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# *Cochliobolus heterostrophus* G-protein alpha and beta subunit double mutant reveals shared and distinct roles in development and virulence

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ABSTRACT

Heterotrimeric G proteins transduce extracellular signals to control development in eukaryotes, including filamentous fungi. Targeted disruption of their α- and β-subunit genes has shown that fungal G proteins play essential roles in sexual and asexual sporulation, hyphal growth pattern and virulence. The G-protein β-subunit gene *CGB1* of *Cochliobolus heterostrophus*, the agent of Southern leaf blight, is essential for virulence and sporulation, while one of the α-subunit genes, *CGA1*, has roles in several developmental pathways. We constructed a strain with insertions/deletions at both *CGA1* and *CGB1*. The double mutant, like the single mutants, was deficient in mating and appressorium formation and its hyphae followed a straight path rather than the typical meandering growth pattern on a hard surface. The two genes shared a combined role in determining pigmentation, surface hydrophobicity, and resistance to different stresses. However, in contrast to the single mutants, the double mutants developed white-gray to completely white colonies that are hydrophilic and form wet, autolytic-appearing patches. These phenotypes resemble some of those conferred by mutations in the MAPK gene *CHK1*, suggesting crosstalk between MAPK and G-protein pathways. Loss of signal-transduction functions, while reducing virulence, increases resistance to some stresses. A model is proposed for the interactions between the signaling pathways.



## 1. Introduction

Heterotrimeric G proteins provide transmembrane signaling in eukaryotic organisms, from protists to mammals. G-protein activation by a signal from a seven-helix transmembrane receptor results in exchange of GDP for GTP by the G protein's α subunit (Gα) and its dissociation from the Gβγ heterodimer [1]. Both GTP-Gα and free Gβγ can activate downstream targets [2]. Filamentous fungi are likely to employ these conserved pathways, but may also provide an opportunity to uncover novel regulatory hierarchies. Activation of a heterotrimeric G protein produces, in fact, two signals, which could provide combined, or perhaps balanced, effects on the output to gene expression and function.

The construction of mutants in Gα and Gβ subunit genes has shown that G-protein signaling is required for virulence in fungal pathogens of plants including, for example, *Magnaporthe grisea* [3], [4], *Magnaporthe oryzae* [5], *Cryphonectria parasitica* [6–8], *Botrytis cinerea* [9], *Trichoderma reesei* (*Hypocrea jecorina*) [10,11], *Fusarium oxysporum* [12,13] and *Fusarium verticillioides* [14]. Gγ subunit genes have been identified in *Neurospora* and *Aspergillus* [15–17] and Gγ interacts with Gβ, as shown by co-immunoprecipitation. Thus it is likely that a Gβγ heterodimer is released upon activation of filamentous fungal G proteins.

In *Cochliobolus heterostrophus*, the agent of Southern Corn Leaf Blight, Gα (encoded by *CGA1*) and Gβ (encoded by *CGB1*) subunits, as well as a mitogen-activated protein kinase (MAPK, encoded by *CHK1*), participate in several developmental pathways [18,19]. Mutations in these genes result in phenotypes that include loss of the normal meandering hyphal growth pattern on hard surfaces, lack of appressorium formation and defects in mating. Loss of the Gβ subunit gene *CGB1* or the MAPK *CHK1* leads to severe loss of virulence [19,20]. Mutants deleted in *CGA1* seem able to cause wild-type (WT) symptoms on maize [18], but careful observation revealed that under some conditions (such as the status of nutrient accessibility, developmental and senescence stages of the host leaf), the *CGA1* mutant is less virulent than the WT strain [21].

Some of the phenotypes conferred by mutations in *CGA1* are found in *CGB1* mutants, suggesting that CGB1 functions together

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with CGA1 in a heterotrimer. Indeed, *CGB1* is the sole Gβ subunit-encoding gene in the genome of this organism. Filamentous fungi have one Gβ- and usually three Gα-encoding genes that belong to three major groups. Encoded proteins in groups I and III are related to the mammalian Gαi and Gαs families, respectively, but group II fungal Gα proteins have no mammalian counterpart [9,20,22–24]. In *C. heterostrophus* there are three Gα-encoding genes (*CGA1*, *CGA2*, and *CGA3*, B. A. Horwitz and B. G. Turgeon, unpublished data) and one Gβ-encoding gene. Mutations in either the *CGA2* or *CGA3* gene result in no obvious phenotypes (unpublished data cited in Ganem et al., 2004), but these mutants should be recharacterized using new criteria, such as surface hydrophobicity, stress-condition resistance, cell-wall integrity, response to light, and more. Nevertheless, additional Gα subunits might contribute to some pathways, or be redundant to CGA1, as found in *Neurospora* [25]. Mutations in either *CGA1* or *CGB1* result in abnormal straight hyphal growth, loss of the ability to make appressoria, and female sterility. In contrast to deletion of *CGA1*, loss of *CGB1* is not ascospore-lethal, indicating that there is a Gβ-independent signaling role for the Gα subunit CGA1 in mating.

An MAPK module is a known target of Gβγ in budding yeast and mammalian cells [26,27]. Loss of the *C. heterostrophus* MAPK CHK1 indeed results in some of the same phenotypes as *cgb1*, including lack of virulence, female sterility and inability to form appressoria [19,20]. Other phenotypes, however, are different. To give two examples, *cgb1* mutants have straight hyphal growth on a hard surface whereas *chk1* mutants have normal wavy growth; *chk1* have decreased pigmentation, whereas *cgb1* overproduce a melanin-like secreted pigment [19,20]. Comparison between the phenotypes of *cga1* or *cgb1* mutants and *chk1* mutants thus reveals that not all of the phenotypes conferred by *chk1* are found in the two G-protein subunit mutants, implying that neither Gα nor the Gβγ heterodimer can be the sole conduit for signals to the MAPK CHK1 module. Furthermore, there must be MAPK-independent roles for the (postulated) CGB1-containing Gβγ dimer. For Gα subunits, adenylate cyclase might be a downstream effector. *C. heterostrophus* adenylate cyclase or protein kinase A mutants are not yet available, but some inferences can be made from other fungi, as by, for example, Lafon et al. [17]. Yet there is evidence that both Gα and Gβ can activate cyclic adenosine monophosphate (cAMP), a downstream adenylate cyclase effector [24,28].

If Gα and Gβγ carry entirely separate signals, a double mutant in *CGA1* and *CGB1* should have a set of phenotypes that is simply the combination of the phenotypes of each mutant alone. If the same set of processes is regulated by both subunits, the double mutant will not identify any new phenotypes. Between these two extremes, mutations in either subunit gene might influence the phenotypes conferred by the other. Characterization of the phenotypes of a double mutant, therefore, can help map the signaling pathways.

In addition to the developmental phenotypes studied in previous work on *C. heterostrophus*, we have introduced two new criteria, as demonstrated recently for MAPKs [29]: sensitivity to stress, and surface hydrophobicity. Studies on the role of signal transduction in response to environmental stresses have been reported for other fungi such as *C. parasitica* [30], *Penicillium chrysogenum* [31] and *Neurospora crassa* [32–34]. Surface hydrophobicity, a consequence of the expression of hydrophobin-encoding genes, is another phenotype that is under the control of environmental signals in other fungi, and there is evidence that G-protein and MAPK pathways modulate hydrophobin expression and secretion [30,35,36].

Starting with two *C. heterostrophus* G-protein subunit genes, *CGA1* and *CGB1*, we studied the influence of a loss-of function mutation in each of these genes on the phenotypes conferred by mutation of the other. The results indicate shared and distinct signaling via the Gα and Gβ subunits.

## 2. Materials and methods

### 2.1. Strains

WT *C. heterostrophus* strains C4 (*MAT1-2*; *Tox1*$^+$ ATCC 48331; abbreviated in figures as WT C4) and C5 (*MAT1-1*; *Tox1*$^-$ ATCC 42332; abbreviated in figures as WT C5) were obtained after six backcrosses [37] and are nearly isogenic. Previously developed [18] strains with mutations in the G-protein α-subunit gene *CGA1* were: C5*cga1* (*MAT1-1 tox cga1*; created by insertion of the hygromycin cassette into the coding region, combined with an 18-bp deletion; hereafter C5*cga1*) and C4*cga1 TSC17* (*MAT1-2 Tox1*$^+$ GFP *cga1*; created by complete deletion of the coding region; hereafter C4*cga1*). Two additional signaling mutants were used: a mutant in the G-protein β-subunit gene (*cgb1*, *MAT1-1; Tox1*$^-$, created by insertion of the hygromycin cassette into the coding region, combined with a 473 bp deletion) [20], and a mutant in the MAPK gene (*chk1*, *MAT1-2*; *Tox1*$^+$, created by replacement of the coding region with the hygromycin resistance cassette) [19]. To test reproductive ability, strain *Δcga1 Δcgb1* (*MAT1-1*; *Tox1*$^-$) was crossed with WT C4 (*MAT1-2*; *Tox1*$^+$) as described previously [37].

### 2.2. Culture conditions and virulence assays

Fungal strains were grown on standard complete medium (CM [38] with or without 50 μg/ml hygromycin B) for about 10 days at room temperature (24–26 °C) under continuous light from cool white and UVA-enriched fluorescent tubes (Philips, Eindhoven, The Netherlands). For hygromycin B (Calbiochem) selection, CM was prepared without salts. For bialaphos (Duchefa, Haarlem, The Netherlands) resistance selection, CM was prepared without yeast extract and casein hydrolysate, to avoid providing amino acids. The final bialaphos concentration was 100 μg/ml. Six CM culture agar disks, 6 mm in diameter, were cut from the margins of 4-day-old colonies of each strain and transferred to a 50 ml polycarbonate screw-capped test tube, containing 20 ml of liquid CM. The cultures were incubated diagonally for 4 days in a rotary shaker at 230 rpm and at a temperature of 30 °C under continuous light from cool white fluorescent tubes. Mycelia were collected by centrifugation (10 min, 600 × *g*) and briefly homogenized (20 s, Polytron, Brinkmann Instruments, Westbury, NY). Mycelia (50 mg wet weight/ml of 0.05% Tween 80) were used to infect leaves.

Maize cv. Grand Jubilee, purchased locally, was grown at 25 °C in a greenhouse. Maize seedlings were 11–13 days old when the third leaf emerged, remaining partly rolled and beginning to expand. Seedlings were inoculated by depositing drops (5 μl) of mycelial suspension, prepared as described above, on the leaves. One drop was applied to the upper third of the first three intact leaves. Alternatively, the whole leaf, still attached to the intact plant, was dipped in the mycelial suspension. The plants were then incubated in a moist chamber (autoclavable plastic bag) for 3 days at 30 °C (unless otherwise indicated) under continuous white light in a growth chamber. For quantitative analysis, leaves were scanned (300 dpi) and the lesion area (calculated using Adobe Photoshop 6.0 and Tina 2.10g software, Raytest, Straubenhardt, Germany) was used to evaluate the severity of the infection.

Media for the stress-response experiments were prepared by adding KCl (to 750 mM) or sorbitol (to 1.5 M) to CM prior to autoclaving. Menadione (2-methyl-1,4-naphthoquinone, Fluka; 125 μM from a 50 mM ethanol stock), which generates superoxide anion radicals [39], or 20 mM hydrogen peroxide was added to autoclaved CM after it had cooled down to 55 °C. Stress media