Suppressor Mutations Bypass the Requirement of fluG for Asexual Sporulation and Sterigmatocystin Production in Aspergillus nidulans

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Manuscript received November 19, 2002
Accepted for publication July 17, 2003

ABSTRACT

Asexual sporulation (conidiation) in the filamentous fungus Aspergillus nidulans requires the early developmental activator fluG. Loss of fluG results in the blockage of both conidiation and production of the mycotoxin sterigmatocystin (ST). To investigate molecular mechanisms of fluG-dependent developmental activation, 40 suppressors of fluG (SFGs) that conidiate without fluG have been isolated and characterized. Genetic analyses showed that an individual suppression is caused by a single second-site mutation, and that all sfg mutations but one are recessive. Pairwise meiotic crosses grouped mutations to four loci, 31 of them to sfgA, 6 of them to sfgB, and 1 each to sfgC and sfgD, respectively. The only dominant mutation, sfgA38, also mapped to the sfgA locus, suggesting a dominant negative mutation. Thirteen sfgA and 1 sfgC mutants elaborated conidiophores in liquid submerged culture, indicating that loss of either of these gene functions not only bypasses fluG function but also results in hyperactive conidiation. While sfg mutants show varying levels of restored conidiation, all recovered the ability to produce ST at near wild-type levels. The fact that at least four loci are defined by recessive sfg mutations indicates that multiple genes negatively regulate conidiation downstream of fluG and that the activity of fluG is required to remove such repressive effects.

ASexual sporulation in Aspergillus nidulans is a continual progression from growth to development and is a precisely timed and genetically programmed event in the life cycle in response to internal and external cues. It involves formation of multicellular reproductive organs termed conidiophores, each of which produces thousands of mitotically derived spores (for review see Adams 1994; Adams et al. 1998). In previous studies, we showed that two antagonistic signaling pathways control growth and asexual development in A. nidulans. Growth signaling is mediated by FadA and SfaD, the α- and β-subunits for a heterotrimeric G protein, respectively. When FadA (Gα) is active, GTP-bound FadA and the heterodimer SfaD(α)/Gγ are dissociated and both signal to enhance proliferative growth, which in turn represses both asexual sporulation and sterigmatocystin (ST) production (Figure 1; Yu et al. 1996; Hicks et al. 1997; Rosen et al. 1999). Constitutive activation of FadA growth signaling results in the fluffy autolytic phenotype. Initiation of asexual development requires the activity of two major genes, flbA and fluG. FluG is responsible for the production of an extracellular factor and it stimulates both development-specific events and activation of FlbA, which in turn inactivates FadA signaling (Lee and Adams 1994b; Yu et al. 1996). FlbA is a regulator of G protein signaling (RGS) domain protein and, like other RGS proteins, is predicted to negatively regulate G protein signaling by facilitating the intrinsic GTPase activity of the Gα (FadA)-subunit (Lee and Adams 1994a; Berman et al. 1996; Koelle and Horvitz 1996; Yu et al. 1996; Hepler et al. 1997). Asexual development requires both inhibition of growth and activation of sporulation.

A key step in the formation of conidiophores is activation of the brlA gene, which encodes a C2H2 zinc finger transcriptional activator required for expression of sporulation-specific genes (Figure 1; Adams et al. 1988; Chang and Timberlake 1992). Genes affecting brlA expression have been identified and characterized and these include fluG, flbA, flbB, flbC, flbD, and flbE. Mutations in all of these genes result in undifferentiated fluffy colonies (Wieser et al. 1994). Two of the delayed conidiation loci, flbC and flbD, are predicted to encode DNA-binding proteins and represent potential direct activators of brlA expression (Wieser and Adams 1995). Because mutations in flbC and flbD have additive effects on development, it has been proposed that these genes control independent steps in a nonlinear pathway (Figure 1). The flbB gene is predicted to encode a bZIP-like transcription factor and flbE does not have clear homologs in the available databases (J. Wieser and T. H. Adams, personal communication). By testing the genetic requirements for the inappropriate conidiation observed

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Genetics 165: 1083–1093 (November 2003)
In this article, we describe the isolation, characterization, and genetic analyses of 40 suppressors of \( \text{fluG} \) (SFGs) that bypass the need of \( \text{fluG} \) in conidiation and production of ST in \( A. nidulans \). Such second site mutations will be extremely useful to further dissect early regulatory mechanisms of asexual sporulation. As the \( \text{fluG} \)-dependent asexual sporulation is independent of and parallel to \( \text{FadA} \) and \( \text{SfaD} \)-mediated growth signaling, no mutations in \( \text{FadA} \) or \( \text{SfaD} \) were able to suppress \( \Delta \text{fluG} \) (Yu et al. 1996; Rosen et al. 1999). Thus, these SFGs will likely identify genes that specifically function in the conidiation regulatory pathway downstream of \( \text{FluG} \). The fact that at least four loci are defined by 39 sfg mutations and all but 1 of the sfg mutations are found to be recessive strongly indicates that genes defined by sfg mutations likely function as negative regulators of conidiation. Moreover, all SFGs are found to produce ST in the absence of \( \text{fluG} \) activity, suggesting that these sfg genes may also negatively affect \( \text{flbA} \) activity and mutations in sfg genes might cause at least partial inhibition of \( \text{FadA} \) growth signaling to allow ST production to occur. A new model for upstream regulation of asexual development and growth is also presented.

**MATERIALS AND METHODS**

**Aspergillus strains, media, growth conditions, and genetic analysis:** The \( A. nidulans \) strains used in this study are listed in Table 1. Genotypes of SFGs are essentially the same (\( \text{pyrG89}, \text{pyrC801}, \text{veA1}; \text{sfg}^+ \)) except for the \( \text{sfg} \) locus and mutant alleles. Standard culture and genetic techniques were employed (Pontecorvo et al. 1953; Käfer 1977). The growth rates of SFGs were checked on minimal medium (MM) from 2 to 5 days. All liquid cultures were inoculated with \( 5 \times 10^7 \) spores in 100 ml of liquid MM or MM with 0.1% yeast extract (YE) with 250 rpm shaking at 37°C. Submerged development in each SFG was observed under a microscope at 1-hr intervals after an initial 18-hr growth period in liquid shake culture. Asexual developmental induction was performed as previously described (Adams et al. 1988) and samples for RNA isolation were collected at designated time points.

All SFGs were crossed with a developmentally wild-type strain, FGSC773. From these crosses, \( \Delta \text{fluG}::\text{sfg} \) strains carrying the \( \text{pyrG89} \) allele were also isolated to carry out pairwise crosses to determine the number of loci and for future gene cloning (Table 1). The dominance or recessiveness of each \( \text{sfg} \) mutation was tested by generating diploid strains between each SFG strain (\( \Delta \text{fluG}::\text{sfg} \)) and RJH128 (\( \Delta \text{fluG}::\text{sfg}^{\text{pyrG89}} \)) or RJA4.4 (\( \Delta \text{fluG}::\text{sfg}^{\text{pyrG89}} \)).

**Mutagenesis and isolation of SFGs:** A \( \Delta \text{fluG} \) strain (TTA127.4) was point inoculated on supplemented solid MM and incubated at room temperature for 7–10 days and conidia were collected for mutagenesis. Approximately \( 10^8 \) conidiospores of TTA127.4 were treated with 1 mg/ml or 10 mg/ml of 4-nitroquinoline-1-oxide (4-NQO; Bal. et al. 1977) for 0, 30, and 60 min, respectively, as previously described (Wieser et al. 1994). The mean survival rate of the 1-mg 4-NQO with a 30-min treatment was 67% and survivors of this condition were further screened.

**ST extraction and TLC analysis:** Spores (\( \sim 10^8 \)) of each SFG were inoculated into 2 ml liquid complete medium (CM) in 8-ml tubes and the stationary cultures were incubated at 37°C for 7 days as previously described (Yu and Leonard 1995). ST was extracted from 7-day-old cultures by adding 1 ml of

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**FIGURE 1.—Genetic model for growth and development in \( A. nidulans \). In this model, the activities of two antagonistic signaling pathways control growth and asexual sporulation (and ST production). Growth signaling is mediated by a heterotrimeric G protein composed of FadA (Ga) and SfaD (Gβγ). Activated FadA-GTP and SfaD-Gγ transduce signals to downstream effectors that include PkaA (a catalytic subunit of protein kinase A; Shimizu and Keller 1995). Activation of proliferative growth represses both asexual sporulation and ST biosynthesis. FlbA is an RGS protein that rapidly turns off FadA-mediated growth signaling likely by acting as a GTPase-activating protein (GAP) for FadA. At least partial inhibition of FadA-mediated growth signaling is required for conidiation and ST production. Activation of asexual sporulation requires the activities of FluG and other downstream developmental genes, \( \text{flbB}, \text{flbC}, \text{flbD}, \text{flbE}, \) and \( \text{brlA} \) (for review see Adams et al. 1998). The following overexpression of \( \text{fluG}, \text{flbA}, \) or \( \text{flbD} \), the gene order, \( \text{fluG} \rightarrow \text{flbE} \rightarrow \text{flbD} \rightarrow \text{flbB} \), has been proposed (Wieser and Adams 1995). As \( \text{fluG} \) functions first in this regulatory network, \( \text{fluG} \) overexpression requires the activities of \( \text{flbC}, \text{flbA}, \) and \( \text{flbD} \) for activating \( \text{brlA} \) and causing conidiation in submerged culture (Lee and Adams 1996). The \( \text{fluG} \) gene encodes a cytoplasmically localized 96-kD (864 amino acid) protein that is present at relatively constant levels throughout the life cycle (Lee and Adams 1994b). The C-terminal half of \( \text{FluG} \) shares \( \sim 30\% \) identity with prokaryotic glutamine synthetase I whereas the N-terminal half of the protein shares no significant similarity with any functionally characterized proteins in the databases. Moreover, the entire N-terminal region could be deleted without affecting sporulation (D’Souza et al. 2001), indicating that \( \text{FluG} \) activity resides in the C-terminal half of the protein, which could be involved in constitutive synthesis of a small diffusible molecule related to glutamine or glutamate (Lee and Adams 1994b). Although \( \text{FluG} \) activity is known to be required for activation of conidiation and for post-transcriptional activation of \( \text{FlbA} \) (Adams et al. 1988; Wieser and Adams 1995; Lee and Adams 1996), specific molecular events responding to \( \text{fluG} \) activity and leading to the developmental switch from vegetative growth remain to be uncovered.
Defects of a loss of FluG took an unbiased approach involving the isolation of the crosses generated matured cleistothecia. If suppression into the molecular events arising from extraction as described above. Figure 5), duplicate samples were prepared and collected from stcU compare ST production with stcU from wild-type (FGSC4) genomic DNA. A 1.48-kb brlA were prepared by amplifying coding regions of brlA and stcU from wild-type (FGSC4) genomic DNA. A 1.48-kb brlA and a 1.12-kb stcU (Brown et al. 1996) amplicon were labeled with 32P-dCTP using a random-primer kit (Promega, Madison, WI) and used as probes for Northern blot analyses. Hybridization was carried out using modified Church buffer (1 mM EDTA, 0.25 mM Na2HPO4, 7H2O, 1% hydrolysated casein, 7% SDS, adjusted to pH 7.4 with 85% H3PO4; Yu and Leonard 1995).

Genomic DNA of wild-type and SFG strains was isolated (SFGs) that restored conidiation to clearly distinguish-by adding 0.6 ml of silica/zirconium beads, 0.5 ml of able levels. These 40 SFG mutants show varying levels breaking buffer [2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA], and 0.5 ml of pheno:chloroform:isoamylalcohol (25:24:1) to mycelial samples. The plate was then developed with toluidine blue:acetic acid (80:10:10, v/v/v), where the Rf value of ST is ~0.65. At this step ST exhibits dark red color under the long-wave UV (320 nm). To enhance visibility and detection limit for ST, aluminum chloride (20% AlCl3 ·6 H2O in 95% ethanol) is sprayed on to the TLC plate and the plate is baked at 80°C for 5 min. The color of ST changes from red to exhibit bright light green by this process (Stack and Rodrick 1971). To compare ST production with stcU mRNA levels (presented in Figure 5), duplicate samples were prepared and collected from days 1–4, one for total RNA isolation and the other for ST extraction as described above.

**Extracellular sporulation defect of ΔfluG by SFGs:** The green conidial ΔfluG strains (RJA23.1 and RJA23.2) were point inoculated at the center of MM with 0.5% YE and three SFGs were inoculated both sides of each ΔfluG strain in duplicate. The strains were incubated at 37°C for ~2–3 days and examined under a stereomicroscope for possible extracellular sporulation as previously described (Lee and Adams 1994).

**Nucleic acid isolation and manipulation:** Total RNA was isolated by adding 0.6 ml of silica/zirconium beads (Biospec, Bartlesville, OK) and 1 ml of Trizol (Invitrogen, San Diego) and homogenizing in a Mini Bead Beater (Biospec) for 2 min and then subsequently following manufacturer’s instructions (Invitrogen). Total RNA (15 μg/lane) was separated by electrophoresis using a 1.1% agarose gel containing 6% formaldehyde and ethidium bromide and the nucleic acids were transferred to a MagnaProbe Nylon membrane (0.45 μm; Osmonics, Minnetonka, MN). Probes for brlA and stcU from wild-type and SFG strains was isolated (SFGs) that restored conidiation to clearly distinguish-by adding 0.6 ml of silica/zirconium beads, 0.5 ml of able levels. These 40 SFG mutants show varying levels breaking buffer [2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA], and 0.5 ml of pheno:chloroform:isoamylalcohol (25:24:1) to mycelial samples followed by homogenizing in a Mini Bead Beater for 2 min. DNA in the aqueous phase was collected and ethanol precipitated. The purified genomic DNA in 50 μl of Tris-EDTA buffer was diluted 10 times for PCR reactions.

**Microscopy:** Photomicrographs were taken using an Olympus BH2 compound microscope with the Kodak MDS290 digital imaging system. All other photographs were taken using a SONY digital camera (DSC-F707).

**RESULTS**

**Isolation of suppressors of fluG:** To gain further insights into the molecular events arising from fluG activity and leading to activation of developmental switch, we took an unbiased approach involving the isolation of second-site mutations that overcome the sporulation defects of a loss of fluG function. Unlike the previous study where a homozygous ΔfluG diploid strain was employed to specifically look for dominant suppressor mutations (D’Souza et al. 2001), we used a ΔfluG haploid strain (TTA127.4), expecting that various recessive and/or dominant suppressor mutations would be isolated. We visually screened ~125,000 survivors from 4-NQO mutagenesis and isolated 40 putative suppressors of ΔfluG (SFGs) that restored conidiation to clearly distinguishable levels. These 40 SFG mutants show varying levels of conidiation recovery and on the basis of the phenotypes on solid MM, the mutants were tentatively grouped into three classes: 9 high sporulators (H), 18 wild-type level sporulators (W), and 13 delayed sporulators (DS). Delayed sporulators initially resemble the ΔfluG mutant for ~2–3 days, but most of them achieve wild-type sporulation levels within 5 days. Representative SFG strains are presented in Figure 2. To be consistent, we designate a mutant strain with SFG#, a mutant allele with sfg#, suppressor mutations collectively with sfg#, and a wild-type allele with sfgWT.

**Individual suppression is caused by a single second-site mutation unlinked to fluG:** As a first step in genetic analyses, each SFG strain was meiotically crossed with a developmentally wild-type strain (FGSC7773) and all of the crosses generated matured cleistothecia. If suppression was caused by a single second-site mutation, segregation of the relevant genotypes (with corresponding
phenotypes) of progeny would be ΔfluG;sfgA (condi-
tiating), fluG⁺;sfgA (probably conjugating like wild type),
ΔfluG;sfgA⁰¹ (fluG⁺ due to ΔfluG), and fluG⁺;sfgA⁰¹ (wild
type), thus generating 25% fluffy progeny. All of our
successful crosses showed ~25% recovery of ΔfluG
(fluffy phenotype) progeny, indicating that a single
gene mutation, not linked to fluG, caused individual
suppression. In addition, no new phenotypes among
the conjugating progeny were evident, indicating that
individual sfgA mutation does not cause readily detect-
able morphological changes with respect to wild-type
FluG function. In these crosses, we have also isolated
multiple recombinant SFG strains carrying the auxotro-
phic marker pyrG89 and these were used to determine
the number of sfg loci (see below). Isolation of ΔfluG;
sfgA; pyrG89 strains was accomplished by examining ~10
independent uracil-requiring conjugating progeny from
each cross for the ΔfluG pattern by genomic DNA PCR.
Conidial strains with the ΔfluG PCR pattern are ex-
pected to be ΔfluG with sfgA (Table 1).

Dominance and recessiveness of sfgA: As a prerequisite
of the SFG gene identification we tested whether each
dsfgA is dominant or recessive to its wild-type allele by
generating diploids that are homozygous for ΔfluG and
heterozygous for sfgA (sfgA⁰¹; sfgA⁰¹) by fusing an individual
SFG strain with another ΔfluG strain, RJH1128 or RA4-4.
Diploid strains of 39 SFGs exhibited a fluffy phenotype
like ΔfluG, indicating that these sfgA mutations are re-
cessive to their wild-type alleles (Table 2). One diploid
strain (dSFG38) sporulated to wild-type level, suggesting
that sfgA38 defines a dominant (interfering) mutation,
which was later found to be an allele of recessive mutants
(see below).

At least four loci are defined by sfg mutations: In an
attempt to determine the number of genes defined by
sfg mutations, each primary SFG strain was meiotically
crossed with recombinant SFG strains carrying different
auxotrophic markers (ΔfluG, sfgA; pyrG89; and/or pyrA4).14.
While pairwise crosses of two allelic (or tightly linked)
sfgA mutations would produce only conidial progeny,
crosses of nonallelic sfgA mutations are expected to
generate ~25% fluffy progeny due to ΔfluG (sfgA⁰¹;sfgA⁰¹ΔfluG).
The results of pairwise crosses showed that 39 sfgA muta-
tions represented at least four linkage groups (sfgA ~ D),
where 31 mapped to sfgA, 6 mapped to sfgB, and 1
each mapped to sfgC and sfgD, respectively (Tables 1 and
2). Although SFG8 could not be assigned to a specific
linkage group due to extreme difficulty in sexual cross-
ings, it was found not to be an allele of sfgA or sfgC.
Isolation of 31 suppressors mapped to sfgA (or a tightly
linked locus) presents a strongly biased distribution of
suppressor mutations in sfgA. Moreover, mutations in
the sfgA locus seem to result in varying ranges of recov-
ered conidiation (Figure 2; Table 2; HS, W, and DS)
indicating that partial loss of sfgA function might be
sufficient to cause suppression of ΔfluG.

Two previously reported mutations, sfdB38, which were originally isolated as suppressors of
ΔfluD, were found to suppress ΔfluG (KELNER and
ADAMS 2002). Thus, it was of interest to see whether these sfd mutations could be mapped to any of four sfg
loci. REK65.13 and REK88.23 were meiotically crossed
with SFG strains from each linkage group and sfdA15
was found not to be linked to sfgA, sfgB, or sfgC. Meiotic
crosses between sfdB38 mutant and selected SFG strains
or sfdA15 with sfgD mutants failed to generate matured
conidiophores.

Mutations in sfgA and sfgC cause submerged conidiation:
One of the phenotypic characteristics of hyperactive
conidiation is the formation of conidiophores in
liquid submerged culture, conditions under which wild-
type strains do not sporulate. To test whether some SFGs
show hyperactive conidiation even in the absence
of FluG activity, we examined an individual SFG strain’s
ability to form conidiophores in liquid shake culture
and found that 14 SFGs elaborated conidiophores in
submerged culture within 25 hr, while 13 and 1 belong
to the linkage groups A and C, respectively. Particularly,
SFG43 (ΔfluG; sfgA43) and SFG44 (ΔfluG; sfgA44) began
to form vesicles at 18 hr and produced complete conidi-
ophores within 20 hr (Table 2; Figure 3). SFG5 (ΔfluG;
sfdC5) produced complete conidiophores within 22 hr
in liquid MM with 0.1% YE, but not in liquid MM alone
(Figure 3). SFG5 exhibited delayed conidiation pheno-
type with enhanced growth (25% more than wild type)
on solid MM (Figure 2 and Table 2). Addition of YE to
SFG5 cultures, however, caused reduced growth (Table
2), slightly increased conidiation on solid medium (not
shown), and inappropriate conidiation in liquid culture
(Figure 3). None of the mutants belonging to linkage
group B or D produced conidiophores in submerged
culture.

We selected four SFG mutants, SFG5, SFG38, SFG44,
and SFG51, and examined accumulation of brlA tran-
script at various stages of the life cycle. As presented in
Figure 4, a wild-type strain (FGSC26) does not show
brlA transcript accumulation during vegetative growth
phase or before 8 hr post-asexual induction. However,
SFG5, which elaborated conidiophores within 22 hr of
vegetative growth, accumulated brlA transcript at 24 hr
in liquid culture and at 12 and 24 hr post-asexual de-
velopmental induction (Figure 4). Almost identical
accumulation patterns were observed for SFG38 (noteach mapped to D fluG) and SFG44 (ΔfluG; sfgA44) began
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Most sfg mutations affect hyphal growth: Asexual de-
Suppressors of *A. nidulans fluG*

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<td>pyrG89;wA3ΔfluG::trpC⁺;pyroA4;sfG3A37</td>
<td>This study</td>
</tr>
<tr>
<td>RSFG38.3</td>
<td>pyrG89;ΔfluG::trpC⁺;pyroA4;sfG3A38</td>
<td>This study</td>
</tr>
<tr>
<td>RSFG44.16</td>
<td>pyrG89;ΔfluG::trpC⁺;sfG4A44</td>
<td>This study</td>
</tr>
<tr>
<td>RSFG45.1</td>
<td>ΔfluG::trpC⁺;pyroA4;sfG4A45</td>
<td>This study</td>
</tr>
<tr>
<td>RSFG47.2</td>
<td>pyrG89,yA2ΔfluG::trpC⁺;sfG4A47</td>
<td>This study</td>
</tr>
<tr>
<td>RSFG51.4</td>
<td>pyrG89;wA3ΔfluG::trpC⁺;sfG5A51</td>
<td>This study</td>
</tr>
<tr>
<td>RSFG52.28</td>
<td>pyrG89;ΔfluG::trpC⁺;sfG5B52</td>
<td>This study</td>
</tr>
<tr>
<td>RSFG53.12</td>
<td>pyrG89,yA2ΔfluG::trpC⁺;sfG5B53</td>
<td>This study</td>
</tr>
<tr>
<td>DSFG⁺⁺⁺⁺</td>
<td>+,+;biA1;methG1;ΔfluG::trpC⁺;alcA(p)::arfR::trpC⁺;+</td>
<td>This study</td>
</tr>
</tbody>
</table>

*All strains are veA1 except FGSC. Fungal Genetics Stock Center.
†SFG1, -2, -4, -6, -11, -12, -13, -14, -15, -18, -22, -23, -26, -30, -32, -33, -34, -35, -36, -37, -38, -42, -43, -44, -45, -46, -47, -48, -50, -51, and -54 are isogenic except for sfG4A# mutant alleles.
‡SFG7, -27, -28, -41, -52, and -53 are expected to carry sfG4B# mutant alleles.
§SFG8 is not mapped to a specific linkage group but is not linked to sfG4A or sfG4C.
‖DSFG# is a diploid strain generated by fusing each SFG with RJJH128 or RJA4.4 (diploid genotype: pyrG89, +, yA2, ΔfluG::trpC⁺;+;+/+, pabaA1, yA2, ΔfluG::trpC⁺;trpC801;sfG#). Each diploid strain is isogenic except for the sfG number.

Development and hyphal growth are antagonistic in that elevation of one process causes downregulation of the other process. The fact that SFGs bypass the requirement for fluG in asexual development and that some SFGs exhibit a hyperactive conidiation phenotype leads us to think that some sfG mutations might also affect hyphal growth, probably due to elevated asexual development. To test this, growth rates of each SFG strain...
### TABLE 2

**Characteristics of SFGs**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Linkage group</th>
<th>Phenotype</th>
<th>Submerged conidiation</th>
<th>Growth rate: mm/hr (% of WT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGSC26</td>
<td>NA</td>
<td>W</td>
<td>–</td>
<td>0.55 ± 0.03 (100)</td>
</tr>
<tr>
<td>TTA127.4</td>
<td>NA</td>
<td>F</td>
<td>–</td>
<td>0.55 ± 0.01 (96.4)</td>
</tr>
<tr>
<td>SFG1</td>
<td>A</td>
<td>HS</td>
<td>–</td>
<td>0.38 ± 0.12 (69.1)</td>
</tr>
<tr>
<td>SFG2</td>
<td>A</td>
<td>HS</td>
<td>–</td>
<td>0.56 ± 0.03 (101.8)</td>
</tr>
<tr>
<td>SFG3</td>
<td>D</td>
<td>DS</td>
<td>–</td>
<td>0.40 ± 0.09 (72.7)</td>
</tr>
<tr>
<td>SFG4</td>
<td>A</td>
<td>W</td>
<td>++</td>
<td>0.45 ± 0.09 (81.8)</td>
</tr>
<tr>
<td>SFG5</td>
<td>C</td>
<td>DS</td>
<td>++</td>
<td>0.69 ± 0.007 (125)</td>
</tr>
<tr>
<td>SFG6</td>
<td>A</td>
<td>DS</td>
<td>–</td>
<td>0.48 ± 0.07 (87.3)</td>
</tr>
<tr>
<td>SFG7</td>
<td>B</td>
<td>DS</td>
<td>–</td>
<td>0.46 ± 0.10 (83.6)</td>
</tr>
<tr>
<td>SFG8</td>
<td>ND</td>
<td>DS</td>
<td>–</td>
<td>0.32 ± 0.03 (58.2)</td>
</tr>
<tr>
<td>SFG11</td>
<td>A</td>
<td>W</td>
<td>++</td>
<td>0.39 ± 0.11 (70.1)</td>
</tr>
<tr>
<td>SFG12</td>
<td>A</td>
<td>HS</td>
<td>–</td>
<td>0.42 ± 0.09 (76.4)</td>
</tr>
<tr>
<td>SFG13</td>
<td>A</td>
<td>W</td>
<td>–</td>
<td>0.42 ± 0.09 (76.4)</td>
</tr>
<tr>
<td>SFG14</td>
<td>A</td>
<td>HS</td>
<td>–</td>
<td>0.45 ± 0.09 (78.2)</td>
</tr>
<tr>
<td>SFG15</td>
<td>A</td>
<td>DS</td>
<td>–</td>
<td>0.38 ± 0.12 (69.1)</td>
</tr>
<tr>
<td>SFG16</td>
<td>A</td>
<td>W</td>
<td>–</td>
<td>0.40 ± 0.11 (72.7)</td>
</tr>
<tr>
<td>SFG17</td>
<td>A</td>
<td>HS</td>
<td>–</td>
<td>0.37 ± 0.13 (67.3)</td>
</tr>
<tr>
<td>SFG18</td>
<td>A</td>
<td>W</td>
<td>–</td>
<td>0.55 ± 0.03 (96.4)</td>
</tr>
<tr>
<td>SFG19</td>
<td>A</td>
<td>DS</td>
<td>–</td>
<td>0.93 ± 0.05 (58.2)</td>
</tr>
<tr>
<td>SFG20</td>
<td>A</td>
<td>DS</td>
<td>–</td>
<td>0.36 ± 0.08 (65.5)</td>
</tr>
<tr>
<td>SFG21</td>
<td>A</td>
<td>W</td>
<td>++</td>
<td>0.42 ± 0.09 (76.4)</td>
</tr>
<tr>
<td>SFG22</td>
<td>A</td>
<td>W</td>
<td>–</td>
<td>0.42 ± 0.09 (76.4)</td>
</tr>
<tr>
<td>SFG23</td>
<td>A</td>
<td>W</td>
<td>–</td>
<td>0.42 ± 0.09 (76.4)</td>
</tr>
<tr>
<td>SFG24</td>
<td>A</td>
<td>W</td>
<td>–</td>
<td>0.42 ± 0.09 (76.4)</td>
</tr>
<tr>
<td>SFG25</td>
<td>A</td>
<td>W</td>
<td>−</td>
<td>0.42 ± 0.09 (76.4)</td>
</tr>
<tr>
<td>SFG26</td>
<td>A</td>
<td>W</td>
<td>−</td>
<td>0.42 ± 0.09 (76.4)</td>
</tr>
<tr>
<td>SFG27</td>
<td>A</td>
<td>W</td>
<td>−</td>
<td>0.42 ± 0.09 (76.4)</td>
</tr>
<tr>
<td>SFG28</td>
<td>A</td>
<td>W</td>
<td>−</td>
<td>0.42 ± 0.09 (76.4)</td>
</tr>
<tr>
<td>SFG29</td>
<td>A</td>
<td>W</td>
<td>−</td>
<td>0.42 ± 0.09 (76.4)</td>
</tr>
<tr>
<td>SFG30</td>
<td>A</td>
<td>W</td>
<td>−</td>
<td>0.42 ± 0.09 (76.4)</td>
</tr>
</tbody>
</table>

*Each SFG mutant phenotype was assigned by growing SFGs on MM with 0.1% of YE at 37°C and then measuring the number of conidia/cm² (data not shown), growth rate, and the degree of fluffy edge (data not shown). F, fluffy with no conidiation; HS, high sporulation; DS, delayed sporulation; W, wild-type level sporulation.

Submerged conidiation was tested in liquid MM at 37°C with 250 rpm shaking. ++, conidiation within 20 hr; ++, conidiation between 21 and 23 hr; +, conidiation between 24 and 25 hr; −, no conidiation observed until 25 hr.

Growth rates were determined by measuring diameters of colonies point inoculated at the center of MM grown at 37°C. Measurement was made at 2–5 days with 24-hr intervals. Two separate experiments were carried out in duplicate. The mean value, the standard deviation, and relative growth to wild type (%) are presented.

Not applicable.

Not determined.
We tested whether any of 40 SFGs could extra-

types or other developmental mutants (H9004) be rescued by growing yA2 strains next to either wild-type strains, (TTA127.4) does not accumulate ST, while the conidiation defect of H9004 fluG::trpC; trpC801; veA1 and produce yellow conidia due to the yA2 mutation. Thus, for efficient rescue experiments, we generated two ΔfluG strains that produce green conidia (RJA23.1 and RJA23.2; see Table 1) and tested all SFGs in duplicate and found that no SFGs were able to rescue the conidiation defect of ΔfluG strains.

All SFG mutants regain the ability to produce ST: Previously, it was shown that FluG is required for the production of the mutagenic and carcinogenic myco-

on solid MM and MM with 0.1% YE (not shown) were measured in triplicate and compared with those of a wild-type strain (FGSC26). As shown in Table 2, SFG mutants show varying levels of growth, ~49–125% of that of wild type, and all SFGs but five (SFG2, SFG5, SFG36, SFG46, and SFG50) show reduced hyphal growth compared to wild type. Particularly, two sfgB mutant alleles, sfgb27 and sfgb52, caused reduction of growth rates to ~50% of wild type on MM. The fact that mutations in sfgB cause reduced growth yet have low levels of conidiation (delayed conidiation) suggests that sfgb might elucidate a new cross-talking network between growth and asexual development.

No sfg mutations extracellularly rescue the conidiation defect of ΔfluG: One of the phenotypic characteristics of ΔfluG strains is that the conidiation defect can be rescued by growing ΔfluG strains next to either wild-type or other developmental mutants (Lee and Adams 1994b). We tested whether any of 40 SFGs could extracellularly rescue the sporulation defect of ΔfluG strains. All primary SFGs originated from TTA127.4 (pabaA1, yA2; ΔfluG::trpC; trpC801, veA1) and produce yellow conidia due to the yA2 mutation. For efficient rescue experiments, we generated two ΔfluG strains that produce green conidia (RJA23.1 and RJA23.2; see Table 1) and tested all SFGs in duplicate and found that no SFGs were able to rescue the conidiation defect of ΔfluG strains.

All SFG mutants regain the ability to produce ST: Previously, it was shown that FluG is required for the production of the mutagenic and carcinogenic myco-

toxin ST in A. nidulans. It has been proposed that this requirement for FluG is via activating FlbA, which in turn inactivates FadA growth signaling (Hicks et al. 1997). To test whether SFGs regain the ability to produce ST, we initially examined 40 SFGs for ST production at 7 days of culture and found that all were able to produce ST at near wild-type levels without fluG activity (data not shown). Two developmentally wild-type strains, FGSC26 and RKH51.117 (an isogenic wild-type strain, Table 1), have been compared with SFG strains and both wild-type strains showed the same ST accumulation patterns. To confirm that ST recovery is through restored stc gene expression we examined the accumulation of ST and stcU mRNA in selected SFGs days 1–4 as previously described (Yu and Leonard 1995). As presented in Figure 5, while the ΔfluG, sfgNT strain (TTA127.4) does not accumulate stcU transcript or ST, four selected SFGs, including dominant SFG38, show certain levels of restored stcU mRNA and ST accumulation. Although until day 4 some SFGs produced less ST than did wild type, they all produced ST at near wild-type levels at 7 days of culture. We also examined stcU transcript accumulation in SFG5, SFG38, wild-type, and ΔfluG strains at various stages of the life cycle. SFG5 does not show stcU transcript accumulation in liquid culture, even though it conidiates and accumulates btlA transcript (Figure 4). Upon induction of asexual development, however, both SFG5 and SFG38 show similar patterns of btlA and stcU transcript accumulation (Figure 4). Recovery of ST production is an important difference between sfg mutations and the previously reported
dominant suppressor of fluG, dsgA1, because dsgA1 cannot bypass the need for fluG in ST production (D’Souza et al. 2001). These results indicate that sfg mutations, even without FluG activity, can (at least partially) inhibit FadA growth signaling and allow ST biosynthesis to occur.

**Dominant negative sfgA38 is different from dsgA1:** At present, only two (sfgA38 and dsgA1) dominant suppressors of fluG have been isolated. As described, sfg38 and 30 other recessive sfg mutations mapped to linkage group A, suggesting that sfgA38 is likely a dominant interfering (negative) mutant allele. We attempted to test whether dsgA1 can be mapped to any of the four sfg linkage groups by meiotic crosses between SFGs and a ΔfluG; dsgA1 strain (HDCD15.1). While HDCD15.1 readily formed heterokaryons with most ΔfluG; sfg2 mutant strains, no matured cleistothecia were formed even under conditions that enhance sexual development (D’Souza et al. 2001).

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**DISCUSSION**

The genus Aspergillus encompasses the most common fungi in our environment. Members of this genus reproduce asexually by forming long chains of conidiospores radiating from a central structure known as a conidiophore. One of the primary questions has been how such a complex structure differentiates from vegetatively growing hyphae. Previous studies in *A. nidulans* showed that FluG is required for this developmental switch by activating the conidiation process and indirectly regulating the G protein-mediated growth-signaling cascade via activating FlbA (Lee and Adams 1994a, 1996; Yu et al. 1996). Balanced control of these two genes represents a reasonable model for the development of other fungi.
Suppressors of A. 

parallel signaling pathways is essential for maintaining the genetically programmed life cycle. For the last decade, heterotrimeric G proteins and their signaling/regulatory mechanisms have been intensively studied and are found to be conserved from yeasts to humans. Such structural and functional conservancy of the G protein signaling components makes reverse genetics and functional genomics favorable approaches. However, genes functioning in the conidiation process and associated regulatory mechanisms are largely unknown primarily due to uniqueness of the conidiation processes in filamentous fungi. Moreover, like FluG, many genes are expected to be novel in their structures and functions. Therefore, we believe that unbiased genetic studies represent the most suitable way to further dissect upstream regulatory mechanisms of conidiation in A. nidulans.

FluG is required for activation of conidiation and it functions upstream of other developmentally specific genes including flbE, flbD, flbC, flbB, and brlA (see Figure 1). Previous genetic studies looking for developmentally defective mutants from a wild-type strain were specifically aimed at the identification of positive regulators of conidiation. A recent study of fluG describing the isolation of dominant suppressors of ΔfluG was also biased to the identification of activating components of conidiation (D’Souza et al. 2001). If negative elements were involved in the regulatory cascade of conidiation, previous studies would have missed these critical components. Thus, to investigate molecular mechanisms associated with the FluG-mediated activation of conidiation in an unbiased way, we have isolated and characterized a large number of SFGs, employing a haploid ΔfluG strain. Because the fluG-dependent initiation of asexual sporulation is independent of and parallel to FadA- and SfaD-Gγ-mediated growth signaling and no mutations in FadA or SfaD are able to suppress ΔfluG (Yu et al. 1996, 1999; Rosen et al. 1999), genes presented by suppressors of fluG are expected to specifically function in the conidiation pathway downstream of FluG.

Characterization and genetic analyses of 40 SFG mutants have been carried out. Because the fluG deletion mutant was used for the isolation of suppressor, all SFGs are expected to be extragenic and bypass suppressors of fluG. Genetic analyses of SFGs can be summarized as follows: (1) each SFG is derived from a single second-site mutation; (2) 39 sfg mutations are recessive to their wild-type alleles and only 1 (sfgA38) is dominant; (3) at least four loci are defined by sfg mutations; (4) 31, 6, 1, and 1 mutations are mapped to linkage groups sfgA, sfgB, sfgC, and sfgD, respectively; and (5) the dominant mutation sfgA38 is an allele of sfgA and is different from dsgA1. The fact that at least four loci are defined by recessive sfg mutations indicates that multiple genes are involved in negative control of conidiation downstream of fluG, which supports our reason for using a haploid ΔfluG strain. In this study, the most interesting findings are that 31 SFGs including the dominant mutant SFG38 are mapped to the sfgA linkage group and that they show varying levels of recovered conidiation (Table 2), where 28 are wild-type level or hyperactive sporulators and 4 are delayed sporulators. These results strongly indicate that sfgA functions as a key negative regulator of conidiation and it might have multiple functional domains. Any mutations causing (at least partial) loss of sfgA function(s) may be sufficient to restore conidiation to certain levels. Depending on the levels of remaining functionality of the SfgA mutant products, varying ranges of suppression, i.e., delayed sporulation to hyperactive sporulation, might result. On the other hand, incremental loss of sfgA function would result in elevated levels of restored conidiation and even hyperactive conidiation. Supporting this idea, a relatively large number (13 of 31) of sfgA mutations are found to cause submerged conidiation in the absence of fluG activity. Unlike sfgA mutations, however, most mutations in sfgB seem to result in delayed conidiation, suggesting that partial (or even complete) loss of sfgB function might not be sufficient to cause full recovery of conidiation. Furthermore, the fact that only one suppressor mutation each has been mapped to sfgC or sfgD after screening 125,000 survivors suggests that only specific mutation(s) in sfgC or sfgD, e.g., a complete loss of function, might bypass fluG function. Similar to sfgA, a complete loss of sfgC function might be sufficient to cause hyperactive conidiation, but only with yeast extract (see Figure 3). However, regardless of levels of recovered conidiation, all SFG mutants regained the ability to produce ST to near wild-type levels, suggesting that all sfg mutations could cause (at least partial) activation of FlbA.

On the basis of our findings, a new genetic model for upstream regulation of asexual sporulation. DsgA is positioned within the developmental-specific functions that do not affect FlbA-mediated ST remediation. FluG functions the most upstream and its primary role is to remove repressive effects imposed by downstream sfg genes. Elimination of Sfg-mediated negative regulation is necessary for activation of conidiation-specific functions and FlbA, which in turn confers ST production.

FIGURE 7.—A new model for upstream regulation of asexual sporulation. DsgA is positioned within the developmental-specific functions that do not affect FlbA-mediated ST remediation. FluG functions the most upstream and its primary role is to remove repressive effects imposed by downstream sfg genes. Elimination of Sfg-mediated negative regulation is necessary for activation of conidiation-specific functions and FlbA, which in turn confers ST production.
genetic interactions among four sfg genes need to be studied, the products of the sfgA, sfgB, sfgC, and sfgD genes, represented as [Sfg], negatively regulate conidiation-specific functions as well as certain FhA functions, which are necessary for ST production through inhibition of FhA growth signaling. We propose that the primary role of FluG is to remove these repressive effects imposed by multiple sfg genes. In the proposed model, a complete loss of negative regulation by the sfg genes would cause a full elimination of repressive effects, resulting in hyperactive sporulation as seen in 13 sfgA and 1 sfgC5 mutants. Conversely, partial functions of the sfg genes would maintain certain levels of negative regulation, which cause low levels of remediation of conidiation, i.e., delayed conidiation.

However, removal of negative regulation is not sufficient to alter developmental competence. Previously, it has been shown that conidiation does not occur until cells have gone through a defined period of vegetative growth (~18 hr), during which cells acquire competence to respond to developmental signaling or induction (for review see Adams et al. 1998). Although many SFGs produced conidiophores in liquid culture in a relatively short time, no SFGs were able to shorten the proposed time (18 hr) for acquisition of developmental competence. Furthermore, no mutations in FhA or SfD result in earlier than 20-hr conidiophore development in liquid culture (Yu et al. 1996, 1999; Rosen et al. 1999). These results are consistent with the observation that overexpression of fluG does not alter the time required for conidiophore development to begin in an air-exposed colony (Lee and Adams 1996). In contrast, dsgA1 causes submerged conidiation between 9 and 11 hr of incubation even without FluG activity, indicating that time required for competence in a dsgA1 mutant is greatly shortened (D’Souza et al. 2001). Collectively, acquisition of developmental competence may be defined by activation of downstream developmentally specific functions, which involve coordination of upstream positive and negative regulatory functions.

It has been proposed that FluG is required for the production of the extracellular sporulation factor. The fact that both FluG mRNA and protein are present at relatively constant levels throughout the life cycle implies that accumulation of the factor above a certain level (threshold) might be necessary to trigger the switch from vegetative growth to conidiation (Lee and Adams 1996; D’Souza et al. 2001). No SFG mutants were able to rescue the conidiation defect of ΔfluG by proximal growth, suggesting that suppression of ΔfluG is due to alterations in intracellular regulation, not through the recovery of the production of the extracellular sporulation factor(s). Overexpression of fluG results in inappropriate production of conidiophores that are remarkably similar to wild-type conidiophores and have all the cell types including stalks, vesicles, metulae, phialides, and conidia, indicating that activation of fluG results in activation of all the genes necessary to form a complete conidiophile (Lee and Adams 1996). Similarly, 14 SFGs produce complete conidiophores within 25 hr of liquid submerged culture, implying that sfg genes likely function immediately downstream of fluG (see Figure 3). Taken together, we propose that sfg genes act to repress conidiation during the early vegetative growth phase and that accumulation of the sporulation factor above certain levels serves as a signal to remove this negative regulation.

We thank Guiping Yang for her assistance on characterization of SFGs and colleagues in the laboratory for helpful discussions and suggestions. Special thanks go to Byron Brehm-Stecher and Ellen Doyle in our institute for critically reviewing the manuscript. This work was supported in part by sponsors of the Food Research Institute and by a Hatch Grant to J.H.Y. from the College of Agriculture & Life Sciences at University of Wisconsin, Madison.

LITERATURE CITED


Communicating editor: J. Loros