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Deletion of the *Aspergillus flavus* Orthologue of *A. nidulans fluG* Reduces Conidiation and Promotes Production of Sclerotia but Does Not Abolish Aflatoxin Biosynthesis

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The *fluG* gene is a member of a family of genes required for conidiation and sterigmatocystin production in *Aspergillus nidulans*. We examined the role of the *Aspergillus flavus fluG* orthologue in asexual development and aflatoxin biosynthesis. Deletion of *fluG* in *A. flavus* yielded strains with an approximately 3-fold reduction in conidiation but a 30-fold increase in sclerotial formation when grown on potato dextrose agar in the dark. The concurrent developmental changes suggest that *A. flavus* FluG exerts opposite effects on a mutual signaling pathway for both processes. The altered conidial development was in part attributable to delayed expression of *brlA*, a gene controlling conidiophore formation. Unlike the loss of sterigmatocystin production by *A. nidulans fluG* deletion strains, aflatoxin biosynthesis was not affected by the *fluG* deletion in *A. flavus*. In *A. nidulans*, FluG was recently found to be involved in the formation of dehydroaustinol, a component of a diffusible signal of conidiation. Coculturing experiments did not show a similar diffusible meroterpenoid secondary metabolite produced by *A. flavus*. These results suggest that the function of *fluG* and the signaling pathways related to conidiation are different in the two related aspergilli.

Aspergillus nidulans has been the model system for studying asexual conidiation at the molecular level. The genes *brlA* (bristle) (1), *abaA* (abacus) (39), and *wetA* (wet-white conidia) (29) are expressed sequentially and form the central developmental pathway of conidiation (17). BrlA is a transcription factor that mediates the budding growth of conidiophores. Mutations in *brlA* prevent vesicle formation, yielding stalks that grow indeterminately and that are unable to bear conidia (1). BrlA activates the expression of *abaA*, a gene that controls phialide differentiation (39). AbaA further mediates transcriptional activation of *wetA* (31) and also expression of *brlA* (3, 4). Efforts to elucidate early events leading to activation of conidiation in *A. nidulans* have identified six genes, *fluG* (fluffy) (2) and *flbA* to *flbE* (fluffy with low *brlA* expression), that are required for normal activation of *brlA* (42). Mutations of these genes result in proliferation of undifferentiated vegetative hyphae that produce fluffy cotton-like colonies. *A. nidulans fluG* is not transcribed in dormant conidia and is significantly downregulated during early vegetative growth; its expression before and after induction of conidiation does not change significantly (2, 7). A positive role of *fluG* in *brlA* activation has been suggested in *A. nidulans*, although the genetic relationship of *brlA* and *fluG* is not well defined. *A. nidulans fluG* mutants do not produce the secondary metabolite sterigmatocystin (20), the penultimate precursor of the carcinogenic aflatoxins. Because contact of the wild-type *A. nidulans* strain with the *fluG* null mutant remediates phenotypic defects (26), it was postulated that FluG synthesizes a diffusible factor that initiates conidiation. The effector molecules have recently been determined to be dehydroaustinol and diorcinol, which form a diffusible complex (36).

Aspergillus flavus, a major producer of aflatoxins, is related to *A. nidulans* in that they share most of the aflatoxin biosynthesis pathway. *A. flavus* normally reproduces and disseminates asexually, mainly through production of conidia, but some strains produce melaninized hyphal aggregates called sclerotia, which serve as an alternative reproductive form and a survival structure (13). Sclerotia are considered to be a vestige of the cleistothecia produced by other sexual aspergilli, including *A. nidulans* (19). Only recently has sexual reproduction under laboratory conditions been demonstrated with *A. flavus* strains of different mating types (21). The relationship between regulatory factors controlling the formation of sclerotia and conidiation is not well understood. SclR, the sclerotial pathway-specific helix-loop-helix transcription factor, was reported to promote sclerotial formation and to regulate hyphal morphology in *Aspergillus oryzae* (23). Deletion of *sclR* yields strains with sparse sclerotia but produces dense conidia. Overexpression of *sclR* results in strains with extremely branched and intertwined aerial hyphae. Conserved global regulators, such as VeA and LaeA, also affect *A. flavus* asexual development. The *veA* deletion strains of *A. flavus* ATCC MYA384 are unable to produce sclerotia and secondary metabolites (16). The *A. flavus laeA* deletion strains also cannot produce sclerotia and exhibit developmental abnormalities, including increased production of conidiophores, reduced conidial chain elongation, and marked reduction in hydrophobicity (11). In *A. nidulans*, VeA acts as a negative regulator of conidiation and a positive regulator of sexual development (25, 32), and LaeA acts as a positive regulator of secondary metabolite production (6). VeA and LaeA of *A. nidulans* are able to form a complex with two other velvet family proteins, VelB and VosA (33), to coordinate light-dependent sexual and asexual development and secondary metabolism (5, 37).

We now report that in *A. flavus* deletion of *fluG* delays and decreases conidiation but elevates sclerotial production, depend-

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ing on the growth medium. These developmental changes were not remediated by coculturing with *fluG*-positive strains. Surprisingly, unlike the loss of the ability to produce sterigmatocystin in *A. nidulans*, the *fluG* deletion strains still retained their ability to produce aflatoxin. These results show that *fluG* functions distinctly in *A. flavus*.

MATERIALS AND METHODS

Fungal strains and media. The *A. flavus* strain CA14 Δ*ku70* Δ*pyrG* was the transformation recipient. This mutant was derived from KuPG no. 1, a strain originally derived from aflatoxigenic wild-type CA14 (10). The *ku70* gene of the nonhomologous end-joining pathway was deleted in the recipient strain to increase the gene-targeting frequency. The Δ*wA* strain used in coculture studies produces white conidia due to the deletion of the polyketide synthase gene, *wA*, involved in conidial pigment synthesis (12). Growth media used for observing morphological changes, such as conidiation and sclerotium formation, were potato dextrose agar (PDA) (EMD, Damstadt, Germany) and Wickerham medium (34) with a minor modification. The medium contained 2.0 g yeast extract, 3.0 g peptone, 5.0 g corn steep solids, 2.0 g dextrose, 30.0 g sucrose, 2.0 g $NaNO_3$, 1.0 g $K_2HPO_4 \cdot 3H_2O$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl, 0.1 g $FeSO_4 \cdot 7H_2O$ (10-fold the original recipe), and 15.0 g agar per liter (pH 5.5).

Deletion of *fluG* and complementation of the *fluG* deletion strain. For the deletion of the *fluG* gene in *A. flavus*, a double-crossover gene knockout strategy was used (12). The complete *fluG* gene sequence of *A. flavus* NRRL3357 (AFL2G_11076.2) was obtained from the *Aspergillus* Comparative Database at Broad Institute (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html). The primer set fluG5Sc, ACAGAGCTCGCCGCGCTCTAC, and fluG5B, CTGTGGATCCCGGCTCACCATA, was used to amplify a 5′ region. The set fluG3S, CGCCTCGTCGACCGCTACTCTC, and fluG3XH, ATTAAGCTTCGCCGATGATGCTCTGCA, was used to amplify a 3′ region. The two PCR fragments, after digestion with appropriate restriction enzymes, were cloned sequentially into the vector pPG15-3, which contains a *pyrG* selection marker. The *fluG* deletion vector was linearized with SacI and XbaI prior to transformation. Putative *fluG* deletion strains were verified by PCR analyses based on the genomic patterns expected from the *fluG* disruption. A confirmed *fluG* deletion strain was used in the *fluG* complementation experiment. For complementation of the *fluG* deletion, the full-length genomic fragment from the start to the stop codons was amplified using AccuPrime *Pfx* PCR Supermix (Invitrogen, Carlsbad, CA) with primers fluGNot, ACTATAGCGGCCGCATGGATCTTACTTCCCTCCAATCC, and fluGRsr, ACTAGACGGTCCGCTAATAACGCTCAAGAAGCCACGTTC. The amplified gene was digested with NotI and ligated into NotI-SmaI-digested pTR1-GPD-TRPC, which contains the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase gene (*gpdA*) promoter and *trpC* terminator and the *A. oryzae* pyrithiamine resistance gene (*ptrA*) as the selection marker.

Generation of *laeA* and *fluG* double-deletion strains. The *laeA* deletion strain was created from *A. flavus* Δ*ku70* Δ*pyrG*, and the uracil auxotrophy was regenerated by growing spores (10^7) of the *laeA* deletion strain on PDA plates supplemented with 2 mg uracil/ml and 2 mg fluoroorotic acid (FOA)/ml to force out the reusable *pyrG* marker (12). The aforementioned *fluG* deletion vector was used to disrupt *fluG* in the resulting *laeA* deletion strain to generate *laeA fluG* double-deletion strains, using *pyrG* as the selection marker.

Conidial and sclerotial production. An aliquot of conidial suspension (10^3) was seeded at the center of each petri dish (Falcon; 60 by 15 mm) of PDA or Wickerham medium agar. For quantitative comparison of the production of conidia and sclerotia, cultures in triplicate were grown in the dark or under white light at 30°C for 1 week. At the end of the growth period, conidia were washed off the agar plates using 0.01% Triton X-100 solution and counted on a hemocytometer. Sclerotia, if present, were counted manually.

qRT-PCR analysis. Cultures for quantitative reverse transcription-PCR (qRT-PCR) analyses were grown on duplicate PDA plates (100 by 15 mm) with single-point inoculation at 30°C for 1 week in the dark. Two 5-mm agar plugs, 1.5 cm from the inoculation site, were cored from each plate with a plastic transfer pipette (Transfertubes; Spectrum, Houston, TX) on day 3, day 5, and day 7. The plugs were placed in a 2-ml microcentrifuge tube on dry ice and stored at −80°C overnight. Total RNA was prepared from the plugs with mycelia using a ZR Fungal/Bacterial RNA MiniPrep kit (Zymo Research, Orange County, CA). Mycelia disrupted with ZR bashing beads were processed in a Mini-Beadbeater-8 cell disrupter (Biospec Products, Bartlesville, OK). Treatment of total RNA with DNase 1 (Ambion, Austin, TX) was performed on the column before cleanup and eluted in RNase- and DNase-free water. qRT-PCR was carried out in a 20-μl reaction volume with the LuminoCT SYBR green qPCR ReadyMix (Sigma-Aldrich, St. Louis, MO) and the Reverse Transcriptase Enzyme Mix (Applied Biosystems, Foster City, CA) in an Applied Biosystems StepOne thermal cycler. The amplification conditions were as follows: an initial step of 48°C for 30 min for reverse transcription, followed by 40 cycles, with each cycle consisting of 95°C for 5 s and 60°C for 20 s. The gene-specific primers were 18S-F (TTCCTAGCGAGCCCAACCT) and 18S-R (CCCGCCGAAGCAACTAAG), brlA-F (TATCCAGACATTCAAGACGCACAG) and brlA-R (GATAATAGAGGGCAAGTTCTCCAAAG), abaA-F (GAGTGGCAGACCGAATGTATGTTG) and abaA-R (TAGTGGTAGGCATTGGGTGAGTTG), wetA-F (CCACAGCAGCCGATCCA) and wetA-R (CCCCTTGCAGGATGTCATG), and sclR-F (TGCCGCACACAACATCATT) and sclR-R (TTCTCCAAGGCCACGAACTT).

Examination of aflatoxin production by semiquantitative TLC. Cultures of PDA plugs for thin-layer chromatography (TLC) analysis were sampled on day 3, day 5, and day 7 and removed at the same time as those used for qRT-PCR analysis. Two plugs in each 2-ml microcentrifuge tube were extracted with 300 μl of methanol for 1 h. The tubes were spun at the maximum speed for 5 min, and 200 μl was drawn off in a 0.5-ml microcentrifuge tube and evaporated to dryness for TLC. The metabolite extracts were redissolved in 20 μl methanol, and 10 μl was spotted and developed on a Si250 silica gel plate (J. T. Baker, Phillipsburg, NJ) with a solvent system of toluene-ethyl acetate-acetic acid (65:35:10 [vol/vol/vol]).

RESULTS

Role of FluG in *A. flavus* conidiation and sclerotial production. When grown on PDA plates in the dark, the *fluG* deletion (Δ*fluG*) strains, compared to the wild-type strain, showed delayed and decreased conidiation but had increased sclerotial production (Fig. 1). The Δ*fluG* strains produced about two-thirds fewer conidia than the wild-type strain and, concomitantly, about 30-fold more sclerotia. The *fluG* complemented strains on PDA exhibited two types of colony morphology, one similar to the wild type and another having much denser conidiation (Fig. 1A; see Fig. S1 in the supplemental material). PCR analyses with location-specific primers suggested that each morphotype corresponded to a homologous integration at a specific *fluG* gene region in the Δ*fluG* recipient strain, which is defective in the nonhomologous end-joining pathway. The two recombinational events resulted in *fluG* being under the control of either the native *fluG* promoter or the stronger *A. nidulans gpdA* promoter, respectively (see Fig. S1 in the supplemental material). Alterations in production of conidia and sclerotia in the dark were remediated in the *fluG* complemented strains (Fig. 1B). The complemented strains with *fluG* under the *gpdA* promoter produced twice the number of conidia produced by the ones with the native *fluG* promoter ($1.18 \times 10^9 \pm 0.17 \times 10^9$ versus $5.43 \times 10^8 \pm 0.54 \times 10^8$ per plate) and did not produce sclerotia on PDA. Conidiation of the Δ*fluG* strains was not affected compared to the wild-type strain when the cultures

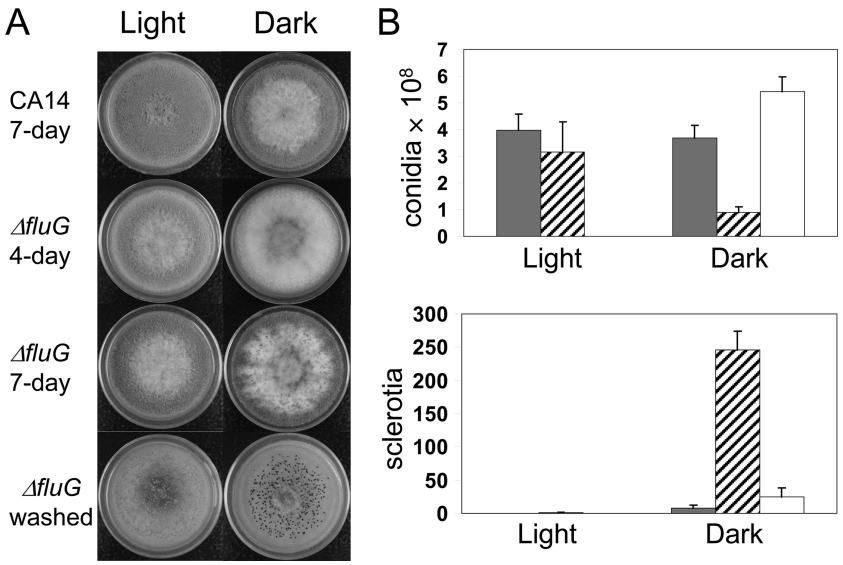

FIG 1 Morphology and production of conidia and sclerotia by *A. flavus* Δ*fluG* strains on PDA. (A) Cultures were grown at 30°C under white light and in the dark for the specified numbers of days. Washed, conidia of the 7-day-old cultures were washed off to expose sclerotia (black aggregates). (B) Quantification of conidia and sclerotia. Gray bars, wild-type CA14; crosshatched bars, Δ*fluG* strains; open bars, *fluG* complemented strains. Conidiation and sclerotial production under light by the *fluG* complemented strains were not determined. The complemented strains shown are those putatively having the native *fluG* promoter (see Fig. S1 in the supplemental material). The numbers of conidia and sclerotia are the total count per plate. The error bars indicate standard deviations.

were grown under light (Fig. 1), and sclerotia were not produced by either the wild-type or the Δ*fluG* strains. Wickerham medium was highly conducive to sclerotial formation and conidiation; 30-fold more sclerotia and 4-fold more conidia were produced by the control strain, KuPG, on this corn steep-containing medium than when grown on PDA. When grown in the dark on Wickerham medium, the Δ*fluG* strains showed a 6-fold decrease in conidiation ($2.35 \times 10^8 \pm 0.15 \times 10^8$ versus $1.53 \times 10^9 \pm 0.21 \times 10^9$) and an approximate 2-fold increase in sclerotial production (916 ± 28 versus 503 ± 125) (Fig. 2). Complemented strains showed almost wild-type levels of conidiation ($1.35 \times 10^9 \pm 0.18 \times 10^9$) and sclerotial production (512 ± 38).

Deletion of *fluG* affects conidial and sclerotial gene expression and aflatoxin production. Transcript levels for the conidiation genes, *brlA*, *abaA*, and *wetA*, and the sclerotial regulatory gene, *sclR*, were measured on the control strain KuPG, two Δ*fluG* strains, and two *fluG* complemented strains at 3, 5, and 7 days after growth on PDA plates. qPCR analyses indicated that in the control strain, *brlA* expression (normalized to 18S rRNA) was the highest on day 3, decreased more than 99.5% (equivalent to an increase of

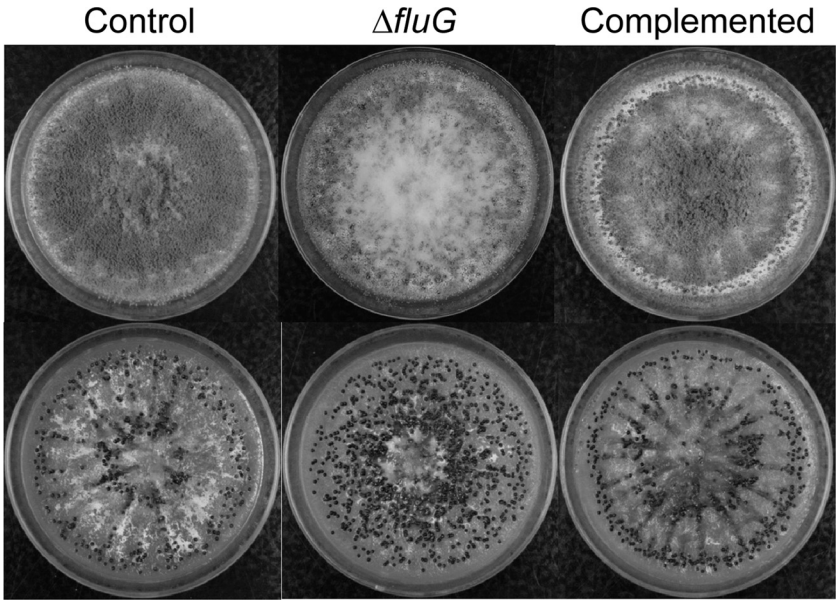

FIG 2 Morphologies of *A. flavus* Δ*fluG* and *fluG* complemented strains on Wickerham medium. (Top) Cultures after 7 days of growth at 30°C in the dark. (Bottom) Conidia of the colonies were washed off to expose sclerotia (black aggregates). The control strain is *A. flavus* KuPG no. 1.

TABLE 1 Transcript levels relative to the control strain level for conidiation and sclerotium regulatory genes in *fluG* deletion and complemented strains[a]

	Expression level on day:											
	brlA			*abaA*			*wetA*			*sclR*		
Strain	3	5	7	3	5	7	3	5	7	3	5	7
Δ*fluG1*	0.25	243.88[b] (0.84)[c]	0.37 (0.80)	0.61	2.46 (0.67)	0.51 (0.82)	0.74	0.41 (2.11)	0.83 (0.38)	0.73	0.14	1.31
Δ*fluG2*	0.70	298.35 (1.02)	0.16 (0.35)	0.14	13.65 (3.70)	0.51 (0.83)	0.85	0.51 (2.64)	1.03 (0.47)	1.15	0.01	1.16
Comp1	0.68	1.64	0.19	0.82	5.02	0.50	2.31	0.27	0.81	0.79	0.64	1.00
Comp2	1.11	2.45	0.01	1.06	5.33	0.05	2.16	0.45	0.92	0.88	0.73	1.06

[a] Independent deletion and complemented strains. The expression of the control strain, KuPG, is 1.00.
[b] Values are expression levels compared to those of the control strain sampled on the same day, that is, 3 day versus 3 day, 5 day versus 5 day, and 7 day versus 7 day.
[c] Values in parentheses are expression levels compared to those of the control strain sampled 2 days earlier, that is, 5 day versus 3 day and 7 day versus 5 day.

more than 8 threshold cycles [C_t]) on day 5, and remained low thereafter. During the same period, *brlA* expression of the Δ*fluG* strains increased approximately 2-fold (ranging from 1.46 to 3.33) on day 5 and decreased more than 99.9% on day 7; the extent of decrease was similar to that of the control strain from day 3 to day 5. Expression of *abaA* and *wetA* of the Δ*fluG* strains also increased on day 5. On day 7, *abaA* transcript levels decreased moderately but *wetA* transcript levels remained the same (data not shown). Table 1 shows that when the Δ*fluG* strains on day 5 were compared to the control strain on day 5, they had an approximately 250-fold increase in *brlA* expression. Because of the delayed conidiation by the Δ*fluG* strains (Fig. 1A), a better comparison is *brlA* expression of the Δ*fluG* strains on day 5 to that of the control strain on day 3. Based on this comparison, the *brlA* transcript levels of the control and Δ*fluG* strains were found to be comparable (Table 1, data in parentheses). Overall, *sclR* expression based on same-day comparison was constant for all strains except the Δ*fluG* strains on day 5, a time corresponding to initiation of conidiation in the Δ*fluG* strains, which had substantially lower levels of expression than on day 3 and day 7. TLC analysis indicated that both Δ*fluG* and *fluG* complemented strains produced aflatoxin B_1, although the amounts varied over the course of 7 days (Fig. 3).

Production of conidia and sclerotia by Δ*laeA* and Δ*laeA* Δ*fluG* strains. A previous study found that *A. flavus* Δ*laeA* strains had developmental abnormalities, including increased conidiophore production and reduced conidial chain elongation when grown on PDA. These alterations gave dense, velvet-like colonies (11). To examine if *fluG* deletion affects development in Δ*laeA* strains, we grew Δ*laeA* Δ*fluG* strains on Wickerham agar plates. The Δ*laeA* strains exhibited the same colony morphology and produced amounts of conidia comparable to those produced by the control strain ($1.68 \times 10^9 \pm 0.09 \times 10^9$ versus $1.53 \times 10^9 \pm 0.21 \times 10^9$), but they did not produce sclerotia (Fig. 4). Deletion of *fluG* in the Δ*laeA* strain caused only a moderate further decrease in conidiation ($1.30 \times 10^9 \pm 0.04 \times 10^9$) compared to that of the Δ*fluG* strains. Like the Δ*laeA* strains, Δ*laeA* Δ*fluG* strains lacked the ability to produce sclerotia (Fig. 4).

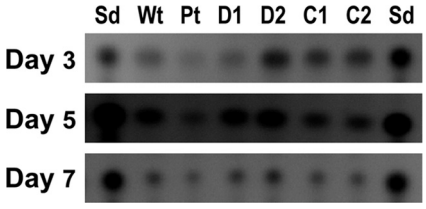

FIG 3 Time course semiquantitative TLC analyses of aflatoxin B_1 production by *A. flavus* Δ*fluG* and *fluG* complemented strains. Wt, wild-type *A. flavus* CA14; Pt, control *A. flavus* KuPG no. 1; D, Δ*fluG* strains; C, *fluG* complemented strains with *fluG* under the *gpdA* (C1) and the *fluG* (C2) promoter, respectively. The numbers indicate independent isolates. Sd, aflatoxin B_1 standard.

Coculture of *fluG*-intact and Δ*fluG* strains has no effect on resulting colony morphology. Coculture of the Δ*fluG* strain with wild-type CA14 or with the CA14-derived white Δ*wA* strain, a strain that produces only a few sclerotia (12), failed to remediate the abnormalities in conidiation and sclerotial production of the Δ*fluG* strain (Fig. 5). At the region of contact, the Δ*fluG* strain still exhibited decreased conidiation and increased sclerotial production.

DISCUSSION

Studies have shown that the *A. nidulans fluG* gene plays an important role in the initiation of conidiation and subsequent sterigmatocystin production (2, 27, 38). Unlike the *A. nidulans* fluffy Δ*fluG* strains, which are unable to conidiate (43), *A. flavus* Δ*fluG* strains, compared to the wild-type strain, show only delayed and decreased conidiation. The delayed conidiation in the *A. flavus* Δ*fluG* strains can be attributed to delayed expression of *brlA*. An approximately 2-day delay in *brlA* expression was observed when the Δ*fluG* strains were compared to the control and complemented strains (Table 1, 5 day versus 3 day). Fluffy mutants of *A. nidulans* exhibit altered *brlA* expression (2, 42); the level of BrlA reduction likely is more severe than that in *A. flavus*, resulting in an inability to form conidiophores. This halt in development in *A. nidulans* Δ*fluG* mutants also explains their inability to make secondary metabolites. For *A. flavus*, the delayed conidiation in the Δ*fluG* strains likely causes metabolic diversion, consequently shifting precursors, such as acetates, normally channeled to conidial pigment production to sclerotial formation (8, 41). The *fluG* complemented strains with the strong *A. nidulans gpdA* promoter conidiate earlier than those complemented strains under the control of the native *fluG* promoter, as evidenced by denser conidiation at and around the inoculation site on PDA (see Fig. S1 in the supplemental material). This earlier initiation of conidiation probably diverts the metabolic flux toward conidial formation, resulting in no sclerotia being produced, in contrast to the small amounts of sclerotia produced by the latter type of *fluG* complemented strains and the wild-type strain (Fig. 1B) (9). Conidiation and sclerotial production probably share common regulatory factors that enable the shift toward a developmental process depen-

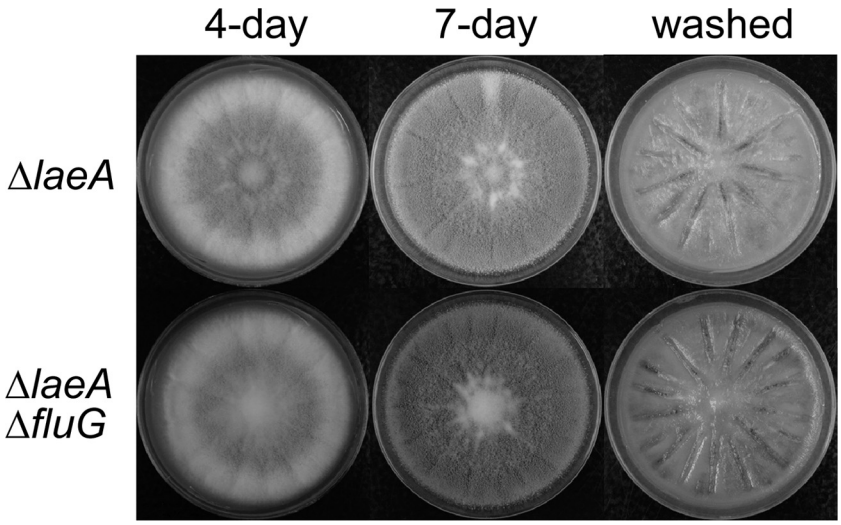

FIG 4 Morphologies of *A. flavus* Δ*laeA* and Δ*laeA* Δ*fluG* strains on Wickerham medium grown at 30°C in the dark for the specified numbers of days.

dent on either physiological or environmental conditions. *A. flavus* FluG may affect a signaling system that has opposite effects on conidial and sclerotial development.

Deletion of *fluG* in the *A. flavus* Δ*laeA* strain decreases conidiation, which further confirms the positive effect of FluG on conidiation. LaeA is a global regulator of secondary metabolism in many fungi, and in *A. nidulans*, it forms a velvet complex with the developmental regulators VeA, VelB, and VosA (5). In *A. nidulans* and *A. flavus*, LaeA does not play a predominant role in the regulation of conidiation (11, 24). However, differences in function have been reported for the two LaeA regulators. *A. nidulans* LaeA inhibits formation of sexual cleistothecia in the light (37), while *A. flavus* LaeA is required for sclerotial formation in the dark (24) (Fig. 4). *A. flavus* FluG probably functions to fine tune the conidiation process by regulating the *brlA* expression level in response to certain physiological or developmental cues (signals). In the presence of LaeA, FluG may indirectly decrease or inhibit sclerotial formation, depending on the metabolic status of the fungus.

The *A. flavus* FluG amino acid sequence shows about 70% identity to those of *A. nidulans* and *A. fumigatus* (see Fig. S2 in the supplemental material). *Aspergillus* FluG is a fusion protein composed of an N-terminal amidohydrolase domain that shares ho-

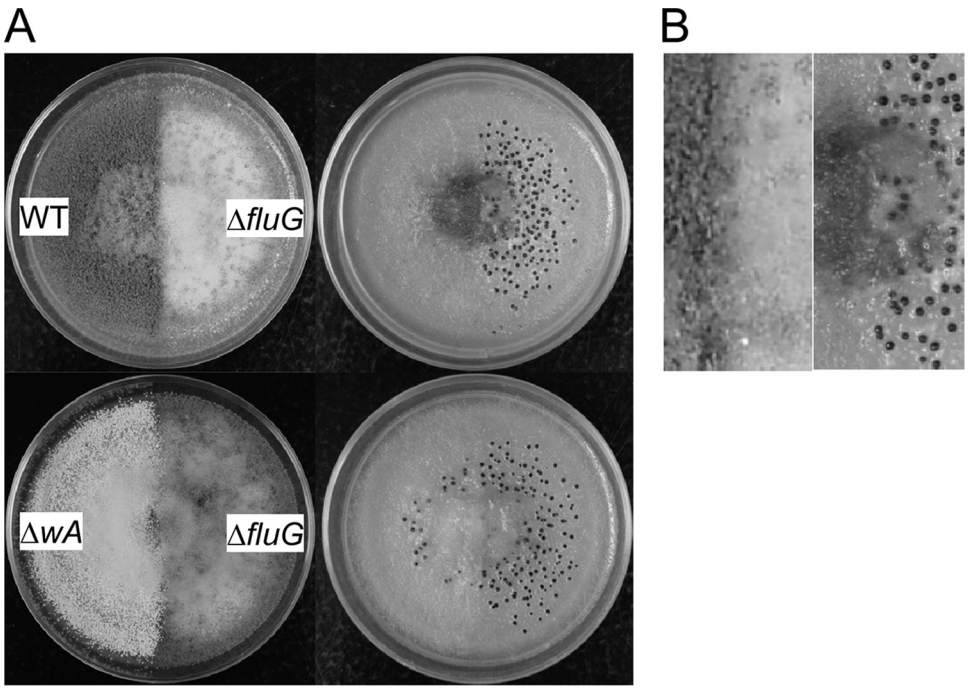

FIG 5 Coculture of *fluG*-positive and Δ*fluG* strains. Cultures were grown on PDA at 30°C for 5 days in the dark. (A) Morphology before (left) and after (right) conidia were washed off the PDA plates. (B) Close-up of the boundary of the wild-type and Δ*fluG* strains.

mology with nodulins (Nod) and a C-terminal prokaryotic glutamine synthetase (GS) type I domain. Proteins similar to FluG are also found in plants (14, 30). FluG is predicted to be capable of carrying out condensation of glutamate with an amino-group-containing molecule and subsequent hydrolysis; the signaling molecule had been proposed to be aminobutyrate (22). However, no catalytic activity has been reported for FluG or FluG-like proteins (35). *A. nidulans* FluG does not directly synthesize but is associated with the synthesis of the diffusible signaling molecule dehydroaustinol (26, 36). The *A. flavus* coculture results suggest that, unlike *A. nidulans*, FluG is not associated with production of a diffusible effector metabolite (Fig. 5). An extensive search for potential dehydroaustinol biosynthesis clusters in the *A. flavus* genome did not find possible candidates. This suggests that the signaling pathways related to conidiation in the two related aspergilli must be different.

A. nidulans fluG fluffy strains have mutations primarily in the GS coding region (26), and the entire N-terminal half of *A. nidulans* FluG is dispensable without affecting conidiation (15). This suggests that FluG activity is mainly associated with the C-terminal GS domain. Since conidiation, sclerotial production, and secondary metabolism in *A. flavus* may be coordinated by the velvet complex and other interacting proteins, we performed tests to determine if FluG is able to interact with LaeA and VeA. Yeast two-hybrid assays showed that the GS domain of *A. flavus* FluG interacts with full-length LaeA and with the N-terminal half of LaeA, but not with VeA (see Fig. S3 in the supplemental material). However, we were unable to substantiate this specific interaction by bimolecular fluorescence complementation (BiFC) in fungal cells (data not shown). In *A. nidulans*, the *fluG*-dependent signal molecule is produced in the dark only in the strain with the *veA1* mutation, and VeA appears to function as a negative regulator of conidiation in the dark at the posttranscriptional level (43). These observations suggest that a FluG-VeA interaction probably exists. Such an interaction may not occur in *A. flavus*, as suggested by the yeast two-hybrid results, because *A. flavus* normally conidiates in the dark. The *Arabidopsis* NodGS protein is part of a large heterogeneous protein complex (14). FluG-dependent conidiation in *A. nidulans* appears to be regulated by a suppressor, SfgA (38). FluG and other FluG-like proteins thus likely engage in protein-protein interactions. Whether FluG is an interacting partner of the *Aspergillus* velvet complex warrants further investigation.

While the downstream central pathway of conidiation regulated by *brlA* (i.e., *abaA* and *wetA*) is common to *A. nidulans* and *A. fumigatus*, the upstream pathways that activate *brlA* are distinct in the two species (28, 40, 44). In both *A. nidulans* and *A. fumigatus*, deletion of *brlA* eliminates conidiation completely, whereas deletion of *fluG* in *A. fumigatus*, as in our study, only reduces conidiation. In addition, in *A. nidulans*, deletion of *fluG* abolishes sterigmatocystin production, but deletion of *fluG* in *A. flavus* did not affect aflatoxin biosynthesis significantly (Fig. 3). Whole-genome comparison has concluded that *A. fumigatus* and *A. oryzae*/*A. flavus* are more closely related to each other than to *A. nidulans* (18). Our results suggest that the function of *fluG* in *A. flavus* is markedly different than in *A. nidulans* and that such potential variability should be taken into account when comparing the roles of developmental regulatory factors in different fungi.

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