculated from 100 iterations. The phylogenetic tree revealed that RFX homologs of filamentous fungi clustered into a separate group relative to those of yeasts and animals (Fig. 1B).

Effects of rfx1 deletion on nucleus and septum formation. The rfx1 deletion strain was provided by a mutant library of TFs in F. graminearum (37). For genetic complementation and GFP tagging of rfx1, a construct containing the rfx1 ORF fused with the GFP cassette was introduced into the rfx1 deletion mutant. The Southern hybridization results showed that the construct replaced the endogenous gene in the genome of the complementation strain, resulting in strain KH23 (see Fig. S1 in the supplemental material). The Δrfx1 strain showed severely reduced radial growth on CM agar plates and did not produce aerial mycelia (Fig. 2 and Table 2).

Strain KH24 (Δrfx1::gen hH1-gfp-hyg) was generated by cross-
ing strain mat1g (\(\Delta mat1-1\):gen hH1-gfp-hyg) and the \(\Delta rfx1\) strain (Table 1). KH24 showed the same phenotypes as the parental \(\Delta rfx1\) strain and exhibited green fluorescence in nuclei. Strain KH25 (\(\Delta rfx1::rfx1-gfp-hyg hH1-rfp-hyg\)) was generated by crossing strain mat1r (\(\Delta mat1-1\):gen hH1-rfp-hyg) and the complementation strain (\(\Delta rfx1::rfx1-gfp-hyg\)). KH25 had both green and red fluorescence in its nuclei. Conidia of the histone H1 (hH1)-GFP strain, KH24, and KH25 were produced in CMC and inoculated into CM to examine the nucleus and septum formation in hyphae. Mycelia were harvested 12 h after inoculation (except for mycelia into CM to examine the nucleus and septum formation in hyphae. Mycelia were harvested 12 h after inoculation (except for mycelia of KH24, which were harvested 24 h after inoculation). Septa and nuclei of hyphae were observed by calcofluor white staining and fluorescent protein images. Scale bar = 20 μm.

The \(\Delta rfx1\) nuclei were irregular in size, and the \(\Delta rfx1\) strain produced small fragmented nuclei, called micronuclei. The level of conidium production of the \(\Delta rfx1\) strain was 9 times lower than that of the wild type (Table 2). In addition, the \(\Delta rfx1\) conidia were abnormally shaped (Fig. 4). The average conidium length in the \(\Delta rfx1\) strain (71 μm) was much longer than that in the wild type (45 μm) (\(P < 0.01\)). Deletion of rfx1 resulted in more severe defects in the nucleus and septum formation of conidia than of hyphae. Most wild-type conidia (~70%) had four or five cells with one nucleus per cell; in contrast, most \(\Delta rfx1\) conidia (~70%) had one or two cells with multiple nuclei. In the \(\Delta rfx1\) conidia, the nuclei were irregularly shaped and many were micronuclei. The hyphal growth and conidial morphology of KH23 were restored to the wild-type phenotypes.

**Virulence and toxin production.** The virulence of the fungal strains was examined by point inoculation of wheat spikelets. The \(\Delta rfx1\) strain was unable to infect the inoculated spikelets, whereas the wild-type and complemented strains readily colonized the inoculated spikelets and spread to the neighboring spikelets. The disease index (number of diseased spikelets per wheat head) of the \(\Delta rfx1\) strain was zero (Table 2). We also examined the production of trichothecene, which is an important virulence factor. Trichothecene biosynthesis was induced in MMA, which contains agmatine as a nitrogen source. GC-MS detected two trichothecenes, deoxynivalenol and 15-acetyldeoxynivalenol. The production of total trichothecenes was not markedly different (\(P > 0.05\)) among the strains on the basis of the biomass (g) of each sample.

**Sexual development.** The \(\Delta rfx1\) strain produced few immature perithecia, whereas the wild-type and the complementation (\(\Delta rfx1::rfx1-gfp\)) strains produced abundant mature perithecia 7 days after sexual induction (Fig. 5). The perithecia did not contain any ascus structures. The heterothallic *F. graminearum* strain carrying the MAT1-1 deletion with histone H1-GFP (mat1g) was crossed with the \(\Delta rfx1\) mutants to investigate the male fertility of

![FIG 2](image_url) Mycelial growth of fungal strains on complete medium plates. Photographs were taken 4 days after inoculation. WT, wild type.

![FIG 3](image_url) Nucleus and septum formation in hyphae. Histone H1 was tagged with green fluorescent protein (GFP) or red fluorescent protein (RFP) to visualize nuclei. Cell wall was stained with calcofluor white. Arrowheads indicate septa, and arrows indicate micronuclei. Both deletion of rfx1 and bleomycin treatment inhibited septum formation and produced micronuclei in hyphae. WT, wild type; WT+BLM, wild-type hyphae grown with 20 μM/ml bleomycin; DIC, differential interference contrast; Nuclei & Septa, overlays of calcofluor white staining and fluorescent protein images. Scale bar = 20 μm.

![FIG 4](image_url) Morphology of \(\Delta rfx1\) conidia changed dramatically. Nuclei in the \(\Delta rfx1\) conidia were fragmented and scattered. WT, wild type; DIC, differential interference contrast; Nuclei, acriflavin staining was used to visualize nuclei. Scale bar = 20 μm.

**TABLE 2 Vegetative growth, virulence, and trichothecene production in *F. graminearum* strains**

| Strain type           | Radial growth (mm)\(^d\) | Conidium formation (10⁵/ml)\(^c\) | Virulence (disease index)\(^d\) | Trichothecene production (mg/g)\(^d\) |
|-----------------------|---------------------------|-----------------------------------|---------------------------------|--------------------------------------|
| Wild type             | 39 ± 2.9 b                | 19 ± 4.7 b                         | 4 ± 2 b                         | 25 ± 2.1 A                           |
| \(\Delta rfx1\)       | 21 ± 1.2 b                | 2.4 ± 0.59 b                       | 0 ± 0 A                         | 20 ± 2.5 A                           |
| \(\Delta rfx1::rfx1-gfp\) | 38 ± 2.2 b               | 17 ± 5.5 b                         | 3 ± 2 b                         | 21 ± 2.7 A                           |

\(^a\) The data presented are average values ± standard deviations. Values within a column with different letters are significantly different (\(P < 0.05\)) based on Tukey’s HSD test.

\(^b\) Radial growth was measured 4 days after inoculation into CM agar plates.

\(^c\) Conidia were counted 5 days after inoculation into CMC.

\(^d\) Disease index (number of diseased spikelets per wheat head) of the strains was measured 21 days after inoculation.

\(^e\) Total trichothecenes (deoxynivalenol and 15-acetyldeoxynivalenol) were analyzed with a gas chromatograph-mass spectrometer. Trichothecene production was quantified on the basis of the biomass (g) of each sample.
We randomly isolated 144 ascospores, but 57 ascospores did not germinate. The genotypes of the germinated 87 ascospores were analyzed. The segregation ratio of the genotypes was 39:27:8:13 (\(rfx1\) h\(H1\)-gfp/\(rfx1\) h\(H1\)/\(rfx1\) h\(H1\)-gfp/\(rfx1\) h\(H1\)), indicating that most ascospores carrying the \(rfx1\) deletion were not viable. Progenies carrying the \(rfx1\) deletion were many fewer than those carrying wild-type \(rfx1\) (21:66). However, the ratio of h\(H1\)-GFP to non-GFP segregation was 1:1, indicating that the outcross underwent normal sexual recombination.

We further investigated role of \(rfx1\) in sexual development, replacing the \(rfx1\) gene with the cytoplasmic GFP gene and thus generating a \(rfx1\)::gfp-hyg strain (KH26). KH27 was generated by crossing mat1r and KH26 (Table 1). A heterothallic \(F.\) graminearum strain carrying the MAT1-1 deletion with histone H1-RFP (mat1r) was crossed with KH27 to examine the fertility of the \(rfx1\) strain as a male (Fig. 6). When mature asci were dissected, four out of eight ascospores expressed GFP, indicating that they were \(rfx1\) deletion mutants. In \(F.\) graminearum, ascospores underwent two mitoses, producing four cells with one nucleus after spore delimitation. Ascospores with the \(rfx1\) deletion were composed of one cell with one or two nuclei, whereas the non-GFP ascospores were composed of four cells with one nucleus. Thus, deletion of \(rfx1\) blocked mitosis in ascospores after spore delimitation. Defects of spore maturation may cause inviability of ascospores carrying the deletion of \(rfx1\).

**Localization of GFP-tagged RFX1 proteins.** To determine the subcellular distribution of RFX1, we complemented the \(rfx1\) strain by introducing the RFX1-GFP-hyg construct, which was GFP tagged at the C terminus of RFX1. The resulting \(rfx1::rfx1-gfp\) strain (KH23) exhibited wild-type phenotypes under all conditions, as previously described. Conidia of KH23 were inoculated into CM and examined after 12 h of incubation. Conidiation of KH23 was induced in CMC and examined 3 days after induction. Although KH23 does not contain any predictable nuclear localization signals, RFX1-GFP localized to the nuclei. Intense fluorescence was found in nuclei of growing hyphae and developing conidiophores (Fig. 7). However, the fluorescence was too weak to detect in mature hyphae and conidia.

**Sensitivity of \(rfx1\) deletion strain to DNA damage.** To investigate the function of \(rfx1\) in the DNA damage response, we examined the germination rate of conidia under conditions of DNA damage (Fig. 8). Conidia of the fungal strains were exposed to UV and several DNA-damaging agents, including hydroxyurea, MMS, and bleomycin. The conditions induced low-level DNA damage, such that the wild-type and \(rfx1::rfx1-gfp\) conidia germinated well. However, the germination rate of \(rfx1::rfx1\) conidia was markedly reduced under all DNA damage conditions, indicating that deletion of \(rfx1\) resulted in sensitivity to DNA damage, regardless of the type of damage.

Interestingly, we found that bleomycin (10 mU/ml), which in-
duced low levels of DNA damage, inhibited septum formation in the wild-type hyphae. Septum formation was severely delayed and micronuclei were found when a higher concentration (20 mU/ml) of bleomycin was used (Fig. 3). Bleomycin is known to induce DNA DSBs ([46, 47]). Both bleomycin and deletion of rfx1 triggered aspetaion and micronuclei in hyphae, implying that deletion of rfx1 might induce spontaneous DNA strand breaks.

Accumulation of spontaneous DNA DSBs. A neutral comet assay was performed to measure the amount of DNA DSBs in fungal cells. The comet assay combines DNA gel electrophoresis with fluorescence microscopy to visualize the migration of DNA from individual agarose-embedded cells ([41]). In the neutral comet assay, the comet head contains undamaged DNA, and the comet tail contains DSB-generated fragments. Therefore, cells with more DSBs show a higher percentage of DNA in the tail. Because bleomycin produces DNA DSBs ([46, 47]), we treated wild-type cells with 20 mU/ml bleomycin as a positive control. The bleomycin-treated cells contained a higher percentage of DNA in the tail than the untreated cells, indicating that the neutral comet assay detected DNA DSBs (Fig. 9). The percentage of DNA in the tail was increased in Δrfx1 cells compared to the result for wild-type cells (P < 0.01). The occurrence of DSBs in Δrfx1 cells was similar to that in bleomycin-treated cells. The results suggested that deletion of rfx1 triggered DNA DSBs in the absence of a DNA-damaging agent.

Effects of rfx1 deletion on genome-wide transcription profiles. We obtained and analyzed genome-wide transcription profiles generated from the RNA sequencing data of the wild-type and Δrfx1 strains. The RPKM values of 13,820 recently reannotated genes in F. graminearum ([48]) were obtained and compared (see Dataset S1 in the supplemental material). Genes with transcript levels showing a 3-fold or greater difference between the Δrfx1 and wild-type strains were functionally characterized ([45]), as shown in Table 3. The results revealed that 38% of all genes were upregulated in the Δrfx1 strain, whereas only 1% were downregulated. These findings implied a role for RFX1 as a transcriptional repressor. There was a significant enrichment of genes in the “Unclassified protein” category, which showed a 3-fold increase in transcript abundance compared to the transcription of the genome as a whole (P < 0.001). However, most of the FunCat categories showed significant underrepresentation of 3-fold-upregulated genes (P < 0.001), except for FunCat categories 32 and 41. The transcript levels of 35 genes involved in DNA repair (FunCat 10.01.05.01) were increased in the deletion mutants. This implied that deletion of rfx1 triggered derepression of many genes whose functions have not been annotated.

There are two complementary mechanisms for DNA DSB repair: homologous recombination (HR) and nonhomologous end joining (NHEJ). We selected several proteins required for HR and NHEJ ([49]), including RAD51, RAD52, and RAD54, which are required for HR, Ku70, Ku80, and DNA ligase IV, which are required for NHEJ, and RAD50, which is required for both HR and NHEJ. Because Δrfx1 cells had spontaneous DNA DSBs, the expression of DSB repair genes was examined in detail (Table 4). The Δrfx1 cells showed greater expression of DSB repair genes than the wild-type cells in RNA-sequencing analysis.

The transcriptome analyses were validated for the selected genes using qRT-PCR (Table 4). Consistent with the RNA sequencing data, the DSB repair genes were upregulated in the Δrfx1 cells in the qRT-PCR analysis. In response to bleomycin, the expression of DSB repair genes was increased in the wild-type and Δrfx1 cells. The Δrfx1 cells with bleomycin exhibited the highest expression of the genes, indicating an additive effect. These find-
ings indicate that both bleomycin and deletion of *rfx1* produced DSBs in DNA and induced the expression of DSB repair genes.

**DISCUSSION**

RFXs are conserved TFs in fungi and animals, but their functions appear to have diversified during evolution (11). Several studies have reported that RFX TFs in fungi are required for DNA damage responses, cell division, and differentiation (2, 5, 16–18). The objective of this study was to investigate *rfx1* in *F. graminearum* and to characterize its role in cell morphogenesis and the DNA damage response. We examined deletion mutants of *rfx1* and found that the loss of *rfx1* triggered multiple defects in the fungal life cycle. The Δ*rfx1* strain lost its pathogenicity in wheat heads (Table 2). As virulence factors of *F. graminearum* in wheat infection, trichothecenes inhibit host defenses (50, 51). Deletion of *Fg.rfx1* did not change the production of secondary metabolites, such as pigments and trichothecenes, in *F. graminearum* (Fig. 2 and Table 2). In contrast, knockdown of *Pc.rfx1* was shown to reduce penicillin biosynthesis in *P. chrysogenum* (13). Thus, the loss of pathogenicity in this study could not be attributed to trichothecene production. It has been reported that hyphae of *F. graminearum* develop mats and appressoria-like structures to penetrate the host cell wall (51). We assume that the Δ*rfx1* strain cannot infect the host cells due to the severe defects in infection-related morphogenesis. The

| Locus       | Putative ortholog of protein in humans* | Fold change of transcript level in RNA-seqb | Relative transcript level of indicated strain grown with or without BLMc |
|-------------|----------------------------------------|--------------------------------------------|-------------------------------------------------|
| FGSG_07420  | RFX1                                    | 0                                          | - BLM  + BLM                                    |
| FGSG_12711  | KU70                                    | 7.3                                        | 1.0  0.85                                      |
| FGSG_06721  | KU80                                    | 8.9                                        | 1.0  7.9                                       |
| FGSG_04154  | DNA ligase IV                            | 6.3                                        | 1.0  2.9                                       |
| FGSG_11814  | RAD50                                   | 4.6                                        | 1.0  1.5                                       |
| FGSG_01157  | RAD51                                   | 21                                         | 1.0  4.6                                       |
| FGSG_10158  | RAD52                                   | 5.5                                        | 1.0  1.8                                       |
| FGSG_07962  | RAD54                                   | 8.5                                        | 1.0  2.5                                       |
| FGSG_05174  | RRM1 (RRN1)                             | 2.0                                        | 1.0  1.0                                       |
| FGSG_05409  | RRM2 (RRN2)                             | 3.4                                        | 1.0  0.9                                       |

*The Fusarium Comparative Database was searched for homologs of human double-strand break (DSB) repair genes using BLAST analysis.

b Fold changes of RPKM (reads per kilobase of exon per million mapped sequence reads) values in the Δ*rfx1* strain compared to the value in the wild type.

c Wild-type and Δ*rfx1* mycelia were grown in complete medium (CM) and subcultured in fresh CM or CM containing 20 mU/ml of bleomycin (BLM). Cultures were incubated for an additional 2 h, and the total RNA was isolated. Transcript levels of target genes were analyzed by quantitative real-time (qRT)-PCR. The endogenous housekeeping gene ubiquitin C-terminal hydrolase (*ubh*; FGSG_01231.3) was used as the reference gene. Data are expressed as arbitrary units, where the transcript level of the wild-type strain not treated with bleomycin was set to 1.
Δrfx1 strain was not able to undergo normal morphogenesis in conidiation and sexual development. We hypothesized that deletion of rfx1 prevented hyphae from developing infection structures required for host infection. Similarly, the knockdown of rfxA resulted in an inability of P. marneffei hyphae to transition to infectious yeast cells (18).

Given the role of RFX TFs in response to DNA damage in S. cerevisiae (2), it is interesting to speculate whether Fgfrx1 is involved in a similar mechanism in F. graminearum, particularly in light of the increased sensitivity of the Δrfx1 strain to DNA damage. UV leads to aberrant covalent bonding between adjacent pyrimidine bases, producing dimers. Hydroxyurea stalls DNA replication because it is an inhibitor of RNRs and thereby depletes dNTP pools (52). The DNA-alkylating agent MMS methylates guanine and adenine to cause base mispairing and replication blocks, respectively (53). Bleomycin binds DNA and produces reactive oxygen species that induce DNA DSBs (46, 47). Disruption of Scrfx1 increased resistance to UV and MMS in S. cerevisiae (5, 6). In C. albicans, the rfxA deletion mutant was significantly resistant to UV irradiation (5). In this study, deletion of Fgfrx1 reduced viability following all of these types of DNA damage in F. graminearum (Fig. 8). Similarly, knockdown mutants of rfxA have been shown to be sensitive to hydroxyurea in P. marneffei (18).

Phylogenetic analysis showed that Scrfx1 and rfxA were grouped in the same clade (Fig. 1B). S. cerevisiae and C. albicans belong to the subphylum Saccharomycotina, while F. graminearum and P. marneffei are members of the subphylum Pezizomycotina. We suggest that RFX TFs have opposite functions in the DNA damage responses of Saccharomycotina and Pezizomycotina. These differences may also reflect specific aspects of the multinucleate and multicellular growth habit that is characteristic of filamentous hyphae in the Pezizomycotina.

We used the neutral comet assay to demonstrate that Δrfx1 cells accumulated DNA DSBs (Fig. 9). RNA-sequencing and qRT-PCR analyses indicated that genes involved in DNA DSB repair were upregulated in the Δrfx1 and bleomycin-treated wild-type strains (Table 4). Bleomycin-treated Δrfx1 cells exhibited the highest transcript levels of DNA repair genes, suggesting a synergistic effect. We suggest that spontaneous DSBs resulted in sensitivity of the Δrfx1 strain to DNA damage. The mutant cells presumably failed to tolerate additional DNA damage because the integrity of their genomes had already been severely compromised by the loss of RFX1. Although DSB repair genes were upregulated in the Δrfx1 cells, we suspect that this was insufficient to cope with the DNA damage caused by agents such as bleomycin. Increased sensitivity to DNA-damaging agents despite the induction of repair activities has also been observed in the Aspergillus nidulans sepB mutant (54).

Deletion of rfX1 inhibited septum formation and produced micronuclei in hyphae and conidia (Fig. 3 and 4). In sexual development, ascospores carrying the rfX1 deletion did not undergo nuclear division or septation after spore delimitation (Fig. 6). Bleomycin treatment also delayed septum formation and produced micronuclei in F. graminearum. Therefore, deletion of rfX1 likely caused DNA DSBs, which prevented septum formation and produced micronuclei. Micronuclei are biomarkers of chromosomal instability because they result from chromosome missegregation caused by the inability to repair DNA DSBs (55). For example, treatment of Neurospora crassa with an inhibitor of DNA topoisomerase I, camptothecin, induced micronuclei formation (56). In the present study, a low concentration of bleomycin inhibited septation but did not inhibit nuclear division. These observations are consistent with the ability of sublethal doses of hydroxyurea to prevent septum formation in A. nidulans (57).

However, hydroxyurea and camptothecin did not delay septum formation or produce micronuclei in F. graminearum (data not shown). These results emphasize the importance of genome integrity in normal cell growth, even if the response to DNA-damaging agents varies between species. In particular, Harris and Kraus suggested that the compartmentalization of hyphae by septa might be exquisitely sensitive to the presence of nuclei that have incurred sublethal DNA damage (57). Because compartmentalized nuclei likely represent a source of nuclei that will populate new branches or developmental structures, this response presumably exists to maximize the likelihood that these nuclei are fully intact.

It is apparent that deletion of rfX1 causes spontaneous DNA DSBs; however, the underlying biochemical mechanism remains to be elucidated. In S. cerevisiae, disruption of Scrfx1 did not induce DNA DSBs but, rather, resistance to DNA damage because of the derepression of RNR1 (5, 6). The transcript levels of the RNR2 homolog were doubled in the F. graminearum Δrfx1 strain compared to the levels in the wild-type, whereas the levels of the RNR1 homolog remained stable (Table 4). However, the observed derepression of RNR2 did not appear to confer resistance to DNA damage. RFX1-GFP localized in nuclei and exhibited high expression levels in growing hyphae and conidiophores, where nuclear division was actively occurring (Fig. 7). We propose that RFX1 functions as a transcriptional repressor that suppresses many genes involved in DNA damage repair and the cell cycle during normal nuclear division. In accordance with this view, RNA-Seq analysis showed that deletion of rfX1 triggered the upregulation of many genes, including those involved in DNA damage repair (Table 3; see also Dataset S1 in the supplemental material). The uncontrolled expression of genes in actively growing cells would presumably disrupt DNA replication and chromosome division, generating DNA DSBs.

In conclusion, the rfX1 gene is important for genome integrity in F. graminearum. Disruption of the rfX1 function resulted in DNA DSBs that, in turn, produced micronuclei and prevented septum formation. Abnormal cell growth caused multiple defects in hyphal growth, conidiation, virulence, sexual development, and the DNA damage response. These results were different from the yeast model proposed in previous studies (2, 6, 7). In the transcriptional rewiring theory, DNA-binding domains of TFs rarely differ substantially across species, but the target genes regulated by TFs can differ considerably (12). Through transcriptional rewiring, ciliary gene promoters were found to acquire RFX TF regulation early in the animal lineage (11). We propose that RFX1 co-opted control of genes involved in genome integrity by a similar process in F. graminearum. However, the target genes directly regulated by RFX1 have not been identified. Transcriptomic analysis of the ΔrfX1 strain indicated that rfX1 directly or indirectly suppressed more than 5,000 genes. Future work, such as chromatin immunoprecipitation-sequencing, will explore downstream genes that RFX1 binds directly. These studies will help to uncover the function of RFX1 and the evolution of its transcriptional network in fungi.
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