GzRUM1*, Encoding an Ortholog of Human Retinoblastoma Binding Protein 2, is Required for Ascospore Development in *Gibberella zeae

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Gibberella zeae (anamorph: *Fusarium graminearum*), a homothallic (self-ferile) ascomycete with ubiquitous geographic distribution, causes serious diseases in several cereal crops. Ascospores (sexual spores) produced by this fungal pathogen have been suggested as the main source of primary inoculum in disease development. Here, we report the function of a gene designated *GzRUM1*, which is essential for ascospore formation in *G. zeae*. The deduced product of *GzRUM1* showed significant similarities to the human retinoblastoma (tumor suppressor) binding protein 2 and a transcriptional repressor, Rum1 in the corn smut fungus (*Ustilago maydis*). The transcript of *GzRUM1* was detected during the both vegetative and sexual stages, but was more highly accumulated during the latter stage. In addition, no *GzRUM1* transcript was detected in a *G. zeae* strain lacking a mating-type gene (*MAT1-2*), a master regulator for sexual development in *G. zeae*. Targeted deletion of *GzRUM1* caused no dramatic changes in several traits except ascospore formation. The Δ *GzRUM1* strain produced perithecia (sexual fruit bodies) but not asci nor ascospores within them. This specific defect leading to an arrest in ascospore development suggests that *GzRUM1*, as *Rum1* in *U. maydis*, functions as a transcriptional regulator during sexual reproduction in *G. zeae*.

Keywords : *Gibberella zeae*, retinoblastoma binding protein, ascospore development, transcription regulator

The ascomycete fungus *Gibberella zeae* (anamorph: *Fusarium graminearum*) is an important pathogen of major cereal plants, causing disease symptoms such as head blight, scab, and stalk or ear rot (McMullen et al., 1997). In addition to direct yield losses, this globally-distributed fungus produces mycotoxins, threatening human and animal health (Desjardins, 2006). To develop a comprehensive strategy for the disease control, many researchers have focused on the ability of *G. zeae* to produce sexual spores, which are

essential for completing the recurrent cycle of the diseases caused by *G. zeae* (Trail et al., 2002). Sexual reproduction by *G. zeae* is a complex developmental process, initiated and controlled by the mating type (*MAT*) locus, the single master regulator of mating (Debuchy et al., 2006; Yun et al., 2000).

In an effort to investigate the regulatory network for sexual development in *G. zeae*, genome-wide approaches have been employed (Güldener et al., 2006; Hallen et al., 2007; Qi et al., 2006). Recently, we identified more than 100 genes that are differentially regulated in this process by comparing the transcriptional and protein profiles between a self-fertile wild-type *G. zeae* strain and self-sterile *MAT*-deleted mutants (Lee et al., 2006, 2008). Among these genes, we were interested in a hypothetical gene (designated *GzRUM1*) whose putative protein product exhibited similarity to human retinoblastoma binding protein 2 (RBP2) (Fattaey et al., 1993; Nevin, 1992). Orthologs of *GzRUM1* are highly conserved in a wide range of filamentous fungi, but their functional roles have not yet been elucidated except in the case of *Rum1* from the corn smut fungus *Ustilago maydis*, which is known to be a transcriptional repressor essential for sporulation (Quadbeck-Seeger et al., 2000). Therefore, the objective of this study was to determine the functional role of *GzRUM1* in *G. zeae*, especially during sexual development.

Materials and Methods

Fungal strains and cultures. The *G. zeae* strains used in this study were Z03643, T43 Δ M1-3, and T43 Δ M2-2. Z03643 is a wild-type self-fertile strain (Lee et al., 2006) belonging to lineage 7 of the *F. graminearum* species complex (O'Donnell et al., 2000). T43 Δ M1-3 (Lee et al., 2008) and T43 Δ M2-2 (Lee et al., 2006) are transgenic self-sterile strains constructed by deletion of the *MAT1-1* and *MAT1-2* idiomorphs, respectively, from the genome of Z03643. Fungal strains from 25% glycerol stock cultures stored at -80°C were maintained on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA). For perithecia

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formation and total RNA extraction, these strains were grown on carrot agar plates as described previously (Lee et al., 2006; Leslie and Summerell, 2006). For vegetative growth, the strains were grown in either PDA or complete medium (CM; Leslie and Summerell, 2006). *Escherichia coli* strains were grown on Luria-Bertani agar or in liquid medium supplemented with 75 µg/ml ampicillin.

Nucleic acid manipulations, PCR, and vector construction. Fungal nucleic acid extractions and other general procedures for restriction endonuclease digestion, agarose gel electrophoresis, gel blotting, ³²P probe labeling, and hybridization were performed as described previously (Lee et al., 2006; Sambrook and Russell, 2001). PCR primers were synthesized by the Bioneer oligonucleotide synthesis facility (Bioneer Corp., Chungwon, Korea), diluted to 100 µM in sterilized water, and stored at –20 °C. PCR was performed as described previously (Kim et al., 2008). Sequences were compared with those available from the NCBI GenBank (<http://www.ncbi.nlm.nih.gov>) and *F. graminearum* genome databases (<http://www.broad.mit.edu/annotation/fungi/fusarium>) using the BLAST algorithm.

A fungal transforming vector for the deletion of *GzRUM1* from the genome of Z03643 was made using an inverse PCR, as described previously (Kim et al., 2008). A 2.8-kb DNA fragment carrying 1.06 kb-5'- and 1.74 kb-3'-flanking sequences of the *GzRUM1* open reading frame (ORF) was amplified by inverse PCR with the primers GC/p2-B (5'-GAAGATCTTCGCGACCTGGTGAAGTTGGAAGC-3') and delRum1/p1 (5'-CAGAGTTGACGGCAGTAAATCCACCAGA-3') using *KpnI*-digested and self-ligated Z03643 genomic DNA as a template. The inverse PCR product was cloned into pGEMT (Promega Corp., Madison, WI, USA), creating pΔGzRUM1 (5.8 kb). Finally, a *SaII*-digested *hygB* cassette, derived from pBCATPH, was cloned into

pΔGzRUM1, resulting in a 7.9-kb plasmid (designated pΔGzRUM1-H). For fungal transformation, pΔGzRUM1-H was linearized by digestion with *KpnI*.

Fungal transformation and virulence testing. For protoplasting, young mycelia derived from fungal conidia were harvested and digested with Drisease in 1 M NH₄Cl. All other PEG-mediated transformation steps were performed as described previously (Kim et al., 2008). The virulence of the fungal strains was determined on young ears of corn as described previously (Han et al., 2004). Fungal mycelia and conidia grown on carrot agar for 2 weeks were scraped using a toothpick and injected into a young ear of corn. The inoculated corn samples were left for 1 week in a plastic box in which the humidity was maintained using several wet paper towels.

Results

Sequence similarity and domain structure of GzRUM1.

GzRUM1 (named based on its similarity to *U. maydis Rum1*) was first identified as a down-regulated gene in *G. zeae* lacking *MAT1-2* (Lee et al., 2006). The deduced product of *GzRUM1* (FGSG_05855.3), interrupted by 6 putative introns, encodes a 1,657 amino acid protein annotated as a hypothetical protein in the *F. graminearum* genome database (http://www.broad.mit.edu/annotation/genome/fusarium_group/MultiHome.html). *GzRUM1* displayed similarities in amino acid sequence to the human retinoblastoma binding protein 2, RBP2 (33% identity over 765 amino acids, GenBank accession no. AAB28544; Fattaey et al., 1993), its human ortholog PLU-1 (32% over 846 amino acids, CAB43532; Aasland et al., 1995), and its fungal orthologs Rum1 from *U. maydis* (32% over 1,398 amino acids, AAG02418; Quadbeck-Seeger et al., 2000)

Fig. 1. Conserved domains in GzRUM1. Domain structure of GzRUM1 compared with that of other RBP2-like proteins. The numbers in the scale bar correspond to amino acid positions.

and Msc1 from the fission yeast *Schizosaccharomyces pombe* (29% over 1,120 amino acids, NP_593431; Ahmed et al., 2004)]. GzRUM1 carries several conserved domains, including jumonji (jnj) N and C domains, plant homeo-domain (PHD) fingers, a zinc-finger domain, and a PLU-1 domain with an overall domain organization similar to that in 3 multi-domain nuclear proteins (Fig. 1). Jumonji domains have been found in the jumonji family of transcription factors (Balciunas and Ronne, 2000). Two PHD fingers in GzRUM1 have a typical C4HC3 zinc-finger like motif and are found predominantly in nuclear proteins associated with chromatin-mediated transcriptional regulation (Aasland et al., 1995). Unlike GzRUM1, the other 3 proteins contain 3 PHD finger domains. The single zinc-finger domain found in the proteins aligned was a C5HC2 zinc-finger motif. In addition, a region previously designated as a PLU domain (Quadbeck-Seeger et al., 2000) between the zinc-finger and second PHD domains was highly conserved in all four proteins (Fig. 1), which is derived from the human PLU-1 protein up-regulated in breast cancer cells (Lu et al., 1999). However, the AT-rich interaction domain (ARID) found in both RBP2 and Rum1 was missing from GzRUM1 and Msc1.

Transcript analysis. The transcript of *GzRUM1* was detected in the wild-type strain Z03643 during both vegetative and sexual development (i.e., perithecial induction) on carrot agar as well as under nutrient-rich conditions (PD broth and CM). However, it was more highly accumulated throughout the entire perithecial stage than during vegetative growth on carrot agar or in liquid media (Fig. 2). Interestingly, *GzRUM1* expression appeared to be reduced

or abolished in those *G. zeae* strains lacking *MAT1-1* or *MAT1-2* during the vegetative and sexual stages.

Targeted gene deletion and complementation. Integration of the linearized plasmid (pΔGzRUM1-H) into the fungal genome via a double crossover resulted in a *G. zeae* strain lacking *GzRUM1* (Fig. 3A). Targeted gene deletion was verified by DNA gel blot analysis (Fig. 3B). Genomic DNA from the Δ*GzRUM1* strains carried a single 7.9-kb hybridizing band when digested with *KpnI*, instead of the 6.8-kb band that hybridized in Z03643, suggesting that the entire *GzRUM1* ORF had been deleted and replaced with *hygB*. Ten out of 18 selected hygromycin B-resistant transformants turned out to be Δ*GzRUM1* strains, and the remaining 8 transformants carried the plasmid DNA at ectopic positions. For genetic complementation, the circular plasmid pGzRUM1 carrying the entire *GzRUM1* ORF and its flanking sequences was introduced into the Δ*GzRUM1* strain (TDGzRUM1-2) along with pII99 (Namiki et al., 2001) carrying a geneticin resistance gene. Five out of 15 geneticin-resistant transformants of the TDGzRUM1-2 strain carried an intact copy of *GzRUM1*, as confirmed by PCR (data not shown).

Sexual defects in the Δ*GzRUM1* strains. To determine the roles of *GzRUM1* in fungal physiology, the phenotypes of the Δ*GzRUM1* strains were compared to those of the wild-type progenitor strain Z03643. Mycelia growth, pigmentation, and asexual sporulation (conidiation) in the Δ*GzRUM1* strains were indistinguishable from those in Z03643. In addition, we examined the pathogenic development of the

Fig. 2. *GzRUM1* expression in the wild-type strain Z03643 (WT), Δ*MAT1-1* strain T43ΔM1-3, and Δ*MAT1-2* strain T43ΔM2-2. RNA gel blots of these strains were hybridized with a 1,473-bp *SalI* fragment within the *GzRUM1* ORF, which was obtained by the enzymatic digestion of the *GzRUM1* product amplified with Rum/p1 (5'-GAACCGCGCCACGACTCCACAC-3') and Rum/p2 (5'-CCCTCGGGCACTCATAGCAC-3'). C and P: total RNA from mycelia grown in CM liquid medium and potato dextrose broth, respectively, for 6 days. The incubation times (days) for vegetative growth and perithecia induction on carrot agar are indicated above the blots. The loading control was ethidium bromide-stained rRNA from Z03643.

Fig. 3. Targeted deletion of *GzRUM1* from the genome of Z03643, and fungal virulence on corn ears. (A) Deletion scheme. WT, genome of Z03643; Δ*GzRUM1*, genome of the Δ*GzRUM1* strain. K, *KpnI* recognition site. (B) DNA gel blot probed with pΔGzRUM1-H. Lane 1, Z03643; lanes 2-5, Δ*GzRUM1* strains; lane 6, transformant carrying pΔGzRUM1-H at an ectopic position. DNA size markers are indicated on the left side of the gel. (C). Necrotic symptoms caused by fungal strains on corn ears. WT, Z03643; Δ*GzRUM1*, a Δ*GzRUM1* strain; Com, a Δ*GzRUM1*::*GzRUM1* strain. Scale bar = 1 cm.

Fig. 4. Sexual development in the Δ GzRUM1 strain. Perithecia formation in Z03643 (A) and a Δ GzRUM1 strain (B) on carrot agar. Ascospores within each ascus (indicated by an arrow) from broken perithecia were observed in Z03643 (C), whereas no ascospores were produced within the smaller perithecia of the Δ GzRUM1 strain (D). Scale bar = 100 μ m.

G. zeae Δ GzRUM1 strains on a host plant. Inoculation with the Δ GzRUM1 strains had similar effects on corn ears as Z03643, resulting in a large area of necrosis around the inoculation points (Fig. 3C). In contrast, the Δ GzRUM1 strains continued to produce conidia after perithecial induction, while asexual sporulation in Z03643 was repressed during sexual development (data not shown). Seven days after perithecial induction on carrot agar, the Δ GzRUM1 strains produced similar numbers of perithecia to Z03643 (Fig. 4A and B). However, the perithecia formed on the Δ GzRUM1 strains examined were much smaller than normalized perithecia from the Z03643 culture, and carried neither asci nor ascospores even 4 weeks after perithecial induction; typically, more than 95% of the perithecia on Z03643 developed into fertile perithecia under the same conditions (Fig. 4C and D). All of the sexual defects in the Δ GzRUM1 strains were recovered to the wild-type level in those Δ GzRUM1 strains carrying an intact copy of *GzRUM1*, confirming that the deletion of *GzRUM1* was the direct cause of the sexual defects in the Δ GzRUM1 strains.

Discussion

Although orthologs of *GzRUM1* exist in the genomes of many filamentous fungi, *U. maydis* *Rum1* is the only one whose function had been characterized (Quadbeck-Seeger et al., 2000). Comparing the phenotypic changes caused by the deletion in both fungi revealed a functional similarity between the two genes, which is specific to fungal sexual development. In *U. maydis*, the deficiency of *Rum1* caused

no defects in asexual development (i.e., morphology and growth of the haploid yeast-like spores [sporidia]) or pathogenicity (i.e., tumor development initiated by mating between sporidia from compatible strains within the host plant). However, the further discrete morphological changes leading to the formation of sexual teliospores, such as hyphal fragmentation and subsequent spore wall deposition (Banuett and Herskowitz, 1996) were not observed within the tumors produced by Δ *rum1* strains, demonstrating that the *rum1* mutation affects a defined stage in sexual development, at least that followed by karyogamy (i.e., nuclear fusion in dikaryotic hyphae) (Quadbeck-Seeger et al., 2000). Moreover, the deletion of *Rum1* caused the derepression of several genes regulated by the homeodomain transcription factors bE and bW located at the mating type locus, leading to a failure to form sexual teliospores. Similarly, the phenotypic change in the *G. zeae* Δ GzRUM1 strains was restricted to the stage of ascospore development within perithecia (sexual fruiting body); other phenotypes such as the initiation of perithecia development as well as asexual hyphal growth and formation of asexual spores (conidia) were indistinguishable from those of the wild-type strain. Although the developmental processes leading to sexual spore formation in both fungi (i.e., haploid ascospores in the ascomycete *G. zeae* and diploid teliospores in the basidiomycete *U. maydis*) are distinct, our results indicate that *GzRUM1* plays important roles in the regulation of specific stages of sexual development, including meiosis, cytokinesis, and asci/ascospore formation. However, it is unknown whether or not the nuclear fusion in ascogenous hyphae is affected by Δ GzRUM1 in *G. zeae*. The proposed functional role of *GzRUM1* could also be supported by cases from its other orthologs. Human PLU-1 is suggested to have a specific role during meiosis in mice (Madsen et al., 2003), and *RUM1* from the human fungal pathogen *Cryptococcus neoformans*, located within the mating-type alleles (Lengeler et al., 2002), is hypothesized to function in meiosis (Fraser et al., 2004).

The way *GzRUM1* operates in gene regulation during sexual development can be inferred from its structural similarity to human and fungal orthologs at the protein level. Each domain that was highly conserved in all proteins (*GzRUM1*, *RPB2*, *Rum1*, and *Msc1*) aligned in this study usually co-occurs in large multi-domain proteins, and is known or thought to be involved in histone-mediated chromatin modification. JmjC-domain proteins may be protein hydroxylases that catalyze a novel histone modification, and they contain PHD fingers (Gregory et al., 1996). PHD finger-containing proteins have been proposed to be involved in protein-protein interactions (Aasland et al., 1995), but they were recently found to have E3 ubiquitin ligase activity, and their likely target is a chromatin-associ-

ated protein (Dul and Walworth, 2007). RBP2 is known to bind to the tumor suppressor (retinoblastoma), and it regulates the expression of several genes essential for cell cycle progression in animals, in particular at the G₁/S transition, by influencing chromatin structure (Nevin, 1992). Msc1 is suggested to play a role in regulating chromatin structure through histone modification since it is required for the global deacetylation of histone H3 (Ahmed et al., 2004). Therefore, it is likely that GzRUM1 is a putative multi-domain nuclear protein involved in the chromatin-level regulation of genes important for ascospore development in *G. zeae*.

It is not yet clear how *GzRUM1* participates in the regulatory network controlling sexual development in *G. zeae*, although *GzRUM1* seems to be regulated by the mating-type locus. A real-time PCR analysis would provide more quantitative expression pattern of *GzRUM1*. A microarray analysis using the Δ *GzRUM1* strain might reveal a set of genes that are either activated or repressed by GzRUM1 during sexual development. Additional studies are necessary to identify the proteins that directly interact with GzRUM1, which may be similar to the binding partners of RBP2 (e.g., human retinoblastoma [Rb] and Rb-related proteins), and to determine if GzRUM1 localizes to the nucleus and is associated with chromatin structure.

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