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Regulators of G-protein signalling in *Fusarium verticillioides* mediate differential host–pathogen responses on nonviable versus viable maize kernels

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SUMMARY

GBB1, a heterotrimeric G-protein β-subunit gene, was shown to be a key regulator of fumonisin B_1 (FB_1) biosynthesis in the maize pathogen *Fusarium verticillioides*. In this study, we performed functional analyses of genes that encode putative RGS (regulators of G-protein signalling) proteins and PhLPs (phosducin-like proteins) in *F. verticillioides*. These proteins are known to regulate heterotrimeric G-protein activity by altering the intrinsic guanosine triphosphatase (GTPase) activity, which, in turn, influences the signalling mechanisms that control fungal growth, virulence and secondary metabolism. Our aim was to isolate and characterize gene(s) that are under the transcriptional control of *GBB1*, and to test the hypothesis that these genes are directly associated with FB_1 regulation and fungal development in *F. verticillioides* on maize kernels. We first identified eight genes (two PhLPs and six RGSs) in the *F. verticillioides* genome, and a subsequent transcriptional expression study revealed that three RGS genes were up-regulated in the *gbb1* deletion (Δgbb1) mutant and one RGS gene was up-regulated in the wild-type. To characterize their function, we generated knockout mutants using a homologous recombination strategy. When grown on autoclaved nonviable kernels, two mutants (ΔflbA2 and ΔrgsB) produced significantly higher levels of FB_1 compared with the wild-type progenitor, suggesting that the two mutated genes are negative regulators of FB_1 biosynthesis. ΔflbA2 also showed a severe curly conidia germination pattern, which was contradictory to that observed in the Δgbb1 strain. Strikingly, when these mutants were grown on live maize kernels, we observed contrasting FB_1 and conidiation phenotypes in fungal mutants, which strongly suggests that these G-protein regulators have an impact on how *F. verticillioides* responds to host/environmental factors. Our data also provide evidence that fungal G-protein signalling is important for modulating the ethylene biosynthetic pathway in maize kernels.

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INTRODUCTION

Fusarium verticillioides (teleomorph *Gibberella moniliformis*) is a maize pathogen that causes seedling blight, root rot, stalk rot and kernel (or ear) rot worldwide (Kommedahl *et al*., 1979; Munkvold and Desjardins, 1997). In particular, kernel rot by *F. verticillioides* not only results in yield loss, but also lower grain quality, because of contamination by mycotoxins, namely fumonisins. Fumonisins are a group of polyketide-derived mycotoxins, which are structurally similar to sphingolipid intermediates, and can lead to the inhibition of ceramide synthase and a disruption of sphingolipid metabolism (Marasas *et al*., 2004; Merrill *et al*., 1996). Fumonisin-contaminated foods and feeds have been linked to a variety of illnesses in animals and humans (Gelderblom *et al*., 1991; Marasas, 2001; Marasas *et al*., 1988; Rheeder *et al*., 1992; Voss *et al*., 2002).

Over 15 structurally related fumonisins are known to date, and they all share a 19- or 20-carbon polyketide backbone, but with variations in functional groups (Proctor *et al*., 1999, 2006; Seo *et al*., 1996). Among these, fumonisin B_1 (FB_1) is the predominant form in nature (Gelderblom *et al*., 1992; Sydenham *et al*., 1990). Fumonisins are synthesized by a cluster of co-regulated genes, designated as the *FUM* cluster (Proctor *et al*., 2003), which spans a region of approximately 45 kb. The cluster harbours over 20 genes, including *FUM1*, a polyketide synthase gene, and *FUM8*, an aminotransferase gene (Proctor *et al*., 1999; Seo *et al*., 2001), which are transcriptionally activated when the fungus encounters environmental conditions conducive to fumonisin production (Brown *et al*., 2005, 2007; Proctor *et al*., 2003). Significantly, one of the key questions that remains is how the fungus perceives these external environmental cues, transduces these signals and ultimately activates the *FUM* cluster. We now have a certain level of understanding of the nature of these fumonisin-conducive conditions and of a select number of regulatory genes associated with toxin production (Bluhm and Woloshuk, 2005; Choi and Shim, 2008b; Flaherty and Woloshuk, 2004; Flaherty *et al*., 2003; Sagaram and Shim, 2007; Shim and Woloshuk, 2001).

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G-protein-mediated signalling is one of the most important mechanisms by which eukaryotic cells sense extracellular signals and integrate them into intrinsic signal transduction pathways (McCudden *et al*., 2005; Neves *et al*., 2002). In fungi, the heterotrimeric G-protein system, with key components such as G-protein-coupled receptors (GPCRs), Gα, Gβ and Gγ subunits, and regulatory proteins, is well recognized as playing a critical role in a variety of cellular functions in response to external signals. Excellent review articles are available discussing the role of these components in cell signalling in fungi (Lengeler *et al*., 2000; Li *et al*., 2007; Yu, 2006; Yu and Keller, 2005). In plant-pathogenic fungi, G-protein signalling components, in particular Gα, Gβ and Gγ subunits, have been functionally characterized in a number of species, and have been shown to play critical roles in virulence, morphogenesis and secondary metabolism (Li *et al*., 2007; Nishimura *et al*., 2003; Sagaram and Shim, 2007; Yu *et al*., 2008). However, we have a limited understanding of how regulatory proteins, namely RGS (regulators of G-protein signalling) and PhLP (phosducin-like proteins), function in pathogenic fungi. RGS proteins regulate cell signalling by acting as negative regulators of heterotrimeric G-protein cascades that enable eukaryotic cells to perceive and respond to external stimuli. These proteins contain a conserved ~130-amino-acid RGS box that interacts with an activated guanosine triphosphate (GTP)-Gα subunit and increases its intrinsic guanosine triphosphatase (GTPase) activity, thereby rapidly turning off the GPCR-mediated signalling pathways (Chidiac and Roy, 2003; McCudden *et al*., 2005). In addition, RGS proteins can enhance G-protein pathway activation, serving as effector antagonists, and can also act as scaffolding proteins to congregate receptors, G proteins, effectors and other regulatory molecules (Zhong and Neubig, 2001). Phosducin and PhLPs are a group of evolutionarily conserved proteins that were initially recognized as negative regulators of Gβγ activity by binding and sequestering Gβγ heterodimer from its interaction with Gα or downstream effectors (Bauer *et al*., 1992; Blüml *et al*., 1997; Flanary *et al*., 2000). However, previous genetic studies with the chestnut blight fungus *Cryphonectria parasitica* (Kasahara *et al*., 2000) and the social amoeba *Dictyostelium discoideum* (Blaauw *et al*., 2003) have shown that PhLPs are positive regulators of Gβγ signalling. Furthermore, biochemical studies of PhLP in humans (Lukov *et al*., 2005) and *D. discoideum* (Knol *et al*., 2005) have clearly demonstrated that PhLP is essential for Gβγ dimer assembly and for normal levels of Gβ and Gγ subunits.

The genome of *Aspergillus nidulans* harbours three PhLPs (PhnA, PhnB and PhnC) and four RGSs (FlbA, RgsA, RgsB and RgsC) (Yu, 2006). One of the earliest known RGSs is *A. nidulans* FlbA, and research has shown that it is required for the control of mycelial proliferation and the activation of asexual sporulation (Yu *et al*., 1996). FlbA transcriptionally and post-transcriptionally regulates the sterigmatocystin transcription factor *aflR* in a PkaA-dependent manner (Shimizu *et al*., 2003). In *Aspergillus fumigatus*, FlbA has been shown to regulate conidiation and hyphal proliferation, indicating a conserved function in *Aspergillus* sp. (Mah and Yu, 2006). Another RGS protein, *A. nidulans* RgsA, downregulates pigment production and conidial germination, but stimulates asexual sporulation (Han *et al*., 2004). In the rice blast fungus *Magnaporthe grisea*, Rgs1 serves as a negative regulator of all three Gα subunits, thus regulating pathogenesis, asexual reproduction and thigmotropism (Liu *et al*., 2007). The deletion of *Cryphonectria parasitica* RGS-1 resulted in reduced growth, sparse aerial mycelium and loss of pigmentation, sporulation and virulence (Segers *et al*., 2004). Furthermore, recently, a PhLP PhnA has been reported to be involved in Gβγ-mediated signalling required for vegetative growth, developmental control and toxin biosynthesis in *A. nidulans* (Seo and Yu, 2006).

In an earlier study, we investigated the role of the heterotrimeric G-protein complex β-subunit gene, *GBB1*, in *F. verticillioides* (Sagaram and Shim, 2007). Our data showed that, although *GBB1* was not involved directly in fungal virulence, it served as a key positive regulator of FB_1 biosynthesis and asexual spore production. Our subsequent question was whether regulatory components of the heterotrimeric G-protein signalling complex are involved directly in FB_1 biosynthesis and conidiation in *F. verticillioides*. In the present study, we identified the regulators of the heterotrimeric G-protein complex, and investigated their roles in the *F. verticillioides*–maize interaction, particularly on nonviable (autoclaved) versus viable (live) maize kernels. In addition to conidiation and FB_1 production, we analysed ethylene emission by live maize kernels when inoculated with fungal mutants.

RESULTS

RGSs and PhLPs in the *F. verticillioides* genome

In silico analysis of the *F. verticillioides* genome revealed the presence of six RGS genes and two PhLP genes: the RGS genes were designated as *RGSA*, *RGSB*, *RGSC1*, *RGSC2*, *FLBA1* and *FLBA2*, and the PhLP genes were designated as *PHNA* and *PHNB*, based on shared homology with the respective proteins in other fungal species (Fig. S1, see Supporting Information). Namely, when we initially compared the similarity of these proteins to the respective proteins in *A. nidulans*, employing SMART (Letunic *et al*., 2009), we recognized that *F. verticillioides* did not contain a PhnC homologue, but had two copies of RgsC and FlbA (Fig. 1A). *Fusarium verticillioides* contains two paralogues of FlbA and, although both conceptually translated proteins are smaller in size than the *A. nidulans* counterpart, FlbA1 and FlbA2 share greater than 40% identity with *A. nidulans* FlbA at the amino acid level and harbour an RGS domain and DEP (dishevelled, Egl-10 and pleckstrin) domain (McCudden *et al*., 2005; Yu,

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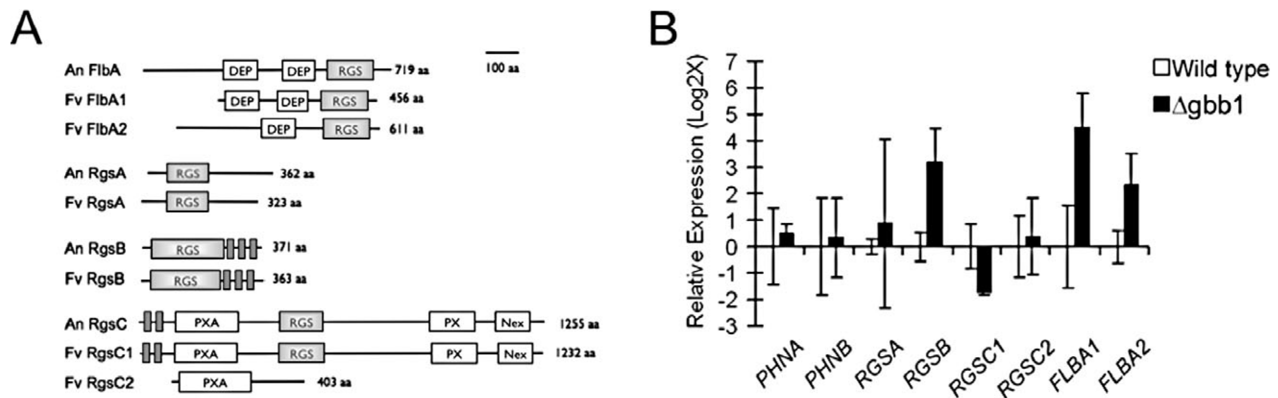

Fig. 1 (A) Comparison of RGS proteins in *Aspergillus nidulans* and *Fusarium verticillioides*. Schematic representation of protein orthologues shows the location and alignment of conserved domains. DEP (dishevelled, Egl-10 and pleckstrin); Nex (nexin family protein C-terminus domain); PX (phox homology domain); PXA (PX association domain); RGS (regulator of G-protein-signalling domain); TM (transmembrane domain). Grey boxes depict transmembrane domains. (B) Expression analysis of G-protein regulator genes in the wild-type and Δgbb1 mutant. Total RNA samples were prepared from fungal strains grown in cracked-corn medium for 10 days, and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis was performed with SYBR-Green as the fluorescent reporter. The levels of transcription were evaluated using the $2^{-\Delta\Delta Ct}$ method, with *TUB2* as the endogenous control. The graph represents the logarithmic fold differences in gene expression. The range of expression was calibrated using $2^{-\Delta\Delta Ct - S} - 2^{-\Delta\Delta Ct + S}$, where S is the standard deviation of the ΔCt value. *Ct*, threshold cycle. Three biological replications and two technical replications were performed.

2006). For RgsC, however, on further analysis, we concluded that *F. verticillioides* RgsC2 cannot be considered as a functional RGS as it lacks a conserved RGS motif. A comparison of *A. nidulans* and *F. verticillioides* RGSs and PhLPs (percentage amino acid identity) and their putative functions are listed in Table 1.

Our next aim was to identify RGSs and PhLPs associated with Gbb1 that were involved in the regulation of FB_1 biosynthesis and conidiation. Published reports have indicated that RGS and PhLP genes are transcriptionally regulated in filamentous fungi (Han *et al*., 2004; Lee and Adams, 1994; Seo and Yu, 2006). Therefore, we studied the relative expression level of these genes in the wild-type and Δgbb1 (*GBB1* deletion) strains grown in cracked-corn medium via quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis. Statistical analyses of qRT-PCR revealed that four genes showed significant differential gene expression between the wild-type and Δgbb1; three genes, *RGSB*, *FLBA1* and *FLBA2*, were up-regulated in the Δgbb1 background, whereas *RGSC1* was up-regulated in the wild-type (Fig. 1B). This outcome suggested that RgsB, FlbA1 or FlbA2 serves as a negative regulator of FB_1 biosynthesis and conidiation. Conversely, RgsC1 serves as a positive regulator.

Generation of RGS gene knockout mutants

To further study gene function, we generated *F. verticillioides* mutants by a double homologous recombination strategy (Sagaram and Shim, 2007). We also used a *F. verticillioides* SF41 strain, a Ku70-deletion strain, to improve homologous recombination efficiency (Choi and Shim, 2008a). We made gene disruption constructs for *flbA1*, *flbA2*, *rgsB* and *RGSC1* by a double-joint PCR strategy with the hygromycin phosphotransferase (*HPH*) gene as the selectable marker (Fig. 2) (Sagaram and Shim, 2007). Hygromycin-resistance transformants were isolated and tested for targeted gene replacement using PCR (data not shown) and Southern analysis (Fig. 2), and we obtained at least three independent knockout mutants for *FLBA1* (ΔflbA1), *FLBA2* (ΔflbA2), *RGSB* (ΔrgsB) and *RGSC1* (ΔrgsC1) (Fig. 2). We selected these mutant strains and the wild-type for phenotypic characterization, focusing on FB_1 biosynthesis and conidiation.

Prior to testing these mutants for toxin and conidia production, we investigated whether these strains had been altered in radial growth (on solid agar) and mycelial mass (in liquid broth). No significant differences were observed between the wild-type, ΔflbA1 and ΔrgsC1 when grown on defined agar medium. However, ΔflbA2 and ΔrgsB showed 10% and 25% reduction in radial growth, respectively, when compared with the wild-type progenitor (Figs 3A,B and S2A, see Supporting Information). In defined liquid culture, only ΔrgsB showed a statistically significant difference (80% reduction) in mycelial mass (dry weight) after 1 week of incubation (Fig. 3C).

Impact of gene deletion on FB_1 biosynthesis, conidiation, stalk rot and sexual mating

Wild-type and mutant strains were grown on autoclaved cracked-corn medium (nonviable kernels) to determine the impact of gene mutation on FB_1 biosynthesis. All mutants grew similarly to the wild-type progenitor on B73-line nonviable kernels and, after a 7-day incubation, FB_1 was extracted from each sample and analysed with a high-performance liquid

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Table 1 The regulators of heterotrimeric G-protein signalling in *Fusarium verticillioides*—PhLPs (phosducin-like proteins) and RGSs (regulators of G-protein signalling).

Aspergillus nidulans protein*	*Fusarium verticillioides* protein	Identity (%)†	Putative function in fungi‡
PhnA: An0082	PhnA: FVEG_10292.3	36.3	Regulation of vegetative growth, development and secondary metabolite biosynthesis
PhnB: An4561	PhnB: FVEG_06475.3	61.1	Apoptosis
PhnC: An8847	—	—	Unknown
RgsA: An5755	RgsA: FVEG_11363.3	63.2	Stress response, conidiation, germination and pigmentation
RgsB: An3622	RgsB: FVEG_09572.3	69.3	Pheromone and cyclic-AMP signalling
RgsC: An1377	RgsC1: FVEG_03826.3	69.9	Unknown
RgsC: An1377	RgsC2: FVEG_05340.3	36.6	Unknown
FlbA: An5893	FlbA1: FVEG_08855.3	44.7	Regulation of conidiation, autolysis and pathogenesis
FlbA: An5893	FlbA2: FVEG_06192.3	71.4	Regulation of conidiation and autolysis

A. nidulans and *F. verticillioides* protein sequences can be found at the Broad Institute (http://www.broadinstitute.org/scientific-community/data) using the locus number listed in the table.

*References for *A. nidulans* genes are: PhnA (Seo and Yu, 2006), PhnB (Seo and Yu, 2006), PhnC (Seo and Yu, 2006), RgsA (Han *et al*., 2004), RgsB (Han *et al*., 2004), RgsC (Yu, 2006) and FlbA (Yu *et al*., 1996).

†Identity (%) is based on amino acid comparison.

‡Putative functions were deduced from recent research and review articles (Li *et al*., 2007; Liu *et al*., 2007; Xue *et al*., 2008; Yu, 2006).

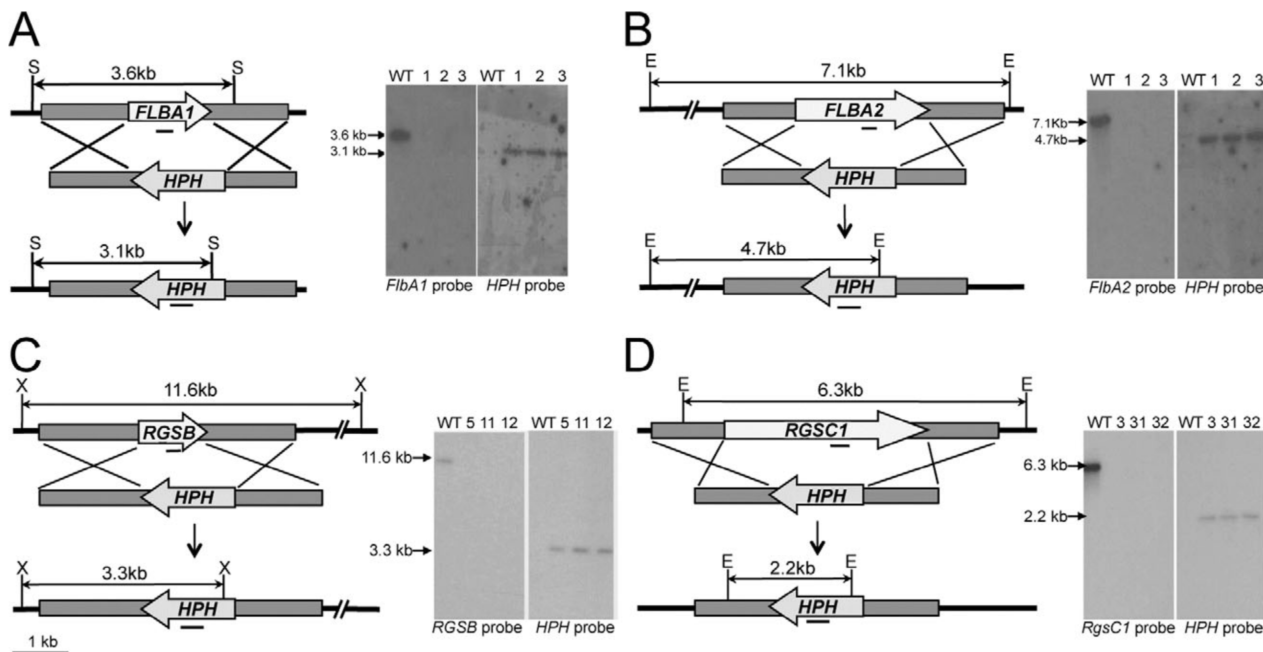

Fig. 2 Targeted gene knockout of *FLBA1* (A), *FLBA2* (B), *RGSB* (C) and *RGSC1* (D) from the genome of *Fusarium verticillioides*. Left of panel: schematic representation of homologous gene recombination strategy resulting in knockout mutant strains. E, *Eco*RI; *HPH*, hygromycin phosphotransferase gene; S, *Sal*I; X, *Xba*I. Solid bar indicates DNA fragment used as the probe for Southern hybridization. Right of panel: Southern analyses of wild-type and three mutants with targeted gene probe and *HPH* probe labelled with ^{32}P. Genomic DNA samples were digested with the restriction enzyme shown on the left. Anticipated band sizes before and after recombination are indicated on the left.

chromatography (HPLC) system. The result showed that two mutants, ΔflbA2 and ΔrgsB, produced higher levels of FB_1 than did the wild-type (Fig. 4A). In particular, the ΔflbA2 strain produced a drastically higher level of FB_1, approximately six times higher than that of the wild-type, on nonviable kernels. However, two other mutants, ΔflbA1 and ΔrgsC1, showed no significant difference in FB_1 level when compared with the wild-type (data not shown). When these strains were grown in liquid medium [bovine serum albumin liquid (BSAL) and 0.2 × potato dextrose broth (PDB)], we observed aberrant pigment production in all four mutant strains (Fig. S2B). The most intense pigmentation was observed in the ΔflbA1 mutant grown in 0.2 × PDB medium.

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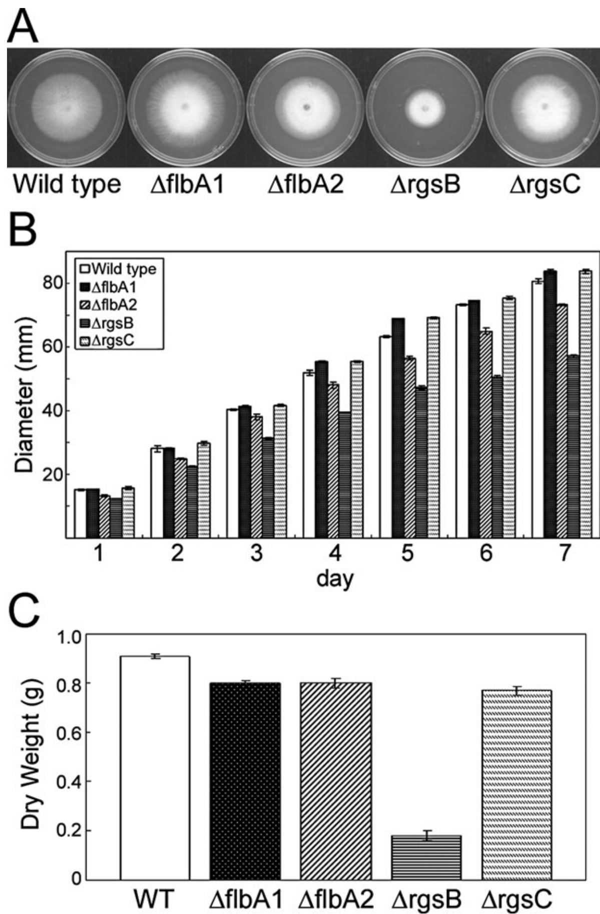

Fig. 3 Growth rate of *Fusarium verticillioides* strains. (A) Hyphal growth rate of wild-type (WT), ΔflbA1, ΔflbA2, ΔrgsB and ΔrgsC1 strains 4 days after inoculation on defined liquid (DL) agar medium. (B) Time-course growth rate analysis of wild-type (WT), ΔflbA1, ΔflbA2, ΔrgsB and ΔrgsC strains. Fungal strains were point inoculated with an agar block (0.5 cm in diameter). Results are the means of three biological replications. (C) Dry weight of wild-type, ΔflbA1, ΔflbA2, ΔrgsB and ΔrgsC1 strains. The mycelia of each strain were harvested after 5 days of incubation in DL medium and dried at 70 °C for 12 h.

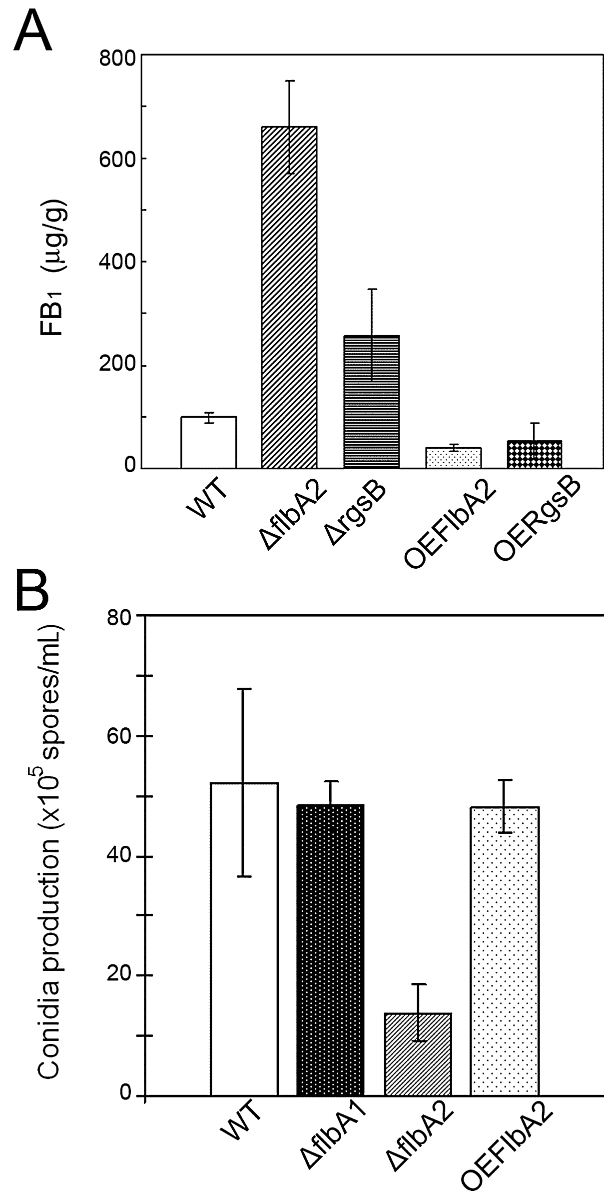

Fig. 4 Quantification of fumonisin B_1 (FB_1) production and conidiation in *Fusarium verticillioides* strains when grown in nonviable autoclaved B73 maize kernels. (A) FB_1 production in wild-type (WT), ΔflbA2, ΔrgsB, OEFlbA2 and OERgsB strains was quantified by high-performance liquid chromatography (HPLC) analysis. Autoclaved cracked corn (2 g) was inoculated with an agar block (0.5 cm in diameter) of WT and mutant strains. After 10 days of incubation at 25 °C, FB_1 was extracted with 10 mL of 50% acetonitrile in water, purified through SPE C18 columns and eluted in 2 mL of 70% acetonitrile in water and quantified by HPLC. All values represent the means of three biological replications with standard deviations shown as error bars. (B) Wild-type (WT), ΔflbA1, ΔflbA2 and OEFlbA2 strains were spot inoculated with an agar block (0.5 cm in diameter) on KCl agar plates and incubated for 7 days at 25 °C under a 14-h light/10-h dark cycle. Conidia were harvested and quantified with a haemocytometer. Three biological replications were performed to obtain standard deviations.

We also studied conidia production in the mutant strains. A fungal agar block (0.5 cm in diameter) was inoculated on KCl agar plates, and the plates were incubated for 7 days. We observed a substantial decrease (>75%) in conidia production in the ΔflbA2 isolate (Fig. 4B), but no statistically significant difference in the three other mutants (data not shown). Germination efficiency was not affected in the mutants when compared with the wild-type progenitor. When incubated in defined liquid medium, the conidia of three mutants, ΔflbA1, ΔrgsB and ΔrgsC, produced standard 'meandering' germ tubes typically observed in the wild-type strain (Fig. 5) (Sagaram and Shim, 2007). Meanwhile, ΔflbA2 conidia germinated in a severe curly, wavy fashion not commonly observed in the wild-type strain (Fig. 5) and quite

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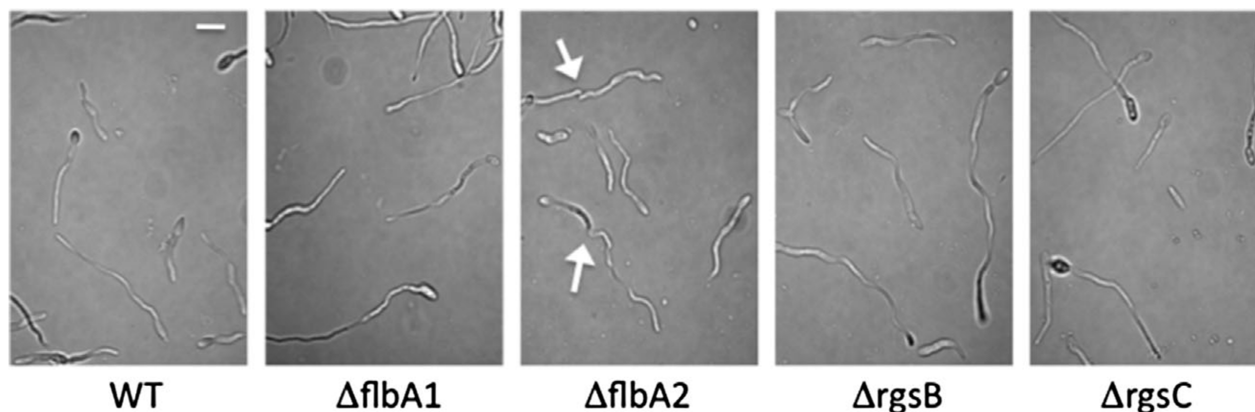

Fig. 5 Effect of RGS (regulators of G-protein signalling) gene mutation on colony hyphal development. Microconidia of the designated strains were allowed to germinate in 0.5 × potato dextrose broth for 24 h at 23 °C. Scale bar, 20 μm. It should be noted that the ΔflbA2 strain produces hyphae that germinate in a severe curly, wavy fashion (as indicated with arrows), not commonly observed in the wild-type and other mutants.

contrary to the 'undeviating straight' hyphal germination seen in the Δgbb1 mutant (Sagaram and Shim, 2007). However, the overexpression of *FLBA2* did not result in Δgbb1-like conidia germination, but rather was similar to that of the wild-type strain (data not shown).

When we investigated whether the mutants were affected with regard to maize stalk rot virulence, we found that the mutants were all capable of colonizing and rotting maize (Fig. S3, see Supporting Information). We measured the vertical length of the rot in every stalk sample tested, and the difference was not statistically significant ($P < 0.05$). We also determined that all mutants could carry out sexual development. The mutants were crossed to the opposite mating type wild-type strain A102 on carrot agar and, after 21 days of incubation under a 14-h light/10-h dark cycle, they produced perithecia with viable ascopores (data not shown).

Effects of *FLBA2* and *RGSB* overexpression in *F. verticillioides*

Deletion of *FLBA2* and *RGSB* resulted in increased FB_1 production (Fig. 4A), but a drastic reduction in conidiation (Fig. 4B) and reduced mycelial growth on solid medium (Fig. 3A,B). This led us to question the impact of the overexpression of these genes. We placed these genes under the control of the GpdA promoter, and transformed them into wild-type protoplasts. The integration and overexpression of these genes were confirmed by PCR and northern analyses (data not shown). The overexpression of *FLBA2* and *RGSB* resulted in suppression of FB_1 production (Fig. 4A), suggesting that these genes are associated with the regulation of FB_1 biosynthesis in *F. verticillioides*. However, the overexpression of *FLBA2* or *RGSB* did not result in hyperconidiation (Fig. 4B) or hypermycelial growth (data not shown), respectively.

FlbA1 and FlbA2 are necessary for mediating the host response during fungal infection in viable maize kernels

To elucidate the potential roles of the RGS gene family in *F. verticillioides*–maize interactions, particularly with viable maize kernels, we inoculated surface-sterilized B73 maize kernels with *F. verticillioides* mutants and analysed conidiation, FB_1 production and endogenous ethylene levels. The disruption of *FLBA1* and *FLBA2* resulted in lower conidiation compared with that of the wild-type strain, whereas ΔrgsB and ΔrgsC1 showed no statistically significant difference in conidiation from the wild-type (Fig. 6A). Quantification of FB_1 in maize kernels for these four strains showed that FB_1 levels in mutant strains were suppressed in comparison with the level of the wild-type strain (Fig. 6B). The role of ethylene as a plant defence hormone is well characterized (for reviews, see Bari and Jones, 2009; Wang *et al*., 2002). A recent study demonstrating that *F. graminearum* exploits host ethylene signalling in wheat and Arabidopsis seeds (Chen *et al*., 2009) raised a question regarding the role of ethylene in the *F. verticillioides*–maize kernel interaction. Here, we tested whether *F. verticillioides* mutants generated in this study could alter the emission of ethylene in infected kernels. For the assay, we quantified ethylene levels in maize kernels in response to fungal strains at designated time points. As shown in Fig. 6C, significantly lower ethylene levels were detected in kernels inoculated with ΔflbA1 and ΔflbA2 mutants at 7 and 10 days post-inoculation when compared with the wild-type and other mutant strains. Taken together, our data indicate a positive correlation between ethylene production in the host and conidiation and mycotoxin production in select fungal mutants (ΔflbA1 and ΔflbA2) when grown on viable maize kernels.

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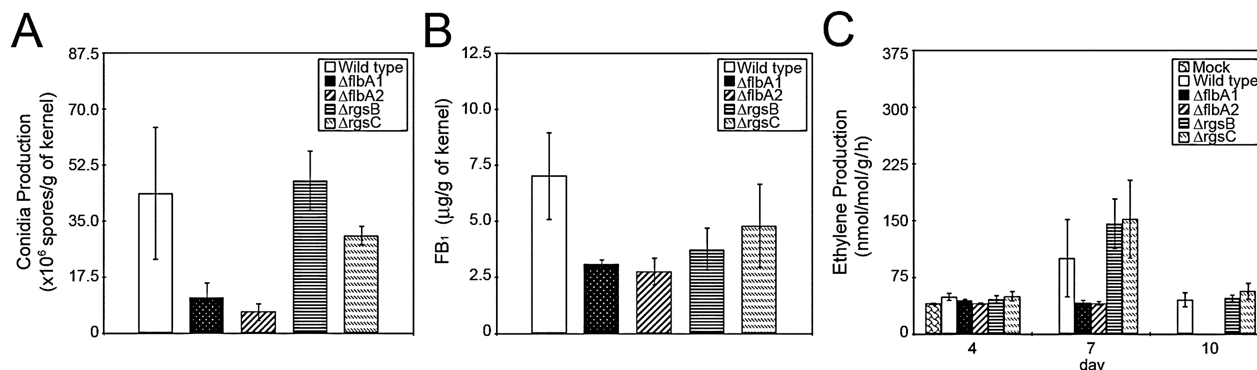

Fig. 6 Fungal infection assay on viable B73 maize kernels by *Fusarium verticillioides* wild-type and mutant strains. (A) Conidiation of wild-type and mutant strains of *F. verticillioides* grown on seeds at 7 days post-inoculation. (B) Fumonisin B_1 (FB_1) was quantified at 7 days after inoculation with *F. verticillioides* wild-type and mutant strains using high-performance liquid chromatography (HPLC). (C) Ethylene was measured at 4, 7 and 10 days post-inoculation with *F. verticillioides* strains. The vials containing infected kernels were tightly sealed for 3 h prior to the withdrawal of headspace gases to allow the detection of ethylene accumulation by gas chromatography. The values are the mean ± standard deviation of four or five replicates per strain.

DISCUSSION

Heterotrimeric G proteins in filamentous fungi were first cloned by Turner and Borkovich (1993) and, since then, researchers have identified and characterized these G proteins in a number of fungal species (Li *et al*., 2007). In the maize pathogen *F. verticillioides*, the Gβ subunit has been shown to be associated directly with mycotoxin FB_1 biosynthesis and hyphal development (Sagaram and Shim, 2007). Gene deletion of *GBB1* did not impact fungal radial growth on solid medium and mycelial mass production in liquid medium. However, HPLC analysis showed that FB_1 production in the mutant (10 μg/g) was drastically suppressed when compared with that of the wild-type progenitor (140 μg/g). In contrast with other plant-pathogenic ascomycetes (Mehrabi *et al*., 2009; Nishimura *et al*., 2003; Yu *et al*., 2008), the mutation of the Gβ subunit did not have an impact on the ability of the fungus to infect and colonize maize kernels and stalks (Sagaram and Shim, 2007). This observation was similar to that in *Ustilago maydis*, where the Gβ subunit regulates cyclic-AMP signalling that ultimately induces pheromone gene expression, but not pathogenicity (Muller *et al*., 2004). However, we know from studies in other fungi that G-protein activities are regulated by RGS proteins and PhLPs (Li *et al*., 2007; Ross and Wilkie, 2000).

If Gβ is associated with the regulation of FB_1 biosynthesis and fungal development in *F. verticillioides*, are these regulated through the same signalling pathway or do they diverge to independent signalling mechanisms? To identify regulatory proteins that mediate G-protein signalling associated with FB_1 production and hyphal development, we searched for RGS and PhLP genes that were transcriptionally impacted by the Gβ mutation (Fig. 1B). As RGS proteins transcriptionally regulate downstream gene activation/suppression (Han *et al*., 2004; Lee and Adams, 1994; Shimizu *et al*., 2003), we reasoned that RGS genes differentially expressed in the Gβ mutant when compared with the wild-type strain could be associated directly with mutant phenotypes, positively or negatively. Based on our results, we hypothesized that RgsB, FlbA1 and FlbA2 are negative regulators of FB_1 production and hyphal germination phenotypes in Δgbb1 (Sagaram and Shim, 2007), as these genes were up-regulated in the mutant strain. RgsC1 would be considered as a positive regulator. Phenotypic analyses of four knockout mutants showed that *RGSB* deletion resulted in FB_1 overproduction but hyphal growth suppression, and that *FLBA2* deletion led to FB_1 overproduction but suppression of conidiation. In contrast with our anticipation, deletion of *FLBA1* and *RGSC1* resulted in no observable phenotypes on synthetic medium and nonviable maize kernels. RGS proteins are GTPase-activating proteins, which are known to interact with specific Gα subunits and promote GTP hydrolysis, thus negatively regulating Gα-mediated cell signalling (Li *et al*., 2007; McCudden *et al*., 2005; Ross and Wilkie, 2000). In addition to the RGS domain, a conserved domain required for Gα interaction, these proteins carry other motifs that may facilitate Gβ binding, Ras binding, GPCR phosphorylation and membrane localization.

In *A. nidulans*, FlbA is known to interact genetically with FadA, a group I Gα subunit, and to promote asexual development and sterigmatocystin biosynthesis (Hicks *et al*., 1997; Tag *et al*., 2000; Yu *et al*., 1996). Hicks *et al*. (1997) showed that a loss-of-function mutation in FlbA blocks sterigmatocystin production and conidiation, and the converse is true when the *FLBA* gene is overexpressed (Hicks *et al*., 1997; Lee and Adams, 1994). However, when we investigated FlbA function in *F. verticillioides*, we discovered that the regulatory circuitry could be quite different from that of *A. nidulans*. First, *F. verticillioides* harbours two FlbA paralogues, both carrying RGS motifs, which suggests

that these FlbA proteins bind to Gα subunits. We hypothesized that the two FlbA proteins bind to group I Gα protein, but physical interaction has yet to be tested. Second, mutation in FlbA1 did not result in any recognizable phenotype on nonviable maize kernels, whereas FlbA2 null mutants resulted in the overproduction of FB_1 (Fig. 4A), but suppressed conidiation (Fig. 4B). Overexpression of *FLBA2* suppressed FB_1 production but, with regard to asexual reproduction, only maintained wild-type level conidiation. The FlbA orthologue in *M. grisea*, Rgs1, physically interacts with group I and II Gα subunits to facilitate intrinsic GTPase activity, and serves as a negative regulator of Gα proteins impacting on asexual development as well as appressoria formation (Liu *et al*., 2007). Mutation in Rgs1 resulted in precocious appressoria formation on noninductive surfaces, and this was suggested to be the result of the unrestrained intracellular cyclic-AMP level. Xue *et al*. (2008) also demonstrated this in the *Cryptococcus neoformans* system. However, our result showed that mutation in FlbA1 and FlbA2 did not have an impact on fungal virulence, positively or negatively. We can postulate that FlbA is associated with fungal development, particularly infection structures, in select pathogenic fungi. Therefore, it is reasonable to predict that FlbA is not associated with pathogenicity in *F. verticillioides*, as *Fusarium* species do not utilize specialized infection structures to penetrate the host.

RgsB proteins can be easily identified *in silico* in filamentous fungi, with a distinct RGS motif and multiple transmembrane domains, but the biological understanding is very limited. RgsB, together with RgsA, is known to be a fungal-specific RGS protein, and participates in the regulation of bipolar budding in *Saccharomyces cerevisiae*. However, in filamentous fungi, the role of RgsB is unclear. In our study, we discovered that the *RGSB* gene is transcriptionally regulated by the Gβ subunit in the *GBB1* deletion, but whether RgsB physically interacts with Gbb1 at the protein level remains to be tested. We also cannot rule out the possibility of Gβ having an indirect impact on *RGSB* gene expression through a dissociated Gα protein that is the target of RgsB. Loss-of-function mutation in RgsB resulted in slower hyphal growth on solid medium and the overproduction of FB_1 (approximately 150% higher level) when compared with the wild-type progenitor (Figs 3A and 4A). Our results suggest that FlbA2 and RgsB work together to regulate the downstream FB_1 biosynthesis machinery; however, they also independently control proper conidiation and hyphal proliferation, respectively, in *F. verticillioides*.

As a result of our qRT-PCR analyses, we identified four RGS genes that were transcriptionally impacted by the deletion of the Gβ subunit. Does this imply then that the other four genes, *PHNA*, *PHNB*, *RGSA* and *RGSC2*, are not involved in *F. verticillioides* development and secondary metabolism via a heterotrimeric G-protein complex? Based on the literature, we can presume that these regulators interact and control downstream signalling pathways at post-translational levels (Liu *et al*., 2007; Xue *et al*., 2008), or that these other genes may be under the transcriptional control of Gα subunits (Han *et al*., 2004; Lafon *et al*., 2005). PhLPs have been shown to serve as molecular chaperones for Gβ and Gγ assembly (Knol *et al*., 2005; Lukov *et al*., 2005), and it is perhaps through these physical interactions that PhLPs perform regulatory roles. For instance, in fungi, Seo and Yu (2006) have shown that PhnA in *A. nidulans* is required for wild-type level fungal biomass and conidiation, phenotypes that are known to be regulated by the Gβ subunit SfaD. With regard to RGS, *A. nidulans* RgsA downregulates pigment production, but stimulates conidiation (Han *et al*., 2004). These cellular responses are triggered when RgsA elicits GTPase activity of GanB, a Gα subunit, which is known to repress *brlA*, a primary regulator of conidiophore development in *A. nidulans*, and, ultimately, conidiation (Chang *et al*., 2004).

One of the intriguing and conceptually important findings of this study is the striking differential response of the fungal mutant strains when grown on either nonviable (autoclaved cracked kernels) or viable (live) maize kernels of the same host genotype. Although the ΔflbA2 mutant grown on cracked kernels produced approximately six times greater FB_1 levels and approximately 4.5 times fewer conidia, this same strain, when grown on live kernels, produced about three- to four-fold less FB_1 and conidia. Similarly, although the ΔflbA1 strain showed no detectable phenotypes associated with FB_1 and conidiation when grown on nonviable kernels, both processes were inhibited when the mutant strain was grown on live maize kernels. These results provide significant evidence for the impact of the fungal G-protein signalling pathway on pathogen-triggered metabolism and/or signalling in the host, which is in contrast with the hypothesis that preformed metabolites in maize seeds regulate pathogen development and secondary metabolism. The importance of signalling cross-talk between hosts and pathogens in determining the outcomes of plant–pathogen interactions has been emphasized recently in several reviews (Christensen and Kolomiets, 2011; Gao and Kolomiets, 2009; Tsitsigiannis and Keller, 2007). Although the requirement for host-derived chemical signals in the regulation of FB_1 biosynthesis and conidia production has been recognized previously (Gao *et al*., 2007), the precise nature of most of these signalling molecules remains obscure. One such potent molecular signal is the plant hormone ethylene. Seed-derived ethylene is implicated in the regulation of *F. verticillioides* growth and conidiation, as fungal growth was reduced on ethylene-deficient maize mutants (M. V. Kolomiets, unpublished data). Interestingly, a recent screen of diverse *Botrytis cinerea* signalling mutants revealed that a Gα null mutant *Δbcg1* was insensitive to exogenous ethylene, and showed considerable changes in expression of the fungal genes when

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compared with the wild-type strain (Chague *et al*., 2006). This result suggests that the fungal pathways mediated by GPCRs may be involved in the sensing and/or response to exogenous ethylene, resulting in transcriptional reprogramming of *B. cinerea*. In our current study, we showed that ΔflbA1 and ΔflbA2 mutants are incapable of triggering the production of normal levels of ethylene by the host, suggesting that the GPCR signalling cascade is important in the modulation of host ethylene biosynthesis.

EXPERIMENTAL PROCEDURES

Fungal strains and media

Wild-type *F. verticillioides* strain 7600 (Fungal Genetics Stock Center, University of Missouri-Kansas City, Kansas City, MO, USA) was stored in 30% glycerol at –80 °C. The fungus was routinely maintained on V8 juice agar (200 mL of V8 juice, 3 g of $CaCO_3$ and 20 g agar per litre) and potato dextrose agar (PDA; Difco, Detroit, MI, USA). For genomic DNA extraction, the fungus was grown in YEPD medium (3 g of yeast extract, 10 g of peptone and 20 g of dextrose per litre) on a rotary shaker (150 r.p.m.) at 25 °C. For RNA isolation, the fungus was grown in defined liquid (DL) medium, pH 4.5–6.0 (1 g $NH_4H_2PO_4$, 40 g sucrose, 3 g KH_2PO_4, 2 g $MgSO_4.7H_2O$ and 5 g NaCl per litre) or BSAL, pH 6.0 (1 g BSA, 40 g sucrose, 3 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$ and 5 g NaCl per litre) (Shim and Woloshuk, 2001) with shaking (150 r.p.m.) at 25 °C. For microconidia counts, 10^4 microconidia were inoculated at the centre of V8 agar or KCl agar (Shim *et al*., 2006) and allowed to grow for 9 days. The spores were harvested in sterile water, passed through sterile Miracloth (Calbiochem, La Jolla, CA, USA) to eliminate mycelia and counted using a haemocytometer.

Nucleic acid manipulation and PCR

Fungal genomic DNA was extracted as described previously (Shim and Woloshuk, 2001). Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Southern and northern analyses were performed following standard procedures described previously (Sagaram and Shim, 2007). The probes used in all hybridization experiments were ^{32}P-labelled with the Prime-It Random Primer Labelling Kit (Stratagene, La Jolla, CA, USA). The primers used in this study are listed in Table S1 (see Supporting Information).

PCR was performed in a GeneAmp PCR system 9700 thermocycler (PE Applied Biosystems, Norwalk, CT, USA). DNA amplification was performed in a 25- or 50-μL volume with either Taq DNA polymerase (New England Biolabs, Ipswich, MA, USA) or Expand Long Polymerase (Roche, Indianapolis, IN, USA). The PCR conditions included 2 min of initial denaturation at 94 °C, followed by 30 cycles of 30 s of denaturation at 94 °C, 30 s of annealing at 55–57 °C (based on primer pair) and 1–5 min (based on length of the anticipated amplicon) of elongation at 72 °C (68 °C for Expand Long Polymerase amplifications). A 10-min final extension was carried out after 30 cycles.

Identification of *F. verticillioides* RGS and phosducin genes impacted by Δgbb1 mutation

qRT-PCR experiments were carried out as described previously (Sagaram *et al*., 2006) with some modifications. All primers used in qRT-PCR for the eight genes (*PHNA*, *PHNB*, *RGSA*, *RGSB*, *RGSC1*, *RGSC2*, *FLBA1* and *FLBA2*) are given in Table S1. For this experiment, the wild-type and the Δgbb1 mutant were grown on cracked-corn medium for 7 days before total RNA samples were extracted using Trizol (Invitrogen). All qRT-PCR experiments were performed in the ABI 7500FAST system (Applied Biosystems, Carlsbad, CA, USA) with the QuantiTech SYBR Green RT-PCR Kit (Qiagen, Valencia, CA, USA). Reactions were carried out with 30 min of RT at 50 °C, followed by 15 min of predenaturation at 95 °C and 35 cycles of 15 s of denaturation at 95 °C, 30 s of annealing at 56 °C and 30 s of extension at 72 °C. The β-tubulin gene (*TUB2*) (GenBank U27303) expression was determined with *TUB2* F1 and *TUB2* R31 primers, and was subsequently used as a reference. The levels of transcription were evaluated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008), with *TUB2* as the endogenous control. Three biological replications and two technical replications were performed.

Gene deletion and overexpression constructs

The individual gene disruption cassettes were constructed by a double-joint PCR strategy (Yu *et al*., 2004). DNA fragments corresponding to the 5′ (1.5 kb) and 3′ (1.5 kb) ends of each gene were amplified from the *F. verticillioides* genomic DNA with the primer pairs listed in Table S1. An *HPH* gene cloned into pBluescript II (Stratagene) was amplified with the primers M13-F and M13-R. Nested primer pairs were used to amplify the amplicon carrying the *HPH* marker fused to the flanking regions of the corresponding individual genes.

FlbA2 and RgsB overexpression strains were generated by transforming the wild-type strain with constructs that harboured the gene of interest under the control of a constitutive GpdA promoter. For FlbA2 overexpression strain generation, the GpdA promoter amplified from the gGFP vector (Maor *et al*., 1998) with the primer pair tTrpF and OEFlbA2revt was fused to FlbA2 (amplified from the *F. verticillioides* genome by the primer pair OEFlbA2for and OEFlbA2rev) by single-joint PCR. The joint-PCR product was amplified with primers tTrpF and OEFlbA2rev. The geneticin-resistant gene (*gen*) cloned into pBluescript II (Stratagene) was amplified with primers M13F and M13R. Two linear

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PCR products were co-transformed in the wild-type strain and the geneticin-resistant colonies were screened for integration of the overexpression cassette. For RgsB overexpression strain generation, we followed the same protocol, except that the *RGSB* gene was amplified with the primer pair OERgsBfor and OERgsBrev and fused to the GpdA promoter.

Fungal transformation

For the generation of high-frequency knockout strains, protoplasts were prepared from *F. verticillioides* KU70 deletion strain SF41 (Choi and Shim, 2008a) and transformed as described previously (Shim and Woloshuk, 2001). Transformants were selected on regeneration medium (Shim and Woloshuk, 2001) containing 150 μg/mL hygromycin B (Calbiochem). Hygromycin-resistant colonies were screened for appropriate knockout construct integration by PCR and further verified by Southern analysis. To obtain overexpression strains, the wild-type *F. verticillioides* protoplasts were used for transformation and the transformants were selected on either 150 μg/mL geneticin G-418 (Cellgro, Herndon, VA, USA) or hygromycin B, depending on the marker used in the experiment. The respective drug-resistant colonies were screened as described above.

FB_1 analysis

For FB_1 analysis, fungal strains were grown on autoclaved cracked-corn medium (B73 line; 2 g dry weight) in a 20-mL glass vial (VWR, West Chester, PA, USA) for 14 days at room temperature (22–23 °C). FB_1 was extracted with acetonitrile–water (1:1, v/v) for 24 h. The crude extracts were passed through equilibrated PrepSep SPE C18 columns (Fisher Scientific, Pittsburgh, PA, USA). The FB_1 concentration of the samples was analysed on a Shimadzu LC-20AT HPLC system (Shimadzu Scientific Instruments, Inc., Kyoto, Japan) equipped with an analytical Zorbax ODS column (4.6×150 mm^2) (Agilent Technologies, Santa Clara, CA, USA) and a Shimadzu RF-20A fluorescence detector. The HPLC system was operated following the protocol described previously (Shim and Woloshuk, 1999). FB_1 was quantified by comparing HPLC peak areas with FB_1 standards (Sigma, St. Louis, MO, USA). The experiment was repeated with three biological replicates.

Maize stalk rot assay

Stalk rot severity was assayed on 8-week-old B73 maize plants as described previously (Shim *et al*., 2006). Internodal regions of the stalk were punctured to approximately 2 mm depth with a sterile needle, and fungal spore suspension (10^8 spores/μL water) was inoculated into the wound with the help of sterile cotton applicators. Plants were incubated in a growth chamber at 25 °C, 70% humidity with a 14-h light/10-h dark cycle. After 14 days of inoculation, stalks were split longitudinally to examine the extent of rot. The experiment was performed thrice with three independent biological replications.

Conidiation and ethylene analyses on viable maize kernels

Maize genotype B73 was used to determine the conidiation ability. Seeds were surface sterilized with Clorox bleach (containing 6% sodium hypochlorite) for 15 min, and rinsed with sterilized, distilled water at least five times. In order to provide an infection site for fungal inoculation, embryos of each kernel were cut longitudinally (0.5 cm) using a razor blade to a depth of about 0.5 mm. Seeds were dried with paper towels and placed in a 20-mL glass scintillation vial (Wheaton Science, Millville, NJ, USA). The fungal suspension (200 μL of 1×10^6 conidia) of the tested strains of *F. verticillioides* was applied to the glass vial. Mock-inoculated kernels were treated with 200 μL of 0.01% Tween-20 solution. The inoculated kernels were placed in a container with moisture and incubated with a 12-h light/12-h dark cycle at 27 °C for 7 days. Incubated kernels were vortexed with 2 mL of sterilized water and the spores were counted using a haemocytometer. Five replicates of maize kernels were used per fungal strain.

The quantification of ethylene production in live maize kernels was carried out as described previously (Gao *et al*., 2008) with some modifications. Conidial suspensions were applied to the kernels as above. Ethylene was quantified at 4, 7 and 10 days post-inoculation (dpi). Vials were sealed with screw caps with septa. One millilitre of the headspace gas was withdrawn from vials by a syringe and analysed using digital gas chromatography (Photovac 10 plus; Perkin-Elmer, Inc., Norwalk, CT, USA) with a photodetector and compressed air (ultra zero grade; Praxair, Inc., Danbury, CT, USA) as carrier gas.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Phylogenetic analyses of *Fusarium verticillioides* RGS (regulators of G-protein signalling) proteins (A) and PhLPs (phosducin-like proteins) (B). Homologues of *F. verticillioides* RGSs and PhLPs in other fungal species were identified in GenBank by BLASTP search. Multiple alignments were performed using MUSCLE software, curated with Gblocks, phylogeny derived using PhyML and tree drawn with TreeDyn on (http://www.phylogeny.fr). Abbreviated names of fungal species in the figure are as follows: Anidulans, *Aspergillus nidulans*; Cglobosum, *Chaetomium globosum*; Cparasitica, *Cryphonectria parasitica*; Fv, *Fusarium verticillioides*; Mgrisea, *Magnaporthe grisea*; Ncrassa, *Neurospora crassa*; Panserina, *Podospora anserina*.

Fig. S2 *Fusarium verticillioides* wild-type and mutant strains grown on solid agar medium (A) to assess the hyphal growth rate and in liquid medium (B) to study pigmentation. (A) *Fusarium verticillioides* strains (wild-type and mutants) were first grown on V8 agar medium for 5 days, and used as an inoculum source. Agar blocks were prepared using a cork borer (0.5 cm in diameter) and inoculated at the centre of Petri plates containing five different solid agar media [defined liquid medium (DL) with glucose, DL with sucrose, DL with sorbitol, 1 × potato dextrose

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agar (PDA) and V8] for 5 days at room temperature before being photographed. (B) *Fusarium verticillioides* strains (wild-type and mutants, 10^7 conidia/mL) were inoculated into 50 mL of bovine serum albumin liquid (BSAL) (DL with BSA as nitrogen source) and 0.2 × potato dextrose broth (PDB) for 7 days with continuous shaking at room temperature. A 2-mL sample of each culture was transferred to a 24-well sterile culture plate and photographed. A noninoculated (blank) sample is shown as a negative control.

Fig. S3 Maize stalk rot assay with *Fusarium verticillioides* wild-type and mutant strains. Eight-week-old maize stalks were inoculated with wild-type (WT) and mutant strains (10^8 spores) at the internodal region, and incubated in a growth chamber for 14 days at 25 °C, 70% humidity with a 14-h light/10-h dark cycle. Subsequently, the stalks were split longitudinally and photographed. Three independent biological replications were used in three independent experiments. Maize stalks inoculated with sterile water are shown as a negative control.

Table S1 Primers used in this study.

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