GPR-4 Is a Predicted G-Protein-Coupled Receptor Required for Carbon Source-Dependent Asexual Growth and Development in *Neurospora crassa*

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The filamentous fungus *Neurospora crassa* is able to utilize a wide variety of carbon sources. Here, we examine the involvement of a predicted G-protein-coupled receptor (GPCR), GPR-4, during growth and development in the presence of different carbon sources in *N. crassa*. Δgpr-4 mutants have reduced mass accumulation compared to the wild type when cultured on high levels of glycerol, mannitol, or arabinoarabinose. The defect is most severe on glycerol and is cell density dependent. The genetic and physical relationship between GPR-4 and the three *N. crassa* Gα subunits (GNA-1, GNA-2, and GNA-3) was explored. All three Gα subunits are defective in mass accumulation when cultured on glycerol. However, the phenotypes of Δgna-1 and Δgpr-4 Δgna-1 mutants are identical, introduction of a constitutively activated gna-1 allele suppresses the defects of the Δgpr-4 mutation, and the carboxy terminus of GPR-4 interacts most strongly with GNA-1 in the yeast two-hybrid assay. Although steady-state cyclic AMP (cAMP) levels are normal in Δgpr-4 strains, exogenous cAMP partially remediates the dry mass defects of Δgpr-4 mutants on glycerol medium and Δgpr-4 strains lack the transient increase in cAMP levels observed in the wild type after addition of glucose to glycerol-grown liquid cultures. Our results support the hypothesis that GPR-4 is coupled to GNA-1 in a cAMP signaling pathway that regulates the response to carbon source in *N. crassa*. GPR-4-related GPCRs are present in the genomes of several filamentous ascomycete fungal pathogens, raising the possibility that a similar pathway regulates carbon sensing in these organisms.

The multicellular fungus *Neurospora crassa* has been used as a model organism for several decades for the study of genetics, biochemistry, and molecular biology (reviewed in reference 8). Like other filamentous fungi, *N. crassa* can grow in diverse environments and thus is confronted with a wide variety of environmental stimuli (8). One of the major systems used by *N. crassa* to sense extracellular changes involves heterotrimeric G proteins. In eukaryotic cells, heterotrimeric G protein signaling pathways are used to sense and respond to environmental stimuli (32, 43). Heterotrimeric G proteins are composed of a Gα subunit and tightly associated Gβ and Gγ subunits (32, 49). A class of plasma membrane proteins known as G-protein-coupled receptors (GPCRs) communicates changes in the environment to heterotrimeric G proteins (49, 74). GPCRs contain seven transmembrane helices that are connected by intracellular and extracellular loops, with the carboxy terminus extending into the cytoplasm (17, 32, 53). Ligand binding to the GPCR activates the G protein by inducing the exchange of GTP for GDP on the Gα and subsequent dissociation of GαGTP from the Gβγ dimer, allowing Gα and/or Gβγ to interact with downstream effector proteins (32).

Three Gα proteins have been identified in *N. crassa*, GNA-1, GNA-2, and GNA-3 (33, 69). GNA-1 was the first heterotrimeric G protein subunit identified in filamentous fungi (69). GNA-1 and GNA-3 play major roles in the regulation of growth and development of *N. crassa* through cAMP-dependent and -independent pathways (28, 31). With regard to cAMP-dependent functions, GNA-1 regulates the activity of adenyl cyclase (CR-1) (36), while GNA-3 controls adenyl cyclase protein levels (29, 31, 33). In contrast to GNA-1 and GNA-3, GNA-2 plays a lesser role, as effects of the gna-2 mutation were only observed in genetic backgrounds also lacking gna-1 or gna-3 (3, 31). *N. crassa* has one Gβ (GNB-1) and one Gγ (GNG-1) subunit, which function as a dimer during signaling and are important for the stability of all three Gα proteins (38, 80).

There are at least 10 predicted seven-transmembrane helix GPCRs in the *N. crassa* genome that fall into five distinct groups (8, 21). Of these 10 GPCRs, 2 are pheromone receptors (34), 3 are similar to predicted GPCRs from *Arabidopsis thaliana*, *Caenorhabditis elegans*, and *Dictyostelium discoideum* (8, 21), 2 are microbial opsins (5, 6), and 2 are related to putative nitrogen sensors in *Schizosaccharomyces pombe* (8).

The fifth group of *N. crassa* GPCRs contains a single member, GPR-4 (G-protein-coupled receptor 4). GPR-4 is similar to a group of putative carbon-sensing GPCRs from yeasts, including *Saccharomyces cerevisiae* Gpr1p, *S. pombe* Git3 and *Candida albicans* Gpr1 (25, 37, 48, 70, 73, 78, 81, 82). In the case of *S. cerevisiae* Gpr1p, glucose and succrose have been implicated as agonist ligands, while mannose acts as an antagonist (42, 56). Gpr1p interacts with Gpa2p, a Gα subunit that regulates pseudohyphal differentiation, invasive growth, and meiosis in *S. cerevisiae* (1, 37, 65, 67, 78, 81, 82). Induction of the Gpr1p pathway leads to elevated intracellular cyclic AMP (cAMP) concentration and activation of the Tpk2p cAMP-
dependent protein kinase (PKA) catalytic subunit, with subsequent increased expression of genes required for filamentation (4). In S. pombe, the GPCR Git3 is coupled to the Gα subunit Gpa2 (24, 73). Git3 and Gpa2 are required for a glucose-triggered increase in cAMP levels which in turn activates PKA (10, 25, 50, 73). In C. albicans, Gpr1 and Gpa2 have been reported to regulate filamentous growth in a cAMP- and PKA-dependent manner (46, 47, 48, 60, 70).

In this study, we present characterization of N. crassa strains used in this study. We created Δgpr-4 mutants and determine effects due to loss of this gene on growth, development, and cAMP metabolism. We also examine epistatic relationships between gpr-4 and the three Gα subunits. Our results demonstrate that GPR-4 physically interacts with the Gα GNA-1 to regulate carbon source-dependent growth and development through a pathway that at least in part involves regulation of cAMP metabolism. We also report the existence of at least two carbon sensory pathways in N. crassa that require the action of heterotrimeric G proteins.

**MATERIALS AND METHODS**

Strains, media, and general molecular procedures. N. crassa strains used in this study are listed in Table 1. Vogel’s minimal medium (designated VM-glycerol) was used for vegetative growth, while synthetic crossing medium (SCM) (75) was used to induce development of female reproductive structures. Sucrose was replaced by other carbon sources in VM medium where indicated (i.e., VM-glycerol). The concentration of the carbon source was 100 mM unless otherwise noted. Sorbose-containing medium (16) was used to facilitate colony formation on plates. If required, hygromycin B (Calbiochem, La Jolla, CA) was added to media at a concentration of 200 μg/ml. Conidia from 5–7-day-old cultures were used as the inoculum for new cultures. Yeast strains were propagated on synthetic dextrose (SD) medium containing the appropriate dropout mixture supplement (US Biological, Swampscott, MA). Plasmids were maintained in Escherichia coli strain DH5α (24).

**TABLE 1. N. crassa strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Comment(s)</th>
<th>Source or reference</th>
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<tbody>
<tr>
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<td>Wild-type mata</td>
<td>FGSC 987</td>
<td>FGSC</td>
</tr>
<tr>
<td>74a-OR8-1a (74a)</td>
<td>Wild-type mata</td>
<td>FGSC 988</td>
<td>FGSC</td>
</tr>
<tr>
<td>7-32</td>
<td>Δgpr-4::hph+ mata</td>
<td>Δgpr-4 homokaryon</td>
<td>This study</td>
</tr>
<tr>
<td>7-33</td>
<td>Δgpr-4::hph+ mata</td>
<td>Δgpr-4 homokaryon</td>
<td>This study</td>
</tr>
<tr>
<td>35-6</td>
<td>Δgpr-4::hph+ mata</td>
<td>Δgpr-4 homokaryon</td>
<td>This study</td>
</tr>
<tr>
<td>35-2</td>
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<td>Δgpr-4 homokaryon</td>
<td>This study</td>
</tr>
<tr>
<td>LA25</td>
<td>pan-2 mata</td>
<td>pan-2 mutant</td>
<td>R. L. Weiss, UCLA</td>
</tr>
<tr>
<td>Rmi2</td>
<td>Δgpr-4::hph+ gpr-4:::his-3+ mata</td>
<td>Complemented Δgpr-4</td>
<td>This study</td>
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<td>FGSC 6103</td>
<td>his-3 mata</td>
<td>his-3 targeting strain</td>
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<td>cr-1 mata</td>
<td>Allele B123</td>
<td>FGSC</td>
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<tr>
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<td>1B8</td>
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<td>29</td>
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<tr>
<td>a1r54</td>
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<td>a1r2</td>
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<td>a12h7</td>
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</tr>
<tr>
<td>a29-1</td>
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<td>33</td>
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<td>a1r2</td>
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</tr>
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<td>a31</td>
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<td>Δgpr-4 gna-1Q204L allele</td>
<td>This study</td>
</tr>
<tr>
<td>a1r1Q204L</td>
<td>Δgna-1::hph+ Δgna-1::hph+ gna-1Q204L::his-3+ mata</td>
<td>Δgpr-4 gna-1Q204L allele</td>
<td>This study</td>
</tr>
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Recombinant DNA procedures, such as plasmid construction. E. coli transformation, and Southern blots, etc., were performed according to standard protocols (59). All PCR products (cloned into the pGem-T vector; Promega Corp., Madison, WI) and recombinant vectors were verified by sequencing (Institute for Integrative Genome Biology, University of California, Riverside).

**RT-PCR analysis.** The tissues used for RNA extraction and isolation of total RNA were as previously described (38, 80). The reverse transcriptase PCR (RT-PCR) was used to assess message levels for various genes. Reactions were performed using 1 μg of total RNA and specific primers with the Access RT-PCR system (Promega Corp.), as recommended by the manufacturer. Reactions were conducted using conditions previously demonstrated to yield quantitative/semiquantitative data for mRNA levels (33). Products were subjected to Southern analysis (59) using specific probes (Table 2).

**gpr-4 intron verification and expression studies.** gpr-4 corresponds to a predicted protein NCU06312.1 in the N. crassa genome database. The gene structure predicted by the automated gene caller contains two exons and one intron (http://www.broad.mit.edu/annotation/genome/neurospora/home.html). For intron

**TABLE 2. Oligonucleotides used in this study**

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<th>Name</th>
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<td>RpRTPCRIIgpr-4</td>
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<td>pGPR-4UF</td>
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<td>pGPR-4UR</td>
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<tr>
<td>pGPR-4DF</td>
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<td>pGPR-4DR</td>
<td>GAGGATCTTGACTGATCGTCCG</td>
</tr>
<tr>
<td>FpNexon2gpr-4</td>
<td>TCAATGTCGCTGTTGATCATTAC</td>
</tr>
<tr>
<td>RpNexon2gpr-4</td>
<td>TTTCCAGGTTGCTCCTCTACGAGTA</td>
</tr>
<tr>
<td>1.5kbFORWARD</td>
<td>CTACCCCTTTGCGGACAACAACTGT</td>
</tr>
<tr>
<td>1.5kbREVERSE</td>
<td>ACTGCACTCCCTGGTACATCATC</td>
</tr>
<tr>
<td>GPR-4RESCUE-FF</td>
<td>GCCGGAATTCTACATCTGGGATTG</td>
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<td>GPR-4RESCUE-RP</td>
<td>TGAATCTGACGCATGAATACGCTAC</td>
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<tr>
<td>Fgpr-4cDNAHPLC</td>
<td>AGTTGGTGTATCTCATCAGAGGT</td>
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<td>CACTCTGAATAAGCAACACTATCAG</td>
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verification, RT-PCR analysis (Access RT-PCR; Promega) was performed on total RNA isolated from conidia, 8- and 16-h submerged cultures, and VM and SCM plates using primers FrpTPCgpr-4 and RpRT-PCITgpr-4 (Table 2) which flank the intron (see Fig. 2A). A genomic control was provided by amplification of a 315-bp PCR product using the same primers, with cosmids pMOcosX X1 B8 as the template (contains the gpr-4 gene) (http://www.broadmit.edu/annotation/genome/neurospora/home.html). The RT-PCRs were electrophoresed on agarose gels and subjected to Southern analysis using the amplified 315-bp genomic fragment as a probe (see Fig. 2A). Analysis of gpr-4 message levels in various tissues in wild-type Δgpr-4 and Δgpr-4 gpr-4 strains was accomplished by RT-PCR-Southern analysis using the same set of primers and probe.

**Western analysis.** For analysis of G protein subunit levels, a fraction enriched for plasma membranes was isolated as described previously (69) from 3-day VM and VM-glycerol plates. For detection of adenylyl cyclase (CR-1), whole-cell extracts were prepared from VM-sucrose or VM-glycerol 16-h shaken submerged cultures as previously described with some modifications (33). Tissue was collected and ground in liquid nitrogen, mixed with extraction buffer, shaken for 20 min at 4°C, and then centrifuged at 1,000 g for 10 min at 4°C. The protein amount was quantified using the Bradford protein assay (Bio-Rad, Hercules, CA). Samples containing 30 μg of total protein were subjected to Western analysis (33). Primary antibodies against GNA-1, GNA-2, GNA-3, GNB-1, and CR-1 were used at 1:1,000, 1:500, 1:1,000, 1:1,000, and 1:5,000 dilutions, respectively (3, 27, 33, 80). A goat anti-rabbit immunoglobulin G horseshard peroxidase conjugate (Bio-Rad) was used as the secondary antibody at a 1:7,500 or 1:10,000 dilution. Detection was performed using the enhanced chemiluminescence method (Amersham Pharmacia Biotech, Little Chalfont, England), as described by the manufacturer. A duplicate gel was electrophoresed and stained with Coomassie blue to verify equal loading of protein samples, as described previously (33, 59).

**Construction of N. crassa strains.** The gpr-4 gene was replaced with a hph gene cassette as follows. The gpr-4 gene replacement construct plL1 was made by ligation of four DNA fragments: vector pGEMZ7Z (+) (Promega Corp.) digested with XbaI and SacI, the HindIII-BamHII fragment from pCSN4 (containing the **hph** gene and Aspergillus nidulans promoter rpoC) (64), and PCR products corresponding to the first 5′ and last 3′ coding region of gpr-4 (see Fig. 2B). The PCR products were amplified using primers 1.5kbFORWARD and 1.5kbREVERSE. The second probe was a 4.9-kb XbaI fragment (see Fig. 2A) containing hph and portions of the gpr-4 5′ and 3′ flanking DNA that was excised from plL2. Heterokaryotic Δgpr-4 strains without ectopic integrations were crossed to wild-type strain 74A (or controls). The mating mixtures were plated on SD minus leucine and tryptophan. No Δgpr-4 strains were obtained, and 1:1 segregants were recovered. The Δgpr-4 strain was transformed using the binary plasmid pGBKT7 (containing the entire gpr-4 ORF, described above) as the template for primers Fgpr4CDNAH1PCL and Rgpr4CDNAH1PCL, and the resulting cDNA clone was designated plL9. A fragment corresponding to the carboxy terminus of GPR-4 (GPR-4CT; amino acids 527 to 654) was amplified from plL9 using primers Fgpr4CT-1 and Rgpr4CT-2 and cloned into pGEM-T to form plL10. plL10 was digested with EcoRI and Ptll to release the carboxy terminus fragment, which was ligated into pGBKT7 and transformed into yeast strain AH109 (Clontech Laboratories, Mountain View, CA) to yield plasmid plL12. this was then transformed into yeast strain Y187 (genotype MATa ura3-323 his2-200 ade2-1 101 trp1-901 leu2-3,112 gal4Δ met1 gal80 URA3::GAL1:GAL1::GAL1::lacZ; Clontech) using the lithium acetate method (Yeast Protocols Handbook; Clontech). Construction of plasmids in which the ORFs of gna-1, gna-2, and gna-3 were inserted in frame into pGD424 (LUE2 Amp; Clontech) and their transformation into yeast strain AH109 (genotype MATa trp1-901 leu2-3,112 his3-200 gal4Δ gal80 URA3::GAL1::GAL1::GAL1::lacZ; Clontech) using the lithium acetate method (Yeast Protocols Handbook; Clontech). Construction of plasmids in which the ORFs of gna-1, gna-2, and gna-3 were inserted in frame into pGD424 (LUE2 Amp; Clontech) and their transformation into yeast strain AH109 (genotype MATa trp1-901 leu2-3,112 his3-200 gal4Δ gal80 URA3::GAL1::GAL1::GAL1::lacZ; Clontech) using the lithium acetate method (Yeast Protocols Handbook; Clontech).
growth assay. Yeast strains were cultured in SD liquid medium lacking leucine and tryptophan at 30°C for 1 day with shaking at 200 rpm. The number of cells in the culture was quantitated and serial dilutions prepared. A 2.5-μl aliquot of each concentration of cells (10^2, 10^3, or 10^4 cells/ml) was spotted onto SD plates lacking adenine, histidine, leucine, and tryptophan containing 5 mM 3-amino-triazole (to increase the stringency of the HIS3 reporter screen). The plate was incubated at 30°C for 3 days.

cAMP assays. Tissues used for measurement of intracellular steady-state cAMP levels were obtained from VM-glucose and VM-glycerol plate cultures incubated at 30°C for 3 days in constant darkness. Mycelia were ground in liquid nitrogen as previously described (29). For analysis of the transient increase in cAMP after glucose addition to glycerol cultures, conidia were inoculated at a final concentration of 1 × 10^5 conidia/ml into 10 ml of VM-glycerol medium in 125-ml Erlenmeyer flasks. There were at least three flasks for each time point/strain. Cultures were incubated at 30°C for 16 h with shaking at 200 rpm, at which time one set of flasks was collected (time = 0). Glucose was then added to the remaining flasks at a final concentration of 100 mM, and samples collected at 30, 60, and 180 s. Cultures were collected using a 2.3-cm metal vacuum filter apparatus with Whatman 2.3-cm filters. The filters were immediately transferred to 2-ml microcentrifuge tubes containing 1 ml of 10% trichloroacetic acid and vortexed briefly, frozen in liquid nitrogen, and then thawed at 4°C with shaking. Samples from two individual flasks were combined during cAMP extraction when needed.

cAMP was extracted from tissue samples as previously described (29). cAMP levels were quantified using a protein binding assay according to the manufacturer’s instructions (Amersham Pharmacia Biotech, Piscataway, N.J.). The protein concentration was determined using the BCA assay (Pierce, Rockford, IL) as described previously (29).

RESULTS

gpr-4 encodes a putative G-protein-coupled receptor that is highly expressed in glycerol-grown cultures. The gpr-4 gene was identified during the initial annotation of the N. crassa genome sequence as NCU06312.1 (21). The predicted GPR-4 protein contains seven transmembrane domains (TMs), characteristic of G-protein-coupled receptors (Fig. 1). GPR-4 is homologous to three putative carbon sensoryGPCRs found in yeasts: Gpr1p from Saccharomyces cerevisiae (ScGpr1p; E = 3e−15), Git3 from Schizosaccharomyces pombe (SpGit3; E = 9e−3) and Gpr1 from Candida albicans (CaGpr1p; E = 2e−17) (Fig. 1; data not shown) (37, 46, 48, 70, 73, 78, 82). N. crassa GPR-4, ScGpr1p, CaGpr1p, and SpGit3 are similar in the transmembrane domains (especially TM1 to TM5) and connecting loops, particularly in the second and third intracellular and second extracellular loops, which are important for coupling to heterotrimeric G proteins and ligand binding, respectively (78, 81). In addition, the third intracellular loops of GPR-4 and ScGpr1p are relatively large (247 and 283 amino acids) (Fig. 1) (37, 73, 78, 82).

N. crassa GPR-4 is also similar to six predicted but uncharacterized proteins containing seven TMs from other ascomycete filamentous fungi. These are CHG04337 (E = 2e−65) from the white rot pathogen Chaetomium globosum, S11G_08243.1 from the necrotrophic pathogen Sclerotinia sclerotiorum, BCI1_03450.1 (E = 4e−41) from the gray mold pathogen Botrytis cinerea, FG05060.1 (E = 3e−41) from the pathogenic filamentous ascomycete Gibberella zeae PH-1 (anamorph, Fusarium gramineum), and MG08803.4 (E = 8e−34) from the rice BLAST fungus Magnaporthe grisea (Fig. 1). These proteins have large third intracellular loops (83 to 348 amino acids) and carboxy-terminal tails (84 to 155 amino acids) and are most similar to GPR-4 in the 7-TM helix regions and connecting loops (especially in the second and third intracellular and second extracellular loops).

The gpr-4 gene structure predicted by the automated gene caller (http://www.broad.mit.edu/annotation/genome/neurospora/home.html) was verified using RT-PCR with gpr-4-specific primers that amplify the region containing the intron (Fig. 2B). The gpr-4 message is of relatively low abundance and could not be detected using Northern analysis (data not shown). To elucidate the expression profile of gpr-4 throughout growth and development, RT-PCR was performed. We began with analysis of total RNA from conidia, 8- and 16-h shaken subcultured cultures, and vegetative (VM) and sexually differentiated (SCM) plate cultures, all with 1.5% sucrose as the carbon source. gpr-4 is expressed to detectable levels in three tissues (16-h subcultured cultures and VM and SCM plates), with the highest level of gpr-4 mRNA present in VM plate cultures (Fig. 2B, left panel). gpr-4 message could also be detected in conidia and 8-h subcultured cultures, although the relative levels of expression were more variable (data not shown). We extended our studies to tissues grown in 1.5% (43.8 mM) and 100 mM sucrose, 100 mM glucose, and 100 mM glycerol, a relatively poor carbon source (Fig. 2B, right panel). The results demonstrate that gpr-4 transcript levels are similar in glucose and both concentrations of sucrose but are highest in glycerol-grown cultures.

Deletion of gpr-4 by targeted gene replacement and isolation of a Δgpr-4 gpr-4+ complemented strain. A Δgpr-4 mutant was created by electroportation of a wild-type strain with a construct in which the Δgpr-4 ORF region was replaced with the hygromycin B resistance marker gene hph (Fig. 2A) (see Materials and Methods). Heterokaryotic primary transformants were identified by Southern analysis (data not shown) and then crossed to a wild-type strain of the opposite mating type. Homokaryotic Δgpr-4 mutants were obtained by selection of the progeny on hygromycin-containing medium and verified by Southern analysis (Fig. 2C).

A complemented Δgpr-4 strain (Δgpr-4::hph+ gpr-4+::his-3+ ) was constructed by targeting a construct containing the wild-type gpr-4 allele to the his-3 locus as described in Materials and Methods and was verified by Southern analysis (data not shown). The gpr-4 mRNA could be detected in Δgpr-4 gpr-4+ and wild-type strains but not in Δgpr-4 mutants (Fig. 2D; data not shown), thus demonstrating restoration of gpr-4 expression in the complemented strains.

Δgpr-4 mutants accumulate less mass than the wild type on poor carbon sources. Extensive phenotypic analysis was performed on the Δgpr-4 mutants. Δgpr-4 strains are fertile as males or females, and ascospores produced from crosses involving Δgpr-4 mutants germinated normally. Δgpr-4 mutants did not exhibit defects during asexual growth and development (colony morphology, aerial hypha height, conidiation, and dry mass) on minimal medium containing sucrose, fructose, or glucose at 30°C (Fig. 3A; data not shown). The apical extension rates of basal hyphae from Δgpr-4 mutants were normal on preferred or poor carbon and/or nitrogen sources (data not shown). Hyphal fusion was normal, as assayed by formation of forced heterokaryons (using strains with different auxotrophic markers) and by microscopic analysis of formation and fusion of conidial anastomosis tubes (55). Growth in VM-sucrose-submerged cultures was normal, as was sensitivity to 0.75 M NaCl, 0.75 M KCl, 1.5 M sorbitol, 5% ethanol, 1% sorbose, or high agar concentration (4%).
FIG. 1. Alignment of GPR-4 (NeGPR-4) with homologous putative G-protein-coupled receptors from ascomycete fungi. The predicted amino acid sequences were aligned using ClustalW with shading by Boxshade (http://www.ch.embnet.org). The predicted transmembrane regions (TM1 to 7) are indicated by numbered lines above the sequences. Abbreviations: NeGPR-4, Neurospora crassa GPR-4 (accession no. NCU06312.1); ScGpr1p, Saccharomyces cerevisiae Gpr1p (accession no. JCS808); Mg08803, Magnaporthe grisea 70-15 hypothetical protein MG08803.4 (accession no. EAA51281); FG05006, Gibberella zeae PH-1 (anamorph Fusarium graminearum) hypothetical protein FG05006.1 (accession no. XP_385182); CHG04337, Chaeotomium globosum hypothetical protein CHG04337.1; SS1G_08243, Sclerotina sclerotiorum hypothetical protein SS1G_08243.1; BC1G_03450, Botrytis cinerea hypothetical protein BC1G_03450.1. An intron in FG05006.1 was misannotated, and the missing amino acid sequence LIMLLIYS was inserted between amino acids 79 and 80 in the original predicted protein sequence (http://www.broad.mit.edu/annotation/fgi/). Amino acids 121 to 137 were removed from the original predicted protein sequences of BC1G_03450.1 and SS1G_08243.1 (http://www.broad.mit.edu/annotation/fgi/), as these were misannotated and are actually part of an intron region (between amino acids 120 and 121 in this figure).
In fact, the only defect observed for Δgpr-4 mutants when cultured on VM-sucrose medium was a slower apical extension rate (~70% of wild type) and less dry mass accumulation at the elevated growth temperature of 42°C.

Since GPR-4 is homologous to putative carbon sensory receptors found in yeasts, we performed further detailed phenotypic analysis in the presence of different carbon sources. When grown on poor carbon sources (100 mM glycerol, mannitol, or arabinose), the dry mass of Δgpr-4 strains was significantly less than that of the wild type (Fig. 3A). The reduced mass accumulation of Δgpr-4 mutants cultured on glycerol solid medium cannot be explained by defects in conidial germination or hyphal fusion. In contrast, we consistently observed that wild-type strains produced more aerial hyphae than the Δgpr-4 mutant at the inoculation point on VM-glycerol plates (data not shown). The reduction in the quantity of aerial hyphae likely explains the reduced biomass accumu-
lation observed in Δgpr-4 mutants relative to the wild type. On the other hand, Δgpr-4 mutants form the same amount of conidia as wild type (data not shown). Thus, Δgpr-4 mutants produce more conidia per aerial hypha, a result which is also supported by microscopic observation.

To investigate the possible cause of the aerial hypha production defect in Δgpr-4 mutants, we next examined the effect of spreading VM-glycerol plates with different amounts of conidia from Δgpr-4 and wild-type strains. At a lower conidial density (1 to 1,000 conidia/cm² agar medium), wild-type and Δgpr-4 strains produce similar amounts of basal and aerial hyphae when cultured on VM-glycerol plates (Fig. 3C). However, at higher amounts of conidia (10⁶, 10⁵, or 10⁴/cm² agar medium), the wild-type strain formed more aerial hyphae than the Δgpr-4 mutant (Fig. 3C). The defects in hypha formation of Δgpr-4 strains led to decreased biomass (Fig. 3C). Taken to-
gether, our results suggest that biomass accumulation inΔgpr-4 mutants when cultured on VM-glycerol medium is dependent on the initial density of conidia used for inoculation, with defects only observed at levels at or above 10^5/cm². Thus, GPR-4 appears to negatively regulate a previously uncharacterized pathway involving formation of aerial hyphae at high inoculation cell densities in N. crassa.

The Gα gene gna-1 is epistatic to gpr-4 with regards to mass accumulation on glycerol medium. As mentioned above, N. crassa possesses three Gα protein genes, gna-1, gna-2, and gna-3. Of these, the protein encoded by gna-3 is most similar to the yeast Gα proteins represented by S. cerevisiae Gpa2p. Our laboratory has previously demonstrated roles for GNA-3 in regulation of adenyl cyclase protein levels and conidiation (31, 33). However, other studies have shown that GNA-1, not GNA-3, regulates the activity of adenyl cyclase in N. crassa (29, 31). GNA-2 appears to play a compensatory role in relation to GNA-1 and GNA-3 (3, 31). To elucidate which Gα protein(s) operates downstream of GPR-4, we conducted epistasis analyses of gpr-4 and the three Gα genes. To control for possible effects of the Δgpr-4 mutation on G protein stability, we first analyzed levels of the three Gα proteins and the Gβ protein in the Δgpr-4 background. Western blot analyses were performed using tissues from VM-sucrose and VM-glycerol plates (Fig. 4A). Levels of GNA-1, GNA-2, GNA-3, and GNB-1 were similar in wild-type and Δgpr-4 strains cultured with the two carbon sources. Thus, G protein levels are similar with sucrose or glycerol as a carbon source and are also not affected by loss of gpr-4 in N. crassa.

We next examined the phenotype of the three Gα mutants on solid medium with glycerol as a carbon source. All of the Gα single mutants possessed approximately the same dry mass as the wild type when cultured with glycerol as a carbon source (data not shown). However, the Δgna-1, Δgna-2, and Δgna-3 mutants accumulated less mass than the wild type when grown on VM-glycerol (Fig. 4B). Levels in Δgna-1 strains showed the greatest reduction, with only 13% of the mass of the wild type, while accumulation in Δgna-2 and Δgna-3 mutants was 51 to 65% of that of the wild type, respectively (Fig. 4B). The observation of reduced mass in Δgna-2 strains cultivated on VM-glycerol is the first report of a phenotype for mutants lacking only the gna-2 gene.

To analyze the relationship between gpr-4 and the three Gα genes, we constructed Δgna-1 Δgpr-4, Δgna-2 Δgpr-4, and Δgna-3 Δgpr-4 double mutants. Examination of mass accumulation on glycerol solid medium showed that Δgpr-4 Δgna-2 and Δgpr-4 Δgna-3 mutants have a more severe defect than the Δgna-2 or Δgna-3 single mutant (Fig. 4B). This result suggests that GNA-2 and GNA-3 do not function downstream of GPR-4 to regulate mass accumulation on glycerol medium. In contrast, Δgna-1 Δgpr-4 double mutants possess the same reduced dry mass as Δgna-1 strains. This finding indicates that gna-1 is epistatic to gpr-4 (Fig. 4B).

We further probed the epistatic relationship between gna-1 and gpr-4 through analysis of a Δgpr-4 strain containing either of two previously characterized, GTPase-deficient, constitutively activated gna-1 alleles, gna-1^Q204L and gna-1^K178C (79). If GPR-4 is a GPCR coupled to GNA-1, then mutational activation of gna-1 should suppress defects caused by the Δgpr-4 mutation. To obtain N. crassa strains containing a gna-1-activated allele in the Δgpr-4 background, the gna-1^Q204L and gna-1^K178C constructs were targeted to the his-3 locus of a Δgna-1 Δgpr-4 his-3 strain and the desired transformants were selected and purified (see Materials and Methods). The resulting homokaryotic Δgpr-4 Δgna-1 gna-1^Q204L or Δgpr-4 Δgna-1 gna-1^K178C strains are identical to Δgna-1 gna-1^Q204L or Δgna-1 gna-1^K178C strains with respect to mass accumulation and colony morphology (Fig. 4B; data not shown), demonstrating that introduction of a constitutively activated gna-1 allele can suppress the defects of the Δgpr-4 mutation. This result further supports the hypothesis that GNA-1 acts downstream of GPR-4.

Our previous results demonstrated that GNA-1 is required for GTP-stimulated adenyl cyclase (encoded by cr-1) activity in N. crassa (79). Based on the epistatic relationship between GPR-4 and GNA-1, we also compared the dry mass of cr-1 mutants to that of Δgpr-4 and wild-type strains. It has previously been reported that cr-1 mutants grow extremely poorly in the presence of several poor carbon sources, including glycerol, mannitol, and arabinose (66). Therefore, we compared the dry mass of cr-1 and wild-type strains cultured with these carbon sources on solid medium. With glucose as the carbon source, the cr-1 mutant has a dry mass that is 44.0 ± 2.8% of that of wild-type strains (data not shown). When grown in the presence of glycerol, mannitol, or arabinose, mass accumulation in the cr-1 strain was only 18.8 ± 2.2%, 25.4 ± 8.0%, or 21.6 ± 3.1% of that of the wild type cultured on the same medium, respectively (Fig. 3A; also data not shown). Correcting for the decreased mass of cr-1 mutants relative to the wild type on glucose (44%), the relative reduction in mass accumulation on glycerol, mannitol, or arabinose is similar for cr-1 and Δgpr-4 mutants. Furthermore, on solid VM-glycerol medium, the dry weight of the cr-1 mutant is similar to that observed for Δgna-1 and Δgpr-4 Δgna-1 strains (Fig. 4B; also data not shown). Thus, the mass accumulation pattern of Δgpr-4, Δgna-1, and cr-1 mutants is consistent with a cAMP-dependent pathway regulating growth on solid medium with glycerol as the carbon source.

GPR-4 physically interacts with GNA-1 in the yeast two-hybrid assay. Since the results of epistasis experiments suggested that GPR-4 acts upstream of GNA-1 during mass accumulation on glycerol medium, we utilized the yeast two-hybrid assay to examine a possible physical interaction between GPR-4 and GNA-1. Assays with GNA-2 and GNA-3 were included as controls. For these tests, the entire ORF for each Gα gene was cloned in frame behind the GAL4 activation domain in pGAD424, while a carboxy-terminal fragment of GPR-4 (amino acids 527 to 654) was inserted in frame behind the GAL4 DNA binding domain in pGBK7T. The corresponding carboxy-terminal region of S. cerevisiae Gpr1p has been demonstrated to interact with Gpa2p in yeast two-hybrid assays (78, 82). We performed the two hybrid assays with the N. crassa proteins using two methods. The activity of the β-galactosidase reporter was measured using filter assays on plates (Fig. 4C, left panel). Expression of the ADE-2 and HIS-3 reporters was monitored by assessing growth on medium lacking adenine and histidine (Fig. 4C, right panel). In β-galactosidase assays of cells containing GNA-1 and the carboxy terminus of GPR-4, a dark blue color developed within 30 min (similar to the positive control), representative of a strong interaction. However, the color was much paler and
took 1 to 2 h to develop in assays of cells containing the GPR-4 carboxy terminus and GNA-2 or GNA-3, indicating a very weak interaction. The growth assays on medium lacking histidine and adenine showed that strains with the GNA-1 and carboxy-terminal GPR-4 vectors grew very well, similar to the positive control (Fig. 4C, right panel). In contrast, cells containing the GPR-4 carboxy terminus and GNA-2 or GNA-3 exhibited growth only at higher cell densities and grew only
slightly better than negative controls (Fig. 4). Thus, the two-hybrid assay provided evidence for a direct interaction between GPR-4 and all three Gα proteins, with the strongest binding to GNA-1. These results further strengthen the notion that GPR-4 is coupled to GNA-1. To our knowledge, there have been no previous reports of a GPCR that physically interacts with a Gα protein related to GNA-1 in filamentous fungi.

GPR-4 is required for a carbon source-dependent transient increase in cAMP levels. A common downstream effector pathway of fungal heterotrimeric G proteins involves cAMP signaling. The enzyme adenylyl cyclase catalyzes the conversion of ATP to cAMP. As mentioned above, our laboratory has previously shown that GNA-1 is required for normal GTP-stimulated adenylyl cyclase activity. The results from epistatic analysis and two-hybrid assays support an interaction between GPR-4 and GNA-1. Taken together, these findings raise the possibility that GPR-4 may regulate adenylyl cyclase activity.

GPR-4 and GNA-1. These results further strengthen the notion that GPR-4 is required for normal GTP-stimulated adenylyl cyclase activity. The results from epistatic analysis and two-hybrid assays support an interaction between GPR-4 and GNA-1. Thus, cAMP addition achieved a partial rescue of the dry mass defects of Δgpr-4 mutants on VM-glycerol medium.

We next measured steady-state cAMP levels in wild-type, Δgpr-4, and Δgpr-4 gpr-4−complemented strains when cultured on VM-sucrose or VM-glycerol plates. Interestingly, cAMP amounts did not vary greatly whether sucrose or glycerol was the carbon source (Table 3). The level of cAMP was also similar in all three strains, indicating that the Δgpr-4 mutation does not significantly affect steady-state intracellular cAMP levels in N. crassa (Table 3).

It has been shown that S. cerevisiae Gpr1p is required for a brief increase in cAMP levels after the addition of glucose to glucose-starved cultures (37, 81). In addition, our laboratory has proposed that GNA-1 may be required for a transient cAMP increase during growth and development, as Δgna-1 mutants have low adenylyl cyclase activity but normal steady-state cAMP levels in shaken submerged cultures (29). In light of these findings, we next analyzed cAMP levels after the transition from a poor carbon source (glycerol) to glucose in wild-type and Δgpr-4 strains.

Consistent with the results presented in Table 3, levels of cAMP were similar in glycerol-grown submerged cultures of wild-type and Δgpr-4 strains at time zero (Fig. 5A). The addition of glucose led to more than a twofold increase in cAMP levels within 30 to 60 s in wild-type cells. In contrast, no significant difference in cAMP amount was observed for the Δgpr-4 mutant over this same time period (Fig. 5A). These results indicate that GPR-4 is required for the glucose-dependent transient increase in cAMP levels, supporting a role for GPR-4 in glucose sensing.

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### TABLE 3. Steady-state intracellular cAMP levels

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Strain</th>
<th>Sucrose</th>
<th>Molar</th>
<th>Glycerol</th>
<th>Molar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>74A</td>
<td>4.66 ± 0.20 (100)</td>
<td>5.65 ± 0.50 (100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δgpr-4</td>
<td>7–32</td>
<td>4.77 ± 0.18 (102.4)</td>
<td>4.62 ± 0.81 (81.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δgpr-4 gpr-4−</td>
<td>Rmi2</td>
<td>3.59 ± 0.66 (77.0)</td>
<td>6.01 ± 0.76 (106.4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Data are from two independent experiments.*

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![Image](http://ec.asm.org/)
We also examined adenyl cyclase (CR-1) protein levels in VM-sucrose- and VM-glycerol-submerged cultures of wild-type and Δgpr-4 strains using a CR-1 antisera (28) (Fig. 5B). CR-1 levels are similar in the wild type and Δgpr-4 mutants cultured on the same medium. This observation is consistent with previous results from our laboratory, indicating that GNA-1 does not influence levels of CR-1 protein (28). Interestingly, the carbon source did affect CR-1 levels, as CR-1 amounts in both strains are greater with VM-sucrose than with VM-glycerol (Fig. 5B).

**DISCUSSION**

Glucose, sucrose, maltose, fructose, and mannose are among the best carbon sources for supporting the growth of *N. crassa*, while arabinose, mannitol, glycerol, and sorbose only allow slow growth and low biomass accumulation (15). The finding that Δgpr-4 mutants have reduced mass accumulation compared to the wild type when cultured with poor carbon sources and lack the transient increase in cAMP levels normally observed during the shift from glycerol to glucose-rich medium suggests that GPR-4 may act as a carbon sensor in *N. crassa*. GPR-4 is most similar to several hypothetical seven-transmembrane helix proteins from pathogenic filamentous fungi. This indicates that the ScGpr1p GPCR superfamily (22, 42) is widely present in pathogenic filamentous fungi and that a carbon-sensing GPCR-Gα protein-adenyl cyclase-cAMP-PKA pathway is likely to be functionally conserved in these organisms. In the filamentous fungus *Aspergillus nidulans*, the heterotrimeric G-protein GanB(α)-S1aD(β)-GpgA(γ) has been implicated in a carbon-sensing cAMP/PKA pathway that regulates conidial germination (12, 20, 41), but as yet, no GPCR has been reported for this pathway. The closest match to *N. crassa* GPR-4 in *A. nidulans* is GprC (23) (AN3765; E = 6e−3). However, GprC is actually more similar to *N. crassa* GPR-1 (E = 5e−05), GPR-2 (E = 3e−03), and GPR-3 (E = 4e−05), predicted GPCRs with similarity to cAMP receptor-like proteins found in *D. discoideum* (8, 54).

The results from epistasis analysis and yeast two-hybrid assays support the hypothesis that GNA-1 interacts with and operates downstream of GPR-4 to regulate the growth and development of *N. crassa* in the presence of poor carbon sources (Fig. 6). The coupling between GPR-4 and GNA-1 illustrates the mechanistic diversity of the G protein signaling pathways that are involved in carbon sensing in fungi. As mentioned above, GPR-4 is homologous to Gpr1p in *S. cerevisiae* and Git3 in *S. pombe*. However, the *N. crassa* homologue of the yeast Gα proteins coupled to these GPCRs is GNA-3, not GNA-1 (33). This variation could stem from several reasons, including regulatory differences due to the presence of three instead of two Gα subunits in ascomycete filamentous fungi versus yeasts. In addition, as mentioned above, both GNA-1 and GNA-3 regulate adenyl cyclase in *N. crassa*, and it has also been demonstrated that the homologue of either GNA-1 or GNA-3 is required for pathogenesis in filamentous fungal species (reviewed in references 7, 32, and 43). Since it has been shown in many cases that modulation of cAMP levels by the Gα protein is an important aspect of pathogenesis, the relative importance of GNA-1 versus GNA-3 to cAMP levels (and pathogenesis) may result from the impact of adenyl cyclase activity versus protein levels in various species.

The GPCRs that are coupled to GNA-2 and GNA-3 during growth on glycerol are currently unknown. Although our results support a scenario in which GPR-4 acts through GNA-1 to regulate mass accumulation on glycerol, we cannot rule out that, under certain circumstances, GPR-4 may also interact with GNA-2 and GNA-3. There are reports of GPCRs that interact with multiple Gα proteins, particularly in mammals (for examples, see references 11, 14, 19, 39, 40, 44, 51, 58, 62, and 76). As mentioned above, GPR-4 is expressed under a variety of growth conditions and, thus, potentially may respond to different environmental signals through coupling to more than one G protein. Such a scenario has been proposed to explain the patterns of G protein coupling to prokineticin receptors in humans (14).

Although the response to glucose is lost in Δgpr-4 mutants, we do not know whether glucose is the in vivo ligand for GPR-4. The correlation between cAMP responses and elicitation of a phenotype is not absolute for the related group of receptors in fungi. In *S. cerevisiae*, data support sucrose and glucose as agonist ligands for Gpr1p, while mannose is an antagonist (42). However, maltose stimulates hyphal growth but does not elicit a cAMP transient after addition (56). In *S. pombe*, Git3 is required for increased cAMP levels after the transfer from glucose starvation to glucose-rich conditions (10, 73). *C. albicans* CaGpr1 has been variously reported to respond to glucose (48) or amino acids, including alanine and methionine (46, 47), for activation of cAMP synthesis. However, cAMP levels are not affected by proline addition, even though this amino acid causes morphological phenotypes (4).

In the filamentous fungus *A. nidulans*, a transient increase in cAMP amount has been reported after glucose addition to starved cultures, although, as mentioned above, the GPCR required for this carbon-sensing cAMP signaling pathway has not been identified (41). In *C. neoformans*, the GPCR Gpr4 is required to sustain a short-lived increase in cAMP levels in response to methionine but not glucose (77). We did not observe any alteration in growth or colony morphology in *N. crassa* after the addition of methionine to media with high or low carbon content (data not shown), suggesting that GPR-4 responds to carbon source(s) and not this amino acid.

Wild-type strains produce much more aerial hyphae than Δgpr-4 mutants when plated at a higher cell density (10^4 conidia/cm²) on glycerol medium. This result is consistent with a scenario in which GPR-4 negatively regulates a cell density-dependent system involving aerial hypha formation in *N. crassa*. Cell density-dependent control of aerial hypha production by GPR-4 could be regulated by small molecules. In *C. albicans*, tyrosol, farnesol, and farnesoic acid have been recently shown to act as quorum-sensing molecules that regulate the switch between yeast and filamentous forms (13, 26, 52). The filamentous fungus *A. nidulans* does not produce farnesol, but exogenous farnesol causes apoptosis in this species (61). Based on these observations, it has been proposed that farnesol production by *C. albicans* may lead to a competitive advantage relative to other fungal species in the environment (26, 61). It will be interesting to determine if quorum-sensing molecules control aerial hypha formation in a pathway involving GPR-4 in *N. crassa*. It is possible that GPR-4 directly binds
both the carbon and cell density signal ligands and even that the same chemical is shared between the two responses.

cAMP is an important regulator of vegetative growth and development in *N. crassa* and other filamentous fungi. Our experiments showed that the cr-1 and Δgpr-4 mutants grow poorly in comparison to the wild type in plate cultures with glycerol, mannitol, or arabinose as the carbon source and that exogenous cAMP can partially reverse the dry mass defect of Δgpr-4 mutants on glycerol solid medium. Although the Δgpr-4 mutation did not lead to a significant difference in steady-state intracellular cAMP levels on VM-glycerol plates, ∆gpr-4 mutants do lack the cAMP transient increase observed in wild-type cells when transferred from glycerol to glucose-rich medium. Taken together, these findings are consistent with a role for GPR-4 in the regulation of cAMP levels in *N. crassa*.

Three glucose sensing/signaling pathways have been identified in *S. cerevisiae*, including the Snf1p protein kinase/Mig1p repressor pathway, the Snf3p and Rgt2p glucose sensors/Rgt1 repressor system, and the Gpr1p/Gpa2p/cAMP pathway (30, 57). Our work supports alignment of GPR-4 with the third pathway, presumably functioning in the presence of poor carbon sources in *N. crassa*. Previous studies have provided evidence for a second carbon sensory pathway, which involves rco-3 (45). RCO-3 is required for high- and low-affinity glucose transport in *N. crassa* (45). Glucose deprivation triggers conidiation in wild-type *N. crassa* cultures; however, rco-3 null mutants conidiate inappropriately in submerged culture in the presence of abundant glucose, suggesting that they have lost sensory capabilities (45). Moreover, the sequence and functional similarity observed between RCO-3 and *S. cerevisiae* Snf3p and Rtg2p supports the hypothesis that RCO-3 functions as a carbon sensor in *N. crassa* (45). Future studies will probe possible cross talk between the GPR-4 and RCO-3 carbon sensory pathways in *N. crassa*.

Δgpr-4 mutants exhibit their most severe mass defects during growth on glycerol, which may have implications for the understanding and control of plant pathogenic fungi. Many studies indicate that glycerol participates in numerous pathways.
and serves diverse cellular roles (9). Glycerol has recently been shown to be a major nutrient obtained by the fungal pathogen from the host plant (72). On the other hand, Δgpr-4 mutants are also defective during growth on arabinose; this sugar is a main component of the monocot cell wall and thus likely to be encountered by fungal plant pathogens in nature (63, 68). As GPR-4 has many close homologues in pathogenic filamentous fungi, the study of GPR-4 may shed light on a possible function encountered by fungal plant pathogens in nature (63, 68). As GPR-4 is a major component of the monocot cell wall and thus likely to be defective during growth on arabinose; this sugar is a main component of the monocot cell wall and thus likely to be encountered by fungal plant pathogens in nature (63, 68).

**ACKNOWLEDGMENTS**

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