Global Survey ofCanonical Aspergillus flavus G Protein-Coupled Receptors

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ABSTRACT G protein–coupled receptors (GPCRs) are transmembrane receptors that relay signals from the external environment inside the cell, allowing an organism to adapt to its surroundings. They are known to detect a vast array of ligands, including sugars, amino acids, pheromone peptides, nitrogen sources, oxylipins, and light. Despite their prevalence in fungal genomes, very little is known about the functions of filamentous fungal GPCRs. Here we present the first full-genome assessment of fungal GPCRs through characterization of null mutants of all 15 GPCRs encoded by the aflatoxin-producing fungus Aspergillus flavus. All strains were assessed for growth, development, ability to produce aflatoxin, and response to carbon sources, nitrogen sources, stress agents, and lipids. Most GPCR mutants were aberrant in one or more response processes, possibly indicative of cross talk in downstream signaling pathways. Interestingly, the biological defects of the mutants did not correspond with assignment to established GPCR classes; this is likely due to the paucity of data for characterized fungal GPCRs. Many of the GPCR transcripts were differentially regulated under various conditions as well. The data presented here provide an extensive overview of the full set of GPCRs encoded by A. flavus and provide a framework for analysis in other fungal species.

IMPORTANCE Aspergillus flavus is an opportunistic pathogen of crops and animals, including humans, and it produces a carcinogenic toxin called aflatoxin. Because of this, A. flavus accounts for food shortages and economic losses in addition to sickness and death. Effective means of combating this pathogen are needed to mitigate its deleterious effects. G protein–coupled receptors (GPCRs) are often used as therapeutic targets due to their signal specificity, and it is estimated that half of all drugs target GPCRs. In fungi such as A. flavus, GPCRs are likely necessary for sensing the changes in the environment, including food sources, developmental signals, stress agents, and signals from other organisms. Therefore, elucidating their functions in A. flavus could identify ideal receptors against which to develop antagonists.
stream signaling cascades. These have been well studied in the model yeast *Saccharomyces cerevisiae*. Sugars stimulate a cAMP-protein kinase A (PKA) pathway in which Gα activates adenylate cyclase, which converts ATP to cAMP. The increased concentration of cAMP results in the release of inhibitory subunits from PKA, thereby activating the enzyme. PKA then phosphorylates downstream proteins to impact the cellular response. Following PKA, thereby activating the enzyme. PKA then phosphorylates cyclase, which converts ATP to cAMP. The increased concentration of cAMP produces a downstream signaling cascade.

The mitogen-activated protein kinase (MAPK) pathway, which is downstream of the cAMP-PKA pathway in which Gα sensing glucose, AtRGS1 interacts with the constitutively active Gα subunit. AtRGS1 interacts with the constitutively active Gα subunit A. thaliana GPA1 (AtGPA1), resulting in hydrolysis of GTP and subsequent deactivation of the G protein (18–20). GPCR-RGS hybrids have since been defined in several species of fungi.

A BLAST search of the amino acid sequences of each of the 16 *A. nidulans* GPCRs against the *A. flavus* genome yielded 77 total hits, 57 of which were unique. GPCRs pass the membrane seven times, so two protein topology prediction tools, TMHMM 2.0 (13, 14) and TopPred 1.10 (15, 16), were used to determine which of these 57 proteins were likely GPCRs. Twenty-nine of the fifty-seven sequences were not predicted to encode any transmembrane (TM) domains, so they were eliminated. Of the remaining 28 proteins, 13 were predicted to have 7 TM domains by at least one of the prediction tools. Two additional proteins that were homologous to *A. nidulans* GprF and GprO were predicted to have fewer than 7 TM domains, but since their *A. nidulans* orthologs were predicted to have 7 TM domains, they were also included with the rest of the *A. flavus* GPCRs, bringing the total to 15 (Table 1).

We also compared the *A. flavus* genome to the reported *A. oryzae* GPCRs (7) since these two species are believed to be the same species, with *A. oryzae* constituting a domesticated clade (17). Similar to what was reported for *A. oryzae*, no orthologs for *A. flavus* GprE, GprI, or GprN were found in the *A. flavus* genome. A novel *A. oryzae* GPCR not found in *A. nidulans* was identified and named GprQ. Its *A. flavus* ortholog (AFLA_132040; 99% identity, with an E value of 7e−115) had only four (TM-HMM) or three (TopPred) predicted TM domains. Since *A. oryzae* GprQ was reported to encode only five predicted TM domains, the *A. flavus* homolog was not included in the set of GPCRs.

The BLAST search also identified two GPCRs that were not previously identified in the aspergilli, GprR and GprS. Like GprK, GprR harbors an RGS (regulator of G protein signaling) domain at the C-terminal end. This type of GPCR was first discovered in *Arabidopsis thaliana* and called *A. thaliana* RGS1 (AtRGS1). AtRGS1 regulates cell proliferation as well as sensitivity to sugars via its functional RGS domain. Unlike canonical GPCRs, it does not trigger the exchange of GTP for GDP on a G protein. Upon sensing glucose, AtRGS1 interacts with the constitutively active Gα subunit *A. thaliana* GPA1 (AtGPA1), resulting in hydrolysis of GTP and subsequent deactivation of the G protein (18–20). GPCR-RGS hybrids have since been defined in several species of fungi. Based on their potential importance to fungal growth and survival, GPCRs have been the subject of numerous bioinformatics studies. As a result, the entire set of GPCRs encoded by various fungi has been predicted for *Aspergillus nidulans*, *Aspergillus fumigatus*, *Aspergillus oryzae*, *Magnaporthe grisea*, *Cryptococcus neoformans*, *Neurospora crassa*, and *Trichoderma asperellum*. This type of GPCR was first discovered in *Arabidopsis thaliana* and called *A. thaliana* RGS1 (AtRGS1). AtRGS1 regulates cell proliferation as well as sensitivity to sugars via its functional RGS domain. Unlike canonical GPCRs, it does not trigger the exchange of GTP for GDP on a G protein. Upon sensing glucose, AtRGS1 interacts with the constitutively active Gα subunit *A. thaliana* GPA1 (AtGPA1), resulting in hydrolysis of GTP and subsequent deactivation of the G protein (18–20). GPCR-RGS hybrids have since been defined in several species of fungi.

**RESULTS**

**A. flavus encodes 15 putative GPCRs.** We began our search for *A. flavus* GPCRs by examining the set of 16 GPCRs reported for *A. nidulans* (7). In an effort to focus on the canonical GPCRs, our study did not include the PTH11-like membrane-spanning proteins, of which *A. nidulans* is predicted to encode at least 25 (7, 8).

**TABLE 1 Overview of *A. flavus* GPCRs**

| Gene | Gene ID (AFLA_x) | No. of amino acids | Class | Conserved domain (note) | No. of TM domains* |
|------|-----------------|--------------------|-------|------------------------|--------------------|
| gprA | 060740          | 374                | I     | STE2 GPCR (S. cerevisiae pheromone receptor) | 6/7                |
| gprB | 061620          | 465                | II    | STE3 GPCR (S. cerevisiae pheromone receptor) | 7/7                |
| gprC | 074150          | 444                | III   | Gt3; Gt3_C (S. pombe glucose receptor) | 7/6                |
| gprD | 135680          | 415                | III   | Gt3; Gt3_C (S. pombe glucose receptor) | 7/7                |
| gprE | 006880          | 300                | IV    | PQ loop repeat (S. pombe nitrogen sensor) | 4/5                |
| gprF | 067770          | 426                | IV    | PQ loop repeat (S. pombe nitrogen sensor) | 7/7                |
| gprG | 006920          | 428                | V     | Secretin family (signal through CAMP pathways) | 7/7                |
| gprH | 127870          | 322                | IV    | PQ loop repeat (S. pombe nitrogen sensor) | 7/7                |
| gprI | 009790          | 560                | VI    | RGS domain (regulator of G protein signaling) | 7/7                |
| gprJ | 075000          | 490                | VII   | [No conserved domains] | 7/7                |
| gprK | 031230          | 282                | VIII  | Hemolysin III related (broad range of ligands) | 6/7                |
| gprL | 088190          | 502                | VIII  | Hemolysin III related (broad range of ligands) | 7/7                |
| gprM | 023070          | 523                | VI    | RGS domain (regulator of G protein signaling) | 7/7                |
| gprN | 006320          | 266                | IV    | PQ loop repeat (S. pombe nitrogen sensor) | 7/7                |
| gprO | 009790          | 560                | VI    | Bacteriorhodopsin-like (photoreactive) | 7/7                |
| gprP | 135680          | 415                | III   | Gt3; Gt3_C (S. pombe glucose receptor) | 7/7                |
| gprQ | 061620          | 465                | II    | STE3 GPCR (S. cerevisiae pheromone receptor) | 7/7                |
| gprR | 074150          | 444                | III   | Gt3; Gt3_C (S. pombe glucose receptor) | 7/7                |
| gprS | 006880          | 300                | IV    | PQ loop repeat (S. pombe nitrogen sensor) | 4/5                |
| gprT | 067770          | 426                | IV    | PQ loop repeat (S. pombe nitrogen sensor) | 7/7                |
| gprU | 006920          | 428                | V     | Secretin family (signal through CAMP pathways) | 7/7                |
| gprV | 127870          | 322                | IV    | PQ loop repeat (S. pombe nitrogen sensor) | 7/7                |
| gprW | 009790          | 560                | VI    | RGS domain (regulator of G protein signaling) | 7/7                |
| gprX | 075000          | 490                | VII   | [No conserved domains] | 7/7                |
| gprY | 031230          | 282                | VIII  | Hemolysin III related (broad range of ligands) | 6/7                |
| gprZ | 088190          | 502                | VIII  | Hemolysin III related (broad range of ligands) | 7/7                |
| spaA | 006320          | 266                | IV    | PQ loop repeat (S. pombe nitrogen sensor) | 7/7                |
| spaB | 009790          | 560                | VI    | Bacteriorhodopsin-like (photoreactive) | 7/7                |

* The first number is predicted by TMHMM; the second is predicted by TopPred.

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A. oryzae
A. flavus
software tool (23) (see Fig. S1 in the supplemental material). From (22), and a phylogenetic tree was generated using the Phylogeny.fr software tool (23), see Fig. S1 in the supplemental material. This analysis indicated that the A. flavus GPCRs, as well as the sequences for GPCRs from A. nidulans, A. fumigatus, A. oryzae, F. graminearum, M. grisea, N. crassa, Trichoderma reesei, and V. dahliae, were aligned using the Clustal Omega software tool (22), and a phylogenetic tree was generated using the Phylogeny.fr software tool (23) (see Fig. S1 in the supplemental material). Based on their domain structures, A. flavus' 15 putative GPCRs were assigned to nine different classes. These classes have been previously described (7, 21), and a few notes on each class are included in Table I. The amino acid sequences for all 15 GPCRs, as well as the sequences for GPCRs from A. nidulans, A. fumigatus, A. oryzae, F. graminearum, M. grisea, N. crassa, Trichoderma reesei, and V. dahliae, were aligned using the Clustal Omega software tool (22), and a phylogenetic tree was generated using the Phylogeny.fr software tool (23) (see Fig. S1 in the supplemental material). From this analysis, it was apparent that the A. flavus GPCRs GprF, GprH, GprR, and GprS are not orthologous to any of A. oryzae's predicted GPCRs, despite the high degree of genetic similarity between the two fungi. Interestingly, of the two GPCRs that were not previously found in the aspergilli, GprS was most closely related to two GPCRs from V. dahliae, while GprR was unique among the fungi analyzed here.

In order to verify the coding sequences of the A. flavus GPCRs, 13 of the 15 GPCRs were amplified, cloned, and sequenced from A. flavus mRNA. DNA for gprF and gprM could not be amplified. Two GPCRs, gprF and gprO, appeared to be misannotated. In both cases, the actual sequence improved the proteins' alignments with their A. nidulans orthologs, and for GprO, it changed the number of predicted TM domains from six to seven (TopPred), with the updated prediction reported in Table I. GprF was only predicted to encode four (TMHMM) or five (TopPred) TM domains, but the percentage of mutant germination versus wild-type germination for each time point (Table 2). Aside from a slightly enhanced germination rate at 6 h by the ΔgprO mutant, the rest of the mutants were either equivalent or impaired in germination compared to the wild type. The most severely deficient mutants were the ΔgprA and ΔgprK strains, with <50% of the wild-type germination rate at nearly all time points measured. Many of the other mutants, including the ΔgprB, ΔgprC, ΔgprH, ΔgprJ, ΔgprP, ΔgprR, and ΔgprS strains, were impaired in germination as well.

(ii) Light-induced sporulation. Rhodopsin is well characterized as a receptor of light, and A. flavus encodes one GPCR, NopA, that belongs to the class IX bacteriorhodopsin-like GPCR family. Since light is an inducer of sporulation, the fungi were exposed to continuous light or continuous dark, and their spores were counted. The wild type and most of the mutants produced significantly more spores in the light versus production in the dark. Two mutants, the ΔgprF and ΔgprR mutants, showed no significant difference between the two conditions. Notably, the ΔnopA mutant exhibited the wild-type response to light (Table 2).

(iii) Quorum sensing. A. flavus undergoes a quorum-sensing-mediated developmental shift in which at low inoculum density, it produces many sclerotia and very few spores. The opposite is observed at high inoculum density (30). Because an exogenous signal mediates this phenomenon, GPCRs are plausible targets for relaying the signal. In fact, two GPCRs in the A. flavus strain NRRL3357, encoded by gprC and gprD, have already been implicated as quorum-sensing receptors based on their inability to respond to a high-density extract (31). Surprisingly, all 15 Δgpr mutants produced more sclerotia and fewer spores at high density.
TABLE 2  Developmental phenotypes of A. flavus Δgpr mutants

| Strain | Germination | AF | Spores | Quorum sensing |
|--------|-------------|----|--------|----------------|
|        | 4h | Sh | 6h | Sh | 8h | L/D | Spores | Sclerotia |      |
| ΔgprA  |     |    |     |     |     |     | YES (+) | (±) | 190% |
| ΔgprB  |     |    |     |     |     |     | YES (+) | (±) | 170% |
| ΔgprC  |     |    |     |     |     |     | YES (+) | (±) | 150% |
| ΔgprD  |     |    |     |     |     |     | YES (+) | (±) | 130% |
| ΔgprE  |     |    |     |     |     |     | YES (+) | (±) | 110% |
| ΔgprF  |     |    |     |     |     |     | YES (+) | (±) | 90%  |
| ΔgprG  |     |    |     |     |     |     | YES (+) | (±) | 70%  |
| ΔgprH  |     |    |     |     |     |     | YES (+) | (±) | 50%  |
| ΔgprI  |     |    |     |     |     |     | YES (+) | (±) | 30%  |
| ΔgprJ  |     |    |     |     |     |     | YES (+) | (±) | 10%  |
| ΔgprK  |     |    |     |     |     |     | YES (+) | (±) |       |
| ΔgprL  |     |    |     |     |     |     | YES (+) | (±) |       |
| ΔgprM  |     |    |     |     |     |     | YES (+) | (±) |       |
| ΔgprN  |     |    |     |     |     |     | YES (+) | (±) |       |
| ΔgprO  |     |    |     |     |     |     | YES (+) | (±) |       |
| ΔgprP  |     |    |     |     |     |     | YES (+) | (±) |       |
| ΔgprR  |     |    |     |     |     |     | YES (+) | (±) |       |
| ΔgprS  |     |    |     |     |     |     | YES (+) | (±) |       |
| ΔgprT  |     |    |     |     |     |     | YES (+) | (±) |       |
| ΔgprU  |     |    |     |     |     |     | YES (+) | (±) |       |
| ΔgprV  |     |    |     |     |     |     | YES (+) | (±) |       |
| ΔgprW  |     |    |     |     |     |     | YES (+) | (±) |       |
| ΔgprX  |     |    |     |     |     |     | YES (+) | (±) |       |
| ΔgprY  |     |    |     |     |     |     | YES (+) | (±) |       |
| ΔgprZ  |     |    |     |     |     |     | YES (+) | (±) |       |

* GPCR mutants were assayed for germination rate at 4 to 9 h postinoculation and for aflatoxin (AF) production on AF-promoting (YES) and AF-repressing (YEP) media. For both assays, shaded squares represent data points that were significantly different from those for the wild type (WT). The color of each box indicates the amount of germinated spores or AF produced as a percentage of WT germination or AF production. The ratios of spores produced under light and dark conditions (L/D), as well as spores and sclerotia produced by high-density (H) and low-density (L) inocula, were determined, and any data point that did not exhibit the same pattern as the WT is shaded gray. For all four experiments, statistical significance was determined by a Student t test, with P < 0.05. Deletions are shown in the “Strain” column.

GPCRs impact growth on various carbon and nitrogen sources. In addition to developmental cues, GPCRs are able to detect the presence or absence of important nutrients. GprC and GprD belong to class III, which is the group that also contains the sugar receptors S. cerevisiae Gpr1 and S. pombe Git3. Gpr1 senses glucose and sucrose (with a stronger affinity for sucrose) and transmits the signal through a G protein/cAMP/PKA pathway (36–38). Other Gpr1 homologs are also carbon source sensors, such as S. pombe Git3 (reviewed in reference 39) and N. crassa GPR-4 (40), although the role of C. albicans Gpr1 in carbon sensing is ambiguous (41, 42). While a carbon-sensing GPCR has not been identified in the aspergilli, it has been shown that carbon sensing is mediated by the heterotrimeric G protein GprA, S. pombe nitrogen starvation sensor Stm1, and A. flavus has four receptors in this group. Nitrogen starvation results in cell cycle arrest in S. pombe, which allows the yeast to remain viable either until nutrition becomes available or mating partners are present.

Class IV receptors are grouped together based on their relatedness to the S. pombe nitrogen starvation sensor Stm1, and A. flavus has four receptors in this group. Nitrogen starvation results in cell cycle arrest in S. pombe, which allows the yeast to remain viable either until nutrition becomes available or mating partners are present.

TABLE 3  Growth of A. flavus Δgpr mutants on various carbon and nitrogen sources

| Strain | Carbon source | Nitrogen source |
|--------|---------------|-----------------|
|        | Glu | Gal | Xyl | Suc | Corn oil | Glucose + Corn oil | Pep | NH4Cl | Pro |
| ΔgprA  |     |     |     |     |       |       |       |       |       |
| ΔgprB  |     |     |     |     |       |       |       |       |       |
| ΔgprC  |     |     |     |     |       |       |       |       |       |
| ΔgprD  |     |     |     |     |       |       |       |       |       |
| ΔgprE  |     |     |     |     |       |       |       |       |       |
| ΔgprF  |     |     |     |     |       |       |       |       |       |
| ΔgprG  |     |     |     |     |       |       |       |       |       |
| ΔgprH  |     |     |     |     |       |       |       |       |       |
| ΔgprI  |     |     |     |     |       |       |       |       |       |
| ΔgprJ  |     |     |     |     |       |       |       |       |       |
| ΔgprK  |     |     |     |     |       |       |       |       |       |
| ΔgprL  |     |     |     |     |       |       |       |       |       |
| ΔgprM  |     |     |     |     |       |       |       |       |       |
| ΔgprN  |     |     |     |     |       |       |       |       |       |
| ΔgprO  |     |     |     |     |       |       |       |       |       |
| ΔgprP  |     |     |     |     |       |       |       |       |       |
| ΔgprR  |     |     |     |     |       |       |       |       |       |
| ΔgprS  |     |     |     |     |       |       |       |       |       |
| ΔgprT  |     |     |     |     |       |       |       |       |       |
| ΔgprU  |     |     |     |     |       |       |       |       |       |
| ΔgprV  |     |     |     |     |       |       |       |       |       |
| ΔgprW  |     |     |     |     |       |       |       |       |       |
| ΔgprX  |     |     |     |     |       |       |       |       |       |
| ΔgprY  |     |     |     |     |       |       |       |       |       |
| ΔgprZ  |     |     |     |     |       |       |       |       |       |

* Strains were grown on a variety of media with different sources of carbon and nitrogen. The radial growth was measured, and mutants were compared to the WT on the same medium. The carbon sources were glucose (Glu), galactose (Gal), xylose (Xyl), sucrose (Suc), corn oil, and corn oil with glucose. The nitrogen sources were peptone (Pep), ammonium chloride, and proline (Pro). Shaded boxes indicate data points that were significantly different from those for the WT, with the color representing the degree of growth inhibition compared to the WT. Significance was determined by a Student t test, with P < 0.05. Deletions are shown in the “Strain” column.
The mutants were exposed to a variety of stressors that inhibit the radial growth of the wild type. The percent inhibition of growth in the presence versus absence of the stressor was compared between the wild type and each mutant, and the changes in inhibition between wild type and the mutants are presented as a heat map (Table 4).

Hydrogen peroxide was used at a concentration of 3 mM to assay oxidative stress. Loss of gprH rendered the fungus more resistant to hydroperoxide, while the ΔgprG mutant was more sensitive. Cell wall stress was created by growing the fungi on Congo red, which weakens the cell wall by binding to growing chitin chains, preventing their attachment to other cell wall components (48, 49). The ΔgprK and ΔgprS mutants were both more sensitive to Congo red. The impact of osmotic pressure was measured with 1 M sodium chloride. Three mutants, carrying the ΔgprK, ΔgprM, and ΔgprR deletions, were more sensitive than the wild type to the hyperosmotic condition. Finally, the strains were grown on media buffered to pH 4 and pH 8. The ΔgprM mutant was more sensitive to both conditions, while the ΔgprK and ΔgprR mutants were more sensitive to acidic and alkaline pHs, respectively. Alternatively, the ΔgprD, ΔgprF, and ΔgprG mutants were more resistant to acidic pH.

**GPCRs involved in response to lipids and oxylipins.** It has long been speculated that oxylipins are detected by fungal GPCRs, since this is their mode of perception in mammalian cells (reviewed in references 3, 5, and 50). To address this, strains were exposed to the oxylipins methyl jasmonate (MeJA) and 13-hydroperoxy-octadecadienoic acid [13(S)-HpODE] and to the

| Strain | MeJA | 13(S)-HpODE | Linoleic acid |
|--------|------|--------------|--------------|
| ΔgprA  | NR   | NR           | NR           |
| ΔgprB  | NR   | NR           | NR           |
| ΔgprC  | NR   | NR           | NR           |
| ΔgprD  | NR   | NR           | NR           |
| ΔgprF  | NR   | NR           | NR           |
| ΔgprG  | NR   | NR           | NR           |
| ΔgprH  | NR   | NR           | NR           |
| ΔgprJ  | NR   | NR           | NR           |
| ΔgprK  | Increase AF | NR           | NR           |
| ΔgprM  | NR   | NR           | NR           |
| ΔgprO  | NR   | NR           | NR           |
| ΔgprP  | NR   | NR           | NR           |
| ΔgprR  | NR   | NR           | NR           |
| ΔgprS  | NR   | NR           | NR           |

Shaded boxes represent data points that did not exhibit the WT response, and the mutant response is indicated ("NR" means "no response"). Statistical significance was determined by a Student’s t test with P < 0.05. Deletions are shown in the "Strain" column.
fatty acid linoleic acid. Any mutant that deviated from the wild-type response to these chemicals is indicated in Table 5 with a shaded box.

The effect of MeJA on AF production varies depending on the experimental setup and concentration of MeJA used (51). Under certain conditions, MeJA has been shown to repress AF production by A. flavus (52), but under others, it has enhanced AF synthesis (53). For this study, an AF-repressive protocol was followed (51) in which cultures were exposed to 10 M MeJA, and AF was measured after 3 days. AF repression was observed for the wild type and five of the gprD mutants. To test whether this represented a general sporulation defect or a specific lack of response to MeJA, 13(HpODE) was lost for the gprR mutant, which actually made more AF when exposed to MeJA compared to the wild type (54). The second (“Wheat/maize”) reports the FC in expression from “Maize/mycelia” reports the FC in expression from “Mycelia/sclerotia” report the FC in expression from untreated A. flavus cultures to those exposed to 5-AC.

### DISCUSSION
Several works have bioinformatically identified the GPCRs encoded by various fungi. These include A. nidulans, A. fumigatus, A. oryzae, M. grisea, C. neoformans, N. crassa, Verticillium spp., and Trichoderma spp. (7–12). Functions have been assigned for a few of these GPCRs. However, this is the first study to identify functional groups for a fungus’ entire set of GPCRs, providing leads on most of the GPCRs that can be pursued further in future studies.

An attempt was made to identify the activating ligands of A. flavus’ GPCRs through heterologous expression in S. cerevisiae. Despite testing more than 60 potential ligands, no ligand-receptor pair was elucidated (data not shown). Individually deleting each GPCR, however, led to an abundance of findings that highlight the complexity of signal transduction networks. Of the 15 deletion mutants, all but two (the gprO and gprP mutants) exhibited aberrancies in multiple processes. This was not unexpected, since there is overlap in many of the pathways that drive various aspects of fungal development. For example, germination in A. nidulans is triggered by a carbon source (28), so without the receptor(s) that detects that signal, the fungus would likely be impaired in both germination and growth on various carbon sources, as was the case with the gprA, gprC, gprD, gprF, gprJ, gprK, gprP, and gprR mutants.

Stress pathways are also interconnected among themselves and with other developmental pathways, making it difficult to decipher which GPCRs are tied to which responses. Nitrogen starvation, for example, creates a stressful environment for cells. Chung...
However, in the case of the yeast pheromone receptor Ste2 and Ste3 homologs paucity of data on the functions of filamentous fungus GPCRs. In the experimental results as presented here, which aligns with the sequence alignment as well as the various processes it may be interrupted.

development pathways, though many more such instances exist, creating a domino effect when a component of one pathway is disrupted.

Table 7 lists each GPCR’s predicted function based on its sequence alignment as well as the various processes it may be involved in based on the functional studies reported here. Interestingly, none of the predicted functions (Table 1) were validated by the experimental results as presented here, which aligns with the paucity of data on the functions of filamentous fungus GPCRs. In the case of the yeast pheromone receptor Ste2 and Ste3 homologs GprA and GprB, a role in mating was not evaluated in this study. However, in *A. nidulans*, gprA and gprB are required for fruiting-body formation during self-fertilization (60). *A. flavus* is a heterothallic fungus, and it undergoes sexual reproduction when the appropriate mating type is present (61). Therefore, GprA and GprB are likely to be important for detecting the presence of mating partners and triggering sexual reproduction.

GprC and GprD were predicted to be carbon source sensors based on their homology with the *S. pombe* glucose receptor Git3. However, only GprC displayed any growth defects on different carbon sources, suggesting that despite their high degree of sequence similarity, GprC and GprD have distinct roles. In addition to GprC, several other putative GPCRs may be involved in carbon source sensing. Deletion of gprA, gprJ, gprK, and gprR also resulted in impaired growth on multiple carbon sources. It was shown in *S. cerevisiae* that its carbon sensor Gpr1 was partially redundant that its carbon sensor Gpr1 was partially redundant with Ras2, a small GTPase, such that a Δgpr1 mutant grew normally but a Δgpr1 Δras2 strain had a severe growth defect (36). Perhaps several of *A. flavus*’ receptors have overlapping functions in carbon sensing with each other and/or with other proteins, such as small GTPases, which would explain why all of the single GPCR mutants were still viable. It is also possible that several of the GPCRs work as heterodimers, as is found in other systems (62), and thus their full role in fungal development will be uncovered only in multiple deletion strains.

Class IV GPCRs are *S. pombe* Stm1 homologs and are therefore predicted to have a role in nitrogen sensing. *A. flavus*’ four class IV GPCRs are GprF, GprG, GprJ, and GprS. However, none of these mutants showed any deviation from the wild-type phenotype on the three nitrogen sources tested. Instead, the ΔgprC, ΔgprD, and ΔgprR mutants were restricted on proline, with the ΔgprR mutant also showing restriction on ammonium chloride. This is reminiscent of the *S. pombe* Stm1 mutant showing premature cell cycle arrest on proline, a poor nitrogen source (44). Further experimentation is required to definitively classify these receptors as nitrogen sensors, though it is interesting that GprC and GprD may have overlapping roles in nitrogen sensing and divergent roles in carbon sensing.

Not much is known about class V receptors. They are predicted to be cAMP sensors, though there is no evidence that this occurs in *Aspergillus*. *A. flavus* encodes only one class V receptor, GprH. It is homologous to *C. neoformans* Gpr4, which is a methionine sensor. The Gpr4 protein is internalized in response to methionine, and a Δgpr4 mutant does not produce a spike in cAMP in the presence of methionine as is seen in the wild type. The Δgpr4 mutant is defective in capsule production and mating (9). Heterologous expression of *A. flavus* GprH in yeast revealed it to be active in the absence of any added ligand (data not shown), although efforts to determine whether this was due to methionine in the media were inconclusive.

NopA belongs to the bacteriorhodopsin-like class of GPCRs (class IX). Rhodopsins consist of an opsin protein and a retinal chromophore and act as light-responsive GPCRs or proton pumps (reviewed in reference 63). In spite of the wealth of knowledge about rhodopsins, the function of many opsins, including NopA, remains enigmatic. The first opsin identified in fungi was *N. crassa* Nop-1 (64), which binds retinal (65). It has no obvious function, though its expression is induced during conidiation, which is a light-responsive process (66). The *Fusarium fujikuroi* Nop-1 ortholog also lacks an obvious function, although it appears to play a role in the regulation of retinal biosynthesis (67).

Based on its sequence, *A. flavus* NopA is an opsin-related protein instead of a rhodopsin, meaning that it lacks a critical lysine residue required for binding retinal. Ospin-related proteins are found throughout the fungal kingdom, though nothing is known about what cofactors, if any, they bind to and what their functions

| Strain description | Predicted role(s) | Observed role(s) |
|--------------------|------------------|-----------------|
| ΔgprA              | Mating           | Germination; AF repression; carbon source sensing; oxylipin sensing |
| ΔgprB              | Mating           | Germination; quorum sensing; MeJA sensing |
| ΔgprC              | Glucose sensing  | Germination; carbon and nitrogen sensing; 13(S)-HpODE sensing |
| ΔgprD              | Glucose sensing  | Nitrogen sensing; ROS, cell wall, acidic pH stress response; 13(S)-HpODE sensing |
| ΔgprF              | Nitrogen sensing | Light sensing; quorum sensing; acidic pH stress response; oxylipin sensing |
| ΔgprG              | Nitrogen sensing | ROS and acidic pH stress responses; oxylipin sensing |
| ΔgprH              | Methionine sensing | Germination; ROS stress response |
| ΔgprI              | Nitrogen sensing | Germination; carbon sensing; 13(S)-HpODE sensing |
| ΔgprK              | Unknown          | Germination; carbon sensing; cell wall, osmotic, and acidic stress response, MeJA sensing |
| ΔgprM              | Unknown          | Carbon sensing; osmotic and pH stress responses; MeJA sensing |
| ΔgprO              | Unknown          | Oxylipin sensing |
| ΔgprP              | Unknown          | Germination; AF repression; carbon sensing; oxylipin sensing |
| ΔgprR              | Unknown          | Germination; light sensing; carbon and nitrogen sensing; osmotic and alkaline pH stress responses; lipid and oxylipin sensing |
| ΔgprS              | Nitrogen sensing | Germination; cell wall stress response; MeJA sensing |
| ΔnopA              | Light sensing    | Unknown |

et al. (44) found that the nitrogen starvation sensor Stm1 was important for growth on different nitrogen sources and that it also appeared to stimulate a stress response pathway. These are just a couple examples of cross talk between different signaling and development pathways, though many more such instances exist, creating a domino effect when a component of one pathway is disrupted.
may be (68). Other than a slight germination defect, the ΔnopA mutant did not appear different from the wild type in any of the development, growth, stress, or lipid response assays described here. Either NopA is functionally redundant with another receptor, or else it has a role that was not addressed by these experiments.

Five GPCRs from three different classes (classes VI, VII, and VIII) had no predicted functions. GprK and GprR both belong to class VI, characterized by hybrid GPCR-RGS proteins. Although the ΔgprK and ΔgprR mutants shared some phenotypes, the ΔgprR mutant was distinct for its defective growth/response in nearly every assay performed. This suggests that GprR might regulate a process that is required for growth, and without it, the mutant is impaired in its response to many other stimuli. GprO and GprP are both class VII receptors. They may share overlapping functions in oxypin sensing, but GprP clearly has additional roles in germination and AF production.

In this study, the Δgpr mutants were evaluated for their basic growth and development, growth on a variety of carbon and nitrogen sources, stress responses, and responses to lipids and oxypins. An area that was not explored but is likely to be extremely important for A. flavus is the roles of its GPCRs during interaction with other organisms. A. flavus is naturally found in the soil, where it encounters many different microbes, and it is also capable of infecting plants and humans. These interactions are mediated by signaling between organisms, and it is quite possible that some of these signals are transduced via A. flavus’ GPCRs.

GPCRs are an attractive antifungal target for their lock-and-key specificity. In fact, approximately half of all drugs target GPCRs (69). Finding antagonists for GPCRs that are required for fungal invasion of a host, for example, could be a very effective way of combating infection. No single GPCR was identified here as being absolutely required for growth in culture, but perhaps certain receptors are required to grow or produce toxins in a host and/or function as heterodimers. By unraveling the functions of A. flavus’ GPCRs, new therapeutic targets may emerge for reducing the impact of this pathogen.

**MATERIALS AND METHODS**

**Culture conditions.** Strains (see Table S2 in the supplemental material) were grown on glucose minimal medium (GMM) (33) with ammonium salts instead of nitrate salts unless otherwise mentioned. In some cases, 5 mM uridine and 5 mM uracil were added, and this is denoted as “plus UU.” For genomic DNA (gDNA) extraction, strains were grown on liquid GM (NH₄⁺) with 0.5% yeast extract added.

**Strain construction.** Strain genotypes and sources are summarized in Table S2 in the supplemental material. All primers used for strain construction are listed in Table S3. GPCRs were disrupted using homologous recombination to replace each gene with pyrG in the parental strain CA14Δku70ΔpyrG (70). Table S3 indicates which primers were used to amplify the GPCR 5’ and 3’ flanks. The pyrG marker gene was amplified from A. fumigatus genomic DNA using primers 61 and 62. In all cases, the 5’ flank forward primer and 3’ flank reverse primer were used to amplify the entire double-joint cassette. CA14Δku70ΔpyrG was also complemented to prototrophy to generate an isogenic “wild type” for comparison with the other mutants. To achieve this, A. fumigatus pyrG was targeted to the nkuA locus. Primers 63 and 64 and primers 65 and 66 were used to amplify the nkuA 5’ and 3’ flanks, respectively. The entire construct was amplified with primers 63 and 66.

All of the above constructs were transformed individually into parental strain CA14Δku70ΔpyrG (70), with the exception of ΔgprR, which was also transformed into strain 3357.5 (71). Transformation of the fungus was carried out as described previously (31). At least 12 independent isolates were screened by PCR, followed by Southern analysis to confirm PCR-positive mutants. The same primers that were used to amplify the full double-joint cassette were used to amplify Southern probes from wild-type gDNA. Confirmed mutants were grown with and without UU to check for marker gene effects (24), and a single isolate of each mutant was selected for subsequent analyses.

**Bioinformatic characterization of GPCRs.** The 15 putative A. flavus GPCRs were identified by a BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search of the A. nidulans GPCRs (7) against the A. flavus genome. The topology prediction software programs TMHMM 2.0 (13, 14) and TopPred 1.10 (15, 16) were used to predict the number of TM domains for all unique hits. GPCRs were aligned using the software program Clustal Omega (22), and a phylogenetic tree was generated using Phylogeny.fr (23).

**Fungal development assays.** (i) Germination assay. Germination was assayed using a 48-well plate, with 400 μl of GMM(NH₄⁺) plus UU plus 0.1% yeast extract containing 10⁶ spores/ml dispensed per well (three replicates per strain). The plate was incubated at 29°C, and germination was measured at 4, 5, 6, 7, 8, and 9 h post inoculation. The plate was imaged on an Eclipse TS100 inverted microscope (Nikon) with an EOS Rebel T3 camera (Canon) fitted into the eyepiece. The Cell Counter tool in the ImageJ program (72) was used to count the number of germinated spores per 100 spores. A spore was counted as germinated when the germ tube extended for at least the length of the spore.

(ii) Aflatoxin analysis. Strains were grown on 10-cm-diameter plates containing 25 ml YES (2% yeast extract and 6% sucrose, pH 5.8) or YEP (2% yeast extract and 6% peptone, pH 5.8) with uridine and uracil (plus UU) and 1.5% agar. Spores (10⁶) in 1 μl of 0.1% Tween 20 were point inoculated onto the center of each plate. Three replicates per strain were placed in the dark at 30°C for 5 days. A 15-mm-diameter core was punched from each plate and homogenized in 3 ml 0.01% Tween 20.

To extract AF, 3 ml of ethyl acetate was added to each tube, and the tubes were shaken vigorously and spun at 3,000 rpm for 10 min. The organic layer was removed, dried down, and resuspended in 1 ml 30:40:10 water-methanol-acetonitrile. Samples were filtered through an Acrodisc syringe filter with a nylon membrane (0.45 μm; Pall Corporation) and run on a PerkinElmer Flexar instrument equipped with a Zorbax Eclipse XDB C₁₈ column (150 by 4.6 mm, 5 μm, 100 Å; Agilent). The column was equilibrated in the running solvent (50:40:10 water-methanol-acetonitrile), and 20 μl was injected and run isocratically for 11 min with 100% running solvent at a flow rate of 0.8 ml/min. AF was detected by a fluorescent detector with an emission wavelength of 455 nm and excitation wavelength of 365 nm.

(iii) Impact of light and dark on sporulation. Strains were grown on 10-cm plates containing 10 ml GMM(NH₄⁺) plus UU (15 g/liter agar). Five hundred spores in 1 μl 0.1% Tween 20 were point inoculated into the center of each plate, and plates were incubated for 3 days at 30°C in either constant light or constant dark. There were three replicates per strain per condition. Following incubation, a 15-mm core was punched from the center of each plate and homogenized in 3 ml 0.1% Tween 20. A 100-μl aliquot was removed and diluted, and spores were enumerated using a hemocytometer.

(iv) Density dependence assay. The impact of GPCRs on density dependence was assayed with the method of Horowitz Brown et al. (30) with some modifications. A 35.3 mM concentration of glutamine (Gln) was used as a nitrogen source since NH₄⁺ does not support the normal density-dependent development pattern. Ten-centimeter plates containing 10 ml GMM(Gln) plus UU plus 2% sorbitol (15 g/liter agar) were overlaid with 10³ (low density) or 10⁶ (high density) spores in 3 ml of the base medium containing 7.5 g/liter agar. Plates were incubated in the dark at 30°C for 6 days. Following incubation, three replicates were processed for sclerotia production, and the other three replicates were processed for spore production.

To measure sclerotia, plates were sprayed with 70% ethanol to wash off
conidia. The sclerotia were scraped from the plates, frozen in liquid nitrogen, and lyophilized. The dry weight was recorded. To measure spore production, three 15-mm cores were punched from each plate and homogenized in 3 ml 0.1% Tween 20. A 100-μl aliquot was removed, diluted, and counted on a hemocytometer.

**Growth on alternative carbon and nitrogen sources.** To assess the effects of a variety of carbon sources, strains were grown on a variety of media, the base being MM(NH₄)₂ plus UU (15 g/liter agar) as described above. The various carbon sources were added at the following concentrations: glucose, galactose, and xylene, 1%; sucrose, 0.95%. To test alternative nitrogen sources, GMM plus UU (15 g/liter agar) with nitrogen-free salts added was the base medium. Three grams per liter peptone or 8.13 g/liter proline was added, and these media were compared to GMM(NH₄)₂ plus UU (15 g/liter agar). Forty milliliters of medium was dispensed into square 10-cm by 10-cm plates and inoculated with 500 spores of each strain in 1 μl 0.1% Tween 20. Plates were incubated at 30°C for 3 days with three replicate plates per condition.

**Fungal stress assays.** Several different media were used to assess the roles of GPCRs in stress responses. The base for this media was GMM(NH₄)₂ plus UU (15 g/liter agar). The following stressors were added to the media after autoclaving: hydrogen peroxide (3 mM), Congo red (200 μg/ml), and sodium chloride (1 M). Media were also buffered to two different pHs before autoclaving: pH 4 (100 mM NaH₂PO₄ and 100 mM NaCl) and pH 8 (100 mM Na₂HPO₄). The pH stress media contained 30 g/liter agar, so they were compared to GMM(NH₄)₂ plus UU with 30 g/liter agar. Forty milliliters of medium was dispensed into square 10-cm by 10-cm plates and inoculated with 500 spores of each strain in 1 μl 0.1% Tween 20. Plates were incubated at 30°C for 3 days with three replicate plates per condition.

**Fatty acid assays. (i) Impact of methyl jasmonate on aflatoxin production.** To test the impact of methyl jasmonate (MeJA) on the ability of the GPCR mutants to synthesize aflatoxin, the method of Meimaroglou et al. (51) was followed with some modifications. One hundred spores were inoculated into 10-cm plates containing 10 ml YES (2% yeast extract and 6% sucrose, pH 5.8) plus UU liquid medium with or without 0.1 mM MeJA (Sigma-Aldrich). Three replicates per condition per strain were incubated in the dark at 30°C for 3 days. The contents of each plate were transferred to a 50-ml conical tube and homogenized.

To extract aflatoxin, 5 ml ethyl acetate was added to each tube for 15 min with periodic shaking. The mixtures were spun at 3,000 rpm for 15 min, and 3 ml of the organic layer was transferred to a glass vial, dried down, and resuspended in 1 ml 50:40:10 water-methanol-acetonitrile. The mixtures were spun at 3,000 rpm for 15 min with periodic shaking. The mixtures were spun at 3,000 rpm for 15 min with periodic shaking. The mixtures were spun at 3,000 rpm for 15 min with periodic shaking.

(ii) Impact of fatty acids on sporulation. Disk assays were carried out to measure the impact of linoleic acid and 13(S)-HpODE (Cayman Chemical) on sporulation, following the method of Calvo et al. (54) with some modifications. Ten-centimeter plates containing 10 ml YGT (0.5% yeast extract, 2% glucose, and 1 ml/liter trace elements) plus UU with 1.5% agar were overlaid with 10² spores per plate in 3 ml YGT plus UU with 0.75% agar. One-centimeter filter disks (Whatman, grade 1) were soaked with either 1 mg linoleic acid in 8 ml ethanol, 1 μl 13(S)-HpODE in 8 μl ethanol, or 8 μl ethanol by itself. The disks were placed at the center of the plates (one disk per plate), and the plates were incubated at 30°C for 3 days (linoleic acid plates) or 4 days [13(S)-HpODE] along with ethanol control plates at each time point.

Following incubation, an 11-mm core containing the disk was removed and discarded. A 23-mm ring was punched out around the removed portion and homogenized in 3 ml Tween H₂O. A 100-μl aliquot was removed and diluted, and spores were counted on a hemocytometer.

**Expression analysis.** Publicly available RNA-seq and microarray expression data for *A. flavus* under various conditions was accessed from the publication itself or from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/), depending on where the data were deposited. The GEO data, the GEO2R Web tool was used to compare different samples within an experiment. The log₂ fold change (FC) values and P values for *A. flavus*’ 15 GPCRs were collected from the various data sets.

**Statistical analysis.** Statistically significant differences were determined by using an unpaired Student’s t test with a two-tailed distribution and a P value of <0.05.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl?doi=10.1128/mBio.01501-14/-/DCSupplemental.

Figure S1, TIF file, 0.8 MB.
Figure S2, TIF file, 1.8 MB.
Figure S3, TIF file, 0.6 MB.
Figure S4, TIF file, 0.6 MB.
Figure S5, TIF file, 0.7 MB.
Table S1, TIF file, 0.3 MB.
Table S2, XLSX file, 0.1 MB.
Table S3, XLSX file, 0.04 MB.

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**REFERENCES**

1. Robens J, Cardwell K. 2003. The costs of mycotoxin management to the USA: management of aflatoxins in the United States. J. Toxicol. Toxin Rev. 22:139–152. http://dx.doi.org/10.1081/ TXR-120024089.
2. Hedayati MT, Pasqualotto AC, Warn PA, Bowyer P, Denning DW. 2007. Aspergillus flavus: human pathogen, allergen and mycotoxin producer. Microbiology 153:1677–1692. http://dx.doi.org/10.1099/ mic.0.2007/007641-0.
3. Xue C, Hsuhe YP, Heitman J. 2008. Magnificent seven: roles of G protein-coupled receptors in extracellular sensing in fungi. FEMS Microbiol. Rev. 32:1010–1032. http://dx.doi.org/10.1111/j.1574-6976.2008.00131.x.
4. Versele M, Lemaire K, Thevelein JM. 2001. Sex and sugar in yeast: two distinct GPCR systems. EMBO Rep. 2:574–579. http://dx.doi.org/10.1039/ embo-reports-kve132.
5. Brodhagen M, Keller NP. 2006. Signalling pathways connecting mycotoxin production and sporulation. Mol. Plant Pathol. 7:285–301. http://dx.doi.org/10.1111/j.1364-3703.2006.00338.x.
6. Ma D, Li R. 2013. Current understanding of HOG-MAPK pathway in *Aspergillus fumigatus*. Mycopathologia 173:13–23. http://dx.doi.org/10.1007/s11046-012-9600-5.
7. Lafon A, Han KH, See JA, Yu JH, d’Enfert C. 2006. G-protein and cAMP-mediated signaling in *aspergilli*: a genomic perspective. Fungal Genet. Biol. 43:490–502. http://dx.doi.org/10.1016/j.fgb.2006.02.001.
8. Kulkarni RD, Thon MR, Pan H, Dean RA. 2005. Novel G-protein-coupled receptor-like proteins in the plant pathogenic fungus *Magnaporthe grisea*. Genome Biol. 6:R24. http://dx.doi.org/10.1186/gb-2005-6-3-r24.
9. Xue C, Bahn YS, Cox GM, Heitman J. 2006. G-protein-coupled receptor Gpr4 senses amino acids and activates the cAMP-PKA pathway in *Cryptococcus neoformans*. Mol. Biol. Cell 17:667–679. http://dx.doi.org/10.1091/mbc.E05-07-0069.
10. Li I, Wright SJ, Krystofosva S, Park G, Borkovich KA. 2007. Heterotrimeric G protein signaling in filamentous fungi. Annu. Rev. Microbiol. 61:423–452. http://dx.doi.org/10.1146/annurev.micro.61.080706.093432.
11. Zheng H, Zhou L, Dou T, Han X, Cai Y, Zhan X, Tang C, Huang J, Wu Q. 2010. Genome-wide prediction of G protein-coupled receptors in *Verticillium* spp. Fungal Biol 114:359–368. http://dx.doi.org/10.1016/j.fimbio.2010.02.008.
12. Gruber S, Omann M, Zeilinger S. 2013. Comparative analysis of the repertoire of G protein-coupled receptors of three species of the fungal genus *Trichoderma*. BMC Microbiol. 13:108. http://dx.doi.org/10.1186/ 1471-2180-13-108.
13. Sonnhammer EL, von Heijne G, Krogh A. 1998. A hidden Markov model for predicting transmembrane helices in protein sequences, Proc. Int. Conf. Intell. Syst. Mol. Biol. 6:175–182.
14. Krogh A, Larsson B, von Heijne G, Sonnhammer EL. 2001. Predicting transmembrane protein topology with a hidden Markov model: applica-
To generate a protein kinase in a G protein signaling pathway regulating morphological and chemical transitions in *Aspergillus nidulans*. Genetics 157:591–600.

Shimizu K, Hicks JK, Huang TP, Keller NP. 2003. Pka, Ras and G protein interactions regulate activity of AflR, a Zn(II)2Cys6 transcription factor in *Aspergillus nidulans*. Genetics 165:1095–1104.

Reese TD, Low B, Beaudry RM, Keller NP, Linz JE. 2004. Regulation of allatostatin synthesis by FadA/CAMP/protein kinase A signaling in *Aspergillus parasiticus*. Mycopathologia 158:219–232. http://dx.doi.org/10.1023/B:MYCO.0000018417.16486.e6

Xue Y, Battle M, Hirsch JP. 1998. GPR1 encodes a putative G protein-coupled receptor that associates with the Gpa2p Galpha subunit and functions in a Ras-independent pathway. EMBO J. 17:1996–2007. http://dx.doi.org/10.1093/emboj/17.7.1996

Kraakman L, Lemaire K, Ma P, Teunissen AW, Donaton MC, Van Dijck P, Winderickx J, De Winde JH, Thevelein JM. 1999. A Saccharomyces cerevisiae G-protein-coupled receptor, Gpr1, is specifically required for glucose activation of the CAMP pathway during the transition to growth on glucose. Mol. Microbiol. 32:1002–1012. http://dx.doi.org/10.1046/j.1365-2958.1999.01417.x

Lemaire K, Van de Velde S, Van Dijck P, Thevelein JM. 2004. Glucose and sucrose act as agonist and mannose as antagonist ligands of the G protein-coupled receptor Gpr1 in the yeast Saccharomyces cerevisiae. Mol. Cell 16:293–299. http://dx.doi.org/10.1016/j.molcel.2004.10.004

Hoffman CS. 2005. Glucose sensing via the protein kinase A pathway in *Schizosaccharomyces pombe*. Biochem. Soc. Trans. 33:257–260. http://dx.doi.org/10.1042/BST0330257

Li Li, Borkovich KA. 2006. GPR-4 is a predicted G-protein-coupled receptor required for carbon source-dependent asexual growth and development in Neurospora crassa. Eukaryot. Cell 5:1287–1300. http://dx.doi.org/10.1128/EC.00109-06

Miwa T, Takagi Y, Shinozaki M, Yun CW, Schell WA, Perfect JR, Kumagai H, Tamaki H. 2004. Gpr1, a putative G-protein-coupled receptor, regulates morphogenesis and hypha formation in the pathogenic fungus *Candida albicans*. Eukaryot. Cell 3:919–931. http://dx.doi.org/10.1128/EC.4.919-931.2004

Maidan MM, De Rop L, Serneels J, Exler S, Rupp S, Tournu H, Thevelein JM, Van Dijck P. 2005. The G protein-coupled receptor Gpr1 and the Galphap protein Gpa2 act through the cAMP-protein kinase A pathway to induce morphogenesis in *Candida albicans*. Mol. Biol. Cell 16:1971–1986. http://dx.doi.org/10.1091/mbc.E05-04-0316

Stroscio A. 1982. Fatty acid composition and triglyceride structure of corn oil, hydrogenated corn oil, and corn oil margarine. J. Food Sci. 47:36–39. http://dx.doi.org/10.1111/j.1152-0489.1982.tb01201.x

Chung KS, Won M, Lee SB, Jang YJ, Hoe KL, Kim DU, Lee JW, Kim KW, Yoo HS. 2001. Isolation of a novel gene from *Schizosaccharomyces pombe*; stml1 encoding a seven-transmembrane loop protein that may couple with the heterotrimeric Galpha protein Gpa2. J. Biol. Chem. 276:40190–40201. http://dx.doi.org/10.1074/jbc.M10034200

Fuchs BB, Mylonakis E. 2009. Our paths might cross: the role of the fungal cell wall integrity pathway in stress response and cross talk with other stress response pathways. Eukaryot. Cell 8:1616–1625. http://dx.doi.org/10.1128/EC.00193-09

Duran R, Cary JW, Calvo AM. 2010. Role of the osmotic stress regulatory pathway in morphogenesis and secondary metabolism in filamentous fungi. Toxins (Basel) 2:367–381. http://dx.doi.org/10.3390/toxins2040367

Selvig K, Alsophaugh JA. 2011. pH response pathways in fungi: adapting to host-derived and environmental signals. Mycobiology 39:249–256. http://dx.doi.org/10.1051/myco/20113.4.249

Roncero C, Duran R. 1985. Effect of calcofluor white and Congo red on fungal cell wall morphogenesis: in vivo activation of chitin polymerization. J. Bacteriol. 163:1880–1885.

Ram AF, Klis FM. 2006. Identification of fungal cell wall mutants using susceptibility assays based on calcofluor white and Congo red. Nat. Protoc. 1:2253–2256. http://dx.doi.org/10.1038/nprot.2006.397

Christensen SA, Kolomietz MV. 2011. The lipid language of plant-fungal interactions. Fungal Genet. Biol. 48:4–14. http://dx.doi.org/10.1016/j.fgb.2010.05.005

Meimaroglou DM, Galanopoulou D, Markaki P. 2009. Study of the effect of methyl jasmonate concentration on allatostatin biosynthesis by *Aspergillus parasiticus* in yeast extract sucrose medium. Int. J. Microbiol. 2009:842626. http://dx.doi.org/10.1155/2009/842626

Goodrich-Tanrikulu M, Mahoney NE, Rodriguez SB. 1995. The plant
growth regulator methyl jasmonate inhibits aflatoxin production by 
Aspergillus flavus. Microbiology 141:2831–2837.
53. Vergopoulos S, Galanopoulou D, Markaki P. 2001. Methyl jasmonate stimulates aflatoxin B1 biosynthesis by Aspergillus parasiticus. J. Agric. Food Chem. 49:3494–3498. http://dx.doi.org/10.1021/jf010074+.
54. Calvo AM, Hinze LJ, Gardner HW, Keller NP. 1999. Sparogenetic effect of polyunsaturated fatty acids on development of Aspergillus spp. Appl. Environ. Microbiol. 65:3668–3673.
55. Georgianna DR, Fedorova ND, Burroughs JL, Dolezal AL, Bok JW, Lin JQ, Zhao XX, Wang CC, Xie Y, Li GH, He ZM. Wu X, Zhou B, Yin C, Guo Y, Lin Y, Pan L, Wang B. 2014. September/October 2014 Volume 5 Issue 5 e01501-14
56. Keller N, Butchko R, Sarr B, Phillips T. 1994. A visual pattern of mycotoxin production in maize kernels by Aspergillus spp. Phytopathology 84:483–488. http://dx.doi.org/10.1094/Phyto-84-483.
57. Wu X, Zhou B, Yin C, Guo Y, Lin Y, Pan L, Wang B. 2014. Characterization of natural antisense transcript, sclerotia development and secondary metabolism by strand-specific RNA sequencing of Aspergillus flavus. PLoS One 9:e97814. http://dx.doi.org/10.1371/journal.pone.0097814.
58. Lin JQ, Zhao XX, Wang CC, Xie Y, Li GH, He ZM. 2013. 5-Azacytidine inhibits aflatoxin biosynthesis in Aspergillus flavus. Ann. Microbiol. 63:763–769. http://dx.doi.org/10.1007/s13213-012-0531-7.
59. Lin JQ, Zhao XX, Zhi QQ, Zhao M, He ZM. 2013. Transcriptomic profiling of Aspergillus flavus in response to 5-azacytidine. Fungal Genet. Biol. 56:78–86. http://dx.doi.org/10.1016/j.fgb.2013.04.007.
60. Seo JA, Han KH, Yu JH. 2004. The gprA and gprB genes encode putative G protein-coupled receptors required for self-fertilization in Aspergillus nidulans. Mol. Microbiol. 53:1611–1623. http://dx.doi.org/10.1046/j.1365-2958.2004.04232.x.
61. Horn BW, Moore GG, Carbene I. 2009. Sexual reproduction in Aspergillus flavus. Mycologia 101:423–429. http://dx.doi.org/10.3852/09-011.
62. Borroto-Escuela DO, Brito I, Romero-Fernandez W, Di Palma M, Oflijan J, Skieterska K, Duchou J, Van Craenenbroeck K, Suarez-Boomgaard D, Rivera A, Guidolin D, Agnati LF, Fuxe K. 2014. The G protein-coupled receptor heterodimer network (GPCR-HetNet) and its hub components. Int. J. Mol. Sci. 15:8570–8590. http://dx.doi.org/10.3390/ijms1508570.
63. Spudich JL. 1998. Variations on a molecular switch: transport and sensory signalling by archaeal rhodopsins. Mol. Microbiol. 28:1051–1058. http://dx.doi.org/10.1046/j.1365-2958.1998.00859.x.
64. Bieszke JA, Braun EL, Bean LE, Kang S, Natvig DO, Borkovich KA. 1999. The nop-1 gene of Neurospora crassa encodes a seven transmembrane helix retinal-binding protein homologous to archaeal rhodopsins. Proc. Natl. Acad. Sci. U. S. A. 96:8034–8039. http://dx.doi.org/10.1073/pnas.96.14.8034.
65. Bieszke JA, Spudich EN, Scott KL, Borkovich KA, Spudich JL. 1999. A eukaryotic protein, NOP-1, binds retinal to form an archaeal rhodopsin-like photochemically reactive pigment. Biochemistry (Mosc.) 38:14138–14145.
66. Bieszke JA, Li L, Borkovich KA. 2007. The fungal opsin gene nop-1 is negatively-regulated by a component of the blue light sensing pathway and influences conidiation-specific gene expression in Neurospora crassa. Curr. Genet. 52:149–157. http://dx.doi.org/10.1007/s00294-007-0148-8.
67. Estrada AF, Avalos J. 2009. Regulation and targeted mutation of opcA, coding for the NOP-1 orthologue in Fusarium fujikuroi. J. Mol. Biol. 387:59–73. http://dx.doi.org/10.1016/j.jmb.2009.01.057.
68. Brown LS. 2004. Fungal rhodopsins and opsin-related proteins: eukaryotic homologues of bacteriorhodopsin with unknown functions. Photochem. Photobiol. Sci. 3:555–565. http://dx.doi.org/10.1039/b315527g.
69. Drews J. 2000. Drug discovery: a historical perspective. Science 287:1960–1964. http://dx.doi.org/10.1126/science.287.5460.1960.
70. Chang PK, Scharfenstein LL, Wei Q, Bhatnagar D. 2010. Development and refinement of a high-efficiency gene-targeting system for Aspergillus flavus. J. Microbiol. Methods 81:240–246. http://dx.doi.org/10.1016/j.mimet.2010.03.010.
71. He ZM, Price MS, OBrian GE, Georgianna DR, Payne GA. 2007. Improved protocols for functional analysis in the pathogenic fungus Aspergillus flavus. BMC Microbiol. 7:104. http://dx.doi.org/10.1186/1471-2180-7-104.
72. Schneider CA, Rasband WS, Elciceri KW. 2012. NIH image to ImageJ: 25 years of image analysis. Nat. Methods 9:671–675. http://dx.doi.org/10.1038/nmeth.2089.