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Functional analyses of regulators of G protein signaling in *Gibberella zeae*

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ABSTRACT

Regulators of G protein signaling (RGS) proteins make up a highly diverse and multifunctional protein family that plays a critical role in controlling heterotrimeric G protein signaling. In this study, seven *RGS* genes (*FgFlbA*, *FgFlbB*, *FgRgsA*, *FgRgsB*, *FgRgsB2*, *FgRgsC*, and *FgGprK*) were functionally characterized in the plant pathogenic fungus, *Gibberella zeae*. Mutant phenotypes were observed for deletion mutants of *FgRgsA* and *FgRgsB* in vegetative growth, *FgFlbB* and *FgRgsB* in conidia morphology, *FgFlbA* in conidia production, *FgFlbA*, *FgRgsB*, and *FgRgsC* in sexual development, *FgFlbA* and *FgRgsA* in spore germination and mycotoxin production, and *FgFlbA*, *FgRgsA*, and *FgRgsB* in virulence. Furthermore, *FgFlbA*, *FgRgsA*, and *FgRgsB* acted pleiotropically, while *FgFlbB* and *FgRgsC* deletion mutants exhibited a specific defect in conidia morphology and sexual development, respectively. Amino acid substitutions in Gα subunits and overexpression of the *FgFlbA* gene revealed that deletion of *FgFlbA* and dominant active GzGPA2 mutant, $gzgpa2^{Q207L}$, had similar phenotypes in cell wall integrity, perithecia formation, mycotoxin production, and virulence, suggesting that FgFlbA may regulate asexual/sexual development, mycotoxin biosynthesis, and virulence through GzGPA2-dependent signaling in *G. zeae*.

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1. Introduction

The ascomycete fungus, *Gibberella zeae* (anamorph *Fusarium graminearum*), is the most common causal agent of Fusarium head blight (FHB), a devastating disease for cereal crops worldwide (Leslie and Summerell, 2006). FHB causes significant crop losses by reducing grain yield and quality, as well as contaminating cereals with mycotoxins. The main mycotoxins produced by *G. zeae* are trichothecene deoxynivalenol (DON) and estrogenic toxin zearalenone (ZEA), both of which are hazardous to humans and livestock (Desjardins, 2006). As the primary and secondary inocula for epidemics of FHB on cereal crops, *G. zeae* produces ascospores (sexual spores) and conidia (asexual spores), respectively. Ascospores are hypothesized to be of greater importance in treating FHB epidemics, since FHB inoculum requires aerial dispersal to the cereal heads through the forcible discharge of ascospores into the air from perithecia (Sutton, 1982; Trail, 2007).

Production of spores and secondary metabolites are frequently linked in filamentous fungi, and require morphological and physiological transitions that are tightly regulated by both environmental signals and intracellular signaling pathways (Brodhagen and Keller, 2006). In *Aspergillus nidulans*, asexual development is usually accompanied by sterigmatocystin (ST) production, and these processes are regulated by G protein signaling pathways (Hicks et al., 1997). G protein signaling has been well studied in eukaryotes, and has important roles in regulating various cellular functions, including mating, development, secondary metabolism, and pathogenicity in fungi (reviewed in Bölker, 1998). Heterotrimeric G proteins consist of Gα, Gβ, and Gγ subunits, and mediate signal transduction between transmembrane G protein coupled receptors (GPCRs) and intracellular effectors such as mitogen-activated protein kinases, adenylyl cyclase-cAMP dependent protein kinases, ion channels, and phospholipases (Neer, 1995; Neves et al., 2002; Simon et al., 1991). For example, upon receiving extracellular stimuli, GPCRs interact with G proteins to induce a substitution of GDP for GTP in the Gα subunit, which leads to dissociation of the activated Gα subunit from the Gβγ subunits. The slow, intrinsic GTPase activity of the activated Gα subunit then hydrolyzes GTP, thereby facilitating the re-association of the GDP-bound Gα subunit with Gβγ and resetting the G protein cycle. The rate of GTP hydrolysis of the Gα subunit determines the intensity of the signaling produced, and plays a key role in controlling the rapid, yet precise signaling responses in the cell (McCudden et al., 2005).

Regulator of G protein signaling (RGS) proteins possess a conserved RGS amino acid domain that interacts with the Gα subunit.

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This interaction greatly facilitates the intrinsic GTPase activity of Gα subunit, and rapidly turns off activated G protein signals, thereby negatively regulating G protein signaling (Chidiac and Roy, 2003). In addition to directly suppressing G protein signaling through GTPase-accelerating protein (GAP) activity, RGS proteins can also enhance the activation of G-protein pathways by acting as effectors, effector antagonists, and scaffolding proteins for receptors, G proteins, or other regulatory molecules (Ballon et al., 2006; Chen and Hamm, 2006; Zhong and Neubig, 2001). Subsequent studies of amino acid substitutions in Gα subunits and disruption of *RGS* genes have provided additional insight into the mechanisms of G protein signaling and novel functions of RGS proteins in fungi (Hicks et al., 1997; Miyajima et al., 1989; Yu et al., 1996; Zhang et al., 2011). For example, in *Saccharomyces cerevisiae*, Sst2p has been found to negatively regulate pheromone signaling via inhibition of the Gα subunit (Dietzel and Kurjan, 1987), while other *S. cerevisiae* RGS proteins, including Rgs2p, Rax1p, and Mdm1p, have been found to be required for stress responses, budding polarity, and mating efficiency (Ballon et al., 2006; Chasse et al., 2006). In *A. nidulans*, FlbA is able to negatively regulate mycelia proliferation and activates sterigmatocystin (ST) production and conidiation (Yu, 2006). RgsA of *A. nidulans* down-regulates the stress response and conidia germination, but stimulates ST production and conidiation (Han et al., 2004). Moreover, in the rice blast fungus, *Magnaporthe oryzae*, Rgs1 has been found to regulate virulence, asexual development, and thigmotropism (Liu et al., 2007), and in *F. verticillioides*, both FlbA2 and RgsB are required to control vegetative growth and fumonisin B_1 production (Mukherjee et al., 2010).

In our previous study of the phytopathogenic fungus *G. zeae*, three putative Gα protein coding genes, *GzGPA1*, *GzGPA2*, and *GzGPA3*, were individually deleted (Yu et al., 2008). When *GzGPA1* was deleted, female sterility was compromised and mycotoxin production was enhanced. When *GzGPA2* was deleted, virulence was reduced and chitin accumulation increased in the cell wall. However, *GzGPA3* null mutants did not exhibit any phenotypic changes. Although the key function of RGS proteins as the negative controller of intracellular signaling through interactions with Gα subunits was demonstrated in other fungi, the functions of RGS proteins in *G. zeae* remain largely unknown. Therefore, in this study, seven *RGS* genes from *G. zeae* were characterized, and mechanistic roles of FgFlbA in G protein signaling pathways were investigated by site-directed mutagenesis of Gα proteins and overexpression studies of the *FgFlbA* gene. The results demonstrate that FgFlbA is involved in various cellular processes including conidiation, sexual development, cell wall integrity, mycotoxin biosynthesis, and virulence via GzGPA2-dependent signaling pathways in *G. zeae*.

2. Materials and methods

2.1. Fungal strains and media

The wild-type strain Z-3639 of *G. zeae* (Bowden and Leslie, 1999) and mutants derived from this strain were maintained in media according to the *Fusarium* laboratory manual (Leslie and Summerell, 2006). Conidia were induced in carboxyl methyl cellulose (CMC) medium (Cappellini and Peterson, 1965) and on yeast malt agar (YMA) (Harris, 2005). All strains were stored as conidia suspensions in 20% glycerol at −70 °C.

2.2. Nucleic acid manipulations, PCR primers, and PCR conditions

Genomic DNA was extracted using a cetyltrimethylammonium bromide procedure (Leslie and Summerell, 2006). Restriction endonuclease digestion, gel electrophoresis, gel blotting, and Southern hybridizations with ^{32}P-labeled probes were performed according to standard procedures (Sambrook and Russell, 2001). PCR primers used for this study (Table S1) were synthesized by the Bionics oligonucleotide synthetic facility (Seoul, Korea). Total RNA was extracted using the Easy-Spin Total Extraction Kit (Intron Biotech, Seongnam, Korea), and first strand cDNA was synthesized with SuperScriptIII reverse transcriptase (Invitrogen, Carlsbad, CA, USA).

2.3. Targeted gene deletion and complementation

For targeted gene deletion, a double joint (DJ)-PCR strategy was used (Yu et al., 2004). Briefly, 5′ and 3′ flanking regions of each target gene were amplified by PCR using the primer pairs, RGS-5for/RGS-5rev and RGS-3for/RGS-3rev, from the wild-type strain, respectively. A geneticin-resistance cassette (*gen*) was amplified from pII99 (Namiki et al., 2001). These three amplicons (e.g., 5′ region, 3′ region, and *gen*) were then mixed at a 1:1:2 M ratio and fused in a second round of PCR. Fused constructs were amplified using the nested primers, RGS-nf and RGS-nr. To complement deletion mutants, *RGS* genes, including native promoter and terminator, were amplified from genomic DNA of the wild-type strain using the RGS-nf and RGS-nr primer pairs. Intact copies of *RGS* genes were then directly transformed into fungal protoplasts of corresponding deletion mutants, along with the vector pUCH1 carrying the hygromycin-resistance gene (*hyg*) as a selective marker (Turgeon et al., 1987). Fungal transformation was performed as previously described (Han et al., 2007).

2.4. Germination test

Germination tests were performed as previously described (Lee et al., 2009b). Briefly, mycelia harvested from potato dextrose broth (PDB) were spread on YMA and incubated for 2 d at 25 °C under near-UV light (wavelength: 365 nm, HKiv Import & Export Co., Ltd., Xiamen, China) to induce conidiation. Conidia were then harvested in distilled water, filtered through a layer of Miracloth (CalBiochem, San Diego, CA, USA), washed twice with distilled water, and centrifuged (5000 rpm, 25 °C, 5 min). Ascospores were harvested by placing carrot agar upside down to capture ascospores discharged by mature perithecia on petri dish covers.

One ml of spore suspension (10^6 conidia/ml) was incubated in 50 ml PDB. The total number of germinated spores at 0, 1, 2, 3, 4, 5, and 6 h were then counted. One hundred conidia for each experiment were also observed by light microscopy, and these experiments were performed twice with three replicates.

2.5. Protoplast production assay

Protoplast production assays for *G. zeae* strains were performed according to Hou et al. (2002) with some modifications. Briefly, fungal conidia produced from CMC cultures were inoculated into YPG liquid medium (3 g yeast extract, 10 g peptone, and 20 g glucose per liter) at 10^6 per ml and grown for 12 h with shaking at 25 °C. Mycelia were harvested by filtration through sterile Whatman No. 2 filter paper and incubated in 1 M NH_4Cl containing 1% Driselase (Karlan Research Products, Santa Rosa, CA, USA) to generate protoplasts for 90 min. Formation of protoplasts was monitored using a DE/Axio Image A1 microscope (Carl Zeiss, Oberkochen, Germany) with a CCD camera.

2.6. Sexual crosses and ascospore discharge

Mycelia grown on carrot agar for 5 d were mock fertilized for selfing as previously described (Leslie and Summerell, 2006). For outcrosses, mycelia of the female strain grown on carrot agar plates were fertilized with 1 ml male strain conidia suspensions

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