DOI: 10.1534/genetics.105.052258

FluG-Dependent Asexual Development in *Aspergillus nidulans* Occurs via Derepression

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Manuscript received October 10, 2005
Accepted for publication December 30, 2005

ABSTRACT

The asexual spore is one of the most crucial factors contributing to the fecundity and fitness of filamentous fungi. Although the developmental activator FluG was shown to be necessary for activation of asexual sporulation (conidiation) and production of the carcinogenic mycotoxin sterigmatocystin (ST) in the model filamentous fungus *Aspergillus nidulans*, the molecular mechanisms underlying the developmental switch have remained elusive. In this study, we report that the FluG-mediated conidiation in *A. nidulans* occurs via derepression. Suppressor analyses of *fluG* led to the identification of the *sfgA* gene encoding a novel protein with the Gal4-type $Zn(II)_2Cys_6$ binuclear cluster DNA-binding motif at the N terminus. Deletion (Δ) and 31 other loss-of-function *sfgA* mutations bypassed the need for *fluG* in conidiation and production of ST. Moreover, both Δ*sfgA* and Δ*sfgA* Δ*fluG* mutations resulted in identical phenotypes in growth, conidiation, and ST production, indicating that the primary role of FluG is to remove repressive effects imposed by SfgA. In accordance with the proposed regulatory role of SfgA, overexpression of *sfgA* inhibited conidiation and delayed/reduced expression of conidiation- and ST-specific genes. Genetic analyses demonstrated that SfgA functions downstream of FluG but upstream of transcriptional activators (FlbD, FlbC, FlbB, and BrlA) necessary for normal conidiation.

REPRODUCTION of fungi results in the formation of enormous numbers of spores that are extremely efficient for genome protection, survival, and propagation. Spores are also the primary means of infecting host organisms for many human and plant pathogenic fungi. Asexual sporulation is a highly successful and effective reproductive mechanism for a diverse group of fungi because vast numbers of spores (called conidia for higher fungi) can be produced from a single colony through repetitive cycles of mitosis (reviewed in Adams 1994).

Members of the genus Aspergillus are the most common fungi and many are beneficial to humans. However, they also include serious human and plant pathogens. Furthermore, certain aspergilli have the capacity to produce one or more toxic secondary metabolites called mycotoxins, which contaminate various plant-based foods/feeds and cause adverse health effects to humans and animals such as liver or kidney damage, cancer, and even death (reviewed in Seo and Yu 2004). All aspergilli produce asexual spores (conidia) as the main means of dispersion and infection. Asexual development (conidiation) and production of certain carcinogenic mycotoxins are intimately associated (reviewed in Calvo *et al.* 2002; Yu and Keller 2005).

Aspergillus nidulans has served as an excellent model system for studying multicellular development and secondary metabolism (Timberlake 1990; Martinelli 1994; Yu and Keller 2005). Conidiation in *A. nidulans* involves many common developmental themes including spatial and temporal regulation of gene expression, specialized cellular differentiation, and intercellular communication. The asexual reproductive cycle of *A. nidulans* begins and ends with the asexual spore called the conidium and can be divided into two distinct phases, growth and development. The vegetative growth phase involves germination of the conidium and formation of an undifferentiated network of interconnected hyphal (vegetative) cells that form the mycelium. After a certain period of vegetative growth, under appropriate conditions, some of the hyphal cells stop normal growth and begin development by forming complex multicellular structures called conidiophores that bear multiple chains of conidia (reviewed in Adams 1994).

A key step in conidiophore development is activation of the *brlA* gene, which encodes a C_2H_2 zinc finger transcription factor (Adams *et al.* 1988; Chang and Timberlake 1992). A series of studies have identified six genes (*fluG*, *flbA*, *flbB*, *flbC*, *flbD*, and *flbE*) that are

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession no. AAY99779.

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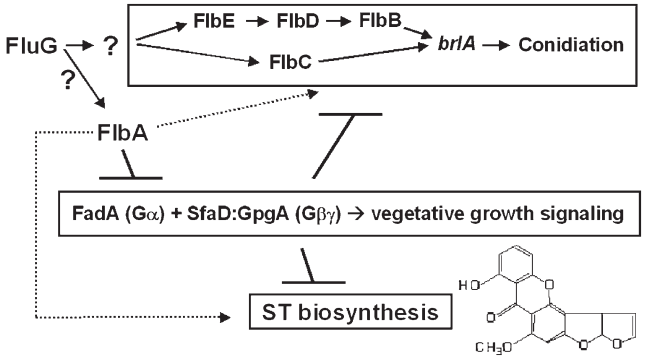


FIGURE 1.—Genetic model for growth and developmental control in *A. nidulans*. Activities of two antagonistic regulatory pathways control growth, development, and ST (structure shown) production. Activation of G-protein-mediated vegetative signaling represses both development and ST biosynthesis (YU *et al.* 1996; HICKS *et al.* 1997). FlbA is an RGS protein that rapidly turns off this growth signaling (LEE and ADAMS 1994a; YU *et al.* 1996). At least partial inhibition of growth signaling is required for development and ST production. Activation of conidiation requires the activities of FluG and other developmental genes, *flbB*, *flbC*, *flbD*, *flbE*, and *brlA* (reviewed in ADAMS *et al.* 1998). FluG is thought to have dual functions, activating conidiation and positively modulating FlbA activity. Missing links are indicated by question marks.

required for proper *brlA* expression and conidiation. Mutations in any of these genes result in "fluffy" colonies that are characterized by undifferentiated cotton-like masses of vegetative cells (reviewed in ADAMS *et al.* 1998). The possible genetic interactions between various *flb* genes were examined and the two genetic regulatory cascades have been proposed (Figure 1), in which the *fluG* gene functions most upstream (WIESER and ADAMS 1995; LEE and ADAMS 1996).

Loss of *fluG* function results in the blockage in both conidiation and production of the carcinogenic mycotoxin sterigmatocystin (ST), the penultimate precursor of the better-known potent carcinogen aflatoxin (LEE and ADAMS 1994b; HICKS *et al.* 1997). Conidiation in the *fluG*$^-$ mutants can be rescued by growing them next to wild-type or other developmental mutants. This observation has led to the proposal that FluG is responsible for the production of an extracellular diffusible conidiation factor (LEE and ADAMS 1994b). The C-terminal half of FluG contains a glutamine synthetase I (GSI)-like domain (LEE and ADAMS 1994b) and the N-terminal half is similar to the product of the early nodulin gene MtN6 in *Medicago truncatula* (MATHIS *et al.* 1999). Overexpression of the full-length or the C-terminal half of *fluG* in vegetative hyphae was sufficient to cause development of conidiophores in liquid submerged culture, where conidial development is normally suppressed (LEE and ADAMS 1996; D'SOUZA *et al.* 2001). Collectively, it has been proposed that FluG is involved in the constitutive synthesis of an extracellular sporulation-inducing factor that is related to glutamine or glutamate (reviewed in ADAMS *et al.* 1998). The two primary consequences of FluG activity were thought to be: (1) activation of development-specific regulatory cascades and (2) positive modulation of the regulator of G-protein signaling (RGS) protein FlbA, which in turn inactivates vegetative growth signaling mediated by a heterotrimeric G protein (see Figure 1; LEE and ADAMS 1994a; YU *et al.* 1996; ADAMS *et al.* 1998). For asexual development to occur both processes must occur. However, the molecular mechanism underlying the FluG-mediated initiation of conidiation remains to be uncovered.

To dissect the molecular events responding to FluG activity, we isolated 40 suppressor mutations (**s**uppressor of ***fluG***, *sfg*) that bypass the need for *fluG* in conidiation and ST production (SEO *et al.* 2003). In this study, we have identified and characterized the key suppressor *sfgA* that is defined by 31 *sfg* mutations. The *sfgA* gene is predicted to encode a novel 601-amino-acid (aa) protein containing the Gal4-type $Zn(II)_2Cys_6$ binuclear cluster DNA-binding motif at the N terminus, suggesting that SfgA is likely a transcription factor. Various mutations have been identified within the *sfgA*-coding region from all 31 SFGA mutants. Deletion of *sfgA* completely bypasses *fluG* in conidiation and ST production. Further studies of *sfgA* have elucidated that conidiation in *A. nidulans* occurs via derepression and the primary role of FluG is to remove intracellular repressive effects imposed by SfgA. Our findings elucidate a new theory for the central regulatory mechanism of conidiation and ST biosynthesis in *A. nidulans*.

MATERIALS AND METHODS

***A.nidulans* strains, media, growth conditions, and genetic analysis:** *A. nidulans* strains used in this study are listed in Table 1. Standard culture and genetic techniques were employed (PONTECORVO *et al.* 1953; KÄFER 1977). All liquid cultures were inoculated with 5×10^7 spores in 100 ml of liquid glucose (1%) minimal medium [pH 6.5; 10 g/liter glucose, 6 g/liter $NaNO_3$, 0.52 g/liter $MgSO_47H_2O$, 0.52 g/liter KCl, 1.52 g/liter KH_2PO_4, and 1 ml/liter of 1000× trace element solution composed of 22 g/liter $ZnSO_47H_2O$, 11 g/liter H_3BO_3, 5 g/liter $MnCl_24H_2O$, 5 g/liter $FeSO_47H_2O$, 1.6 g/liter $CoCl_25H_2O$, 1.6 g/liter $CuSO_45H_2O$, 1.1 g/liter $(NH_4)_6Mo_7O_244H2O$, 50 g/liter Na_2EDTA] with supplements (simplified as MMG) or MMG with 0.1% (w/v) yeast extract (YE) and incubated at 37°, 250 rpm. Submerged development of individual strains was observed under a microscope at 1-hr intervals after an initial 18-hr growth period in liquid culture. Asexual developmental induction was performed as previously described (ADAMS *et al.* 1988). As a prerequisite for the identification of the *sfgA* gene, Δ*fluG sfgA11 pyrG89* (RSFGA11) and Δ*fluG sfgA44 pyrG89* (RSFGA44) strains were generated by meiotically crossing Δ*fluG sfgA11* and Δ*fluG sfgA44* strains with the developmentally wild-type strain FGSC773 (Table 1) and by examining 20 sporulating progeny that required uracil for growth and carried the Δ*fluG* genotype. Such conidial strains with the Δ*fluG* Southern blot hybridization pattern are expected to have the Δ*fluG sfgA11 pyrG89* or Δ*fluG sfgA44 pyrG89* genotypes.

To examine the effect of overexpression of *sfgA* by an ectopic copy of *sfgA* under the *alcA* promoter, control (TSR2.1) and *alcA*(p)::*sfgA* (TJAS5.7; Table 1) strains were

TABLE 1

***A. nidulans* strains used in this study**

Strain	Genotype[a]	Source
FGSC4	*veA*+	FGSC[b]
FGSC26	*biA1*	FGSC
FGSC237	*pabaA1, yA2*; *trpC801*	FGSC
FGSC773	*pyrG89*; *wA3*; *pyroA4*	FGSC
PW1	*biA1*; *argB2*; *methG1*	P. Weglenski
RMS011	*pabaA1, yA2*; Δ*argB::trpC*$^+$; *trpC801*	Stringer *et al.* (1991)
RJYE07	*biA1*, Δ*flbA::argB*$^+$; *methG1*; Δ*fadA::argB*$^+$, *trpC801*	Hicks *et al.* (1997)
TTA127.4	*pabaA1, yA2*; Δ*fluG::trpC*$^+$; *trpC801*	Lee and Adams (1994b)
RJA56.25	*pabaA1, yA2*	Seo *et al.* (2004)
TSR2.1	*pabaA1, yA2*; pSH96*::trpC*$^+$	Han *et al.* (2004b)
SFGA1[c]	*pabaA1,yA2*; *sfgA1*; Δ*fluG::trpC*$^+$; *trpC801*	Seo *et al.* (2003)
RSFGA11	*pyrG89*; *sfgA11*; *pyroA4*; Δ*fluG::trpC*$^+$	This study
RSFGA44	*pyrG89*; *sfgA44*; Δ*fluG::trpC*$^+$	This study
RJA4.4	*pyrG89, yA2*; Δ*fluG::trpC*$^+$	This study
TYGN2	*pabaA1, yA2*; Δ*sfgA::argB*$^+$	This study
RYG3	*pabaA1, yA2*; Δ*sfgA::argB*$^+$; Δ*fluG::trpC*$^+$; *trpC801*	This study
TJAS5.7	*pabaA1, yA2*; *alcA*(p)*::sfgA::trpC*$^+$, *trpC801*	This study
RJA46.3	*biA1*; Δ*sfgA::argB*$^+$; Δ*flbA::argB*$^+$	This study
RJA47.29	Δ*sfgA::argB*$^+$; Δ*brlA::argB*$^+$	This study
RJA48.1	Δ*sfgA::argB*$^+$; *methG1*; Δ*flbB::argB*$^+$	This study
RJA50.9	Δ*sfgA::argB*$^+$; *methG1*; Δ*flbD::argB*$^+$	This study
RJA51.4	*pabaA1, yA2*; Δ*sfgA::argB*$^+$; Δ*flbE::argB*$^+$	This study
RJA52.16	*pyrG89, yA2*; Δ*sfgA::argB*$^+$; Δ*fluG::trpC*$^+$; *trpC801*	This study
RJA53.11	*pyrG89, pabaA1, yA2*; Δ*sfgA::argB*$^+$; Δ*flbC::argB*$^+$	This study
RHS94.4	*methG1*; Δ*brlA::argB*$^+$	S.-T. Han
TJW113	*biA1*; *methG1*; Δ*flbB::argB*$^+$	Kellner and Adams (2002)
RGM612	*pabaA1*; *methG1*; Δ*flbC::argB*$^+$	Kellner and Adams (2002)
TJW110	*biA1*; Δ*flbE::argB*$^+$; *methG1*	Kellner and Adams (2002)
REK1003.2	*biA1*; *methG1*; Δ*flbD::argB*$^+$	Kellner and Adams (2002)
REK1056	*argB2*; *methG1*; Δ*flbD::trpC*$^+$; *trpC801*	Kellner and Adams (2002)

[a] All strains carry the *veA1* mutation except FGSC4.
[b] Fungal Genetics Stock Center.
[c] All 31 *sfgA* mutants have the same genotype except for the *sfgA* alleles (see Figure 3).

grown in liquid MMG at 37°, 250 rpm for 14 hr. Subsequently, mycelia were collected, rinsed with liquid medium without a carbon source, divided into two equal parts, transferred into solid (1.6% agar added to the above composition) MMG (noninducing conditions) or solid MM with 100 mM threonine as a sole carbon source (MMT) (inducing overexpression of *sfgA*; composition the same as above except for adding 11 g/liter threonine instead of 10 g/liter glucose), and further incubated at 37°. Progression of asexual development of control and *alcA*(p)*::sfgA* strains was examined under a microscope, and samples were collected at 3, 6, 9, 12, and 24 hr post-transfer and subjected to total RNA isolation.

Cloning of *sfgA*: Cloning of *sfgA* was carried out employing the pRG3-AMA1-based wild-type genomic DNA plasmid library with *pyr4* of *N. crassa* as the selective marker (Osherov and May 2000). Each Δ*fluG sfgA11 pyrG89* and Δ*fluG sfgA44 pyrG89* mutant strain was transformed with the library and the fluffy progeny were screened visually. After screening >5000 transformants for each case, two to three Δ*fluG*-like fluffy transformants were isolated. Total DNA was isolated from individual transformants and used to transform *Escherichia coli* DH10B by electroporation. From each transformant, the pRG3-AMA1 plasmids carrying an ~7.0-kb insert were isolated. These plasmids were then reintroduced into Δ*fluG sfgA11 pyrG89* and Δ*fluG sfgA44 pyrG89* recipient strains and were confirmed to restore the fluffy phenotype at a high (~60%) frequency. The responsible gene within the 7.0-kb insert was identified by a transposon (Tn)-mediated mutagenesis using an *in vitro* transposon kit (GPS-1 system; New England Biolabs, Beverly, MA). Twenty Tn-tagged clones were analyzed for Tn integration within the 7.0-kb insert and such Tn-tagged clones were introduced into Δ*fluG sfgA11 pyrG89* and Δ*fluG sfgA44 pyrG89* recipient strains. About 70% of the Tn-tagged clones failed to restore the fluffy phenotype, indicating that Tn had disrupted the responsible gene. Most of the Tn-tagged plasmids were sequenced, and the assembled sequences were used for blastn search of the *A. nidulans* genome (the Broad Institute: http://www.broad.mit.edu/annotation/fungi/aspergillus/index.html).

Construction of *sfgA* mutants: The double-joint PCR method was used to generate the *sfgA* deletion mutant (Yu *et al.* 2004). Briefly, using wild-type (FGSC4) genomic DNA as a template 5′- and 3′-flanking regions (~1 kb) of *sfgA* were amplified with the primer pairs of OYG7-9 and OYG10-11, respectively. The *argB*$^+$ marker was amplified with OKH60-61 (Han *et al.* 2004a). The three amplicons were fused as described (Yu *et al.* 2004). The final *sfgA* deletion construct was amplified with OYG8 and OYG12 (Table 2) and introduced into PW1. Resulting transformants were randomly screened for deletion of *sfgA* by PCR analysis using OYG21-22 followed by Southern blot. The Δ*sfgA*

TABLE 2

Oligonucleotides used in this study

Primer	Sequence	Position/purpose
OKH60	gac tct ata cca ccg tac gcc gat at	Forward primer for *argB*$^+$
OKH61	cac cgg gtg cga ttt gcc cca ttt cc	Reverse primer for *argB*$^+$
OYG7	ggt gct cat ccc gtg tca aac a	5′-Flanking forward primer of *sfgA*
OYG8	gct aat tgg tat ttt gcc tct a	5′-Flanking nested forward primer of *sfgA*
OYG9	agt caa atg agg cct cta aac tgg tc a aga gct gta agg gag aga cat g	5′-Flanking reverse primer of *sfgA* with *argB* tail (underlined)
OYG10	agc caa ggt aga tcc agg cct aac ac a gac cat aca cct cgc act ttc t	3′-Flanking forward primer of *sfgA* with *argB* tail (underlined)
OYG11	cat gga ctg aat aat gtg gct c	3′-Flanking reverse primer of *sfgA*
OYG12	cta tct ata att aag cca agc a	3′-Flanking nested reverse primer of *sfgA*
OYG13	acc tcg ccc ctc cat gtc tct c	Sequencing of *sfgA*, RT–PCR
OYG21	cta tat aga cgc cgt aac caa c	Confirmation of Δ*sfgA*, RT–PCR
OYG22	atc tca gat cat ctc atc aag a	Confirmation of Δ*sfgA*
OJA107	aca atc aat gtt caa tgt ac	5′ end of *alcA*(p)
OJA106	ttt gag gcg agg tga tag gat tgg a	3′ end of *alcA*(p)
OJA108	***cg gga tcc*** agt ggt tcg gta atc	*alcA*(p) 5′ nested with *Bam*HI tail (boldface italic)
OYG28	tcc aat cct atc acc tcg cct caa a atg gaa gct cgt acg atg gt	3′ end of *alcA*(p) (underlined) + 5′ forward of *sfgA* ORF
OYG29	***cg ggatc c***ta tct ata att aag cca agc a	3′ nested of *sfgA* with *Bam*HI (boldface italic)
OYG30	atg tgc tac cag tat gct	5′ forward of *flbD*
OYG31	tcg tag cgt gcc tag gta	3′ reverse of *flbD*
OYG38	acttgatcatcgctccaa	5′ forward of *flbC*
OYG39	gaaagccgaatcgtacac	3′ reverse of *flbC*
OJA133	gag att cga gcc tgt gc	5′ forward of *flbA*
OJA134	ctg tca tga acg ttg tg	3′ reverse of *flbA*
OJA142	ctg gca ggt gaa caa gtc	5′ forward of *brlA*
OJA143	aga agt taa cac cgt aga	3′ reverse of *brlA*
OJA152	aac tct cga gct gac atg	5′ forward of *yA*
OJA153	ctt gga cat tat gta ggt	3′ reverse of *yA*

mutant was meiotically crossed with RJA4.4 (*pyrG89*, *yA2*; Δ*fluG::trpC*$^+$) and the Δ*sfgA* Δ*fluG* double mutant was subsequently identified among the progeny. The *sfgA* overexpression mutant was generated by a PCR-assisted method as described (Yu *et al.* 2004). Briefly, the *alcA* promoter (Gwynne *et al.* 1987) and the *sfgA* ORF with terminator were amplified using the primer pairs OJA106-107 and OYG11-28, respectively, and fused as described (Yu *et al.* 2004). The *alcA*(p)::*sfgA* fusion product was amplified with OJA108 and OYG29, and the final amplicon was digested with *Bam*HI and ligated into the *Bam*HI cut pSH96 plasmid (Wieser and Adams 1995). The sequence verified that the *alcA*(p)::*sfgA* plasmid was introduced into FGSC237 (Table 1). The single-copy integration of the plasmid into the *trpC* locus was confirmed by PCR. Multiple *alcA*(p)::*sfgA* transformants were isolated and further examined.

ST analysis: Conidia (~10^6) of relevant strains (Table 1) were inoculated into 2 ml liquid complete medium in 8-ml test tubes. The stationary cultures were incubated at 37° for 7 days and ST was extracted as described (Yu and Leonard 1995). Approximately 5 μl of a concentrated sample was applied onto a thin-layer chromatography (TLC) silica plate containing a fluorescence indicator (Kiesel gel 60; Merck, St. Louis). ST standard was purchased from Sigma. The TLC plate was developed in toluene:ethyl acetate:acetic acid (80:10:10, v/v/v). Enrichment of ST visualization was achieved by spraying the TLC plate with 20% $AlCl_2$ in 95% ethanol followed by incubation at 80° for 5 min (Stack and Rodricks 1971).

Nucleic acid isolation and manipulation: Genomic DNA and total RNA isolation and Northern blot analyses were carried out as described (Seo *et al.* 2003). To examine the *sfgA* mRNA levels in wild type, samples from liquid submerged and postdevelopmental induction cultures were collected as described (Han *et al.* 2004a). Briefly, the conidia (5×10^7/ml) of FGSC4 were inoculated in 100 ml liquid MMG with 0.1% YE in 250-ml flasks and incubated at 37°, 250 rpm. For the vegetative growth phase, samples were collected from liquid submerged cultures at 14, 18, and 22 hr, squeeze dried, and stored at −80° until subjected to total RNA isolation. For sexual and asexual developmental induction, 18-hr vegetatively grown mycelia were transferred to solid MMG and the plates were either air exposed for asexual developmental induction or tightly sealed and shielded from light to induce sexual development. Samples were collected at various time points after developmental induction and subjected to total RNA isolation. Approximately 8 μg per lane of total RNA was separated by electrophoresis using a 1.1% agarose gel containing 6% formaldehyde and ethidium bromide and the nucleic acids were transferred to the MagnaProbe Nylon membrane (0.45 μm; Osmonics, Minnetonka, MN).

To examine the effects of overexpression of *sfgA* on mRNA levels of developmental or ST biosynthetic genes, total RNA (8 μg/lane) of control and *alcA*(p)::*sfgA* strains was isolated from the samples mentioned above. Probes for *flbD*, *flbC*, *brlA*, *yA*, and *stcU* mRNA examination were prepared by amplifying coding regions of individual genes (see Table 2 for primers) from wild-type (FGSC4) genomic DNA. Individual amplicons were labeled with ^{32}P-dCTP and used for Northern blot hybridization as described (Yu and Leonard 1995).

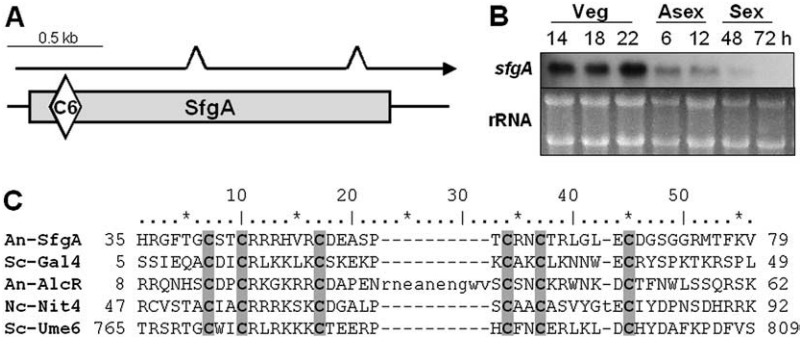


FIGURE 2.—The *sfgA* gene structure, expression, and alignment. (A) The *sfgA* ORF (shaded box) of 1908 bp with 49- and 53-bp introns (shown by discontinuity in the arrow) is presented. Approximate position of the $Zn(II)_2Cys_6$ domain is marked as C6. (B) The steady-state mRNA levels of *sfgA* during the life cycle of *A. nidulans* are shown. Numbers indicate the time (hours) of incubation in liquid submerged culture (Veg) and postasexual (Asex) or sexual (Sex) developmental induction. Equal loading of total RNA was evaluated by ethidium-bromide-stained rRNA. (C) Alignment of the C6 region of An-SfgA (accession no. AAY99779) and Sc–Gal4 (LAUGHON and GESTELAND 1984), An–AlcR (FELENBOK *et al.* 1988), Nc–Nit4 (YUAN and MARZLUF 1992), and Sc–Ume6 (STRICH *et al.* 1994) is shown. The conserved six Cys residues are in boldface type and shaded (An, *A. nidulans*; Sc, *S. cerevisiae*; Nc, *Neurospora crassa*).

Microscopy: The colony photographs were taken using a Sony DSC-F707 digital camera. Photomicrographs were taken using an Olympus BH2 compound microscope installed with an Olympus DP-70 digital imaging system.

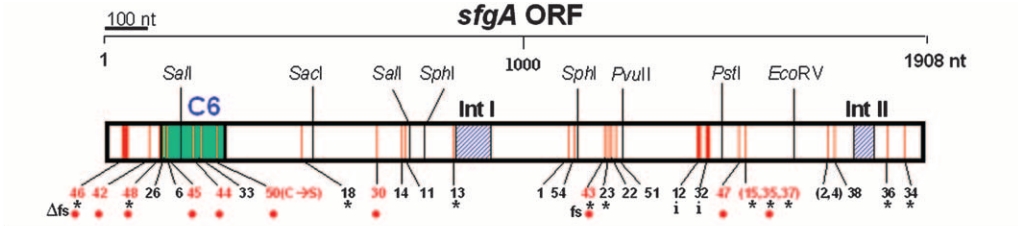
RESULTS

The *sfgA* gene encodes a novel protein with the $Zn(II)_2Cys_6$ DNA-binding motif: Previously we found that 31 of 40 *sfg* mutations mapped to linkage group A (*sfgA*) and thereby proposed that SfgA might play a key role in controlling conidiation in *A. nidulans* (SEO *et al.* 2003). The recessive nature of the *sfgA* suppressor mutations suggests that suppression of Δ*fluG* results from loss-of-function mutations in the *sfgA* locus. The *sfgA* gene was isolated by transformation-based complementation employing a wild-type genomic DNA library (see MATERIALS AND METHODS). Blastn search of the *A. nidulans* genome database (the Broad Institute: http://www.broad.mit.edu/annotation/fungi/aspergillus/index.html) using the sequences derived from the Tn-tagged plasmids has identified a contig that maps to chromosome II. We also searched the *A. nidulans* EST database (http://www.genome.ou.edu/asper_blast.html) and found an EST clone that could define the 3′ end of the *sfgA* mRNA coding region. Positions of Tn integration, sequence analyses of the RT–PCR product, and the EST information (10b04a1.f1) led to the identification of the *sfgA* ORF composed of 1908 bp with 49- and 53-bp introns (Figure 2A). The 2.7-kb *sfgA* mRNA accumulates at high levels during the vegetative growth phase and decreases at later stages in asexual and sexual development (Figure 2B), implying that *sfgA* is subjected to transcriptional regulation.

The predicted SfgA protein is a 68-kDa (601-aa) protein with the fungal-specific $Zn(II)_2Cys_6$ (or C_6) DNA-binding domain at the N terminus. This DNA-binding domain is found in a number of regulatory proteins including Gal4p (LAUGHON and GESTELAND 1984), AlcR (FELENBOK *et al.* 1988), Ume6p (STRICH *et al.* 1994), and Nit4 (YUAN and MARZLUF 1992; see Figure 2C). The highly conserved six cysteine residues bind two zinc atoms, forming a structure (Zn_2Cys_6) that is required for the recognition of specific DNA sequences (PAN and COLEMAN 1990; MARMORSTEIN *et al.* 1992). The presence of such a conserved DNA-binding domain indicates that SfgA may function as a transcription factor (TF). Furthermore, SfgA contains a nuclear localization signal at the N terminus (~31st–48th aa) and has a 82.6% probability of nuclear localization [predicted by PSORT II (psort.nibb.ac.jp/form2.html)]. While no dimer probability was detected, potential (homo/hetero) dimerization of SfgA cannot be excluded.

Analyses of *sfgA* alleles: To verify that the identified gene truly defines *sfgA*, the coding region of *sfgA* from the original 31 *sfgA* mutants (*sfgA*^{S} Δ*fluG*) was PCR amplified and each amplicon was sequenced directly. Analyses of sequences revealed that all the *sfgA* mutants contain mutations, which are distributed throughout the *sfgA* ORF (Figure 3). In summary, the *sfgA* mutant alleles are derived from 18 missense and 9 nonsense mutations, two insertions and two deletions followed by frameshift and early termination (Figure 3, fs*). Interestingly, some mutant alleles (2 and 4; 15, 35, and 37) are identical, suggesting possible mutation hot spots. All of the nine nonsense *sfgA* mutant alleles resulted in the fully restored conidiation. The fact that even the *sfgA34* and *sfgA36* mutations causing truncation of only 14 and 30 aa from the C-terminal end, respectively, could restore conidiation to the wild-type level in the absence of *fluG* indicates that the full-length SfgA is required for its proper function.

Twelve (6 nonsense and 6 missense) *sfgA* mutants produced conidiophores in liquid submerged culture, which normally suppresses sporulation (for reference see Figure 4C). Among these, 5 mutants have mutations clearly affecting the C6 domain. The *sfgA46* and *sfgA48* mutant alleles have nonsense mutations prior to the C6 domain, which essentially nullify *sfgA*. The *sfgA45*, *sfgA44*, and *sfgA50* mutant alleles have missense mutations within the C6 domain that are predicted to abolish the DNA-binding ability. The *sfgA45* allele has a substitution of the 40th aa Gly to Cys (*sfgA*^{G40C}), where Gly40 is just prior to the first Cys (the 41st aa), forming the zinc



Allele	Position (nt#)	Mutations		Allele	Position (nt#)	Mutations	
1 (HS)	1107	GAC → TAC	D353Y	33 (W)	154	GAT → CAT	D52H
2 (W)	1648	TGG → TCG	W533S	34 (HS)	1863	TGG → TAG	W587*
4 (W)	1648	TGG → TCG	W533S	• 35 (W)	1487	GAA → TAA	E480*
6 (DS)	118	GGT → CGT	G40R	36 (W)	1814	GAG → TAG	E571*
11 (DS)	741	AAC → AAG	N247K	• 37 (W)	1487	GAA → TAA	E480*
12 (HS)	i1365	iTACAACGATTAG	i439QRLV	38 (W)	1669	CGG → CCG	R540P
13 (W)	889	GAG → TAG	E297*	• 42 (HS)	95	CGA → CCA	R32P
14 (HS)	721	GGA → AGA	G241R	• 43 (W)	1172	CG → T	375fs*
• 15 (W)	1487	GAA → TAA	E480*	• 44 (HS)	144	CATGTC→CAATTC	HV48QF
18 (W)	469	CAG → TAG	Q157*	• 45 (W)	118	GGT → TGT	G40C
22 (HS)	1197	GGA → AGA	G383R	• 46 (W)	Δ40~52	ΔCCTCAACCTCTAC	Δ14PQPL→fs*
23 (W)	1184	TGG → TGA	W378*	• 47 (W)	1483	TGC → TTC	C478F
26 (DS)	116	ACC → ATC	T39I	• 48 (W)	109	GGA → TGA	G37*
• 30 (W)	662	CGA → CCA	R221P	• 50 (W)	182	TGT → TCT	C61S
32 (W)	i1382	iCGATTAGTACAA	i444RLVQ	51 (DS)	1207	AGT → AAT	S386N
				54 (W)	1116	GGA → AGA	G356R

• : submerged sporulation DS: delayed sporulation W: wild type level sporulation HS: hyper sporulation
fs: frame shift *: stop i: insertion Δ: deletion fs*: frame shift followed by stop

FIGURE 3.—Summary of the 31 *sfgA* mutant alleles. The *sfgA* ORF composed of 1908 nucleotides and each *sfgA* mutation is presented. In the table, allele number, position, and nature of the mutation and resulting amino acid change(s) for each *sfgA* mutant allele are indicated. Phenotypes of the *sfgA* mutants are divided into three groups on the basis of the levels of restored conidiation on solid medium: wild type (W), hypersporulation (HS), and delayed sporulation (DS). Twelve mutants that elaborated conidiophores in liquid submerged culture are marked by red dots under (or before) the allele number.

clustered binuclear structure. Although the amino acid conservation of that position is low (see Figure 2C), it is possible that such G40C substitution resulting in the two repeated Cys residues might disturb proper binuclear formation. The *sfgA50* allele has a G to C transversion, causing a substitution of the 61st aa Cys to Ser (*sfgA*^{C61S}). The 61st aa Cys of SfgA is the last one of the six Cys residues, suggesting that the *sfgA50* mutation may cause an effect similar to that caused by the null allele (see Figure 4). The *sfgA44* allele is defined by two nucleotide changes (TG → AT) affecting two amino acids at the 48th and 49th positions, *sfgA*^{H48QV49F}. Crystallographic analysis of Gal4p has shown that the Lys-18 (equivalent to His-48 in SfgA) residue is necessary for formation of multiple sequence-specific bonds with the Gal4-binding site (MARMORSTEIN *et al.* 1992). Thus, such His (basic) to Gln (uncharged polar) substitution alone would likely abolish the DNA-binding ability of SfgA, resulting in a (complete) loss of function. Both SFGA50 and SFGA44 mutants elaborated complete conidiophore structures within 20 hr of liquid culture (Figure 4C; SEO *et al.* 2003). The fact that the *sfgA30*, *-42*, and *-47* mutations defined by R221P, R32P, and C478F substitutions, respectively, resulted in development of conidiophores in liquid submerged culture suggests that such amino acid changes may completely abolish SfgA function.

Those *sfgA* mutants with partially restored conidiation exhibited delayed sporulation phenotypes on solid medium, indicating that each mutant allele might have

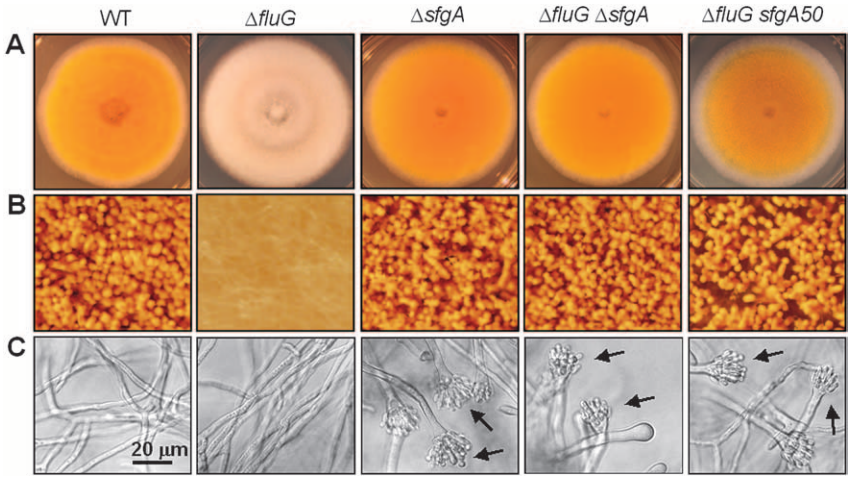


FIGURE 4.—Suppression of Δ*fluG* by Δ*sfgA* and *sfgA50*. The conidia of wild-type (WT; RJA56.25), Δ*fluG* (TTA127.4), Δ*sfgA* (TYGN2), Δ*fluG* Δ*sfgA* (RYG3), and Δ*fluG sfgA50* (SFGA50) strains were point inoculated on solid medium (A and B) and colony photographs were taken at 3 days incubation at 37° (see B for close-up views). All strains carried a spore color marker (*yA2*) and, thus, produced yellow conidia. (C) Conidiophore formation in liquid submerged culture was photographed at 22 hr of incubation. Note that Δ*sfgA*, Δ*fluG* Δ*sfgA*, and Δ*fluG sfgA50* strains showed fully restored conidiation (A and B) as well as conidiophore formation (arrows) in liquid submerged culture (C).

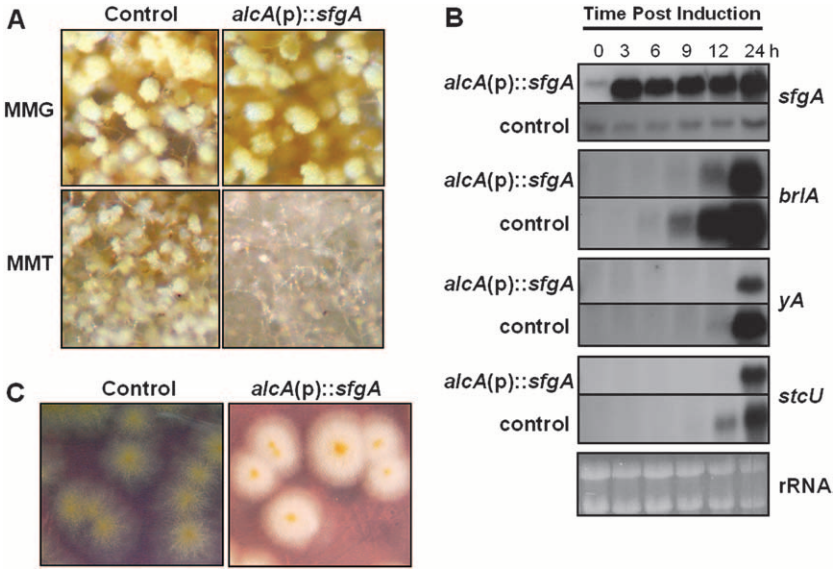


FIGURE 5.—Overexpression of *sfgA* inhibits conidiation. (A) Control (TSR2.1) and *alcA*(p)::*sfgA* (TJAS5.7) strains were grown in liquid glucose medium for 14 hr and then transferred onto solid medium with glucose (MMG; noninducing) or threonine (MMT; inducing) as a sole carbon source. Photographs were taken at 24 hr post-transfer. (B) Samples were collected from control and *alcA*(p)::*sfgA* strains at designated time points postinduction on solid threonine medium (MMT; shown in A). Total RNA was isolated and subjected to Northern blot analyses for the levels of *sfgA*, *brlA*, *yA*, and *stcU* mRNA. Equal loading of total RNA was evaluated by ethidium bromide staining of rRNA. (C) The conidia of control and *alcA*(p)::*sfgA* strains were directly inoculated (streaked) onto solid MMT and incubated at 37° for 3 days. Each colony was derived from a single conidium. Note that *alcA*(p)::*sfgA* colonies exhibit white undifferentiated hyphae (fluffy) with low levels of conidiation in the center.

different remaining repressive activity. All four partial suppressor mutants (SFGA6, -11, -26, and -51) have missense mutations. The *sfgA26* and *sfgA6* alleles have T39I and G40R substitutions within the C6 domain, respectively, that might not be as detrimental as the above-mentioned *sfgA45*^{G40C} mutation. The *sfgA11* and *sfgA51* alleles have N247K and S386N missense mutations, respectively.

Deletion of *sfgA* eliminates the need for *fluG* in conidiation and ST production: To verify that elimination of *sfgA* function is sufficient to bypass the *fluG* function, we generated the *sfgA* deletion mutant with *argB*$^+$ as a selective marker. Several Δ*sfgA* transformants were isolated and subsequently crossed with a Δ*fluG* strain (RJA4.4). Three independent Δ*fluG* Δ*sfgA* strains were isolated and were found to behave identically. The Δ*fluG* Δ*sfgA* double mutant exhibited conidiation levels similar to those of wild type and was indistinguishable from the *fluG*$^+$ Δ*sfgA* single mutant (Figure 4, A and B). As did the *fluG*$^+$ Δ*sfgA* and Δ*fluG sfgA50* mutants, the Δ*fluG* Δ*sfgA* mutant produced conidiophores within 20 hr in liquid submerged culture (Figure 4C). Moreover, the *fluG*$^+$ Δ*sfgA*, Δ*fluG sfgA50*, and Δ*fluG* Δ*sfgA* mutants accumulated *brlA* mRNA as early as 18 hr in liquid submerged culture (not shown). Finally, the Δ*fluG* Δ*sfgA* mutant regained the ability to produce ST and restored the expression of ST-specific genes (Brown *et al.* 1996) at wild-type levels (not shown). These results indicate that SfgA plays a key role in negative regulation of conidiation and ST production, and that the primary role of FluG is to remove SfgA-mediated repression of conidiation.

To further address a potential additional positive role of FluG in conidiation, we examined levels of conidiation (and growth) of wild-type, Δ*sfgA fluG*$^+$, and Δ*sfgA* Δ*fluG* strains in various ways. If FluG plays an additional role in conidiation, Δ*sfgA fluG*$^+$ would likely cause higher levels of sporulation than Δ*sfgA* Δ*fluG*. Conversely, if FluG's main role were to remove the SfgA-mediated repressive effects, both mutants would exhibit the same phenotypes. We compared multiple strains for: (i) timing and levels of conidiation in liquid submerged culture, (ii) timing of the first conidiophore formation from a single conidium on various solid media, and (iii) number of conidia per square centimeter and per colony on various solid media. The two mutants showed no differences in levels (or timing) of conidiation or vegetative growth (radial growth and dry mass), confirming that FluG's primary role is to remove the repressive effects imposed by SfgA.

Overexpression of *sfgA* inhibits conidiation: To further verify the repressive role of *sfgA* in conidiation and ST production we generated the *sfgA* overexpression mutant by fusing the *sfgA* ORF with the inducible *alcA* promoter (Gwynne *et al.* 1987). Multiple strains with a single-copy integration of the *alcA*(p)::*sfgA* fusion construct were generated and expression of *sfgA* was controlled by varying carbon sources: *e.g.*, glucose (noninducing) or threonine (inducing). Phenotypic changes caused by overexpression of *sfgA* were evaluated in two ways and the *alcA*(p)::*sfgA* mutant exhibited repressed conidiation levels under all inducing conditions (Figure 5, A and C). To correlate phenotypic changes caused by overexpression of *sfgA* with the molecular events, we examined mRNA levels of selected developmental or ST regulators (or indicators) including *brlA*, *yA*, and *stcU* (reviewed in Adams *et al.* 1998). Overexpression of *sfgA* resulted in delayed and/or reduced levels of mRNA of all genes examined (Figure 5B). This result indicates that the elevated level of *sfgA* mRNA is sufficient to inhibit the expression of developmental and ST genes. Constitutive level accumulation of *flbD* (Wieser and

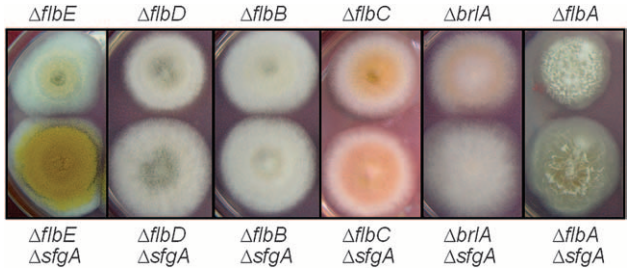


FIGURE 6.—Phenotypes of various double mutants. Colonies of designated single- and double-deletion mutants grown on solid glucose medium for 3 days at 37° are shown. Note partial suppression of Δ*flbE* by Δ*sfgA*.

ADAMS 1995) and *flbC* (this study) mRNA was not affected by overexpression of *sfgA* (not shown), indicating that *sfgA*-mediated repression of conidiation does not occur by altering mRNA levels of *flbD* or *flbC*.

Genetic position of *sfgA* in the FluG-dependent conidiation pathway: To determine the genetic position of *sfgA* in the FluG-initiated asexual developmental regulatory cascade, a series of double-mutant analyses was carried out. As shown in Figure 6, deletion of *sfgA* could not bypass the need for *flbA*, *flbD*, *flbB*, *flbC*, or *brlA* in conidiation, indicating that *sfgA* functions upstream of these genes. In contrast, Δ*sfgA* suppressed Δ*flbE* to some degree (note the fluffy nonsporulating region at the growing edge of the colony in Figure 6), implying that SfgA functions downstream or at the same level as FlbE. The *flbE* gene is predicted to encode a novel protein (201 aa) that is conserved in many filamentous fungal species (J. K. WIESER and T. H. ADAMS, personal communication). On the basis of the genetic data, two new genetic models are proposed (see DISCUSSION and Figure 7).

DISCUSSION

While asexual sporulation is a common reproductive mode for many filamentous fungi, the mechanisms underlying the initiation of asexual spore formation are largely unknown. In this study, based on the useful framework established by the previous studies (see Figure 1; reviewed in ADAMS *et al.* 1998; SEO *et al.* 2003), we unveiled that the FluG-dependent activation of asexual development in the model fungus *A. nidulans* requires removal of negative regulation imposed by the novel protein SfgA.

SfgA belongs to a family of Zn binuclear cluster proteins (or C6 factors) that are transcriptional regulators of genes involved in a wide variety of cellular/physiological processes including carbon/nitrogen utilization, secondary metabolism, and multicellular development (reviewed in TODD and ANDRIANOPOULOS 1997). This family includes >50 and 100 different *Saccharomyces cerevisiae* (Sc) and *A. nidulans* (An) proteins, respectively. The classical examples are Sc–Gal4p and An–AlcR that activate the genes of the galactose and alcohol utilization pathway, respectively, permitting the use of galactose and alcohol as the sole source of carbon (LAUGHON and GESTELAND 1984; FELENBOK *et al.* 1988). Biosynthesis of certain secondary metabolites including aflatoxins, sterigmatocystin (reviewed in YU and KELLER 2005), and fumonisins (FLAHERTY and WOLOSHUK 2004) is also controlled by C6 factors. In most cases, C6 factors are known to regulate (activate) a set of genes specific for a given metabolic or physiological pathway and thus are often called pathway-specific transcription factors. Recent studies have identified C6 factors that are involved in regulation of sexual development in *A. nidulans* (VIENKEN *et al.* 2005) or asexual development in *Neurospora crassa* (RERNGSAMRAN *et al.* 2005).

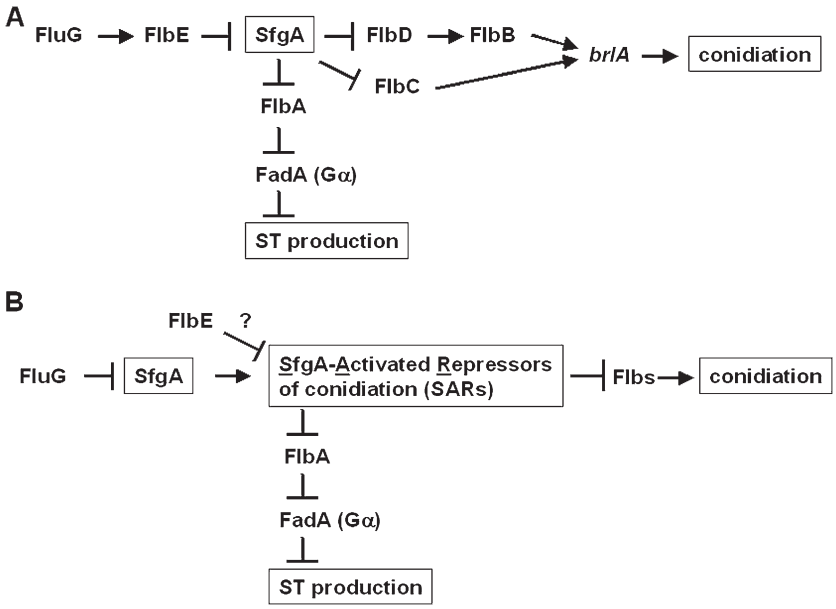


FIGURE 7.—Genetic models for regulation of conidiation and ST production in *A. nidulans*. (A) A simple genetic model in which SfgA functions downstream of FlbE. This model is not sufficient to explain the partially restored conidiation phenotype of the Δ*flbE* Δ*sfgA* mutant or the production of ST by the Δ*flbE* mutant (HICKS *et al.* 1997). (B) A complex working model. The fact that most C6 factors function as transcriptional activators (reviewed in TODD and ANDRIANOPOULOS 1997) led to the proposal that SfgA activates a group of genes (*S*fgA-*A*ctivated *R*epressor*s* of conidiation, SARs) that negatively control conidiation, and FlbE competes with SfgA to inhibit expression of SARs. Accordingly, deletion of both *sfgA* and *flbE*, *i.e.*, lack of both activation and inhibition, may cause the elevated basal level expression of SARs, which partially represses conidiation, resulting in the Δ*flbE* Δ*sfgA* phenotype (Figure 6).

It is evident that SfgA has multiple functional domains and the full-length SfgA is required for its functionality. As revealed by allele analyses, the integrity of the C6 domain is crucial for SfgA function. Mutations predicted to affect the C6 domain result in hyperactive conidiation phenotypes essentially identical to those caused by deletion of *sfgA*. Furthermore, any mutations causing at least partial loss of *sfgA* function(s) seem to be sufficient to restore conidiation to certain levels (Figure 3). Deletion of *sfgA* eliminates the requirement of FluG in conidiation and ST production. These data clearly suggest that the primary role of FluG is to remove repressive effects imposed by SfgA.

One of the key hypotheses in *A. nidulans* asexual developmental control is that FluG is responsible for the production of an extracellular factor that is required for developmental initiation. Earlier studies suggested that this factor is <6000–8000 Da in size (Lee and Adams 1994b). Levels of the *fluG* mRNA and protein are relatively constant throughout the life cycle (Lee and Adams 1994b), implying that the amount of the FluG factor may increase gradually in vegetative cells. As overexpression of *fluG* did (Lee and Adams 1996; D'Souza *et al.* 2001), deletion and 12 other *sfgA* mutant alleles resulted in formation of conidiophores in liquid submerged cultures that have all the cell types including stalks, vesicles, metulae, phialides, and conidia even in the absence of *fluG*. Taken together, it is speculated that during the early vegetative growth phase the level of the FluG factor in cells is low and SfgA-mediated repression of conidiation dominates. Once accumulation of the FluG factor reaches a certain level in cells, it removes SfgA-mediated negative control of conidiation (possibly involving downregulation of *sfgA* transcription), which triggers the initiation of conidiophore development.

Another key role of FluG is thought to be post-transcriptional activation of FlbA (Lee and Adams 1996), which in turn attenuates vegetative proliferation signaling mediated by a heterotrimeric G protein composed of FadA (Gα), SfaD (Gβ), and GpgA (Gγ) (Yu *et al.* 1996; Rosén *et al.* 1999; Seo *et al.* 2005). Constitutive activation of this FadA signaling blocks development and production of ST (see Figure 1; Yu *et al.* 1996, 1999; Hicks *et al.* 1997). It was demonstrated previously that mutational inactivation of FadA bypassed the requirement of FluG in ST biosynthesis but not in conidiation (Hicks *et al.* 1997). This observation has led to the hypothesis that the primary role of FluG in activating ST is to activate FlbA, which in turn inactivates FadA signaling, shifting the physiological balance in favor of development. However, the role of FluG in activating FlbA has remained elusive. Our findings that deletion and all 31 *sfgA* mutations restored ST production in the Δ*fluG* mutant may also provide a missing link between FluG and FlbA. It can be speculated that the end result of SfgA activity is negative regulation of conidiation and FlbA, and removal of SfgA-mediated repression by FluG confers both activation of conidiation and inhibition of FadA signaling (see Figure 7). Further characterization of SfgA coupling with G-protein signaling will elucidate a regulatory network coordinating the balance between growth and development.

Genetic data clearly demonstrate that *sfgA* functions downstream of *fluG* but upstream of *flbD, flbB, flbC*, and *brlA* (Figure 6). In contrast, the genetic interaction between *sfgA* and *flbE* cannot be easily resolved. While a simple model for *sfgA* functioning downstream of *flbE* can be proposed (Figure 7A), this model cannot explain the incomplete suppression of Δ*flbE* by Δ*sfgA* as well as the ability of the *flbE*$^{-}$ mutant to produce ST (Hicks *et al.* 1997). Thus, we propose an alternative model that FlbE and SfgA function at the same level and compete for the expression of downstream genes (Figure 7B). Regardless of the position of *sfgA*, our study elucidates a new concept for the FluG-dependent central regulatory mechanism of conidiation in the model fungus *A. nidulans*.

The authors are grateful to the Broad Institute for the genome database and to Amy Wong and Ellin Doyle for critically reviewing this manuscript. Special thanks are given to Thomas Adams, Jenny Wieser, Ellen Kellner, BeeNa Lee, Sang-Tae Han, and other fungal developmental biologists. This work was supported by Hatch grant WIS-04667 and National Science Foundation grant MCB-0421863 to J.H.Y.

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Communicating editor: J. J. Loros