G-Protein β Subunit of Cochliobolus heterostrophus Involved in Virulence, Asexual and Sexual Reproductive Ability, and Morphogenesis

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Previous work established that mutations in mitogen-activated protein (MAP) kinase (CHK1) and heterotrimeric G-protein α (Gα) subunit (CGA1) genes affect the development of several stages of the life cycle of the maize pathogen Cochliobolus heterostrophus. The effects of mutating a third signal transduction pathway gene, CGB1, encoding the Gβ subunit, are reported here. CGB1 is the sole Gβ subunit-encoding gene in the genome of this organism. cgb1 mutants are nearly wild type in vegetative growth rate; however, Cgb1 is required for appressorium formation, female fertility, conidiation, regulation of hyphal pigmentation, and wild-type virulence on maize. Young hyphae of cgb1 mutants grow in a straight path, in contrast to those of the wild type, which grow in a wavy pattern. Some of the phenotypes conferred by mutations in CGA1 are found in cgb1 mutants, suggesting that Cgb1 functions in a heterotrimeric G protein; however, there are also differences. In contrast to the deletion of CGA1, the loss of Cgb1 is not lethal for ascospores, evidence that there is a Gβ subunit-independent signaling role for Cga1 in mating. Furthermore, not all of the phenotypes conferred by mutations in the MAP kinase CHK1 gene are found in cgb1 mutants, implying that the Gβ heterodimer is not the only conduit for signals to the MAP kinase CHK1 module. The additional phenotypes of cgb1 mutants, including severe loss of virulence on maize and of the ability to produce conidia, are consistent with CGB1 being unique in the genome. Fluorescent DNA staining showed that there is often nuclear degradation in mature hyphae of cgb1 mutants, while comparable wild-type cells have intact nuclei. These data may be genetic evidence for a novel cell death-related function of the Gβ subunit in filamentous fungi.

Filamentous fungi recognize and respond to signals from the environment and from host organisms by altering their growth and development. The creation of mutants deficient in signaling is key to understanding the regulation of the disease process of plant pathogens. The loss of a signaling element controlling a coordinated set of downstream events can result in an avirulent phenotype. Following the isolation of a G-protein α (Gα) subunit gene from Neurospora crassa (35), a large number of such genes were identified in filamentous fungi (4) and classified into groups according to conserved motif sequences (13). Most ascomycetes for which there are data (e.g., N. crassa) have three Gα subunit genes (10). One fungal Gα subunit group, defined by N. crassa GNA1, is most similar to the mammalian Goi subunit class; this class includes consensus myristoylation sequences and the target of pertussis toxin ADP ribosylation (35). Mutations in members of this class often lead to defects in development and virulence. Less is known about the functions of members of the other two main classes of fungal Gα subunits (4, 11, 12, 13, 20, 44). The different classes are similar enough that functional redundancy can occur. In N. crassa, for example, loss of Gna-2 results in only subtle phenotypes, while deletion of GNA-2 from a strain that is already null for GNA-1 accentuates the phenotypes (2). A recurrent observation is that a particular Gα subunit may have different functions in different fungal species. For example, the N. crassa Gα subunit Gna-3 apparently belongs to a pathway that represses conidiation (16), while a different Goi subunit, the Goi subunit homolog FadA, has this function in Aspergillus nidulans (1).

Fungal Gα subunits, like those in animal cells, likely form heterotrimers that are composed of α, β, and γ subunits and that, upon interaction with an activated heptahelical transmembrane receptor, dissociate from the βγ heterodimer. Either part of the dissociated trimer can activate downstream effectors. The presence of a Gβγ dimer has not yet been demonstrated in filamentous fungi, and relatively little is known about the signaling functions of the Gβ subunit. Among the plant pathogens, Gβ subunit genes are known to be required for the virulence and development of the chestnut blight fungus Cryphonectria parasitica (15), the rice blast agent Magnaporthe grisea (28), and Fusarium oxysporum f.sp. cucumerinum, which causes cucumber wilt (14). The homologs in A. nidulans (29), N. crassa (42), and the opportunistic basidomycete human pathogen Cryptococcus neoformans (37) are also involved in developmental pathways. The C. neoformans or-
tholog is required for mating but is dispensable for virulence (37), again emphasizing how different signaling pathways are put to different uses, depending on the fungal species.

In budding yeast cells, the mating pheromone signal is transduced by the Gpγ dimer STE4/STE18, while the corresponding α subunit serves to sequester the dimer. A mitogen-activated protein kinase (MAPK) cascade transmits the signal downstream to drive the expression of target genes; specificity is maintained despite the fact that the same module can signal for mating or filamentous growth (23, 31). Filamentous growth is mediated by MAPK cascades operating in parallel to or downstream from the G proteins Ras and Gpa2 (17, 26, 30). This is mediated by MAPK cascades operating in parallel to or downstream from the G proteins Ras and Gpa2 (17, 26, 30). The pathways of yeast cells can provide insight into how filamentous fungi, including pathogens, transduce the particular signal that controls their development (3). In C. parasitica, both α subunit and β subunit genes have been studied by targeted gene disruption. Both genes are required for pathogenesis, but the phenotypes conferred by mutations in the genes encoding the two subunits are not identical (15). The β subunit is required for the regulation of development in A. nidulans. A loss of function of the Gβ subunit can suppress the phenotypes resulting from the overactivation of the Ga subunit that occurs in mutants lacking the RGS (regulator of G-protein signaling) protein Fh1A (29). The C. neoformans Gβ subunit gene GPB1 is required for mating and signals through an MAPK cascade analogous to that in budding yeast cells, while the Ga subunit Gpα1 signals through a distinct, cyclic AMP-dependent pathway (37).

The maize pathogen Cochliobolus heterostrophus attacks the host leaf, forming small appressoria that do not appear essential for penetration (13), which may occur through stomata or directly through the epidermis. Mutants in which the Ga subunit gene CGA1 is disrupted or deleted still cause disease symptoms on corn leaves (13), despite their altered growth pattern and nearly total loss of the capacity to form appressoria. Furthermore, once inside the leaf, CGA1 mutants are able to grow and overcome host defenses. One of several possible explanations for the ability of a signaling mutant to retain at least partial virulence is redundancy in the pathways that transduce signals from the host. At least two pathways are thought to transduce host cell signals leading to the pathogenic development of M. grisea (6). In all filamentous fungal species for which sufficient information is available, several Ga subunit genes have been found (C. heterostrophus has three, CGA1, CGA2, and CGA3) (B. A. Horwitz and B. G. Turgeon, unpublished data). To help distinguish between redundancy in G-protein signaling pathways and alternate signaling routes through additional pathways, we have isolated and disrupted the sole C. heterostrophus Gβ subunit gene, CGB1. The phenotypes conferred by the loss of CGB1 overlap only partially with those of cga1 mutants, providing insight into heterotrimeric G-protein signaling pathways.

MATERIALS AND METHODS

Fungal strains, growth conditions, and nucleic acid isolation. Wild-type (WT) C. heterostrophus strains C4 (MAT1-2 toxin-1; ATCC 48331) and C5 (MAT1-1 toxin-1; ATCC 48332) were described previously (33). Three original cgb1 mutant strains generated from strain C4 (C4ΔGβ1, C4ΔGβ1, and C4ΔGβ9) were purified by isolation of single conidia. One purified cgb1 mutant strain, C4ΔGβ9, was backcrossed to WT strain C5, and WT (N52-R-1, N52-R-2, and N53-R-6) and mutant (N52-R-3, N52-R-4, and N52-R-5) progeny were collected and screened for the cgb1 mutation. Progeny strain N52-R-5 (cgb1 MAT1-2 toxin-1) was backcrossed again to strain C5 (cross 1400), and a tetrad was collected. One progeny mutant from the tetrad from cross 1400 (1400-1-3; cgb1 hygB tox1 MAT1-1 ALB1) was crossed to strain C4 to confirm cegosegregation of the hygB marker and the cgb1 mutation. To test reproductive ability, strain 14-1-3 was also crossed to strain C12 (MAT1-2 alb1) (cross 1421). Four progeny strains (two black and two albino) from a tetrad from cross 1421 were backcrossed to WT test strains of the opposite color. Pigmented cgb1 progeny were also crossed to albino cgb1 progeny of the opposite mating type to test reproductive ability in a cgb1 homozygous cross.

Media, standard growth conditions, and mating assays were described previously (18, 36, 39). To examine the ability to conidiate, total conidia were collected from plates (100 by 15 mm) containing complete medium with xylene (CMX; three replicates were used) and counted by using a hemocytometer.

Genomic DNA was isolated from mycelium ground in liquid nitrogen as described for plant tissue (8) and used for PCR analysis.

Isolation of CGB1. An internal fragment of the CGB1 gene was isolated by PCR amplification from genomic DNA of strain C4. In the first reaction, the following primers, designed to match peptide sequences iYAMHHW and GDDNRY, respectively, were used: forward, 5'-AT(A/T)/CTY/ATC/GC/CGATTT; reverse, 5'-ACGC/CG/AT/GC/AT/GCTTC/GC/GC. A second, nested reaction was carried out with a 1-μl aliquot from the first reaction as a template and with the following primers, designed to match the peptide sequences VTMCAY and ATGSD, respectively: forward, 5'-GTNT/G CGACT/G/GT/GC/GC; reverse, 5'-ATCGT/AC/AT/GC/TCG/CG/GC. The resulting plasmid was digested with SalI and cloned into pBluescript (Stratagene) under the regulation of the A. nidulans TrpC promoter (excised from vector pUCATPH [22] with XbaI and SalI) were cloned into pBluescript (Stratagene).

Confirmation of a gene replacement event at CGB1. Genomic DNA was isolated from mycelium ground in liquid nitrogen as described for plant tissue (8) and used for PCR analysis.

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Construction of a gene replacement vector. The CGB1 disruption construct (see Fig. 2A) was prepared in two steps. First, a 693-bp fragment excised from GWS5 with HindIII and XbaI and the gene for resistance to hygromycin B (hygB) under the regulation of the A. nidulans TrpC promoter (excised from vector pUCATPH [22] with XbaI and SalI) were cloned into pBluescript (Stratagene) digested with HindIII and SalI. The resulting plasmid was digested with SalI and ligated to a 460-bp SalI fragment excised from GWS3. The desired orientation of this 3'-flanking region was confirmed by restriction enzyme digestion. The final construct was designated GbetaDXO. A 3.5-kb linear fragment that included the flanking regions and a selectable marker was obtained by PCR amplification with T7 and T7 primers and with GbetaDXO as a template. A total of 20 μg of this fragment was used to transform protoplasts as described previously (39), except that the enzyme Novozym 234 was replaced with β-glucanase (catalog no. 0439-1; InterSpx, San Mateo, Calif.) at a concentration of 2.9 mg/ml. After 24 h of recovery on regeneration medium (1 M sucrose, 0.1% yeast extract, 0.1% casin hydrolysate) (34), the plate was overlaid with water agar containing hygromycin B (Calbiochem) so that the final concentration of hygromycin B in the plate was 50 μg/ml.

Confirmation of a gene replacement event at CGB1. To test the insertion of the transforming DNA and the concomitant deletion of 473 bp of CGB1 by a double-crossover event, the following primers were used in PCRs with genomic DNAs from WT and transformant strains as templates: Beta3as, 5'-GAATAAG GGGCAACAGTGC-3'; Gj321for, 5'-CACACATGACGGTCTAC-3';
Mycelial mats of 7-day-old fungal cultures were scraped from plates (100 by 15 mm) of CMX agar, inoculated into 100 ml of liquid CM at room temperature with shaking for 48 h, and culture extracts were collected by filtration first through four layers of cheesecloth and then through a 0.2-μm-pore-size filter (Corning Glass Works, Corning, N.Y.). For pigmentation evaluation in liquid, strains were incubated using a Photomicroscope II microscope (Carl Zeiss, Oberkochen, Germany) with bright-field optics. For pigmentation evaluation on solid agar substrates, strains were grown on CMX agar plates for 7 days. Mycelial mats then were scraped from the plates and fragmented in 50 ml of sterile distilled water in a sterile stainless steel blender chamber with 16 h of light per day. Lesions were assessed daily, and photographs were taken at 5 days postinfection.

**Microscopy.** For microscopic observation of vegetative hyphae and appressorium formation, WT (C5) and cgb1 mutant (14-1-3) strains were grown on CM agar plates for 7 days. Mycelial mats then were scraped from the plates and fragmented in 50 ml of sterile distilled water in a sterile stainless steel blender cup (ground for 30 s). About 50 μl of mycelial fragment suspension was placed on a microscope glass slide (Fisherbrand Plain; Fisher Scientific, Pittsburgh, Pa.) and incubated at 32°C in a mini-mist chamber (100- by 15-mm petri dish padded with water-saturated filter paper) for 6, 8, 14, and 24 h. Three replicates were used for each treatment. After each period of incubation, fungal material was stained with cotton blue.

For pigment evaluation on solid agar substrates, strains were grown on CMX agar plates, and fungal aerial masses were scraped from the surfaces of the plates 2 weeks after inoculation. A cross section of the agar substrate was cut from a pigmented region by using a surgical blade, and the section was viewed by using bright-field optics. For pigmentation evaluation in liquid, strains were incubated in 100 ml of liquid CM at room temperature with shaking for 48 h, and culture extracts were collected by filtration first through four layers of cheesecloth and then through a 0.2-μm-pore-size filter (Corning Glass Works, Corning, N.Y.).

To examine nuclear conditions, germinated mycelial fragments (obtained by scraping) were fixed with 100% ethanol followed by washing with 75% ethanol and then with 60% ethanol. For microscopic observation of vegetative hyphae and appressorium formation, WT (C5) and cgb1 mutant (14-1-3) strains were grown on CM agar plates for 7 days. Mycelial mats then were scraped from the plates and fragmented in 50 ml of sterile distilled water in a sterile stainless steel blender cup (ground for 30 s). About 50 μl of mycelial fragment suspension was placed on a microscope glass slide (Fisherbrand Plain; Fisher Scientific, Pittsburgh, Pa.) and incubated at 32°C in a mini-mist chamber (100- by 15-mm petri dish padded with water-saturated filter paper) for 6, 8, 14, and 24 h. Three replicates were used for each treatment. After each period of incubation, fungal material was stained with cotton blue.

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To examine nuclear conditions, germinated mycelial fragments (obtained by using the methods described above) were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich Co., St. Louis, Mo.). For this, specimens were fixed with 100% ethanol followed by washing with 75% ethanol and then with 60% ethanol.

**FIG. 2.** CGB1 gene replacement construct. (A) Construct strategy. The top line shows the chromosomal region carrying CGB1 (light gray arrow) and the 473-bp region replaced by the hygB resistance marker in the event of a double-crossover integration. Also shown are the 5' (GW5'-hygB- GW3'), the hygB resistance cassette from pUCATPH (22) cloned between the two flanks as a SalI/XbaI fragment. Primers used for gene amplification and for checking integration events are indicated by arrows. (B) Confirmation of a double-crossover integration event at CGB1. Genomic DNAs from the strains indicated above the panels were used as templates for amplification with the primer pairs indicated below the panels. Strains 1400-1-3 and 1400-1-4 are cgb1 progeny from the backcross described in Materials and Methods (see also Fig. 3). In parentheses below each panel are the expected sizes of PCR products obtained with each primer pair.
water. DAPI-stained specimens were viewed by using the Photomicroscope II microscope with UV excitation filter BG3, beam splitter 450, and barrier filter 41. Images were captured on Elite Chrome 400 film (Kodak, Rochester, N.Y.) at exposure times of 5 to 10 s.

TUNEL staining. Terminal deoxynucleotidyltransferase-mediated dUTP-fluorescein nick end labeling (TUNEL) was performed by using an in situ cell death detection kit (fluorescein; Roche Applied Science, Indianapolis, Ind.). Mycelia from test strains C5 (WT) and 1400-1-3 (cgb1 mutant) were scraped from CMX agar plates (7 days old), fragmented in a stainless steel blender, and inoculated into 200 ml of liquid CM. Mycelia were harvested after 20 h of incubation at room temperature with shaking. Fungal protoplasts were prepared by following the same procedure as that used for C. heterostrophus transformation (34), except for the replacement of Novozyme 234 with Glucanex (in a liquid preparation; kindly provided by C. M. Hjort, Novo Nordisk, Bagvaerd, Denmark) in the enzyme-osmoticum solution (2 mg/ml). After adjustment of the final concentration to 10^6/ml in STC (1.2 M sorbitol, 10 mM Tris, pH 7.5, 50 mM CaCl_2), protoplasts were stored at -80°C and thawed on ice just before use.

For the TUNEL assay, thawed protoplasts (40 μl each) were placed as drops on glass slides (Fisherbrand Colorfrost Plus; Fisher Scientific), air dried in a laminar hood for 1 h, and then fixed with 2.5% paraformaldehyde in phosphate-buffered saline (PBS [pH 7.4]) at 4°C overnight. Fixed protoplasts were washed three times with PBS, dehydrated in an ethanol series (50, 70, 90, and 100%), and air dried for 2 h. Just before the TUNEL assay, the dried protoplasts were rehydrated in an ethanol series (100, 90, 70, and 50%) and then resuspended in 10 mM sodium citrate (pH 6.0), permeabilized with 0.1% Triton X-100 in 10 mM sodium citrate (pH 6.0) at room temperature for 3 min, and washed three times with PBS. TUNEL labeling was performed according to the manufacturer’s instructions, except that the incubation at 37°C was increased to 2 h. A negative control (labeling buffer only, without terminal deoxynucleotidyltransferase) and a positive control (pretreated with DNase I) were included for both strains.

After the TUNEL assay, the slides were washed three times with PBS, air dried briefly, mounted in mounting medium from a Prolong Antifade kit (Molecular Probes, Eugene, Oreg.), covered with a coverslip, and sealed with nail polish. Specimens were examined by using an Olympus BH-2 microscope. Fluorescence images were recorded on Kodak Elite Chrome 400 film at exposure times of 30 to 60 s with ×40 or ×100 fluorescence objectives.

Nucleotide sequence accession number. The sequence of CGB1 has been deposited in GenBank with accession numberAY211190.

RESULTS
Isolation of CGB1. Nested PCR, hybridization with a full-length C. parasitica Cpgb-1 cDNA clone (15), and use of the GenomeWalker kit led to the cloning of the C. heterostrophus CGB1 gene (ChCGB1). The complete coding sequence was obtained from the clone. Four introns were located by comparison with the cDNA sequence obtained by RT-PCR. The four ChCGB1 introns are conserved in the A. nidulans SfaD gene, while introns 1, 2, and 4 are conserved in the N. crassa GNB-1 gene; the third intron is missing. An additional sequence overlapping and extending the genomic sequence by 1.5 kb upstream from the start codon and downstream from the stop codon was obtained by using CGB1 to query the C. heterostrophus genome database (provided by Celera Genomics for Torrey Mesa Research Institute [TMRI]/Syngenta). The deduced amino acid sequence of 351 amino acids shows high homology to those of other filamentous ascomycete G8 subunits (e.g., 81% identity to A. nidulans SfaD [AAC33436], 80% identity to C. parasitica Cpgb-1 [AAC49838], and 76% identity to N. crassa GNB-1 [AAM53552]). The phylogenetic relationships among fungal G8 subunits and several plant and animal G8 subunits are shown in Fig. 1A. The ChCgb1 protein grouped with the other fungal G8 subunits, STE4 of Saccharomyces cerevisiae grouped separately from G8 subunits of the other ascomycetes and even other fungi.

The ChCgb1 protein was used to query a database of WD repeat proteins (BioMolecular Engineering Research Center

FIG. 3. Purification of strains carrying the cgb1 mutation. (A) Morphology of WT strain C5 (left) and C4AΔCgb4 (right), a hygB<sup>+</sup> transformant recovered from transformation of protoplasts with the CGB1 gene disruption vector. Strains were grown on CMX for 5 days. The WT was dark green (left); the double mutant was dark green with a white border (right). Both formed conidia. (B) Segregation of progeny from a tetrad from a cross between the WT and mutant strains shown in panel A. The mutant strain carried a second, untagged mutation, as revealed by segregation analysis (four types of progeny are shown). Progeny were grown on CMX (left) or CM (minus salts) plus hygromycin B (right) for 4 days. Progeny segregated 1:1:1:1 (dark green:dark gray:white:light gray with a white border). Dark gray progeny (strains 1400-1-3 and 1400-1-4; arrows) and white progeny were hygB<sup>+</sup>, whereas dark green progeny and progeny that were light gray with a white border were hygB<sup>−</sup>. Note that when first isolated from pseudomycelia and transferred to CMX, the double-mutant parental progeny were white, but they darkened (to look like those in panel A, right) when transferred from the CMX plate on which they were originally isolated to a new CMX plate (not shown). If a cross is done again between this type of progeny and the WT, tetratype progeny result again. Thus, in the tetratype, parental-type progeny are dark green (hygB<sup>+</sup>) and white (hygB<sup>−</sup>), while non-parental-type progeny are dark gray (hygB<sup>+</sup>) and light gray with a white border (hygB<sup>−</sup>). PCR analysis confirmed that the dark gray (hygB<sup>+</sup>) progeny carried the CGB1 mutation. (C) Progeny from a cross between the WT and dark gray (hygB<sup>−</sup>) non-parental-type progeny (strain 1400-1-3; panel B, arrows) segregated 1:1 for dark green (hygB<sup>+</sup>) (WT):dark gray (hygB<sup>−</sup>), indicating a single mutation. Progeny were grown on CMX (left) or CM (minus salts) plus hygromycin B (right) for 4 days.
This analysis confirmed that the predicted ChGb1 protein has seven repeats (probability of 1.0). WD repeats typically contain a GH dipeptide 11 to 24 residues from the start of the domain and a terminal WD dipeptide, which together represent the WD signature (Fig. 1B). There is flexibility in even the most conserved positions in the profile. For example, in repeat 2, YN appears instead of WD, but these are among the most common replacements found in the WD repeat database (Fig. 1B, bottom panel). A search of the C. heterostrophus genome database (provided by Celera Genomics for TMRI/Syngenta) did not reveal any other Gβ subunit genes, although other genes predicted to encode WD repeat-containing proteins were present (B.-N. Lee and B. G. Turgeon, unpublished data). Known Gβ subunits share homology in the N-terminal region preceding the WD repeat domain (32). When the protein database was searched (BLASTP) by using the 62 N-terminal amino acids of ChGb1, the fungal homologs were the top hits, followed by those of Dictyostelium discoideum and metazoans. This region displayed significant homology to other Gβ subunits (45% identity [significance score, E = 0.001] to mouse and human Gβ subunit 5).

**Identification of cgb1 mutant strains.** To construct cgb1 mutants, protoplasts of C. heterostrophus strain C4 were transformed with linear DNA obtained from construct GbetaDXO. The 3.5-kb fragment consisted of the two ChCGB1 homologous regions flanking the selectable marker (Fig. 2A). Following plating of protoplasts on regeneration medium and selection for hygromycin B resistance, 12 candidate transformants were isolated from two transformation experiments. These were purified by isolation of single conidia and screened by PCR for those carrying a deletion at ChCGB1. Such events were confirmed for three transformants (C4ΔGb1, C4ΔGb4, and C4ΔGb9). A double-crossover integration event at GGB1 would be expected to replace 473 bp of the GGB1 coding region (amino acids 101 to 258, including all of WD repeats 2, 3, and 4 and a large part of repeat 5) (Fig. 1B) with 2,107 bp.

![Fig. 4. The cgb1 mutant has increased pigmentation in vegetative mycelia. (A to F) Pigmentation of mycelia on the agar substrates of WT strain C5 (A to C) and cgb1 mutant strain 1400-1-3 (D to F). Fungal aerial masses were scraped off the surfaces of plates 2 weeks after inoculation. The agar substrate of the WT was only lightly pigmented (A), whereas that of the mutant was completely dark (D). Microscopic images of cross sections of agar substrates of the WT (B and C) and the mutant (E and F) showed that the increased pigmentation of the cgb1 mutant occurred within vegetative hyphae. Note that vegetative hyphae of the cgb1 mutant were often fragmented (F, arrows); the inset shows a higher magnification of the area with the arrows. (G and H) Liquid cultures of the same two strains as those shown in panels A and D. Note that the liquid culture of the cgb1 mutant (right) was much darker than that of the WT (left), but the filtrates showed no difference.](image-url)
FIG. 5. The cgb1 mutant has abnormal hyphal growth and fails to produce appressoria. Mycelial fragments of WT strain C5 (A, C, and E) and cgb1 mutant strain 1400-1-3 (B, D, and F) were suspended in water, drops were placed on glass slides and incubated for 14 h at 32°C, and slides were stained with cotton blue-lactophenol. Photographs show mycelia from the older parts of the hyphae (A and B), the young hyphae at the growing edge (C and D), and the hyphal tips (E and F). Note that young hyphae of the WT were often curved and that appressoria formed frequently at the tips (E, arrow), whereas young hyphae of the mutant tended to be straight and no appressoria-like structures formed at the tips (F, arrow). Incubation for 6 or 24 h gave the same results (not shown).

containing the hygB selectable marker (Fig. 2A). PCR amplification with primer combinations Gβ3215for-TTrpC and PTrpC-Beta3as and with genomic DNAs from two candidate cgb1 mutant strains (1400-1-3 and 1400-1-4; see Materials and Methods for the origins of these strains) and the WT strain generated 1,488- and 841-bp products from the mutant strains, respectively (Fig. 2B). DNA from the WT strain did not yield products with these primer pairs. Primer pair GSP7-GSP12 generated a 411-bp product from the WT strain which was missing from the mutant strains (Fig. 2B). PCR products corresponding to the 5’ and 3’ sides of the integration (Fig. 2B) were sequenced for one transformant, confirming the double-crossover event.

Backcrosses (1402, 1404, and 1400) between WT strain C5 and three hygromycin B-resistant cgb1 progeny strains (N52-R-3, N52-R-4, and N52-R-5, respectively) recovered from the cross between original transformant C4ΔGβ4 and C5 showed that all three mutant progeny strains carried a second mutation (Fig. 3; shown here is the cross between N52-R-5 and C5). This second mutation was not linked to the cgb1 mutation, as demonstrated by the tetrad depicted in Fig. 3B. Four types of progeny were observed: dark green (hygB^cgb1), light gray with white edges (hygB^cgb1), gray (hygB^cgb1), and white (hygB^cgb1) (Fig. 3B). A backcross of one of the gray (hygB^cgb1) progeny strains (Fig. 3B, 1400-1-3) to WT strains C4 (cross 1408) and CB12 (cross 1421) and analysis of both random and tetrad ascospore progeny revealed that the second mutation had been eliminated, leaving a strain with a single mutation at CGB1 (Fig. 3C). Two of the purified cgb1 strains from backcross 1400 (1400-1-3 and 1400-1-4) were used for further detailed characterization of strains with deletions at CGB1.

Characterization of cgb1 mutants. (i) Growth and pigmentation. The radial rate of growth of cgb1 mutants was not significantly different from that of the WT (six replicates measured over 7 days); however, fewer aerial hyphae were produced. One striking difference was that the mutants accumulated considerably more dark pigment in older cultures whether grown on solid medium (Fig. 4A to D) or in liquid medium (Fig. 4G and H). The increased pigmentation of the mutants was contained in the hyphae, not secreted into the medium (Fig. 4G and H), and the older hyphae of the mutants were often fragmented (Fig. 4F).

Hyphae of the WT growing on a glass slide followed a meandering or wavy pattern (Fig. 5A and C) and often produced appressoria at their tips (Fig. 5C and E). Hyphae of the cgb1 mutants grew along a straight path (Fig. 5B and D) and did not make appressoria (Fig. 5F).

(ii) Asexual reproduction. cgb1 mutants made fewer than 100 conidia per plate, in contrast to the WT, with 10^7 to 10^8. The few conidia produced were normal in shape; however, in contrast to the WT conidia, which germinated and produced appressoria by 6 h at 32°C, the mutant conidia did not germinate. The second mutation carried by the original isolates suppressed the cgb1 conidiation phenotypes; original isolates (C4ΔGβ1, C4ΔGβ4, and C4ΔGβ9) and progeny of a cross between C4ΔGβ4 and C5 (N52-R-3, N52-R-4, and N52-R-5) showed a reduced but still significant ability to produce conidia. Partial conidiation was not restored upon subsequent transfers of these lines. The second mutation likely originated during transformation.

(iii) Sexual reproduction. To check reproductive ability, four progeny (two black and two albino) from a tetrad recovered from cross 1421 were backcrossed again to WT test strains of opposite color. Pigmented cgb1 progeny were also crossed to albino cgb1 progeny of the opposite mating type to test reproductive ability in a cgb1 homozygous cross.

In crosses between albino and pigmented WT test strains, both white (Fig. 6A) and black pseudothecia formed. In crosses between an albino cgb1 mutant and a pigmented WT test strain, no albino pseudothecia (the color of the cgb1 mutant) were observed (Fig. 6B), indicating that the mutant was female sterile. The progeny of crosses between two cgb1 mutants were completely sterile (Fig. 6C): no pseudothecia formed. The fertility of pseudothecia that did form was normal in terms of numbers of asci and ascospores produced, and complete tetrads could be found (compare Fig. 6E with Fig. 6D). On average, asci produced by mutant strains (Fig. 6G) were smaller than those produced by WT strains (Fig. 6F).

(iv) Virulence. Since very few conidia were produced by cgb1 mutants and those that were found did not germinate, mycelial fragments were used to inoculate maize plants. No disease symptoms were found on plants inoculated with cgb1 mutants, while WT controls produced large lesions under these conditions (Fig. 7). T-toxin production was normal in cgb1 Tox1^- progeny (data not shown), indicating that T-toxin production does not depend on CGB1.
(v) Distribution of nuclei. Mycelia of WT and mutant strains were stained with DAPI to determine whether the alteration from a wavy to a straight growth pattern in cgb1 mutants was related to an abnormal distribution of nuclei. In young hyphae, the number and distribution of nuclei appeared normal in the mutants. Mature hyphae of the mutants often showed an abnormal DAPI staining pattern (Fig. 8B to D). In the WT, nuclei could be clearly observed as punctate dots in the cytoplasm (Fig. 8A), while in the mutant, DAPI-stained material was diffuse and patchy in most of the cell, indicating the possible breakup of nuclei (Fig. 8C and D).

(vi) TUNEL. The apparent breakup of nuclei indicated by the DAPI staining pattern might reflect a greater incidence or an earlier appearance of programmed cell death in cgb1 mutants. To test this notion, the free 3’OH termini of low- and high-molecular-weight DNAs with strand breaks can be labeled by terminal transferase. TUNEL staining of nuclei, which is based on this labeling reaction, is thus a hallmark of apoptosis. In the reaction used here, terminal transferase added fluorescein-labeled dUTP to these ends.

Attempts to use mycelium for the in situ TUNEL reaction were unsuccessful. We tried several procedures previously de-
scribed for other filamentous fungi, including permeabilization by freezing in liquid nitrogen (24) and treatment with cell wall-degrading enzymes plus proteinase (21), but failed to obtain the expected results from positive controls, suggesting that the TUNEL methods developed for one system may not be suitable for another. We found that protoplasts prepared for transformation (34) could be used for the TUNEL assay relatively easily. Protoplasts of both the WT (Fig. 9E) and the cgb1 mutants (data not shown) pretreated with DNase I (positive control) showed typical TUNEL-positive cells at the same rates and intensities, while those from the negative control showed background staining only (Fig. 9A). TUNEL-positive cells were frequently observed in cgb1 mutant protoplasts even without DNase I treatment (Fig. 9C). In contrast, TUNEL-positive cells were only occasionally found in WT protoplasts without DNase I treatment (Fig. 9A). The fraction of TUNEL-positive WT protoplasts was 0.036 (329 protoplasts counted from three photographs; 99% confidence interval, 0.026). The fraction of TUNEL-positive cgb1 mutant protoplasts was 0.204 (240 protoplasts counted from four photographs; 99% confidence interval, 0.067). The TUNEL-positive staining in the mutants showed a fragmented pattern (Fig. 9C), while the positive control-stained nuclei appeared intact (Fig. 9E).

These data suggest that cells of cgb1 mutants may undergo DNA fragmentation during vegetative growth.

**DISCUSSION**

One can infer from the known functions of signaling proteins in other eukaryotes that a G-protein heterotrimer detects extracellular ligands through as-yet-unidentified receptors. The next step in the signal cascade is for the activated GTP-bound form of the Gα subunit, as well as the free Gβγ heterodimer, to transmit the signal to downstream effectors. Mutants carrying mutations in these subunits can provide insight into the functions of G-protein-coupled pathways. One should keep in mind, however, that the roles of the products of signaling gene orthologs often differ even between closely related
The high level of identity of \textit{CGB1} with other fungal \textit{G}\beta genes and the structure of the predicted polypeptide (Fig. 1) are good evidence that \textit{ChCGB1} is a \textit{G}\beta subunit-encoding gene. An exhaustive search of the \textit{C. heterostrophus} genome sequence (TMRI/Syngenta) indicated that there is only one in the genome; therefore, disruption of this gene creates a strain that cannot form G-protein heterotrimerics. Thus, we can conclude that G-protein heterotrimerics are not essential for survival. There are a total of three \textit{G}\alpha subunit-encoding genes in the genome (Lee and Turgeon, unpublished; M. Giloh and B. A. Horwitz, unpublished data; TMRI/Syngenta \textit{C. heterostrophus} genome BLAST search). It was shown previously that one of these (\textit{CGA1}) is essential for several developmental pathways of the corn pathogen \textit{C. heterostrophus}. A loss of \textit{ChCGA1}, which belongs to the fungal \textit{G}\alpha subunit class, results in a loss of the ability to form appressoria and female sterility, while virulence on corn is retained (13). A loss of \textit{CGA1} does not block asexual sporulation, and conidia of \textit{cga1} mutants are viable; however, \textit{cgb1} mutants produce very few conidia, and these do not germinate. A loss of \textit{CGA1} is lethal for ascospores, and in \textit{cga1} homozygous crosses, no pseudothecia are formed. In contrast, \textit{cgb1} mutants, although female sterile, can be crossed to the WT, and mutant ascospores are viable. We predict that functions lost in \textit{cga1} but not \textit{cgb1} mutants will be attributed to specific interactions of the \textit{G}\alpha subunit \textit{Cga1} with a downstream effector.

The isolation of \textit{cgb1} mutants was accompanied by the discovery of an additional unlinked mutation (Fig. 3) which permitted increased conidiation. This finding was also reported for the \textit{M. grisea} ortholog \textit{MGB1}, although in that case, spontaneous mutations occurred at low rates (27). This observation may be further studied by the identification of the relevant gene combined with the construction of additional mutant alleles of the \textit{G}\beta subunit gene in both species. The gene corresponding to the second mutation is of interest because it may encode an interaction partner of the \textit{G}\beta subunit.

A loss of the MAPK Ch\textit{CHK1} results in a broad set of phenotypes, including a drastic loss of virulence and autolysis of mature mycelial colonies (19). A loss of the \textit{G}\beta subunit does not result in age-related colony autolysis. These data show that at least some of the functions of Ch\textit{CHK1} are independent of the \textit{G}\beta\gamma dimer. \textit{chkl} mutants grow in the normal wavy pattern on a glass or hard plastic surface, in contrast to \textit{cgb1} and \textit{cga1} mutants, which grow in a straight path. All three types of mutants produce few or no appressoria. \textit{ChCHK1} mutants, like

\begin{table} 
\centering 
\caption{Summary of phenotypes associated with signal transduction gene mutations in \textit{C. heterostrophus}} 
\begin{tabular}{|l|c|c|c|} 
\hline 
Characteristic & Chk1 (MAPK) & \textit{Cga1} (\textit{G}\alpha subunit) & \textit{Cgb1} (\textit{G}\beta subunit) \\ 
\hline 
Conidiation & None & Nearly normal & None or extremely rare \\ 
Mating & Female sterile & Female sterile, ascospore lethal & Female sterile \\ 
Virulence & Greatly reduced & Nearly normal & Greatly reduced \\ 
Colony morphology & Age-dependent autolysis & Altered & Altered \\ 
Hyphal growth pattern on surface & Wavy (normal) & Linear & Linear \\ 
Appressoria & None & None or few & None or few \\ 
Pigmentation & Decreased & Nearly normal & Increased \\ 
\hline 
\end{tabular} 
\begin{flushright} 
\textsuperscript{a} Nearly normal means that the function was not lost, but quantitative differences remained to be characterized in more detail. Female sterile means that pseudothecia were formed in a cross between the mutant and a WT tester, but the hyphae composing the pseudothecia were derived from the WT parent. Data are from this work and earlier studies (12, 18). Altered colony morphology means that colonies appeared different from WT colonies but that the overall growth pattern was retained. 
\end{flushright} 
\end{table}
Appressorium formation and branch formation may share loss of polarity that occurs when an appressorium is formed. An example of such remodeling is the momentary be needed for the output, i.e., the remodeling of the growing hyphal tip. An example of such remodeling is the momentary loss of polarity that occurs when an appressorium is formed. Appressorium formation and branch formation may share some features. The small appressoria of *C. heterostrophus* cells contain multiple nuclei, and in this respect they are like normal hyphal cells, except that they are wider and rounded, presumably because of a transient loss of the normal hyphal tip polarity. In budding yeast cells, the activity of the small rho-type G-protein Cdc24p determines the budding frequency (5), but there is no evidence to implicate heterotrimeric G proteins in the control of polarity. Although they are defective in certain morphogenetic transitions, G-protein mutants still essentially follow the normal fungal growth pattern. Mutants with defects in the establishment and maintenance of polarity have more drastic phenotypes and often must be studied with the help of temperature-sensitive alleles (25). Thus, heterotrimeric G-protein pathways, while being dispensable for the polar growth of hyphae, may modulate or interact with the machinery that sets up the main polar axes, changing them in response to extracellular signals or internally produced developmental cues. This scenario could explain the phenotypes of altered conidiation and appressorium formation and the absence of a wavy growth pattern for G-protein mutants. All may involve a transient or a permanent loss of polar growth in response to signals. Novel downstream effectors may be required to transmit such signals.

We observed a staining pattern suggesting nuclear disintegration in vegetative hyphae (Fig. 8) and also found a marked increase in TUNEL staining of protoplasts obtained from mycelia of the cgb1 mutant relative to the WT (Fig. 9). Nuclear disintegration is characteristic of apoptosis and indicates that the mutant may undergo premature or abnormal cell death. This is a novel phenotype for fungal signal transduction mutants. TUNEL is nevertheless an indirect method, and the logical defects shown by conidia germinating on a hard surface appear the inhibition of apoptosis by G-protein signaling pathways. Novels signal transduction mutants will be a valuable asset in efforts to define groups of genes whose expression is specific to the conidiation and disease development pathways.

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**REFERENCES**

1. Adams, T. H., J. K. Wieser, and J. H. Yu. 1998. Asexual sporulation in Aspergillus nidulans. Microbiol. Mol. Biol. Rev. 62:35–54.
2. Baasiri, R. A., X. Lu, P. S. Rowley, G. E. Turner, and K. A. Berkoевич. 1997. Overlapping functions for two G protein alpha subunits in Neurospora crassa. Genetics 147:137–145.
3. Banuett, F. 1996. Signaling in the yeasts: an informational cascade with links to the filamentous fungi. Microbiol. Mol. Biol. Rev. 62:249–274.
4. Boelker, M. 1998. Sex and crime: heterotrimeric G proteins in fungal mating and pathogenesis. Fungal Genet. Biol. 25:143–156.
5. Caviston, J. P., S. E. Tcheperegine, and E. Bi. 2002. Singularity in budding: a role for the evolutionarily conserved small GTPase Cdc42p. Proc. Natl. Acad. Sci. USA 99:12185–12190.
6. Choi, W., and R. Dean. 1997. The adenylate cyclase gene MAC1 of Magnaporthe grisea controls appressorium formation and other aspects of growth and development. Plant Cell 9:1973–1983.
7. Coca, M. A., R. Dansa, D. J. Yun, P. M. Hasegawa, R. A. Bressan, and M. L. Narasimhan. 2000. Heterotrimeric G-proteins of a filamentous fungus regulate cell wall composition and susceptibility to a plant PR-5 protein. Plant J. 22:61–69.
8. Edwards, K., C. Johnstone, and C. Thompson. 1991. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res. 19:1349.
9. Felsenstein, J. 1993. PHYLIP (phylogenetic inference package), version 3.57c. Department of Genetics, University of Washington, Seattle.

10. Galagan, J. E., S. E. Calvo, K. A. Borkovich, E. U. Selker, N. D. Read, D. Jaffe, W. FitzHugh, L. J. Ma, S. Smirnov, S. Purcell, B. Behnam, T. Elkins, R. Engel, S. Wang, C. B. Nielsen, J. Butler, M. Endrizzi, D. Quit, P. Ianakiev, D. Bell-Pedersen, M. A. Nelson, M. Werner-Washburne, C. P. Selitrennikoff, J. A. Kinsey, E. L. Braun, A. Zelter, U. Schulte, G. O. Kothe, G. Jedd, W. Mews, C. Staben, E. Marchotte, D. Greenberg, A. Roy, K. Foley, J. Naylor, N. Stange-Thomann, R. Barrett, S. Gierme, M. Kamal, M. Kamvysselis, E. Mauceli, C. Bielek, S. Rudd, D. Frishman, S. Krystofova, C. Rasmussen, R. L. Metzenberg, D. Perkins, S. Kroken, C. Cognoni, G. Macino, D. Catcheise, W. Li, R. J. Pratt, S. A. Osmani, C. P. DeSouza, L. Glass, M. J. Orbach, E. A. Berglund, R. Voelker, O. Yarden, M. Plamann, S. Seiler, J. Dunlap, A. Radford, R. Aramayo, D. O. Natvig, L. A. Alex, G. Mannmann, D. J. Ebbole, M. Freitag, I. Paulsen, M. S. Sachs, E. S. Lander, C. Nussbaum, and B. Birren. 2003. The genome sequence of the filamentous fungus Neurospora crassa. Genet. Biol. 5:231–243.

11. Giambarella, U., T. Yamatsuji, T. Okamoto, T. Matsu, T. Ikezu, Y. Muramaya, M. A. Levine, A. Katz, N. Gautam, and I. Nishimoto. 1997. G protein βγ complex-mediated apoptosis by familial Alzheimer’s disease mutation. EMBO J. 16:381–390.

12. Gronover, C. S., D. Kasulke, P. Tudzynski, and B. Tudzynski. 2003. The role of G protein alpha subunit subunits in the infection process of the gray mold fungus Botrytis cinerea. Mol. Plant-Microbe Interact. 16:1264–1269.

13. Horwitz, B. A., A. Sharon, S.-W. Lu, V. Ritter, T. Sandrock, B. G. Turgeon, and O. C. Yoder. 1999. A G protein alpha subunit gene of Cochliobolus heterostrophus involved in mating and appressorium formation. Fungal Genet. Biol. 26:19–32.

14. Jain, S., K. Akiyama, T. Kan, T. Ohguchi, and R. Takata. 2003. The G protein β subunit FGB1 regulates development and pathogenicity in Fusarium oxysporum. Curr. Genet. 43:75–86.

15. Kasahara, S., and D. L. Nuss. 1997. Targeted disruption of a fungal G-protein β-subunit gene results in increased vegetative growth but reduced virulence. Mol. Plant-Microbe Interact. 10:984–993.

16. Kays, A. M., P. S. Rowley, R. A. Baasirii, and K. A. Borkovich. 2000. Regulation of conjugation and adenylyl cyclase levels by the G protein alpha subunit gene from Cochliobolus heterostrophus involved in mating and appressorium formation. Fungal Genet. Biol. 30:181–191.

17. Lu, B. C., N. Gallo, and U. Kues. 2000. White-cap mutants and meiotic differentiation in Neurospora crassa. Mol. Gen. Genet. 263:1301–1311.

18. Lu, S., L. Lyngholm, G. Yang, C. Bronson, and O. Yoder. 1994. Tagged genes at the Tox1 locus of Cochliobolus heterostrophus by restriction enzyme-mediated integration. Proc. Natl. Acad. Sci. USA 91:12649–12653.

19. Madehni, H. D., C. A. Styles, and G. R. Fink. 1997. MAP kinases with distinct inhibitory functions impart signal specificity during yeast differentiation. Cell 91:673–684.

20. Marek, S., J. Wu, N. Glass, D. Gillechrist, and R. Bostock. 2003. Nuclear DNA degradation during heterokaryon incompatibility in Neurospora crassa. Funct. Genet. Biol. 40:126–137.

21. Momany, M. 2002. Polarity in filamentous fungus: establishment, maintenance and new axes. Curr. Opin. Microbiol. 5:580–585.

22. Mosech, H.-U., R. L. Roberts, and G. R. Fink. 1996. Ras2 signals via the Cdc24/Ste20/mitogen-activated protein kinase module to induce filamentous growth in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA 93:5352–5356.

23. Narasimhan, M. L., B. Damsz, M. A. Coca, J. I. Ibeas, D. J. Yun, J. M. Pardo, P. M. Hasegawa, and R. A. Bressan. 2001. A plant defense response effector induces microbial apoptosis. Mol. Cell 8:921–930.

24. Nishimura, M., G. Park, and J. R. Xu. 2003. The G-beta subunit MGB1 is involved in regulating multiple steps of infection-related morphogenesis in Magnaporthe grisea. Mol. Microbiol. 50:231–243.

25. Rosen, S., J. H. Yu, and T. H. Adams. 1999. The Aspergillus nidulans sfd gene encodes a G protein beta subunit that is required for normal growth and repression of sporulation. EMBO J. 18:5592–5600.

26. Rupp, S., E. Summers, H. J. Lo, H. Madhani, and G. R. Fink. 1999. MAP kinase and cAMP filamentation signaling pathways converge on the unusually large promoter of the yeast FLO11 gene. EMBO J. 18:1257–1269.

27. Sathbang, W., J. R. Flatauer, A. J. Bardwell, and L. Bardwell. 2001. Specificity of MAP kinase signaling in yeast differentiation involves transient versus sustained MAPK activation. Mol. Cell 8:683–691.

28. Smith, T. F., C. Gaitatzes, K. Saxena, and E. J. Neer. 1999. The WD repeat: a common architecture for diverse functions. Trends Biochem. Sci. 24:181–185.

29. Turgeon, B. G., H. Bohlmann, L. M. Ciuffetti, S. K. Christiansen, G. Yang, W. Schäfer, and O. C. Yoder. 1993. Cloning and analysis of the mating type genes from Cochliobolus heterostrophus. Mol. Gen. Genet. 238:270–284.

30. Turgeon, B. G., R. C. Garber, and O. C. Yoder. 1987. Development of a fungal transformation system based on selection of sequences with promoter activity. Mol. Cell. Biol. 7:3297–3305.

31. Turner, G. E., and K. A. Borkovich. 1993. Identification of a G protein alpha subunit from Neurospora crassa that is a member of the Gi family. J. Biol. Chem. 268:14805–14811.

32. Tzeng, T. H., L. K. Lyngholm, C. F. Ford, and C. R. Bronson. 1992. A restriction fragment length polymorphism map and electrophoretic karyotype of the fungal maize pathogen Cochliobolus heterostrophus. Genetics 136:81–96.

33. Wang, P., J. R. Perfect, and J. Heitman. 2000. The G-protein beta subunit GPB1 is required for mating and haploid fruiting in Cryptococcus neoformans. Mol. Cell. Biol. 20:352–362.

34. Willard, F. S., and M. F. Crouch. 2000. Nuclear and cytoskeletal translocation and localization of heterotrimeric G-proteins. Immol. Cell Biol. 78:387–394.

35. Wirsel, S., B. G. Turgeon, and O. C. Yoder. 1996. Deletion of the Cochliobolus heterostrophus mating-type (MAT) locus promotes the function of MAT transgenes. Curr. Genet. 29:241–249.

36. Wu, H. C., P. H. Huang, C. Y. Chiu, and C. T. Lin. 2001. G protein β2 subunit antisense oligonucleotides inhibit cell proliferation and disorganize microtubule and mitotic spindle organization. J. Cell. Biochem. 83:136–146.

37. Xu, J. R. 2000. MAP kinases in fungal pathogens. Fungal Genet. Biol. 31:137–152.

38. Yang, Q., S. Poole, and K. A. Borkovich. 2002. A G-protein beta subunit required for sexual and vegetative development and maintenance of normal G alpha protein levels in Neurospora crassa. Eukaryot. Cell 1:278–300.

39. Zhu, W. Z., M. Zheng, W. J. Koch, R. J. Lefkowitz, B. K. Kobilka, and R. P. Xiaob. 2001. Dual modulation of cell survival and cell death by beta(2)-adrenergic signaling in adult mouse cardiac myocytes. Proc. Natl. Acad. Sci. USA 98:1607–1612.

40. Zuber, S., M. J. Hynes, and A. Andrianopoulos. 2002. G-protein signaling mediates ascus development at 25 °C but has no effect on yeast-like growth at 37 °C in the dimorphic fungus Penicillium mameffei. Eukaryot. Cell 1:440–447.