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Upstream and Downstream Regulation of Asexual Development in *Aspergillus fumigatus*†

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The opportunistic human pathogen *Aspergillus fumigatus* produces a large quantity of asexual spores (conidia), which are the primary agent causing invasive aspergillosis in immunocompromised patients. We investigated the mechanisms controlling asexual sporulation (conidiation) in *A. fumigatus* via examining functions of four key regulators, GpaA (Gα), AfFlbA (RGS), AfFluG, and AfBrlA, previously studied in *Aspergillus nidulans*. Expression analyses of *gpaA*, Af*flbA*, Af*fluG*, Af*brlA*, and Af*wetA* throughout the life cycle of *A. fumigatus* revealed that, while transcripts of Af*flbA* and Af*fluG* accumulate constantly, the latter two downstream developmental regulators are specifically expressed during conidiation. Both loss-of-function Af*flbA* and dominant activating GpaA^{Q204L} mutations resulted in reduced conidiation with increased hyphal proliferation, indicating that GpaA signaling activates vegetative growth while inhibiting conidiation. As GpaA is the primary target of AfFlbA, the dominant interfering GpaA^{G203R} mutation suppressed reduced conidiation caused by loss of Af*flbA* function. These results corroborate the hypothesis that functions of G proteins and RGSs are conserved in aspergilli. We then examined functions of the two major developmental activators AfFluG and AfBrlA. While deletion of Af*brlA* eliminated conidiation completely, null mutation of Af*fluG* did not cause severe alterations in *A. fumigatus* sporulation in air-exposed culture, implying that, whereas the two aspergilli may have a common key downstream developmental activator, upstream mechanisms activating *brlA* may be distinct. Finally, both Af*fluG* and Af*flbA* mutants showed reduced conidiation and delayed expression of Af*brlA* in synchronized developmental induction, indicating that these upstream regulators contribute to the proper progression of conidiation.

The genus *Aspergillus* represents the most widespread fungi in the environment and includes industrially, agriculturally, and medically important species. All aspergilli reproduce in asexual mode, which involves the formation of multicellular organs termed conidiophores bearing thousands of mitotically derived asexual spores (conidia). The study of asexual development (conidiation) in the model fungus *Aspergillus nidulans* has provided important information on the mechanisms controlling growth and development (reviewed in references 3 and 4).

Conidiation in *A. nidulans* is a continual sequence from vegetative growth to asexual development. It is a precisely timed and genetically programmed event responding to internal and external cues. Previous studies demonstrated that vegetative growth signaling is primarily mediated by a heterotrimeric G protein system composed of FadA, SfaD, and GpgA (Gα, Gβ, and Gγ subunits, respectively); PhnA (a Gβγ activator); and the cyclic AMP (cAMP)-dependent protein kinases PkaA and PkaB (23, 25, 28, 29, 32, 39; reviewed in reference 43). Activation of this G protein signaling stimulates hyphal proliferation, which in turn represses conidiation and production of the mycotoxin sterigmatocystin (ST; Fig. 1A) (10, 25, 39). Constitutive activation of FadA signaling causes uncontrolled accumulation of hyphal mass and the absence of sporulation, resulting in the fluffy autolytic phenotype (39). Initiation of conidiation requires both inhibition of this G protein signaling and activation of development-specific functions. FlbA is an RGS (regulator of G protein signaling) domain protein, which plays a crucial role in antagonizing vegetative growth signaling, likely by facilitating the intrinsic GTPase activity of FadA (15, 39).

FluG is a key upstream activator of conidiation and is associated with the production of a small diffusible molecule (16). Loss-of-function *fluG* mutants form colonies exhibiting the nonsporulating fluffy phenotype (16). Our recent study showed that this FluG-dependent commencement of development in *A. nidulans* occurs via removal of the negative regulation imposed by the novel $Zn(II)_2Cys_6$ domain protein SfgA (30; reviewed in reference 44). Derepression of conidiation caused by FluG activity leads to the activation of the key downstream developmental activator *brlA* encoding a C_2H_2 zinc finger transcription factor, which activates expression of other genes required for asexual development (Fig. 1A) (1, 8; reviewed in references 4 and 44). Further genetic and biochemical studies identified two additional regulators of conidiation, *abaA* and *wetA*, that function downstream of *brlA*. The *abaA* gene encodes another developmental regulator that is activated by *brlA* during the middle stages of conidiophore development (5). The *wetA* gene functions in the late phase of conidiation for the synthesis of crucial cell wall components (19, 31). These three genes act in concert with other genes to control conidiation-specific gene expression and determine the order of gene

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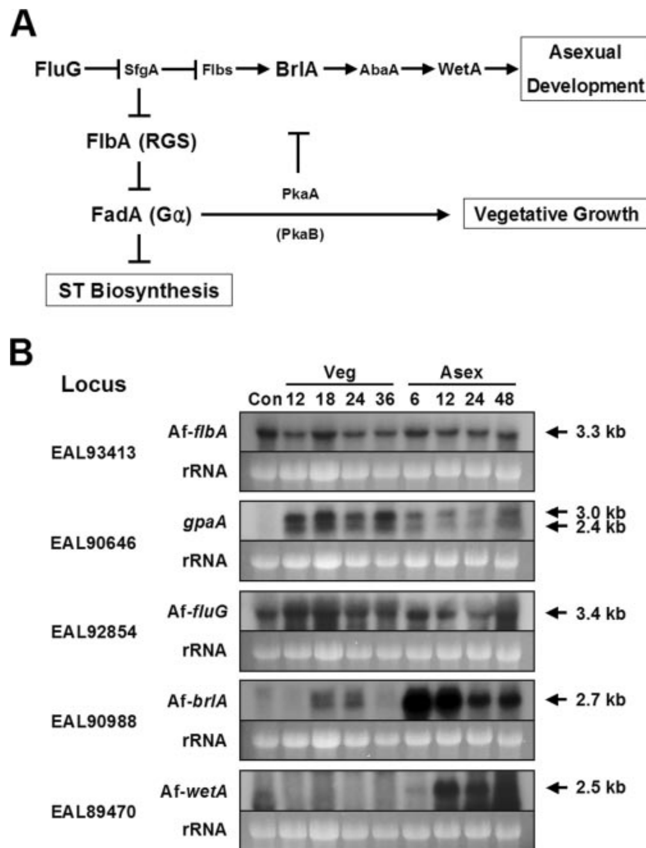


FIG. 1. Model for growth and developmental control in *A. nidulans* and expression of major regulators in *A. fumigatus*. (A) Activation of FadA-mediated vegetative signaling represses asexual development and ST production (10, 39). FlbA is an RGS protein that down-regulates FadA signaling, which in turn allows asexual development to proceed. Commencement of conidiation requires the activities of FluG and other developmental genes (reviewed in reference 4; see also reference 44). Key regulators examined in this study are shown in a larger font. (B) Northern blots showing mRNA levels of Af*flbA*, *gpaA*, Af*fluG*, Af*brlA*, and Af*wetA* throughout the life cycle including conidia (Con) of the WT strain AF293. Numbers indicate time (hours) of incubation in liquid-submerged culture (Veg) and synchronized asexual developmental induction conditions (Asex). Equal loading of total RNA was evaluated by ethidium bromide staining of rRNA.

activation during conidiophore development and spore maturation (Fig. 1A) (22, reviewed in reference 4).

The opportunistic human pathogen *Aspergillus fumigatus* is the most prevalent airborne fungal pathogen, and it causes severe and usually fatal invasive aspergillosis in immunocompromised patients (reviewed in reference 14). Moreover, the airborne *A. fumigatus* conidia are the primary agent for allergic bronchopulmonary aspergillosis, a severe pulmonary complication resulting from hypersensitivity to *A. fumigatus* proteins. Probably, among many characteristics, the ability to produce a massive number of small (2- to 3-μm-diameter) hydrophobic conidia and the thermotolerant nature of this fungus have contributed significantly to its ubiquitous presence and fitness in the environment (14). However, the mechanisms controlling asexual sporulation in *A. fumigatus* are largely unknown.

Based on the useful framework obtained from the study of *A. nidulans* development, we attempted to dissect the upstream and downstream regulatory mechanisms of conidiation in *A. fumigatus*. The facts that these two fungi are distantly related (9) and are different in the reproductive processes, i.e., *A. fumigatus* lacks a sexual cycle and produces a structurally different conidiophore (lacking metulae), led us to the hypothesis that the two aspergilli may have both conserved and distinct mechanisms controlling the conidiation process. Via comparative genome analyses, we have identified the homologues of FadA, FlbA, FluG, BrlA, and WetA in *A. fumigatus*, which are designated GpaA (17), AfFlbA, AfFluG, AfBrlA, and AfWetA, respectively. Deletion and additional genetic analyses in conjunction with expression and phenotypic studies revealed that AfFlbA and GpaA constitute the crucial G protein signaling components that coordinate vegetative growth and asexual development, implying that functions of these signaling elements are conserved in both species. Moreover, as found in *A. nidulans*, AfBrlA is essential for conidiophore formation, and AfFlbA and AfFluG are necessary for proper conidiation and Af*brlA* expression in *A. fumigatus*. However, somewhat distinct from *A. nidulans*, AfFlbA or AfFluG is not absolutely required for conidiation or activation of Af*brlA* in *A. fumigatus*. Taken together, we propose that, whereas a G protein (GpaA) signaling pathway and its regulation (AfFlbA) as well as downstream activation of conidiation are conserved in these distantly related model and pathogenic aspergilli, the imperfect fungus *A. fumigatus* has distinct and persistent mechanisms activating conidiation through AfBrlA.

MATERIALS AND METHODS

***Aspergillus* strains, growth conditions, and transformation.** *A. fumigatus* strains used in this study are listed in Table 1. Both *A. fumigatus* AF293 (wild type [WT] [7]) and AF293.1 (Af*pyrG1* [36]) strains were used as WT. Standard culture and genetic techniques for *A. nidulans* were used (12, 24). The composition of minimal medium was as follows (per liter): 10 g glucose, 6 g $NaNO_3$, 0.52 g $MgSO_4 \cdot 7H_2O$, 0.52 g KCl, 1.52 g KH_2PO_4, and 1 ml of the 1,000× trace element solution [22 g/liter $ZnSO_4 \cdot 7H_2O$, 11 g/liter H_3BO_3, 5 g/liter $MnCl_2 \cdot 4H_2O$, 5

TABLE 1. *Aspergillus* strains used in this study

Strain name	Relevant genotype	Source or reference
A. fumigatus		
AF293	Wild type	7
AF293.1	Af*pyrG1*	36
ΔAf*fluG*1	Af*pyrG1* ΔAf*fluG*::Af*pyrG*$^+$	This study
ΔAf*flbA*4	Af*pyrG1* ΔAf*flbA*::Af*pyrG*$^+$	This study
ΔAf*brlA*7	Af*pyrG1* ΔAf*brlA*::Af*pyrG*$^+$	This study
mGF01	Af*pyrG1* Af*flbA1 white1*	This study
mGF24[a]	Af*pyrG1* Af*flbA24*	This study
tJH5.01	Af*pyrG1* Af*flbA88* Af*flbA*$^+$ Af*pyrG*$^+$	This study
tJH4.02	Af*pyrG1* Af*pyrG*$^+$	This study
tJH4.04	Af*pyrG1* *gpaA*^{Q204L} Af*pyrG*$^+$	This study
tJH3.06	Af*pyrG1* Af*flbA88* Af*pyrG*$^+$	This study
tJH3.09	Af*pyrG1* Af*flbA88* *gpaA*^{G203R} Af*pyrG*$^+$	This study
tJH6.05	Af*pyrG1* Af*flbA88* *gpaA*^{G203R} Af*pyrG*$^+$	This study
A. nidulans		
FGSC26	*biA1 veA1* (wild type)	FGSC[b]
RJA4.4	*pyrG89 yA2* Δ*fluG*::*trpC veA1*	26
RJA5.9	*pyrG89* Δ*flbA*::*argB*$^+$ *pyroA4 veA1*	27
AJC11.32	*biA1 trpC801 brlA42 veA1*	11

[a] mGF24, -49, -50, -80, -88, -104, -112, -114, -115, -129, -130, -131, and -132 are likely isogenic except for the Af*flbA* mutant allele (Fig. 2C).
[b] FGSC, Fungal Genetic Stock Center.

g/liter $FeSO_4 \cdot 7H_2O$, 1.6 g/liter $CoCl_2 \cdot 5H_2O$, 1.6 g/liter $CuSO_4 \cdot 5H_2O$, 1.1 g/liter $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 50 g/liter Na_2EDTA]. The mixture was then pH adjusted to 6.5 with 1.0 N NaOH. All strains were inoculated on solid (or liquid) minimal medium with appropriate supplements (5 mM uridine and 10 mM uracil; simplified as MM) and incubated at 37°C. If needed, yeast extract (YE) was added (0.1% or 0.5% final concentration). To observe development in liquid-submerged culture, all strains were inoculated with 5×10^5 conidia/ml in 100 ml liquid MM and incubated at 250 rpm at 37°C. The mycelial aggregates of each strain were observed microscopically every 3 h starting at 18 h of growth in liquid culture. Under these experimental conditions AF293 and AF293.1 elaborated conidiophores consistently. Standard *A. nidulans* transformation techniques (21, 37) were used.

For synchronized asexual developmental induction, about 1×10^8 conidia of WT and relevant mutant strains were inoculated in 100 ml liquid MM with 0.1% YE and incubated at 37°C and 250 rpm for 18 h (0 h for developmental induction). Then, mycelia were harvested by being filtered through Miracloth (CalBiochem, California), transferred to solid MM with 0.1% YE, and further incubated at 37°C. Samples for RNA isolation were collected (conidia, 12, 18, 24, and 36 h of liquid culture and 6, 12, 24, and 48 h post-asexual developmental induction; Fig. 1B), squeeze dried, stored at −80°C, and subjected to total RNA isolation.

The Af*fluG*, Af*flbA*, and Af*brlA* null (deletion) mutants were generated by transforming AF293.1 with the individual PCR-generated deletion constructs (see below). The *gpaA*^{Q204L} mutants were generated by transformation of AF293.1 with the PCR-generated *gpaA*^{Q204L} construct along with the wild-type Af*pyrG* gene. The *gpaA*^{G203R} mutants were generated by introducing the *gpaA*^{G203R} construct and Af*pyrG*$^+$ together into AF293.1 or mGF88 (Af*flbA88*; Table 1). The relevant genotype of each mutant was confirmed by PCR amplification of the coding regions followed by restriction enzyme digestion of the amplicons.

Mutagenesis and isolation of Af*flbA* loss-of-function mutants. About 10^5 conidia of AF293.1 were inoculated on solid MM with 0.5% YE and supplements (5 mM uridine and 10 mM uracil) and incubated at 37°C for 4 days, and the conidia were collected for mutagenesis. Approximately 10^8 conidia of AF293.1 were treated with 1 μg/ml or 10 μg/ml (final concentration) of 4-nitroquinoline-1-oxide (6) for 0, 30, and 60 min, respectively, as previously described (26). The mean survival rate of a treatment with 1 μg/ml 4-nitroquinoline-1-oxide for 30 min was ~70%, and more than 110,000 survivors of this condition were screened for morphological abnormalities.

Nucleic acid isolation and manipulation. Genomic DNA isolation was carried out as previously described (41). Briefly, about 10^5 conidia of individual strains were inoculated in 2 ml liquid MM with 0.5% YE in 10-ml test tubes and incubated at 37°C for 18 h (stationary culture), and the mycelial mats were harvested and squeeze dried. Samples (0.2 to 0.5 g) were transferred to microcentrifuge tubes containing 400 μl of 0.5-mm zirconia/silica beads (BioSpec Products, Oklahoma), 500 μl of breaking buffer (2% Triton X-100, 1% sodium dodecyl sulfate, 0.1 M NaCl, 10 mM Tris-Cl [pH 8.0], 1 mM EDTA), and 500 μl of phenol-chloroform-isoamyl alcohol (25:24:1) and ground with a Mini Bead-Beater (BioSpec Products) for 2 min. The aqueous phase was separated by centrifugation, and genomic DNA was isolated as described previously (41).

Total RNA isolation and Northern blot analyses were carried out as described previously (26, 41). Total RNA was isolated from individual samples (about 0.2 g) by adding 400 μl of 0.5-mm zirconia/silica beads and 1 ml of Trizol reagent (Invitrogen, California) and grinding the mixture in a Mini Bead-Beater for 2.5 min. Subsequent RNA isolation was performed following the manufacturer's instructions. Total RNA (6 μg/lane) was separated by electrophoresis using a 1.1% agarose gel containing 6% formaldehyde. The nucleic acids were transferred to the MagnaProbe nylon membrane (0.45 μm; Osmonics, Minnesota). Probes were prepared by amplifying the coding regions of the individual genes from WT (AF293) genomic DNA. Primers are listed in Table S1 in the supplemental material. Each amplicon (1.43-kb Af*flb*A, 1.65-kb *gpaA*, 1.45-kb Af*fluG*, 1.51-kb Af*brlA*, and 1.34-kb Af*wetA*) was labeled with [^{32}P]dCTP using the Prime-a-Gene system (Promega, Madison, WI) and used as a probe for Northern blot analyses. Hybridization was carried out using modified Church buffer (1 mM EDTA, 0.25 M $Na_2HPO_4 \cdot 7H_2O$, 1% hydrolyzed casein, 7% sodium dodecyl sulfate; adjusted to pH 7.4 with 85% H_3PO_4) as previously described (38).

Deletion constructs of Af*fluG*, Af*flbA*, and Af*brlA* were generated employing the double-joint PCR method (41). OKH237 and OKH238, OKH243 and OKH244, and OJH75 and OJH88 primer pairs (see Table S1 in the supplemental material) were used to amplify the 5′-flanking regions (~1 kb) of Af*fluG*, Af*flbA*, and Af*brlA*, respectively. The 3′-flanking regions (~1 kb) of the individual genes were amplified with OKH239 and OKH240 (Af*fluG*), OKH245 and OKH246 (Af*flbA*), and OJH89 and OJH78 (Af*brlA*). Primers away from the open reading frames (ORFs) contained 22 bases of homologous sequences overlapping with the ends of Af*pyrG*$^+$. The selective marker Af*pyrG*$^+$ was amplified with OKH235 and OKH236 (for ΔAf*fluG* and ΔAf*flbA*) or OJH84 and OJH85 (for ΔAf*brlA*). Three amplicons were mixed in a 1:2:1 ratio, and the second round of PCR was carried out (41). Using the second-round PCR products as templates, the final deletion constructs were generated with nested primer pairs OKH241 and OKH242 (ΔAf*fluG*), OKH247 and OKH248 (ΔAf*flbA*), and OJH79 and OJH80 (ΔAf*brlA*) and used for transformation of AF293.1.

For sequencing analyses of the Af*flbA* mutant alleles, the Af*flbA* coding regions from 14 Af*flbA*$^-$ mutants were amplified by PCR using OJH68 and OJH42 (for the N-terminal region) and OJH43 and OKH246 (for the C-terminal region). The *gpaA* coding region was amplified with OJH12 and OJH17. The resulting amplicons were sequenced directly.

The *gpaA*^{Q204L} dominant activating mutant allele was generated by site-directed mutagenesis with oligonucleotides OJH69 (paired with OJH12 for 5′-flanking region) and OJH70 (paired with OJH17 for 3′-flanking region). These oligonucleotides introduced a BglII site for screening convenience. The *gpaA*^{G203R} dominant interfering mutant allele was generated with OJH71 (paired with OJH12 for 5′-flanking region) and OJH72 (paired with OJH17 for 3′-flanking region), which introduced a NaeI site. The resulting amplicons were joined as described previously (41), and each construct was confirmed by digestion with BglII and NaeI. The final *gpaA*^{Q204L} and *gpaA*^{G203R} amplicons were cointroduced with Af*pyrG*$^+$ to AF293.1 (and Af*flbA88* for *gpaA*^{G203R}). Transformants were screened for the presence of the mutant (*gpaA*^{Q204L} or *gpaA*^{G203R}) allele by PCR followed by restriction enzyme digestion.

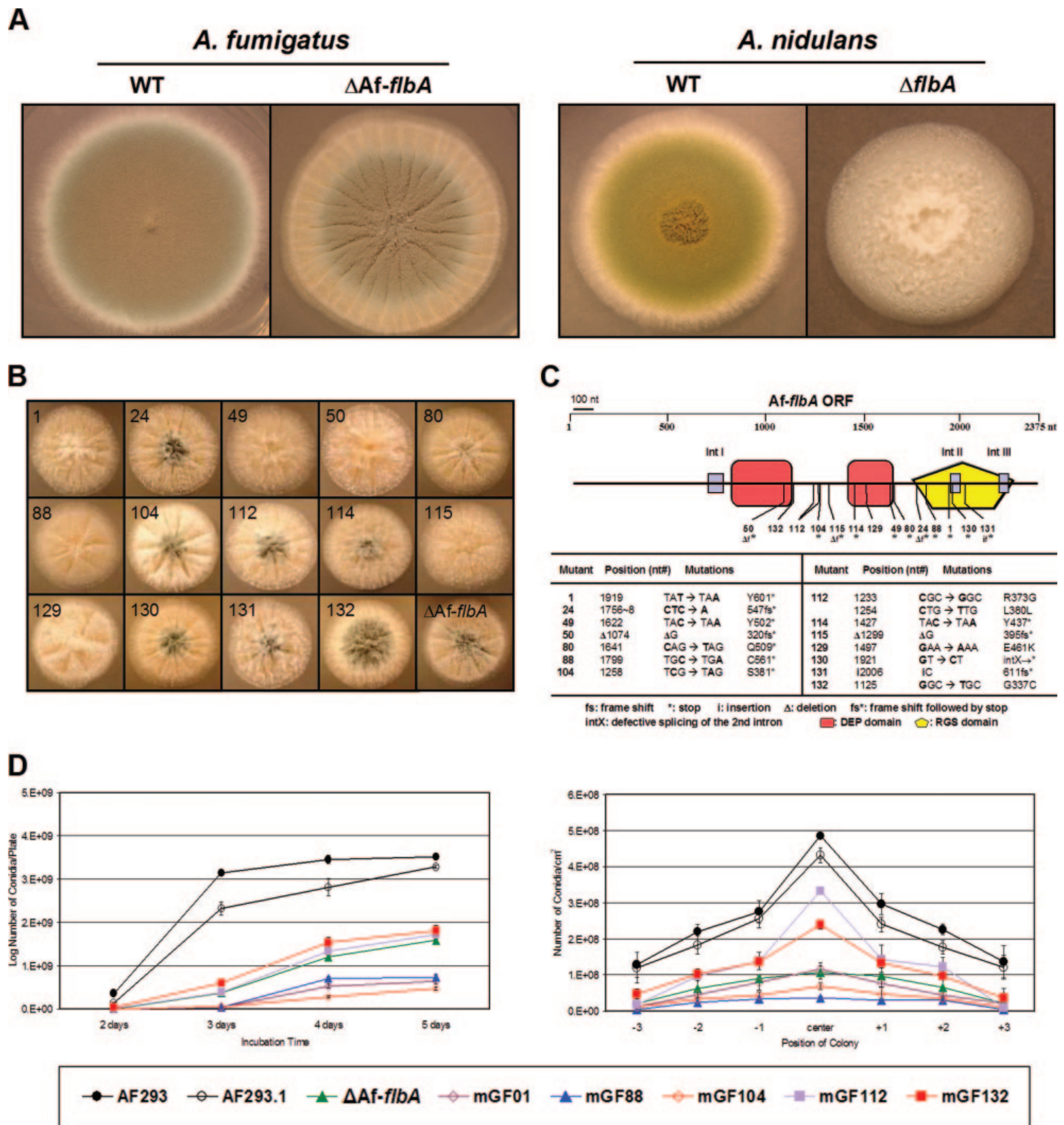
Quantitative analyses of conidiation levels. The numbers of conidia in various strains were determined in two ways: incubation time and the age of the colony section (distance from the center of the colony). In both cases, WT and relevant mutant strains were point inoculated and grown on solid MM with 0.5% YE at 37°C. The conidia were collected in 0.01% Tween 20 at 2, 3, 4, and 5 days of incubation from the entire plate (colony) and counted. To measure spores in the different regions of the colony grown for 5 days, conidia were collected from the center, middle, and edge of the colonies and counted using a hemocytometer.

Microscopy. The colony photographs were taken using a Sony DSC-F828 digital camera. Photomicrographs were taken using an Olympus BH2 compound microscope installed with an Olympus DP-70 digital imaging system.

RESULTS

Identification and expression of key developmental regulators in *A. fumigatus*. To begin to understand the regulatory mechanisms of growth and development in *A. fumigatus*, we first identified the *A. fumigatus* homologues of the five key *A. nidulans* regulators (Fig. 1A). Blastp analyses of the *A. fumigatus* genome (TIGR [http://www.tigr.org/tdb/e2k1/afu1/]) using the *A. nidulans* FadA, FlbA, FluG, BrlA, and WetA proteins as queries have identified GpaA (97% identity, 98% similarity with FadA in *A. nidulans* [17]), AfFlbA (79% identity, 85% similarity), AfFluG (69% identity, 83% similarity), AfBrlA (68% identity, 77% similarity), and AfWetA (56% identity, 66% similarity). The locus numbers are presented in Fig. 1B.

To check whether these genes are expressed, we examined the mRNA levels of individual genes throughout the life cycle of an *A. fumigatus* WT strain (AF293) by Northern blot analyses. As shown in Fig. 1B, the Af*flbA* and Af*fluG* genes were found to encode 3.3-kb and 3.4-kb transcripts, respectively, which were present at relatively constant levels throughout the life cycle. Hybridization with the *gpaA* probe resulted in the detection of two (~3.0-kb and 2.4-kb) transcripts, whose levels are high in vegetative growth, low in conidiation, and absent in conidia. While it can be speculated that the *gpaA* gene may encode two transcripts, a potential cross-hybridization cannot be excluded due to the high nucleotide level identity between *gpaA* and *gpaB* (EAL90625; 63% identity) or Af*ganA* (EAL92343; 61% identity). The Af*brlA* and Af*wetA* genes were specifically expressed during conidiation. The Af*brlA* transcript reached the highest level at 6 h



Mutant	Position (nt#)	Mutations	
1	1919	TAT → TAA	Y601*
24	1756~8	CTC → A	547fs*
49	1622	TAC → TAA	Y502*
50	Δ1074	ΔG	320fs*
80	1641	CAG → TAG	Q509*
88	1799	TGC → TGA	C561*
104	1258	TCG → TAG	S381*
112	1233	CGC → GGC	R373G
	1254	CTG → TTG	L380L
114	1427	TAC → TAA	Y437*
115	Δ1299	ΔG	395fs*
129	1497	GAA → AAA	E461K
130	1921	GT → CT	intX→*
131	i2006	iC	611fs*
132	1125	GGC → TGC	G337C

FIG. 2. Phenotypes of various Af*flbA* mutants. (A) WT (AF293 and FGSC26) and Δ*flbA* (ΔAf*flbA4* and RJA5.9) strains of *A. fumigatus* and *A. nidulans* were point inoculated on solid MM with 0.5% YE and incubated for 3 days at 37°C. Note the differences between the *A. nidulans* and *A. fumigatus flbA* mutant phenotypes. (B) Fourteen Af*flbA* mutant strains and the Δ*flbA* mutant were point inoculated on solid MM with 0.5% YE and incubated for 2 days at 37°C. Numbers indicate mutant strains and alleles. (C) The Af*flbA* ORF composed of 2,375 nucleotides (nt) (including three introns, IntI, IntII, and IntIII) and the approximate position of each Af*flbA* mutation are schematically presented. In the table, the allele number, position, and nature of the nucleotide and resulting amino acid change(s) for each Af*flbA* mutant allele are presented. (D) Two graphs present the results of quantitative analyses of conidiation in WT (AF293 and AF293.1) and selected Af*flbA* mutant strains inoculated on solid MM with 0.5% YE. Error bars indicate standard deviations calculated from biological triplicates. Note that mutations in Af*flbA* result in a reduced number of conidia in both cases.

and then decreased, whereas the Af*wetA* mRNA began to accumulate at 12 h post-developmental induction and continued to accumulate. In accordance with the occurrence of conidiophore formation in WT, Af*brlA* transcripts (reviewed in reference 4) were clearly visible at 18 and 24 h of liquid MM-submerged culture conditions (see below).

Deletion and 14 additional loss-of-function Af*flbA* mutations cause reduced conidiation. We first attempted to dissect

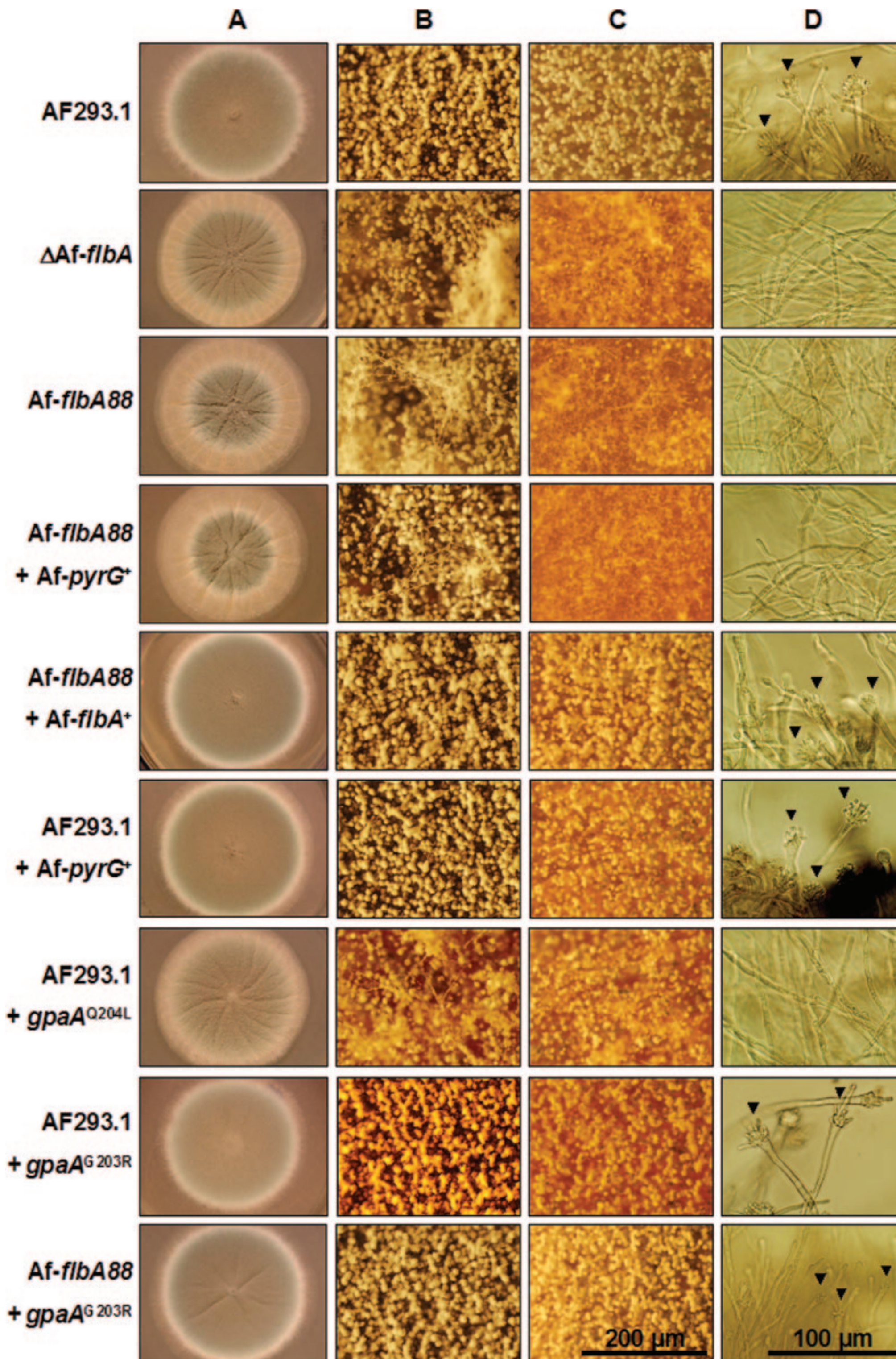


FIG. 3. GpaA is the cognate Gα for AfFlbA. WT (AF293.1 and AF293.1 with Af*pyrG*$^+$), ΔAf*flbA*, Af*flbA88*, Af*flbA88* with Af*pyrG*$^+$ alone, Af*flbA88* with Af*flbA*$^+$, AF293.1 with *gpaA*^{Q204L}, AF293.1 with *gpaA*^{G203R}, and Af*flbA88* with *gpaA*^{G203R} strains were point inoculated on solid MM with 0.5% YE (A, B, and C) and incubated for 3 days at 37°C. (A to C) Entire colonies (A) and close-up views of the center (B) and the edges (C) of individual colonies (bar, 200 μm). (D) Developmental status of tested strains in liquid-submerged culture (MM) was photographed at 24 h of incubation. Note that, while WT (AF293.1 and AF293.1 with Af*pyrG*$^+$), Af*flbA88* with Af*flbA*$^+$, AF293.1 with *gpaA*^{G203R}, and Af*flbA88* with *gpaA*^{G203R} strains form conidiophores (marked by arrowheads), ΔAf*flbA*, Af*flbA88*, Af*flbA88* with Af*pyrG*$^+$ alone, and AF293.1 with *gpaA*^{Q204L} strains do not sporulate (bar, 100 μm).

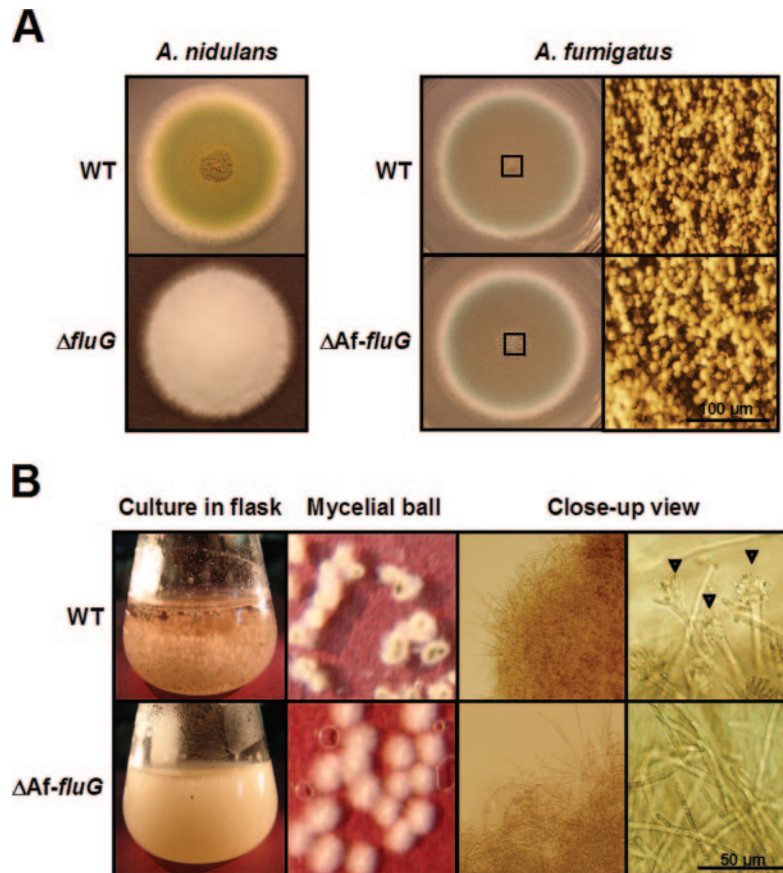


FIG. 4. Phenotypes of the ΔAf*fluG* mutant. (A) WT (FGSC26 and AF293) and Δ*fluG* (RJA4.4 and ΔAf*fluG1*) strains of *A. nidulans* and *A. fumigatus* were point inoculated on solid MM with 0.5% YE and incubated at 37°C for 3 days. Note that deletion of *fluG* in *A. nidulans* caused the fluffy nonconidial phenotype, whereas deletion of Af*fluG* in *A. fumigatus* did not affect conidiation severely (bar, 100 μm). The rightmost panel shows the close-up views of the centers of the colonies (squares). (B) Morphology of the mycelial aggregates and developmental status in liquid MM-submerged culture were photographed at 24 h of incubation. Note that the ΔAf*fluG* mutant forms less compact mycelial aggregates and does not sporulate (bar, 50 μm). The arrowheads indicate conidiophores elaborated by the wild type.

the role of AfFlbA in developmental regulation by generating the ΔAf*flbA* mutant. Multiple ΔAf*flbA* mutants were isolated and examined for phenotypic changes. Similar to the *A. nidulans* Δ*flbA* mutant, the ΔAf*flbA* mutant exhibited the fluffy phenotype during the first 2 days of growth. In contrast, while the *A. nidulans* Δ*flbA* mutant continues to accumulate hyphal mass without development, resulting in autolysis of the colony (15), the ΔAf*flbA* mutant started to produce conidiophores from the center of the colony and did not undergo hyphal disintegration (Fig. 2A). However, the levels of conidiation (and spore pigmentation) in the ΔAf*flbA* mutant were dramatically reduced (~30% of WT), indicating that AfFlbA is necessary for the normal levels of conidiation in *A. fumigatus*, but the fungus can overcome the developmental defect caused by the lack of a key RGS protein.

To further confirm that reduced conidiation is due to loss of Af*flbA* function, we isolated 14 mutants exhibiting phenotypes similar to those of the ΔAf*flbA* mutant via random chemical mutagenesis (see Materials and Methods). These mutants showed various levels of enhanced vegetative growth (at the early phase of growth) with reduced sporulation (Fig. 2B). To test whether such phenotypic changes were related to loss of Af*flbA* function, the Af*flbA* coding regions from all 14 mutants were PCR amplified and the individual amplicons were directly sequenced. As presented in Fig. 2C, sequence analyses revealed that all 14 mutants had mutations within the Af*flbA* ORF.

Among 14 mutants, mGF104, mGF114, mGF49, mGF80, mGF88, and mGF1 are derived from nonsense mutations and exhibit phenotypes similar to those of the Af*flbA* deletion mutant. Three mutants that have missense mutations prior to the RGS domain show various degrees of sporulation. mGF129 has a G-to-A transition, causing a mutation of the 461st amino acid Glu (acidic) to Lys (basic), which likely abolishes AfFlbA function. mGF112 has both missense (Arg373Gly) and silent (Leu380Leu) mutations, where the R373G substitution is likely responsible for the mutant phenotype. Sporulation of mGF132 that had the G337C substitution was affected less severely than that of other mutants. mGF24, mGF50, and mGF115 are all derived from deletion followed by frameshift and early termination within or before the RGS domain. The mGF131 mutant has a C insertion followed by frameshift and early termination. mGF130 is derived from a GT-to-CT transversion, which likely blocks the splicing of the second introns, resulting in frameshift and early termination.

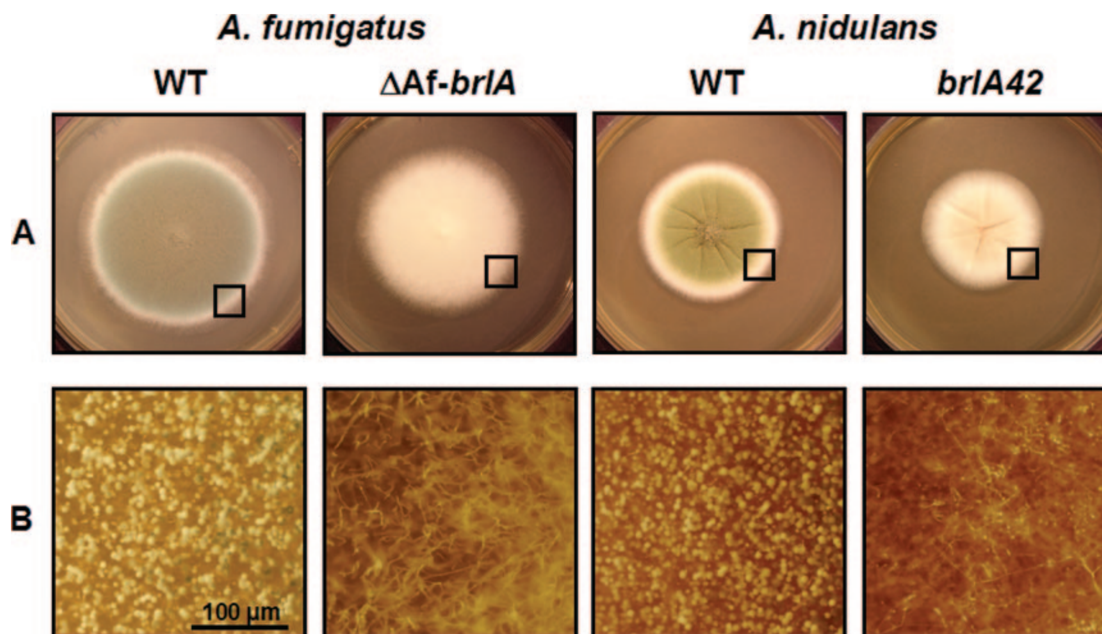


FIG. 5. Requirement of Af*brlA* in *A. fumigatus* conidiation. (A) WT (AF293 and FGSC26) and *brlA* mutant (ΔAf*brlA*7 and AJC11.32) strains of *A. fumigatus* and *A. nidulans* were point inoculated on solid MM with 0.5% YE and incubated at 37°C for 3 days. (B) Close-up views of the edges of the colonies (marked by squares; bar, 100 μm). Note that the Af*brlA* deletion mutant fails to produce conidiophores.

In order to confirm whether the mutations in Af*flbA* are solely responsible for the phenotype, mGF01, mGF88, and mGF104 were transformed with the wild-type Af*flbA* gene and Af*pyrG*$^+$, and 40%, 54%, and 22% of the transformants in each case restored the WT phenotype (Fig. 3 shows results for Af*flbA88* [mGF88] and Af*flbA88* with Af*flbA*$^+$). Interestingly, introduction of Af*flbA*$^+$ into mGF01, which produces white conidia, restored conidiation, but not spore pigmentation, to the WT level, indicating that mGF01 may have two mutations (Af*flbA1* and *white1*; Table 1).

Due to the evident reduction in conidiation levels of various Af*flbA*$^-$ mutants, quantitative analyses of spore formation were carried out by measuring the number of conidia produced by WT, ΔAf*flbA*, and five selected Af*flbA*$^-$ strains. This was accomplished in two ways: (i) counting conidia from the entire point-inoculated colony grown for 2 to 5 days and (ii) counting conidia from the center (old region), middle, and edge (actively growing region) of the 5-day-old colony. In both cases, all Af*flbA*$^-$ mutants tested exhibited reduced (10 to 70% of WT) levels of conidiation (Fig. 2D). Moreover, unlike WT, Af*flbA*$^-$ mutants did not produce conidiophores in liquid-submerged culture (Fig. 3D). These results indicate that, while it is not absolutely required for conidiation, AfFlbA is needed for proper asexual sporulation in *A. fumigatus*.

GpaA is the primary target of AfFlbA. High (97%) identity between GpaA (17) and FadA led us to hypothesize that GpgA is the primary target of AfFlbA and that uncontrolled activation of GpaA-mediated signaling causes reduced conidiation. We tested this hypothesis by generating the constitutively active (Q204L) and dominant interfering (G203R) mutant GpaA alleles (references 39 and 40 and references therein) and examining the phenotypic changes caused by these mutations. If AfFlbA regulates GpaA negatively, GpaA^{Q204L} should cause the phenotypic alterations similar to those resulting from loss of Af*flbA* function and GpaA^{G203R} should suppress the altered sporulation caused by Af*flbA* mutations in a dominant manner. Keeping this in mind, we first generated *gpaA*^{Q204L} and *gpaA*^{G203R} mutant strains that are heterozygous for *gpaA* by cointroducing each construct with Af*pyrG*$^+$ into a WT strain (AF293.1, *pyrG1*). To generate the Af*flbA*$^-$ *gpaA*^{G203R} double mutant, mGF88 (Af*flbA88*; *pyrG1*) was transformed with the *gpaA*^{G203R} construct and Af*pyrG*$^+$ or with Af*pyrG*$^+$ alone. As shown in Fig. 3, introduction of the *gpaA*^{Q204L} allele into AF293.1 yielded the colonies exhibiting reduced conidiation and the absence of conidiophore formation in liquid-submerged culture as observed in Af*flbA*$^-$ mutants. Moreover, somewhat similarly to those found in *A. nidulans* (39), the *gpaA*^{G203R} mutants showed reduced radial growth with normal conidiation levels. Importantly, the introduction of the *gpaA*^{G203R} mutant allele into the Af*flbA88* mutant restored conidiation in both air-exposed and liquid-submerged culture conditions (Fig. 3, bottommost panel). Collectively, these results corroborate the hypothesis that GpaA is the cognate Gα for AfFlbA and that GpaA signaling stimulates hyphal growth while inhibiting asexual sporulation in *A. fumigatus*.

A potential role of Af*fluG* in sporulation. As the upstream developmental activator FluG is required for the commencement of conidiation in *A. nidulans*, the Δ*fluG* mutant exhibits the nonconidial fluffy (but not autolytic) phenotype (Fig. 4A) (16). To test whether the *A. fumigatus* FluG homologue is needed for conidiation in *A. fumigatus*, the Af*fluG* deletion mutant was generated. Somewhat unexpectedly, the Af*fluG* deletion mutant could sporulate normally like WT but formed slightly increased levels of aerial hyphae in air-exposed culture (solid medium) conditions (Fig. 4A), indicating that activation of *A. fumigatus* conidiation in the presence of air does not

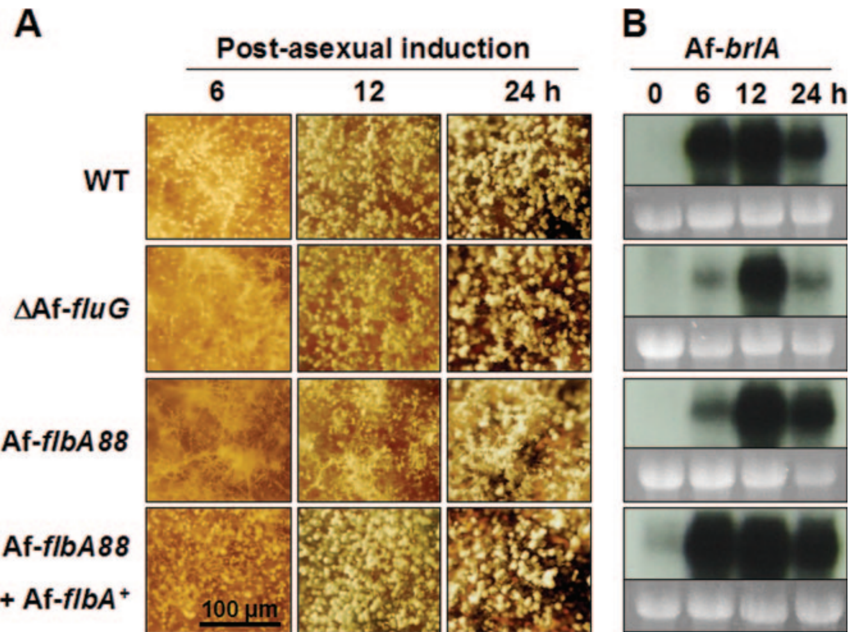


FIG. 6. Af*fluG* and Af*flbA* are required for proper activation of Af*brlA*. (A) Progression of conidiation during synchronous asexual developmental induction is shown (bar, 100 μm). Numbers indicate time (hours) of post-asexual developmental induction. Note that Af*fluG* and Af*flbA* mutant strains exhibit enhanced formation of aerial hyphae and reduced (and delayed) sporulation. (B) Corresponding Northern blots of the samples shown in panel A (0 h = 18 h in culture submerged in liquid MM with 0.1% YE). Equal loading of total RNA was evaluated by ethidium bromide staining of rRNA. Note that mutational inactivation of Af*fluG* or Af*flbA* causes delayed and reduced Af*brlA* mRNA accumulation. The Af*flbA88* with Af*flbA*$^+$ strain shows slightly elevated Af*brlA* expression levels, which is likely due to the presence of two copies of Af*flbA*$^+$ (data not shown).

require the activity of Af*fluG*. However, while *A. fumigatus* WT strains sporulated within 24 h in liquid-submerged culture, the Af*fluG* deletion mutant never produced conidiophores up to 32 h. Moreover, the Af*fluG* deletion mutant formed less compact mycelial aggregates (Fig. 4B). Taken together, it can be speculated that, while AfFluG may play a certain role in conidiation, the presence of air can bypass the need for AfFluG in conidiophore development in *A. fumigatus*. A potential role of AfFluG in conidiation was further tested by examining the expression of Af*brlA* (see below).

AfBrlA is required for conidiophore formation. The result suggesting that *A. fumigatus* may have a distinct upstream regulatory mechanism(s) for the activation of sporulation led us to test whether downstream regulation of conidiation by BrlA is divergent in the two aspergilli. To test this, multiple Af*brlA* deletion mutant strains were generated. As shown in Fig. 5, deletion of Af*brlA* completely eliminated asexual development in *A. fumigatus*, resulting in the colonies displaying elongated aerial hyphae and increased hyphal mass. These characteristics of the ΔAf*brlA* mutant are more similar to those of the *A. nidulans* fluffy mutants than the *A. nidulans brlA* mutants, which form flat colonies. In any case, it is clear that Af*brlA* is essential for the formation of conidiophores in *A. fumigatus* and that the role of the key downstream developmental activator BrlA in asexual development is conserved in the two aspergilli.

AfFluG and AfFlbA are required for proper expression of Af*brlA*. In *A. nidulans*, both *fluG* and *flbA* are required for the expression of *brlA* (15, 16). The facts that Af*brlA* is necessary for conidiophore formation and that accumulation of its transcript(s) is specifically coupled with conidiation (Fig. 1B) led us to test whether AfFlbA and AfFluG affect Af*brlA* expression. Synchronous developmental induction of WT (AF293), ΔAf*fluG*, Af*flbA88*, and Af*flbA88* with Af*flbA*$^+$ strains was carried out, and changes in the development and *brlA* expression patterns were examined. We found that the ΔAf*fluG* and Af*flbA88* mutants, but not WT or Af*flbA88* with Af*flbA*$^+$, exhibited delayed conidiation with increased aerial hyphae during the early phase (6 to 12 h) of post-asexual developmental induction (Fig. 6A). Furthermore, while WT and Af*flbA88* with Af*flbA*$^+$ strains accumulated high levels of Af*brlA* transcript at 6 h post-developmental induction, the ΔAf*fluG* and Af*flbA88* mutants showed the highest level of Af*brlA* accumulation at 12 h (Fig. 6B). These results indicate that AfFluG and AfFlbA (at least partially) function in conidiation by influencing expression of Af*brlA*. However, in contrast to *A. nidulans*, these two upstream regulators are not absolutely required for activation of Af*brlA* expression or conidiation in *A. fumigatus*.

DISCUSSION

The ease of genetic analyses and the availability of various experimental tools have made *A. nidulans* an excellent model system for studying signal transduction, multicellular development, and secondary metabolism (20, 34, 42, 43). While the study of developmental regulation in *A. nidulans* has provided valuable information, the potential use of such knowledge in dissecting the mechanisms controlling growth and development in other aspergilli remained to be tested. In this study, we examined the roles of the four key *A. nidulans* regulators in

controlling development of the pathogenic fungus *A. fumigatus* and demonstrated that these two *Aspergillus* species have conserved Gα-RGS signaling components and a core downstream activator of sporulation, but *A. fumigatus* may have distinct upstream mechanisms activating Af*brlA*.

We previously showed that the *A. nidulans* RGS protein FlbA (*fl*uffy *l*ow *brlA* locus *A*) has a major role in determining the balance between vegetative growth and development through its ability to down-regulate FadA (39). When FadA-dependent signaling is activated in response to some unknown factor, it stimulates growth and blocks both asexual and sexual development. Attenuation of FadA-mediated vegetative growth signaling by FlbA allows development to occur. Inactivation of FlbA or constitutive activation of FadA, e.g., by the G42R, R178L, G183S, R178C, or Q204L mutation predicted to cause reduced (or lack of) intrinsic GTPase activity of FadA, results in uncontrolled FadA signaling and leads to proliferation of undifferentiated aerial hyphae that autolyze as colonies mature (35, 39, 40). In contrast, overexpression of *flbA* or a dominant interfering mutation in FadA (G203R) results in inhibited hyphal growth coupled with hyperactive conidiation (15, 39). The *flbA* loss-of-function or dominant activating *fadA* mutations result in the fluffy-autolytic phenotype regardless of the *veA1* or veA^{+} alleles (our unpublished data). VeA is a novel multifunctional protein balancing sexual and asexual development in *A. nidulans* and influencing production of pigments and secondary metabolite in other aspergilli (see reference 42 and references therein).

Due to such a critical function of FlbA in *A. nidulans*, we first investigated the role of the FlbA homologue AfFlbA in *A. fumigatus*. Deletion and 14 other loss-of-function Af*flbA* mutations resulted in reduced levels of conidiation and conidial pigmentation. Furthermore, it also caused increased hyphal proliferation during the early period of growth (up to 2 days), and the mutant colonies exhibited an expanded growing edge with delayed conidiation, while WT colonies showed vigorous production of conidiophores (Fig. 3A and C). In addition, the ΔAf*flbA* or Af*flbA88* mutants did not produce conidiophores in liquid-submerged culture conditions, whereas WT and Af*flbA* complemented (Af*flbA88* with Af*flbA*$^{+}$) strains elaborated conidiophores abundantly. Collectively, these findings suggest that AfFlbA functions in down-regulation of hyphal proliferation and (indirect) activation of development in *A. fumigatus*, too. However, there is a noticeable difference between the phenotypes of the *A. nidulans* Δ*flbA* and ΔAf*flbA* mutants, where the latter never undergoes autolysis. Such a difference can be explained by a speculation that *A. fumigatus* has multiple mechanisms activating development, which bypass the requirement of AfFlbA in sporulation and allow the Af*flbA* mutants to escape hyphal disintegration. It is important to note that the *A. nidulans* Δ*flbA* mutant cannot proceed to development. This speculation is further studied by examining the role of AfFluG (see below).

As the FadA homologue GpaA is the primary target of AfFlbA, the constitutively active $GpaA^{Q204L}$ mutation caused increased hyphal proliferation and reduced sporulation in a dominant manner. Moreover, the dominant interfering $GpaA^{G203R}$ mutation restored conidiation in the Af*flbA88* mutant to the WT level in both air-exposed and liquid-submerged culture conditions (Fig. 3). The G203R mutation is predicted to block the conformational change in the switch II region of GpaA and thereby prevent dissociation of GTP-Gα from Gβγ (39, 40). These results indicate that inactivation of GpaA signaling circumvents the need for AfFlbA in proper progression of conidiation and corroborate the idea that GpaA and AfFlbA constitute a Gα-RGS pair, which functions as a major coordinator of growth and development in *A. fumigatus*. Interestingly, the levels of *gpaA* mRNA(s) appeared to be low during asexual development and absent in conidia in comparison to those observed during vegetative growth. If this is a part of the means by which the level of GpaA signaling is controlled, it indicates that the two aspergilli may have different regulatory mechanisms, because both the mRNA and the protein levels of *fadA* were relatively constant throughout the life cycle of *A. nidulans* (39).

In *A. nidulans*, both the FadA and GanB (another Gα) signaling pathways are involved in activation of cAMP-dependent protein kinase A (PKA [13, 32]). Two PKA catalytic subunits, PkaA and PkaB, have been shown to stimulate vegetative growth, where PkaA plays a primary role (23, 32). Deletion of *pkaA* resulted in hyperactive conidiation with restricted vegetative growth and suppressed developmental defects caused by Δ*flbA* as well as the dominant activating $fadA^{G42R}$ mutation. Furthermore, overexpression of *pkaA* led to reduced sporulation with elevated hyphal proliferation (32). Later, PkaA was also shown to function downstream of GanB for conidial germination (13). Similarly, in *A. fumigatus*, GpaB (GanB homologue)-mediated signaling is associated with activation of the predominant PKA catalytic subunit PkaC1 (18). However, one critical difference between two fungi is that, while deletion of *pkaC1* also resulted in restricted hyphal growth, it caused drastically reduced sporulation (18). These findings indicate that, whereas the Gα-RGS level regulatory mechanism is conserved, asexual development is regulated differently at the level of PKA in the two aspergilli. The potential involvement of PkaC1 in the GpaA signaling pathway remains to be investigated.

The study of asexual development in *A. nidulans* has identified a number of genes required for the activation of conidiation, where FluG functions most upstream (reviewed in reference 4; see also reference 44). The FluG-dependent sporulation requires the key downstream transcription factor BrlA, a C_2H_2 zinc finger DNA-binding protein, which activates development-specific gene expression beginning at the time of conidiophore vesicle formation (1, 2). Since FluG and BrlA represent key upstream and downstream activators of conidiation, we attempted to characterize the homologues of these regulators in *A. fumigatus*. Expression patterns of Af*fluG* and Af*brlA* were almost identical to those found in *A. nidulans* (reviewed in reference 4). However, mRNA of Af*brlA* accumulates highly at 6 h post-developmental induction, whereas it takes about 12 h for the *A. nidulans brlA* mRNA to reach the same level (Fig. 1B) (30). Moreover, Af*brlA* mRNA(s) started to accumulate as early as 18 h of vegetative growth in liquid-submerged culture conditions, at which time no *A. nidulans brlA* mRNA is clearly detectable. These results are in agreement with our observations that *A. fumigatus* WT strains sporulate in liquid-submerged culture and produce conidiophores much faster than *A. nidulans* does under synchronous developmental induction conditions (not shown).

Deletion of Af*brlA* completely eliminated conidiation in all conditions tested, indicating that the activation of Af*brlA* expression early in conidiophore development also represents a foremost and essential control point for initiating the conidiation pathway in *A. fumigatus* and that the two aspergilli have a common core downstream activator for conidiophore development. However, somewhat unexpectedly, AfFluG is found to be dispensable for conidiation in the presence of air, which is in contrast to the necessity for FluG in *A. nidulans* conidiation. On the other hand, we also found that AfFluG (at least partially) contributes to the commencement of development under different culture conditions, i.e., liquid MM-submerged culture and synchronous developmental induction, through affecting expression of Af*brlA* (Fig. 4 and 6). We also demonstrated that AfFlbA is necessary for the proper expression of Af*brlA* and thereby progression of conidiation. Collectively, our phenotypic, expression, and genetic studies all suggest that the pathogenic fungus *A. fumigatus* may have more than one mechanism activating Af*brlA* and unique and powerful strategies for its asexual reproduction. In *A. nidulans*, both inhibition of G protein-mediated growth signaling by FlbA and activation of developmental functions by FluG must occur in order for the development to proceed (reviewed in reference 4). Thus, together with the fact that both AfFluG and AfFlbA are required for proper expression of Af*brlA*, it will be interesting to test whether removal of both Af*flbA* and Af*fluG* functions would cause additive detrimental effects on development of *A. fumigatus*.

Regarding a possible FluG-independent developmental activation branch, it is noteworthy that the newly identified *A. nidulans tmpA* gene regulates conidiation independently of the FluG pathway (33). The TmpA protein belongs to a novel family of putative membrane flavoproteins that may be involved in the synthesis of a (different) developmental signal. The absence of *tmpA* resulted in decreased *brlA* expression and conidiation on solid medium, and overexpression of *tmpA* tagged alleles caused conidiation in liquid-submerged culture. Three lines of evidence indicate that TmpA and FluG regulate conidiation through independent pathways: (i) conidiation of the Δ*tmpA* mutant could be restored by juxtaposed growth with WT or the Δ*fluG* mutant, (ii) overexpression of *fluG* induced conidiation independently of *tmpA*, and (iii) the Δ*tmpA* Δ*fluG* double mutants exhibited an additive fluffy phenotype (33). If the homologue of TmpA plays a similar role in *A. fumigatus* conidiation, it can be speculated that the presence of either AfFluG or AfTmpA function alone may be sufficient to confer the progression of conidiation in *A. fumigatus*.

Finally, based on our findings, we present a genetic model for regulation of asexual development and vegetative growth in *A. fumigatus* (Fig. 7). In this model, similar to the one proposed for *A. nidulans*, AfFlbA functions as the major negative regulator of GpaA-mediated signaling that stimulates vegetative growth, which in turn inhibits sporulation. The GpaB-PkaC signaling pathway has been proposed to induce both hyphal growth and conidiation (18). AfBrlA is essential for the activation of conidiophore formation, and its expression is influenced in part by AfFluG and AfFlbA. The potential presence of an upstream mechanism(s) activating Af*brlA* that is independent of AfFluG is indicated. The roles of other G protein components and FLB genes in conidiation as well as the involvement of negative regulators of conidiation including SfgA in *A. fumigatus* remain to be studied. Experiments testing the roles of these *A. fumigatus* developmental regulators in gliotoxin production (reviewed in reference 14) and virulence establishment are currently being carried out.

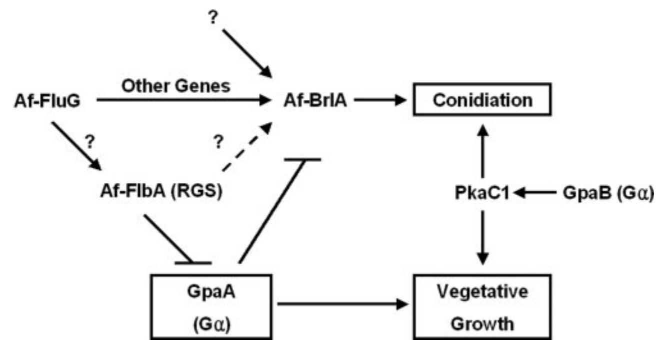


FIG. 7. Model for growth and developmental control in *A. fumigatus*. Activation of GpaA stimulates hyphal growth and represses conidiation. This GpaA-dependent signaling pathway is attenuated by AfFlbA. A potential role of AfFluG in activating AfFlbA is indicated by a question mark. Activation of conidiation does not absolutely require the activity of AfFluG or AfFlbA. The potential existence of an AfFluG-independent pathway(s) activating Af*brlA* is indicated. The GpaB-PkaC1 pathway has been proposed to be responsible for both conidiation and vegetative growth (18).

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