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Cloning and Functional Analysis of the Gβ Gene *Mgb1* and the Gγ Gene *Mgg1* in *Monascus ruber*[§]

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The ascomycetous fungus *Monascus ruber* is one of the most well-known species widely used to produce *Monascus*-fermentation products for natural food colorants and medicine. Our previous research on the Gα subunit Mga1 and the regulator of G protein signaling MrflbA indicated that heterotrimeric G protein signaling pathways were involved in aspects of growth, sporulation and secondary metabolite production in *M. ruber*. To better understand the G protein signaling pathways in this fungus, a Gβ subunit gene (*Mgb1*) and a Gγ subunit gene (*Mgg1*) were cloned and investigated in the current study. The predicted Mgb1 protein consisted of 353 amino acids and Mgg1 consisted of 94 amino acids, sharing marked similarity with *Aspergillus* Gβ and Gγ subunits, respectively. Targeted deletion (Δ) of *Mgb1* or *Mgg1* resulted in phenotypic alterations similar to those resulting from Δ*Mga1*, i.e., restricted vegetative growth, lowered asexual sporulation, impaired cleistothecial formation, and enhanced citrinin and pigment production. Moreover, deletion of *Mgg1* suppressed the defects in asexual development and in biosynthesis of citrinin and pigment caused by the absence of MrflbA function. These results provide evidence that Mgb1 and Mgg1 form a functional Gβγ dimer and the dimer interacts with Mga1 to mediate signaling pathways, which are negatively controlled by MrflbA, for growth, reproduction and citrinin and pigment biosynthesis in *M. ruber*.

***Keywords*:** *Monascus ruber*, G-protein beta gamma, growth, reproduction, citrinin, pigment

Introduction

The genus *Monascus* was classified and named in 1884 by French scientist van Tieghem (van Tieghem, 1884). It belongs to the family Monascaceae, order Eurotiales, class Ascomycetes, and phylum Ascomycota. This fungus can reproduce either vegetatively with filaments and conidia, or sexually by the formation of asci and ascospores, which are associated with cleistothecia (Patakova, 2013). Today, there are nine *Monascus* species, and the best known species are *M. ruber*, *M. purpureus* and *M. pilosus*, which are widely used to produce *Monascus*-fermentation products (MFPs) (Lee and Pan, 2012). MFPs have been employed as food colorants and medicine for more than 1,000 years in Asian countries. To date, MFPs have been proven to be effective for the management of blood cholesterol, diabetes, blood pressure, obesity and Alzheimer's disease, and for the prevention of cancer development, due to the variety of bioactive metabolites produced by *Monascus* spp., including pigments, polyketide monacolins, dimerumic acid, γ-aminobutyric acid, and some other chemical components that have not been purified and identified (Lee and Pan, 2011, 2012; Hsu and Pan, 2012; Shi and Pan, 2012; Feng *et al.*, 2013). However, a safety issue has emerged since 1995 when Blanc *et al.* (1995a, 1995b) detected a hepatotoxic and nephrotoxic mycotoxin, citrinin, in some MFPs. Due to concerns of citrinin contamination, many of the recent investigations on *Monascus* spp. are focused on enhancing the production of useful metabolites and eliminating the production of citrinin (Wang *et al.*, 2005; Lee *et al.*, 2007; Pattanagul *et al.*, 2008; Jia *et al.*, 2010).

Recently, G protein signaling pathways have been demonstrated to be involved in fungal secondary metabolite biosynthesis (Li *et al.*, 2007; Sagaram and Shim, 2007; Yu *et al.*, 2008; Shin *et al.*, 2009). One important element of the G protein signaling pathway is the heterotrimeric G protein composed of α, β, and γ subunits. Heterotrimeric G proteins not only regulate biosynthesis of secondary metabolites, but also influence fungal germination, vegetative growth, mating, morphogenesis and pathogenicity in various ways (Li *et al.*, 2007).

Most characterized filamentous fungi possess five G protein-encoding genes, three for Gα, one for Gβ and one for Gγ (Li *et al.*, 2007; Servin *et al.*, 2012). In the model filamentous fungus *Aspergillus nidulans,* all of the five heterotrimeric G protein subunits, FadA (Gα), GanA (Gα), GanB (Gα), SfaD (Gβ), and GpgA (Gγ), have been identified, and two independent RGS-Gα signaling pathways have been proposed: (1) the FlbA-FadA signaling pathway controls vegetative growth, which in turn inhibits both development and toxin production; (2) the RgsA-GanB signaling pathway controls stress response, carbon sensing and germination (Han *et al.*, 2004; Lafon *et al.*, 2005; Yu, 2006). FlbA and RgsA are specific regulators of G protein signaling (RGS), respectively controlling FadA-mediated signaling and GanB-mediated signaling, by enhancing the intrinsic GTPase activity of the

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corresponding Gα protein FadA or GanB (Yu *et al.*, 1996; Han *et al.*, 2004; Yu, 2006) .The Gβ subunit SfaD has been presumed to function as a heterodimer with the Gγ subunit gpgA. The SfaD::GpgA dimer is involved in both of the RGS-Gα signaling pathways via the formation of two different heterotrimers FadA-SfaD::GpgA and GanB-SfaD::GpgA (Rosen *et al.*, 1999; Seo *et al.*, 2005; Seo and Yu, 2006). Additionally, SfaD and GpgA are hypothesized to be the primary signaling components for sexual development in *A. nidulans*, as deletion of either *sfaD* or *gpgA* resulted in the absence of sexual fruiting body formation after self-fertilization and caused a severe impairment in sexual development in outcrosses (Seo *et al.*, 2005).

In our previous study, we investigated the roles of the FadA (Gα) homologue gene *Mga1* and the FlbA (RGS protein) homologue gene *mrflbA* in the homothallic ascomycetous fungus *M. ruber* and demonstrated that both *Mga1* and *mrflbA* were involved in regulating growth, reproduction, and citrinin and pigment production (Li *et al.*, 2010; Yang *et al.*, 2012). To further understand the G protein mediated growth and development, particularly secondary metabolite production in the industrially important *Monascus* spp., we have cloned and functionally analyzed the SfaD (Gβ) homologue gene *Mgb1* (*Monascus* G protein beta-subunit 1) and the GpgA (Gγ) homologue gene *Mgg1* (*Monascus* G protein gamma-subunit 1) in *M. ruber*, providing further understanding of G protein mediated signaling in this economically important group of microorganisms.

Materials and Methods

Strains and media

The *Monascus ruber* wild-type strain (WT) M7 was used to generate the gene knockout strains Δ*Mgb1* and Δ*Mgg1*. The resulting Δ*Mgb1* strain was then used to construct the double deletion strain Δ*Mgb1*Δ*Mgg1*. The Δ*mrflbA* strain (Yang *et al.*, 2012) was used to generate the double deletion strain Δ*mrflbA*Δ*Mgg1*. PDA (potato dextrose agar), CYA (Czapek yeast extract agar) and G25N (25% glycerol nitrate agar) were used for strain growth and maintenance. YES (yeast extract sucrose) broth was used to produce citrinin and pigments (Li *et al.*, 2010; Yang *et al.*, 2012). If required, hygromycin B and G418 were added to the medium at a concentration of 30 μg/ml and 15 μg/ml, respectively.

Cloning of the *Mgb1* and *Mgg1* genes

The procedures for cloning the Gβ subunit gene and the Gγ subunit gene were performed as described by Li *et al.* (2010), and the primers for degenerate PCR and single oligonucleotide nested (SON)-PCR (Antal *et al.*, 2004) are listed in Table 1. The degenerate primer pairs GBF/GBR and GGF/GGR were designed to amplify the conserved regions of the Gβ subunit-encoding gene *Mgb1* and the Gγ subunit-encoding gene *Mgg1*, respectively. Nested primer sets B31/B32 and B51/B52/B53 were designed based on the sequenced *Mgb1* fragment to isolate its flanking regions. Primer B52 was used as the inner SON-PCR primer for the first amplification, as well as the outer SON-PCR primer for the second amplification of the 5′-end of *Mgb1* fragment. Nested primers G31 and G32, and G51 and G52 were designed based on the sequenced *Mgg1* fragment to isolate its flanking regions. The optimal annealing temperature of degenerate PCR was determined by the gradient PCR method. The PCR products were purified using a DNA gel extraction kit (Sangon Biotech, China), cloned into pMD18-T (TaKaRa, Japan) and sequenced. The sequences obtained were compared to the GenBank database using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Vector construction and transformation

The *Mgb1* and *Mgg1* deletion cassettes were constructed via the double-joint PCR (DJ-PCR) method (Yu *et al.*, 2004) using primers indicated in Table 1. Primer pairs *mgb*K5f$_1$/*mgb*K5r and *mgg*K5f$_1$/*mgg*K5r were used to amplify the 5′-flanking regions of *Mgb1* and *Mgg1* respectively from WT genomic DNA. The 3′-flanking regions of the individual genes were amplified with *mgb*K3f/*mgb*K3r$_1$ (*Mgb1*) and *mgg*K3f/*mgg*K3r$_1$ (*Mgg1*). The selective marker *hph* (hygromycin B resistance gene) was amplified from plasmid pSKH (He *et al.*, 2013) with primers hygF and hygR, and *neo*^{R} (neomycin resistance gene) was amplified from plasmid pKN1 (He *et al.*, 2013) with primers G418F and G418R. The *mgb*K5r and *mgb*K3f primers contained 25 bases of homologous sequences overlapping with the ends of the *hph* fragment, while *mgg*K5r and *mgg*K3f contained 20 bases of homologous sequences overlapping with the ends of the *neo*^{R} fragment. The amplified fragments from the flanking regions of *Mgb1* were fused with *hph* to form a 3.9 kb recombined fragment, and then amplified with the primer pair *mgb*K5f$_2$/ *mgb*K3r$_2$, which carried *Sal*I and *Kpn*I restriction enzyme sites, respectively, facilitating insertion into the *Sal*I-*Kpn*I sites of the Ti plasmid pCAMBIA3300 to generate the *Mgb1* gene replacement vector pMgb1. The amplified fragments from the flanking regions of *Mgg1* were fused with *neo*^{R} to form a 3.5 kb recombined fragment, and then amplified with the primer pair *mgg*K5f$_2$/*mgg*K3r$_2$, which carried *Hin*dIII and *Kpn*I restriction enzyme sites, respectively, facilitating insertion into the *Hin*dIII-*Kpn*I sites of pCAMBIA3300 to generate the *Mgg1* gene replacement vector pMgg1.

The plasmids pMgb1 and pMgg1 were transformed into *Agrobacterium tumefaciens* EHA105 using the freeze-thaw method. Then the transformed *A. tumefaciens* were used to carry out the *A. tumefaciens*-mediated transformation of *M. ruber* strains to generate the relevant gene deletion mutants.

The Δ*Mgb1* and Δ*Mgg1* strains were generated by direct transformation of *M. ruber* wild-type strain M7. The double deletion mutant strains Δ*Mgb1*Δ*Mgg1* and Δ*mrflbA*Δ*Mgg1* were generated by the transformation of Δ*Mgb1* and Δ*mrflbA* strains, respectively. The genotypes of Δ*Mgb1,* Δ*Mgg1,* Δ*Mgb1* Δ*Mgg1* and Δ*mrflbA*Δ*Mgg1* were confirmed by PCR amplification and Southern hybridization.

Southern hybridization

Southern blotting was performed according to the protocol of the DIG-high prime DNA labeling & detection starter kit I (Roche, Germany). To prepare probes, fragments from the open reading frame (ORF) of *Mgb1*, *Mgg1*, *mrflbA* and

the selective marker gene *hph* and *neo*^{R} were amplified with primer pairs *mgb*F/*mgb*R, *mgg*F/*mgg*R, *mrflbA*F/*mrflbA*R, hygF/hygR, and neoF/neoR (Table 1), respectively, and then labeled with digoxin after purification with the DNA gel extraction kit (Sangon Biotech, China).

Citrinin and pigment analysis

Freshly harvested conidiospores (10^5 conidia/ml) were inoculated into liquid YES medium and incubated at 28°C without agitation for 16 days. To detect citrinin in liquid culture, we followed a protocol from Li *et al.* (2010). The fermentation liquid was passed through filter paper. The filtrate was extracted with an equal volume of toluene-ethyl acetate-formic acid (7: 3: 1 by volume). The organic phase was collected and diluted with methanol. Citrinin was determined by HPLC (Waters e2695) with a Kromasil C_{18} column (250×4.60 mm; particle size, 5 μm; Elite) using acetonitrile and water (pH 2.5) (65:35 v/v) as the mobile phase. The flow rate of the mobile phase was maintained at 1.0 ml/min and the detection was carried out at 330 nm with a photodiode array detector (PDA, Waters 2998). Standard

Table 1. **Primers used in this study**

Name	Sequence (5′→ 3′)	Description
GBF GBR	ATCTAYGCYATGCACTGGTC GAGAAMGCRACRGAHGTGAT	Degenerate primers used to amplify the Gβ-subunit gene
GGF GGR	GTNAAGAASAAGAAGCAGAG TCVCGCTTGTCRACCTG	Degenerate primers used to amplify the Gγ-subunit gene
B31	TCACGAATCCGACATCAATGC	Outer SON-PCR primer for amplification of the 3′-end of *Mgb1* fragment
B32	CCTGTCGCCTCTTCGATATTC	Inner SON-PCR primer for amplification of the 3′-end of *Mgb1* fragment
B51	TGATGAAGCGACAGCAGGAGA	Outer SON-PCR primer for the first amplification of the 5′-end of *Mgb1* fragment
B52	GAATGGCGTGGACCTTGTTAG	Inner SON-PCR primer for the first amplification of the 5′-end of *Mgb1* fragment Outer SON-PCR primer for the second amplification of the 5′-end of *Mgb1* fragment
B53	TGAGAAGAGAGCCCACCGAG	Inner SON-PCR primer for the second amplification of the 5′-end of *Mgb1* fragment
G31	TTCCGGTCGCATCGTCTAATC	Outer SON-PCR primer for amplification of the 3′-end of *Mgg1* fragment
G32	TCGCTAGATAGCACATATTCA	Inner SON-PCR primer for amplification of the 3′-end of *Mgg1* fragment
G51	TGCTTGCCAGCTCCAACAGAT	Outer SON-PCR primer for amplification of the 5′-end of *Mgg1* fragment
G52	GCTCTGTCAACCGCCGATACT	Inner SON-PCR primer for amplification of the 5′-end of *Mgg1* fragment
*mgb*K5f$_1$	AGATTCATTCGGACCCTATT	For amplification of the 1214 bp 5′-flanking region of *Mgb1* ORF
*mgb*K5r	AGTGCTCCTTCAATATCATCTTCTGCCTGCG CTTAACTAGATTGT	
*mgb*K3f	GTTTAGAGGTAATCCTTCTTTCTAGTATTCT CCGAGGCGATGTTG	For amplification of the 667 bp 3′-flanking region of *Mgb1* ORF
mgbK3r$_1$	ACTACTTCCCCAATCTCACC	
*mgb*K5f$_2$	ACGCGTCGACCAGAGGCGTTTGT	For amplification of the 3910 bp *Mgb1* replacement cassette
*mgb*K3r$_2$	CGGGGTACCAGAGGCAATAGAC	
hygF	CAGAAGATGATATTGAAGG	For amplification of the 2132 bp *hph* cassette from plasmid pSKH
hygR	CTAGAAAGAAGGATTACCTC	
*mgg*K5f$_1$	GACGGCATCCTTGACGCTGG	For amplification of the 1121 bp 5′-flanking region of *Mgg1* ORF
*mgg*K5r	GTTCGATGGGGTTGAGTTGGGGGAGGAGGT AACGCAATTC	
*mgg*K3f	CATGCATGTTGCATGATGATTACGCCGTCAT CCGACATAC	For amplification of the 1213 bp 3′-flanking region of *Mgg1* ORF
*mgg*K3r$_1$	GTTGAGAGGAGTTCTTGGGC	
*mgg*K5f$_2$	CCCAAGCTTCGGCCGTGACTTTGGCCCTC	For amplification of the 3191 bp *Mgg1* replacement cassette
*mgg*K3r$_2$	CGGGGTACCGTCTGCTTCCGGTGTGGGTT	
G418F	CCAACTCAACCCCATCGAACCGTAACCCCAT	For amplification of the 1221 bp *neo*^{R} cassette from plasmid pKN1
G418R	ATCATCATGCAACATGCATG	
*mgb*F	AGACGCAGAAAGGAGGAGTT	For amplification of a 552 bp fragment in the *Mgb1* ORF
*mgb*R	CAGGTTGTAGATGGAGCAGA	
*mgg*F	CCGAGCTGGCGATGTTAGGA	For amplification of a 354 bp fragment in *the Mgg1* ORF
*mgg*R	TAGGGATCGTCACGCTTGTC	
*mga*F *mga*R	neoF ATGATTGAACAAGATGG neoR TCAGAAGAACTCGTCAAG	For amplification of a 795 bp fragment in the *neo*^{R}ORF
*mrflb*AF *mrflb*AR	CTTGAAGTTGGAATCGCATCGTGT GCGAGGAAAGCATTGTAGAGG	For amplification of a 1121 bp fragment in the *mrflbA* ORF

The underlined sequences are restricted enzyme sites.

citrinin purchased from Sigma was applied to confirm the HPLC analysis.

To detect pigments in liquid culture, the filtered broth was diluted with distilled H_2O without organic extraction and then measured on a scanning spectrophotometer (Shimadzu, Japan) in the wavelength region from 300 nm to 600 nm as described by Li *et al.* (2010). The production of pigment was expressed as OD units per milliliter of liquid culture multiplied by the dilution factor.

Conidial germination analysis

Pre-sterilized, glass microscope slides were briefly immersed into sterile molten PDA medium (50–60°C) and then cooled on pre-sterilized stands to form a thin layer of agar medium on the surface. Freshly harvested conidiospores (10^7 conidia/ml) were dropped onto the surface of the PDA-coated glass slides and incubated at 28°C in moist sterile plates. The slides were removed every hour after an initial 4 h-incubation to observe the emergence of germ tube under the microscope. Photomicrographs were taken using a Leica DME compound microscope (LEICA DME, Germany).

Results

Cloning and sequence analysis of *Mgb1* and *Mgg1*

PCR amplification with degenerate primers GBF/GBR resulted in a product of 765 bp, which was homologous to the fungal Gβ subunit based on similarity analysis of amino acid sequences deduced from the sequenced PCR fragments. PCR amplification with degenerate primers GGF/GGR resulted in a product of 333 bp, which was homologous to the fungal Gγ subunit. SON-PCR was then performed to amplify the flanking sequences of the two fragments with primers listed in Table 1, generating a 5,222 bp DNA fragment containing the ORF of the Gβ subunit gene (1,511 bp), and a 3,849 bp DNA fragment containing the ORF of the Gγ subunit gene (417 bp). The two genes were named *Mgb1* (*Monascus* G protein beta-subunit 1) and *Mgg1* (*Monascus* G protein gamma-subunit 1), and deposited in GenBank under the accession numbers FJ640859 (*Mgb1*) and FJ640860 (*Mgg1*).

Sequence analysis revealed that the *Mgb1* ORF was interrupted by four putative introns and was predicted to encode a 353-amino-acid protein with typical WD repeats, which are common to Gβ proteins. BLAST searches showed that the predicted Mgb1 protein shared marked similarity with Gβ subunits from other organisms, including *A. fumigatus* (99%, FJ842005) (Shin *et al.*, 2009), *A. nidulans* (97%, AAC33436) (Rosen *et al.*, 1999), *Magnaporthe grisea* (93%, BAC01165) (Nishimura *et al.*, 2003), *Cryphonectria parasitica* (92%, AAC49838) (Kasahara and Nuss, 1997), and *Neurospora crassa* (92%, AAM53552) (Yang *et al.*, 2002). The ORF of *Mgg1* was interrupted by two putative introns and was predicted to encode a 94-amino-acid protein, which contained a typical G protein gamma like domain (GGL) and a conserved C-terminal CAAX box (CLVM). It exhibited 95% and 94% similarity to the Gγ subunit from *A. fumigatus* (ABG73390) (Shin *et al.*, 2009) and *A. nidulans* (ABG73391) (Seo *et al.*, 2005), respectively. Southern blot analyses indicated that both *Mgb1* and *Mgg1* were single copy genes in the *M. ruber* M7 genome (Fig. 1C and 1E).

Targeted deletion of *Mgb1* and *Mgg1* in *M. ruber*

To investigate the functional roles of *Mgb1* and *Mgg1* in *M. ruber*, the gene deletion mutants, Δ*Mgb1*, Δ*Mgg1*, and Δ*Mgb1*

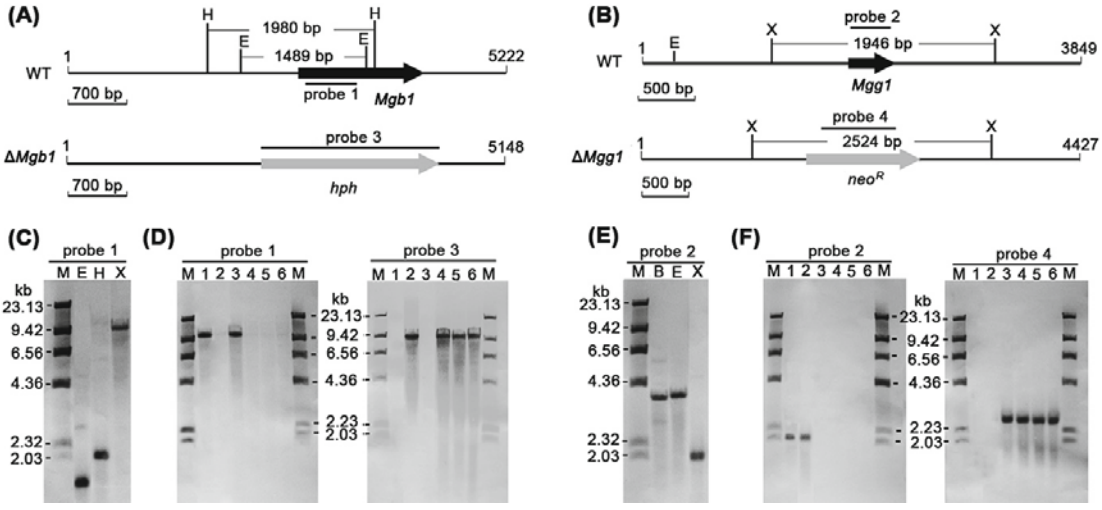

Fig. 1. Results of Southern blot analysis. (A) and (B) Schemes of the *Mgb1* and *Mgg1* genomic locus from the wild-type, Δ*Mgb1* and Δ*Mgg1* strains. Probes used for Southern blot are shown as black lines, and the relative positions of the *Xba*I (X), *Hin*dIII (H), *Eco*RI (E), and *Bam*HI (B) restriction sites are indicated. (C) and (E) Southern blot analysis of the copy number of *Mgb1* and *Mgg1* in the genome of *M. ruber* M7 using the *Mgb1* probe (probe 1) or the *Mgg1* probe (probe 2). For *Mgb1*, genomic DNAs were digested with *Xba*I (X), *Hin*dIII (H), and *Eco*RI (E), repectively. For *Mgg1*, genomic DNAs were digested with *Xba*I (X), *Bam*HI (B), and *Eco*RI (E), repectively. (D) and (F) Southern blot analyses of transformants using the *Mgb1* probe (probe 1) and the *hph* probe (probe 3), or the *Mgg1* probe (probe 2) and the *neo*^{R} probe (probe 4). Genomic DNAs were digested with *Xba*I. Lanes: 1, wild-type strain M7; 2, a Δ*Mgb1* strain; 3, a Δ*Mgg1* strain; 4-6, Δ*Mgb1*Δ*Mgg1* strains. Size markers are from a *Hin*dIII digested λ DNA.

Δ*Mgg1*, were generated by replacing the *Mgb1* ORF with the *hph* marker and the *Mgg1* ORF with the *neo*^{R} marker via *Agrobacterium*-mediated transformation and homologous recombination. Successful targeted gene deletion events were confirmed through a PCR-based screen using primers located in the *Mgb1* and *Mgg1* ORF, and Southern hybridization. As shown in Fig. 1, when hybridized with a probe containing the ORF of the *Mgb1* gene (probe 1), no hybridizing band was detected in Δ*Mgb1* and Δ*Mgb1*Δ*Mgg1* mutants (Fig. 1D), while with a probe containing the ORF of the *Mgg1* gene (probe 2), no band in Δ*Mgg1* and Δ*Mgb1*Δ*Mgg1* mutants was detected (Fig. 1F). Meanwhile, a single hybridizing band was detected in Δ*Mgb1* and Δ*Mgb1*Δ*Mgg1* mutants with the selective marker gene *hph* (probe 3, Fig. 1D), and in Δ*Mgg1* and Δ*Mgb1*Δ*Mgg1* mutants with the selective marker gene *neo*^{R} (probe 4, Fig. 1F), revealing that all the deletion mutants carried a single copy of the *Mgb1* and/or *Mgg1* disruption construct.

Effects of deletion of *Mgb1* and *Mgg1* on fungal growth and reproduction

The effects of *Mgb1* and *Mgg1* deletion on vegetative growth and reproduction were analyzed, and as shown in Fig. 2, the most prominent phenotype of the Δ*Mgb1*, Δ*Mgg1*, and Δ*Mgb1*Δ*Mgg1* mutants was the severely restricted vegetative growth. The radial growth rates of the Δ*Mgb1*, Δ*Mgg1*, and Δ*Mgb1*Δ*Mgg1* mutant colonies were approximately 20%–30% of WT on PDA medium, and approximately 50% of WT on CYA medium. However, these extreme defects in vegetative growth by the deletion of *Mgb1* or *Mgg1* were not obvious in G25N medium (containing 25% glycerol, Fig. 2E), indicating that high osmolality might restore vegetative growth of the mutants. Moreover, the hyphae of all the deletion strains were short and highly branched, causing the colonies to exhibit a compact appearance, which was far different from that of the wild-type (Fig. 2G). With regard to reproduction, no typical cleistothecium was found in the *Mgb1* or *Mgg1* deletion strains, while large quantities of cleistothecia were observed in the wild-type (Fig. 2B). Additionally, the conidia- forming ability of all the disruptants was found to be reduced when compared to the WT strain (about 20%–30% of WT, Fig. 2D and 2F). These results suggested that *Mgb1* and *Mgg1* were required for normal vegetative growth, and for sexual and asexual reproduction of *M. ruber*.

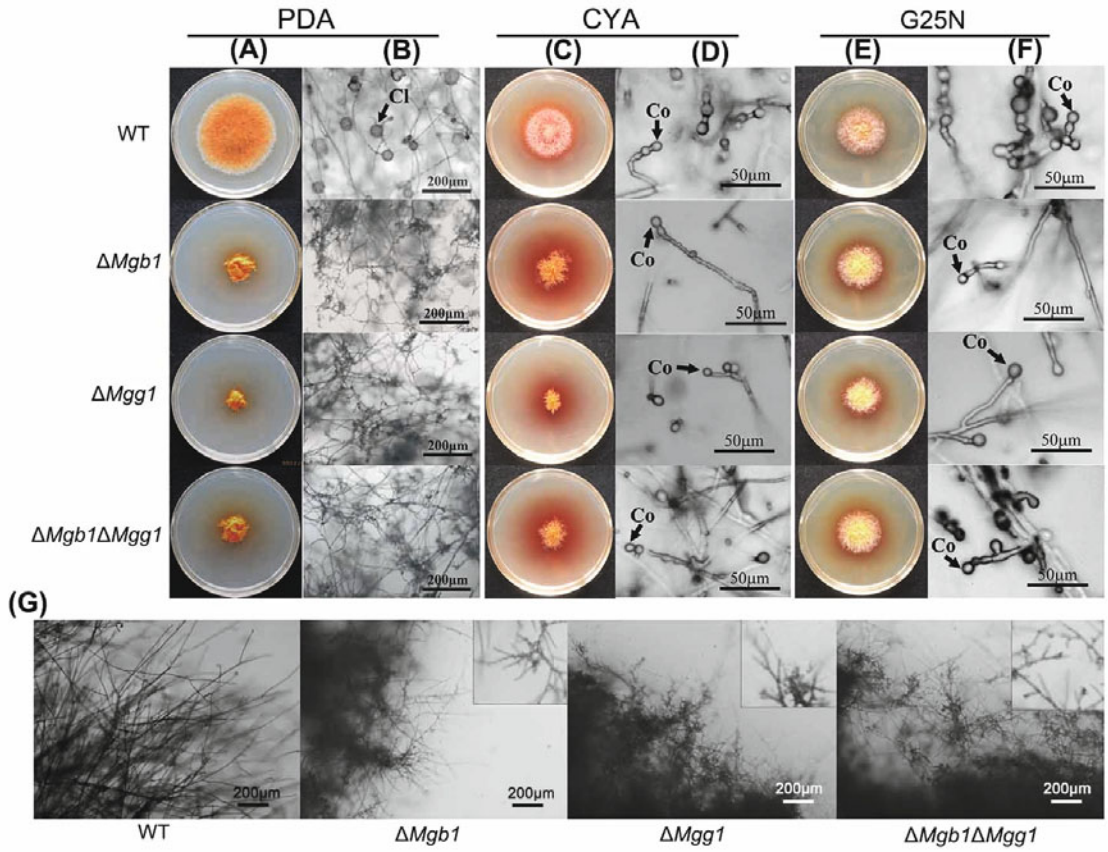

Fig. 2. Comparison of the wild-type and deletion mutant strains for morphology and spore production. Equal numbers of conidia from the wild-type strain M7 (WT) and deletion mutant strains Δ*Mgb1*, Δ*Mgg1*, and Δ*Mgb1*Δ*Mgg1* were inoculated onto the center of plates containing PDA (A, B, G), CYA (C, D) or G25N (E, F) medium and cultivated at 28°C for 10 days. Photographs of the whole colony (A, C, E), close-up views of the center (B, D, F) and of the edge (G) of individual colonies are shown. Cleistothecia (Cl) and conidia (Co) are indicated by black arrows. The short and highly branched hyphae are accentuated by a consistent amplification at the top-left corner of each photograph (G).

Effects of deletion of *Mgb1* and *Mgg1* on the production of pigment and citrinin

To test whether *Mgb1* and *Mgg1* were involved in secondary metabolite biosynthesis, pigment content and citrinin content in the YES culture filtrate were analyzed by UV-Visible spectrophotometry and HPLC, respectively. The results of UV-Visible scan are shown in Fig. 3A and the HPLC data are shown in Supplementary data Fig. S1 and S2. There were two absorption peaks in the UV-Visible spectra of the pigment from all strains (Fig. 3A). The maximum absorbing wavelengths were almost the same, around 375 nm and 485 nm, respectively. To calculate the differences in pigment concentration, the absorbance value at 485 nm, the maximum absorbing wavelength in the UV-Visible spectra, was determined. After 16 days of cultivation, the pigment production of the Δ*Mgb1*, Δ*Mgg1*, and Δ*Mgb1*Δ*Mgg1* mutants was 19.41±0.90, 20.46±0.29, and 38.83±0.52 U/ml, respectively (Fig. 3B), an increase of approximately 2–4 fold, in comparison to the wild-type strain (8.82±0.41 U/ml). The citrinin production of Δ*Mgb1*, Δ*Mgg1*, and Δ*Mgb1*Δ*Mgg1* mutants was 38.80±2.56, 41.27±2.76, and 34.70±1.23 μg/ml, respectively (Fig. 3B), about 1.6–2 times the amount detected in the wild-type (21.46±0.25 μg/ml).

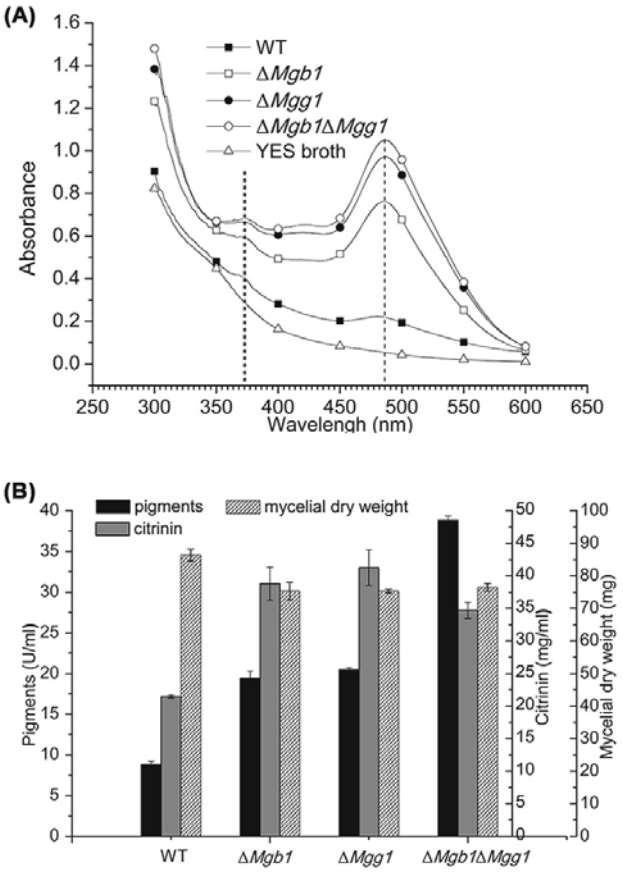

Fig. 3. Roles of *Mgb1* and *Mgg1* in the biosynthesis of citrinin and pigment. The wild-type (WT) and gene targeted deletion strains (Δ*Mgb1*, Δ*Mgg1*, and Δ*Mgb1*Δ*Mgg1*) were incubated in liquid YES medium at 28°C for 16 days without agitation. (A) The UV-Visible spectra of pigments excreted into the culture medium. Data from YES broth without inoculation were taken as the control. (B) Black column, pigment detected at 485 nm; gray column, citrinin; grid column, mycelial dry weight.

In contrast to the significantly enhanced citrinin and pigment production, the vegetative proliferation of Δ*Mgb1*, Δ*Mgg1*, and Δ*Mgb1*Δ*Mgg1* mutants under the same condition was slightly reduced in comparison to the WT strain (about 90% of the WT strain, Fig. 3B). These results are consistent with our previous finding that deletion of *Mga1* (Gα) in *M. ruber* caused reduced vegetative growth and increased citrinin and pigment production (Li *et al.*, 2010). Conversely, absence of MrflbA, an RGS protein likely to negatively control Mga1-mediated signaling by enhancing the intrinsic GTPase activity of Mga1, resulted in uncontrolled vegetative growth followed by autolysis, and an inhibition of pigment and citrinin production in *M. ruber* (Yang *et al.*, 2012). These observations led to the hypothesis that signaling mediated by the G protein Mga1-Mgb1::Mgg1 promotes vegetative growth, and in turn, inhibits pigment and toxin production.

Deletion of *Mgg1* suppressed defects resulting from the absence of MrflbA

In *A. nidulans*, the Gγ subunit GpgA functions in vegetative growth signaling controlled by the RGS protein FlbA (Yu, 2006). In our previous study, a FlbA-like protein encoding gene *mrflbA* was isolated and found to be involved in the modulation of aerial hyphal development and pigment/citrinin production in *M. ruber* M7 (Yang *et al.*, 2012). To provide genetic evidence of the involvement of Mgg1 and MrflbA in signaling transduction in *M. ruber*, we generated the Δ*mrflbA*Δ*Mgg1* double mutants by introducing Δ*Mgg1* into the Δ*mrflbA* background. As expected, we found that the fluffy-autolytic phenotype, delayed germination and severe inhibition of pigment/citrinin production caused by the loss of *mrflbA*, were suppressed by the deletion of *Mgg1*. The phenotype of Δ*mrflbA*Δ*Mgg1* double mutants was identical to that of the Δ*Mgg1* single mutants (Fig. 4).

Discussion

The roles of heterotrimeric G protein signaling pathways in various important processes in a number of model and pathogenic fungi have been intensively investigated in the past two decades (Li *et al.*, 2007; Servin *et al.*, 2012). In this study, we report the cloning and characterization of a Gβ subunit-encoding gene (*Mgb1*) and a Gγ subunit-encoding gene (*Mgg1*) from the ascomycete *M. ruber*, which is employed to produce commercial products in industrial fermentation due to its beneficial metabolites.

To date, data regarding the biological function of Gβ and/or Gγ is available for about 15 different fungal species (Table 2). These detailed studies revealed that despite considerable sequence similarity among these signaling proteins, in some cases their functions showed distinct variations among species. For instance, in *Cryphonectria parasitica*, deletion of the *cpgb*-1 (Gβ) or *cpgg*-1(Gγ) resulted in increased growth rate and reduced conidiation (Kasahara and Nuss, 1997; Fan, 2007, see Table 2), while in *A. fumigatus*, deletion of *AfusfaD* (Gβ) or *AfugpgA* (Gγ) caused the opposite effect, with re-

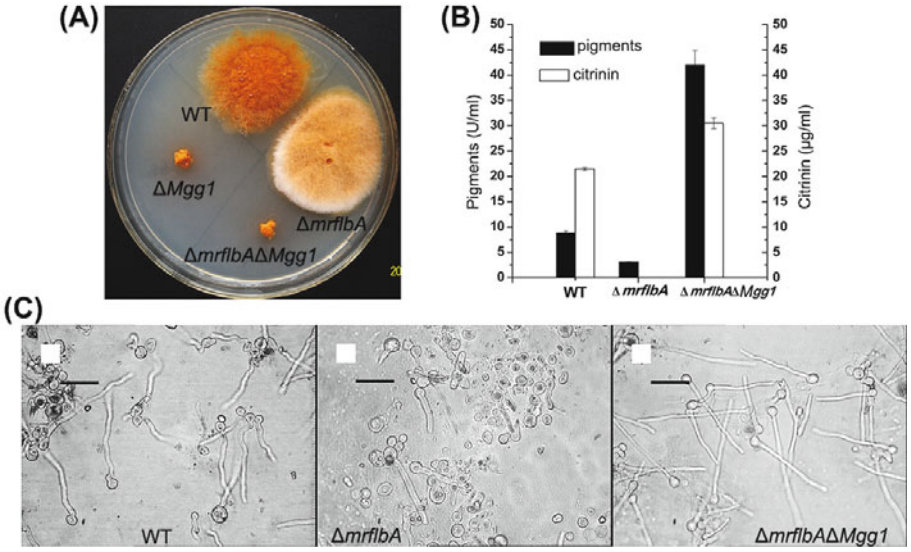

Fig. 4. Comparison of the Δ*mrflbA* and Δ*mrflbA*Δ*Mgg1* strains for colony morphology (A), citrinin and pigment production (B) and conidial germination (C). Photomicrographs of colonies were taken after 7 days on PDA medium. Photomicrographs of germ tubes were taken after 7 h on PDA-coated glass slides at 28°C. Citrinin and pigment were detected after 16 days in liquid YES medium at 28°C without agitation.

duced vegetative growth and induced hyperactive sporulation (Shin *et al*., 2009). In this study, deletion of the orthologous genes *Mgb1* and *Mgg1* in *M. ruber* M7 reduced both vegetative growth and conidiation. Our findings are consistent with the model proposed in *M. grisea* (Nishimura *et al*., 2003; Liang *et al*., 2006). Moreover, like the Gβγ mutants in *A. nidulans* (Rosen *et al*., 1999; Seo *et al*., 2005), the *Mgb1* or *Mgg1* mutants in *M. ruber* are blocked in cleistothecium formation. In addition, the roles of Gβ and Gγ subunits in mycotoxin synthesis have also been reported. In *A. nidulans*, the Δ*sfaD* (Gβ), Δ*gpgA* (Gγ), and Δ*sfaD*Δ*gpgA* mutants were unable to produce sterigmatocystin, and in *A. fumigatus*, no gliotoxin was detected in the $CHCl_3$ extracts of Δ*AfusfaD* (Gβ) and Δ*AfugpgA* (Gγ) strains (Seo and Yu, 2006; Shin *et al*., 2009). However, in *M. ruber*, we found that deletion of *Mgb1* (Gβ) and/or *Mgg1* (Gγ) enhanced the production of the mycotoxin citrinin, which is consistent with findings in *G. zeae* that deletion of *GzGPB1* (Gβ) resulted in increased deoxynivalenol and zearalenone production (Yu *et al*., 2008).

In this study, we found that Δ*Mgb1* and Δ*Mgg1* showed identical phenotypes under the conditions tested, suggesting that the two G protein subunits might strictly function as heterodimers in *M. ruber*. Moreover, the phenotypic alterations observed in Δ*Mgb1*, Δ*Mgg1*, and Δ*Mgb1*Δ*Mgg1*, i.e., restricted vegetative growth, lowered asexual sporulation, impaired cleistothecial formation, and enhanced citrinin and pigment production, were consistent with those in Δ*Mga1* (Li *et al*., 2010). In addition, the fluffy-autolytic phenotype and severe inhibition of pigment/citrinin production caused by the loss of MrflbA were suppressed by the deletion of *Mgg1* (Fig. 4). These results support the hypothesis that the heterodimer Mgb1::Mgg1 interacted with Mga1 to form a heterotrimeric G protein and the heterodimer functioned in the Mga1-mediated signaling pathway, which was negatively controlled by MrflbA (Fig. 5).

Recently, it has been reported that most characterized filamentous fungi possess three Gα proteins, but only a single Gβγ heterodimer. The unique Gβγ heterodimer is presumed to form a complex with each of the three GDP-bound Gα subunits, and plays a global, positive role in maintaining

Table 2. Gβ and Gγ subunit encoding genes in filamentous fungal species

Species	Gβ genes	References	Gγ genes	References
Cryphonectria parasitica	*cpgb-1*	Kasahara and Nuss (1997)	*cpgg-1*	Fan (2007)
Aspergillus nidulans	*sfaD*	Rosen *et al.* (1999)	*gpgA*	Seo *et al.* (2005)
Cryptococcus neoformans	*gpb1*	Wang *et al.* (2000)	*gpg1, gpg2*	Palmer *et al.* (2006)
Neurospora crassa	*gnb-1*	Yang *et al.* (2002)	*gng-1*	Krystofova and Borkovich (2005)
Phytophthora infestans	*pigpb1*	Maria Laxalt *et al.* (2002)		
Magnaporthe grisea	*mgb1*	Nishimura *et al.* (2003)	*mgg1*	Liang *et al.* (2006)
Fusarium oxysporum	*fgb-1*	Jain *et al.* (2003); Delgado-Jarana *et al.* (2005)		
Ustilago maydis	*bpp-1*	Muller *et al.* (2004)		
Cochliobolus heterostrophus	*cgb-1*	Ganem *et al.* (2004)		
Botrytis cinerea	*Bcgb1*	Osada *et al.* (2005)		
Fusarium verticillioides	*GBB1*	Sagaram and Shim (2007)		
Gibberella zeae	*GzGPB1*	Yu *et al.* (2008)		
Setosphaeria turcica	*Stgb-1*	Hao *et al.* (2010)	*Stgγ-1*	Wu (2009)
Mycosphaerella graminicola	*MgGpb1*	Mehrabi *et al.* (2009)		
Aspergillus fumigatus	*AfusfaD*	Shin *et al.* (2009)	*AfugpgA*	Shin *et al.* (2009)

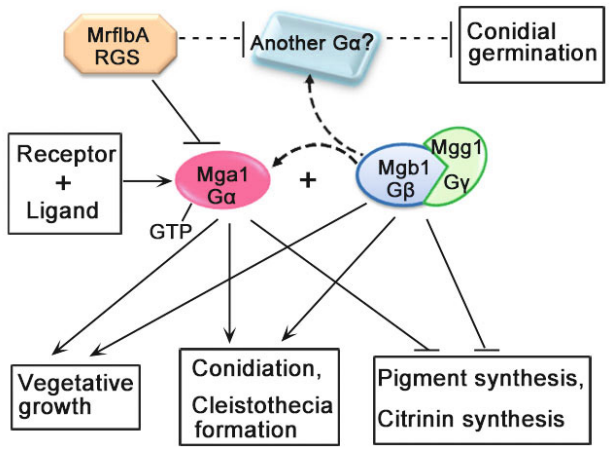

Fig. 5. Proposed model for the roles of Mgb1 and Mgg1 in *M. ruber*. It is presumed that Mga1 (Gα), Mgb1(Gβ) and Mgg1(Gγ) constitute a heterotrimeric G protein. However, their physical association has not been directly demonstrated. Ligand binding to a receptor(s) triggers GDP/GTP exchange on Mga1 and dissociation from the Mgb1::Mgg1 heterodimer, resulting in activating vegetative growth, conidiation and cleistothecial formation, while repressing biosynthesis of pigment and citrinin. The signaling pathway mediated by Mga1-Mgb1::Mgg1 is negatively regulated by the RGS MrflbA, which is required for normal growth, sporulation and production of pigment and citrinin in *M. ruber* (Yang *et al.*, 2012). However, the regulatory roles of MrflbA in germination may be mediated by another Gα (not Mga1), and the Mgb1::Mgg1 heterodimer may play a positive role in maintaining normal levels of the Gα protein involved in germination, since deletion of *Mgg1* in Δ*mrflbA* reversed the germination defect caused by the absence of *MrflbA*. Arrows indicate positive regulation, and blocked arrows indicate negative regulation. Solid lines indicate genetically determined steps. Dashed lines indicate postulated steps.

normal levels of all the three Gα proteins possibly by post-transcriptional regulation (Yang *et al.*, 2002; Parsley *et al.*, 2003; Chang *et al.*, 2004; Li *et al.*, 2007). These findings and hypotheses may provide an explanation to the question of why only Δ*mrflbA* exhibited changes in the germination rate among all the mutants investigated in this study (Δ*Mga1*, Δ*Mgb1*, Δ*Mgg1*, Δ*Mgb1*Δ*Mgg1*, Δ*mrflbA*, and Δ*mrflbA*Δ*Mgg1*), and why the germination defect in Δ*mrflbA* strains was restored in Δ*mrflbA*Δ*Mgg1* strains. The regulatory roles of the RGS MrflbA in germination might be mediated by another Gα (not Mga1), and the absence of Gβγ-signaling, caused by deletion of *Mgg1*, might lead to a reduced protein level of the Gα involved in germination. A proposed model summarizing the potential roles of *Mgb1* and *Mgg1* in controlling vegetative growth, reproduction, conidial germination and secondary metabolite production is presented in Fig. 5.

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