NsdD Is a Key Repressor of Asexual Development in *Aspergillus nidulans*

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ABSTRACT Asexual development (conidiation) of the filamentous fungus *Aspergillus nidulans* occurs via balanced activities of multiple positive and negative regulators. For instance, FluG (+) and SfgA (–) govern upstream regulation of the developmental switch, and BrlA (+) and VosA (–) control the progression and completion of conidiation. To identify negative regulators of conidiation downstream of FluG–SfgA, we carried out multicopy genetic screens using *sfgA* deletion strains. After visually screening >100,000 colonies, we isolated 61 transformants exhibiting reduced conidiation. Responsible genes were identified as AN3152 (*nsdD*), AN7507, AN2009, AN1652, AN5833, and AN9141. Importantly, *nsdD*, a key activator of sexual reproduction, was present in 10 independent transformants. Furthermore, deletion, overexpression, and double-mutant analyses of individual genes have led to the conclusion that, of the six genes, only *nsdD* functions in the FluG-activated conidiation pathway. The deletion of *nsdD* bypassed the need for *fluG* and *flbA*~*flbE*, but not *brlA* or *abaA*, in conidiation, and partially restored production of the mycotoxin sterigmatocystin (ST) in the Δ *fluG*, Δ *flbA*, and Δ *flbB* mutants, suggesting that NsdD is positioned between FLBs and BrlA in *A. nidulans*. Nullifying *nsdD* caused formation of conidiophores in liquid submerged cultures, where wild-type strains do not develop. Moreover, the removal of both *nsdD* and *vosA* resulted in even more abundant development of conidiophores in liquid submerged cultures and high-level accumulation of *brlA* messenger (m)RNA even at 16 hr of vegetative growth. Collectively, NsdD is a key negative regulator of conidiation and likely exerts its repressive role via downregulating *brlA*.

THE filamentous ascomycete *Aspergillus nidulans* has served as an excellent model system for studying cell biology, asexual development (conidiation), and secondary metabolism (Timberlake 1990; Martinelli 1994; Yu and Keller 2005). The *A. nidulans* asexual reproductive cycle can be divided into two distinct phases: growth and development. The growth phase involves germination of an asexually derived spore called a conidium and formation of an undifferentiated network of interconnected hyphal cells that form the mycelium. After a certain vegetative growth period, under appropriate conditions, some of the hyphal cells stop

normal growth and begin development by forming complex structures called conidiophores that bear multiple chains of conidia (Adams *et al.* 1988; Park and Yu 2012a).

A key and essential step for conidiophore development in *Aspergillus* is the activation of *brlA*, which encodes a C₂H₂ zinc-finger transcription factor (TF) (Figure 1A) (Adams *et al.* 1988; Chang and Timberlake 1993). Further genetic and biochemical studies identified the additional key regulators *abaA* and *wetA* that function during the middle and late stages of conidiation, respectively (Figure 1A) (Sewall *et al.* 1990; Andrianopoulos and Timberlake 1991; Marshall and Timberlake 1991). These three genes have been proposed to define a central regulatory pathway that acts in concert with other genes to control the spatial and temporal specificity of gene expression during conidiophore development and spore maturation (Mirabito *et al.* 1989; Yu 2010; Park and Yu 2012a).

Activation of *brlA* requires activities of various upstream elements including *fluG*, *flbA*, *flbB*, *flbC*, *flbD*, and *flbE*. Mutations in any of these genes result in “fluffy” colonies

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that are characterized by undifferentiated cotton-like masses of vegetative cells (Adams *et al.* 1988). FlbA is a regulator of G-protein signaling (RGS) protein, and *flbA* mutants are distinguished from the others by the fluffy autolytic phenotype (Lee and Adams 1994b; Yu *et al.* 1996). FlbC is a putative TF with two C₂H₂ zinc-finger DNA-binding domains and is thought to directly control *brlA* expression (Kwon *et al.* 2010a). FlbE is a 201-aa-length polypeptide with two conserved yet uncharacterized domains and colocalizes with FlbB, a basic leucine zipper (b-zip) TF, at the hyphal tip in an actin cytoskeleton-dependent manner (Garzia *et al.* 2009). FlbE physically interacts with FlbB, and they activate FlbD interdependently (Garzia *et al.* 2009; Kwon *et al.* 2010b). FlbD (a cMyb-type TF) and FlbB then cooperatively function in activating expression of *brlA* (Garzia *et al.* 2010). Both of these two independent conidiation activation cascades (Figure 1A) are necessary for full activation of *brlA* expression and proper conidiation (Wieser and Adams 1995; Garzia *et al.* 2010; Kwon *et al.* 2010a). Importantly, loss of *fluG* or *flbA* 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 aflatoxins (Lee and Adams 1994a; Hicks *et al.* 1997).

FluG is required for the synthesis of the extracellular sporulation-inducing factor (a diorcinol-dehydroaustinol adduct) that triggers the commencement of conidiation in *A. nidulans* (Lee and Adams 1994a; Rodriguez-Urra *et al.* 2012). The two genetic responses to FluG activity were thought to be (1) activation of the FLBs → BrlA development-specific regulatory cascades and (2) positive modulation of FlbA, which in turn inhibits vegetative growth signaling mediated by a heterotrimeric G protein composed of FadA and SfaD::GpgA (Lee and Adams 1994a; Yu *et al.* 1996; Yu 2006). Our studies have revealed that both processes might involve the removal of a key repression of conidiation imposed by SfgA, which is a putative TF with a Gal4-type Zn(II)₂Cys₆ binuclear DNA-binding domain (Seo *et al.* 2003, 2006). SfgA acts as an important upstream negative regulator of conidiation functioning downstream of FluG, but upstream of FlbA, FlbB, FlbC, FlbD, and BrlA (Seo *et al.* 2006). The deletion of *sfgA* causes hyperactive conidiation and eliminates the need for *fluG* in conidiation and ST production. These led us to propose that the developmental transition from vegetative growth in *A. nidulans* occurs via removal of the negative regulator SfgA, which inhibits precocious activation of *brlA* during proliferation, thereby allowing proper vegetative growth.

During conidiogenesis, the spatial and temporal expression of *brlA*, *abaA*, and *wetA* is tightly controlled (Boylan *et al.* 1987; Mirabito *et al.* 1989; Adams *et al.* 1998; Ni *et al.* 2010). For instance, transcripts of *brlA* and *abaA* specifically accumulate during the early-to-middle phases of conidiophore formation and quickly disappear upon the formation of conidia. Our gain-of-function (multicopy) genetic screen has identified VosA as a key negative feedback

regulator of *brlA* (Ni and Yu 2007). In conjunction with the differentiation of conidia, *vosA* is activated by AbaA (and WetA). This, in turn, represses *brlA* and, together with WetA, promotes maturation of conidia and trehalose biogenesis in spores (Figure 1A) (Ni and Yu 2007). VosA localizes in the nucleus of mature conidia and contains the velvet DNA-binding domain recognizing 11 nucleotide sequences and a transcriptional activation domain, indicating it is a TF (Ni and Yu 2007; Ahmed *et al.* 2013).

In the present study, we specifically aimed to identify key negative regulators of conidiation that acted downstream of FluG-SfgA and likely upstream of *brlA*. Employing two types of *sfgA* deletion strains (*veA+* or *veA1*) as recipients, we screened a pRG3-AMA1-based wild-type (WT) genomic DNA library (Osherov *et al.* 2000) for the genes inhibiting conidiation when present in multiple copies. Among 100,000 transformants screened, 61 candidates were isolated and 6 responsible genes, AN3152 (*nsdD*), AN7507, AN2009, AN1652, AN5833, and AN9141, were identified. Notably, the previously reported GATA-type TF NsdD activating sexual development in *A. nidulans* (Han *et al.* 2001) was identified by 10 independent transformants. Further studies indicate that NsdD is a key negative controller of conidiation, likely acting at the *brlA* level in *A. nidulans*, and also influences ST biosynthesis.

Materials and Methods

Aspergillus strains and culture conditions

Aspergillus strains used in this study are listed in Table 1. Individual strains were inoculated into liquid or on solid 1% glucose minimal medium (MMG) with appropriate supplements and incubated at 37°. If needed, 0.5% yeast extract (YE) was used (Pontecorvo *et al.* 1953; Kafer 1977). For liquid-submerged culture, 5 × 10⁵ conidia·ml⁻¹ were inoculated into 100 ml liquid MMG and incubated at 37°, 220 rpm. Samples were taken at 1-day intervals for up to 6 days of cultivation. Standard *A. nidulans* transformation techniques were used as described previously (Szewczyk *et al.* 2006; Park and Yu 2012b). Characterization of phenotype was performed by point inoculating each strain on solid MMG at 37° for up to 4 days. Entire colonies and close-up views of the middle zone of individual colonies were examined and photographed under a microscope. Expression of target genes under the control of the *niiA* promoter was controlled by nitrate source: repression on MMG plus 0.2% ammonium tartrate or induction on MMG plus 0.6% sodium nitrate (Arst *et al.* 1979). To examine the effect of the overexpression (OE) of *fluG* and *sfgA* by the ectopic copy of the gene under control of the *alcA* promoter (Waring *et al.* 1989), strains were grown through stationary culture in noninducing liquid MMG (MMG plus 0.5% YE) and inducing liquid MMG (MMT: 1.1% threonine plus 0.5% YE) and incubated at 37° for 3 days. Northern blot analyses during vegetative growth and postdevelopmental induction were

carried out as described in Seo *et al.* (2003; Ni and Yu 2007). Briefly, 2×10^6 conidia ml^{-1} were inoculated into 100 ml liquid MMG and cultured at 37° for 18 hr. The mycelium was collected at designated time points from liquid-submerged cultures and squeeze-dried. For sexual and asexual developmental induction, cultured mycelia were harvested and transferred on solid MMG and the plates were air exposed for asexual developmental induction or tightly sealed and blocked from light for sexual developmental induction. Samples for RNA isolation were collected at designated time points after transfer. The *Escherichia coli* DH5 α strain was cultured in Luria-Bertani medium with 50 $\mu\text{g/ml}$ of ampicillin (Sigma-Aldrich) for plasmid amplification.

Nucleic acid isolation and manipulation

The oligonucleotides used in this study are listed in supporting information, Table S1. Genomic DNA isolation was carried out as described in Yu *et al.* (2004). About 10^6 ml^{-1} conidia of WT and mutant strains were inoculated into 2 ml liquid MMG with 0.5% YE and stationary cultured at 37° for 1 day. The hyphal mat was collected and squeeze-dried and genomic DNA was isolated. Total RNA samples were prepared at various time points after liquid-submerged culture and postdevelopmental induction as described in Han *et al.* (2004; Mah and Yu 2006). For Northern blot analysis, $\sim 10 \mu\text{g}$ of total RNA was separated by electrophoresis, using a 1% agarose gel containing 6% formaldehyde, and ethidium bromide and the nucleic acids were transferred to the Hybond-N+ membrane (0.45 μm , GE Healthcare Life Sciences). The DNA probes were prepared by PCR amplification of the coding regions of individual genes with appropriate primer pairs (all primers listed in Table S1) from *A. nidulans* WT (FGSC4) genomic DNA. Individual PCR amplicons were labeled with ^{32}P -dCTP and used for Northern blot hybridization as described in Yu and Leonard (1995).

Multicopy screening of pRG3-AMA1-based genomic DNA library

For multicopy screening, two types of *sfgA* deletion (Δ) mutants were generated and used. Briefly, the 5'- and 3'-flanking regions of *sfgA* with the *pyroA*⁺ marker tails were amplified with the primer pairs of oNK397;oNK398 and oNK399;oNK400, and the *A. nidulans pyroA*⁺ gene was amplified with primer pair oNK395;oNK396 from *A. nidulans* WT (FGSC4) genomic DNA. The joined *sfgA* deletion construct was amplified with the nested primer pair oNK401;oNK402, and the final PCR amplicon was introduced into RJMP1.59 (*pyrG89*; *pyroA4*; *veA*⁺) and RNIW3 (*pyrG89*; *pyroA4*; *veA1*). The generated *sfgA* deletion strains TNJ30 (*pyrG89*; Δ *sfgA::pyroA*⁺; *pyroA4*; *veA*⁺) and TNJ134 (*pyrG89*; Δ *sfgA::pyroA*⁺; *pyroA4*; *veA1*) were then transformed with the pRG3-AMA1-NotI genomic DNA library with the *Neurospora crassa pyr4*⁺ marker (Osherov *et al.* 2000; Park and Yu 2012b). Sixty-one transformants showing fluffy or repressed conidiation were isolated. Genomic

DNA was isolated from individual transformants and electroporated into *E. coli* DH5 α to rescue the total 36 recombinant plasmids. Inserts of these plasmids were then directly sequenced with the primer pair oMN33 and oMN35, and the sequence data were matched with the *A. nidulans* genome [Broad Institute (Cambridge, MA) and AspGD]. Thirteen genes were defined by sequencing the inserts, and each recombinant plasmid with the insert was introduced back to the recipient *A. nidulans* Δ *sfgA* strains to verify their ability to inhibit conidiation when present in multicopy. Collectively, the six potential multicopy repressors of conidiation were identified.

Construction of overexpression strains

To generate the fusion constructs under the *niiA* promoter, i.e., *niiA*(p)::AN1652, *niiA*(p)::AN2009, *niiA*(p)::AN7507, *niiA*(p)::AN3152, *niiA*(p)::AN5833, and *niiA*(p)::AN9141, each ORF derived from *A. nidulans* WT genomic DNA was PCR amplified using the primer pairs oNK754; oNK755 (OE1652 with *EcoRI* and *NotI*), oNK872; oNK747 (OE2009 with *BamHI* and *NotI*), oNK867; oNK739 (OE7507 with *HindIII* and *NotI*), oNK892; oNK893 (OE3152 with *BamHI* and *HindIII*), oNK890; oNK891 (OE5833 with *EcoRI* and *HindIII*), and oNK932; oNK925 (OE9141 with *HindIII* and *NotI*). The PCR products were then double digested with the enzymes shown above and cloned into pHS11, which contains the *A. nidulans niiA* promoter and the *trpC* terminator (Park *et al.* 2012). For the *alcA*(p)::*fluG* and *alcA*(p)::*sfgA* fusion constructs, each ORF derived from *A. nidulans* WT genomic DNA was amplified using the primer pairs oNK126; oNK127 (ORF of *fluG* with *BamHI* and *NotI*) and oNK47; oNK991 (ORF of *sfgA* with *BamHI* and *NotI*). The PCR product was then double digested with the enzymes shown above and cloned into pHS3, which contains the *A. nidulans alcA* promoter and the *trpC* terminator (Kwon *et al.* 2010a). The resulting recombinant DNA was then introduced into TNJ36.1. The overexpression strains among transformants were screened by Northern blot analysis, using each ORF probe (primer pairs to generate overexpression constructions), followed by PCR confirmation (Yu *et al.* 2004).

Generation of deletion mutants

The 5'- and 3'-flanking regions of each gene were amplified from genomic DNA of FGSC4, using designated primer pairs. For the deletion mutants of AN1652, AN2009, AN7507, *nsdD* (AN3152), AN5833, and AN9141, the flanking regions were amplified using primer pairs oNK748;oNK749 (5' AN1652 with *AfupyrG* tail), oNK750;oNK751 (3' AN1652 with *AfupyrG* tail), oNK740;oNK741 (5' AN2009 with *AfupyrG* tail), oNK742;oNK743 (3' AN2009 with *AfupyrG* tail), oNK732;oNK733 (5' AN7507 with *AfupyrG* tail), oNK734; oNK735 (3' AN7507 with *AfupyrG* tail), oNK12;oNK913 (5' *nsdD* with *AfupyrG* tail), oNK914;oNK915 (3' *nsdD* with *AfupyrG* tail), oNK906;oNK907 (5' AN5833 with *AfupyrG* tail), oNK908;oNK909 (3' AN5833 with *AfupyrG* tail), oNK896;oNK897 (5' AN9141 with *AfupyrG* tail), and oNK898;

oNK899 (3' AN9141 with *AfupyrG* tail). The *A. fumigatus pyrG* gene was amplified from *A. fumigatus* WT (AF293) genomic DNA with the primer pair oJH84;oJH85. The final PCR fragments were amplified using the nested primer pairs oNK752;oNK753 (AN1652), oNK744;oNK745 (AN2009), oNK736;oNK737 (AN7507), oNK916;oNK917 (*nsdD*), oNK910;oNK911 (AN5833), and oNK900;oNK901 (AN9141). The deletion cassettes were introduced into RJMP1.59 protoplasts generated by VinoTaste Pro lysing enzyme (Novozymes) (Szewczyk *et al.* 2006; Park and Yu 2012b). The recipient deletion mutants were used to generate double-deletion mutants with $\Delta fluG$ with the *pyroA*⁺ allele by subsequent transformation. For the deletion mutants of *fluG*, *sfgA*, *flbA*, *flbB*, *flbD*, and *rgsA* with *A. fumigatus pyrG*⁺ as the marker, each flanking region was PCR amplified using primer pairs oNK788;oWS7 (5' *fluG* region), oWS8;oNK791 (3' *fluG* region), oNK397;oNK612 (5' *sfgA* region), oNK613;oNK400 (3' *sfgA* region), oNK142;oNK1032 (5' *flbA* region), oNK1033;oNK145 (3' *flbA* region), oNK522;oNK523 (5' *flbB* region), oNK524;oNK525 (3' *flbB* region), oNK1017;oNK1018 (5' *flbD* region), oNK1019;oNK1020 (3' *flbD* region), oNK540;oNK1030 (5' *rgsA* region), and oNK1031;oNK543 (3' *rgsA* region), using *A. nidulans* WT genomic DNA as a template. The final PCR constructs were amplified with the nested primer pairs oNK792;oNK793 ($\Delta fluG$), oNK401;oNK402 ($\Delta sfgA$), oNK146;oNK147 ($\Delta flbA$), oNK526;oNK527 ($\Delta flbB$), oNK1021;oNK1022 ($\Delta flbD$), and oNK544;oNK545 ($\Delta rgsA$). The deletion cassettes were introduced into RJMP1.59 protoplasts. The *flbB*, *flbD*, *flbE* (TNJ32), *flbC* (TNJ31), *abaA* (TNJ37), *brlA* (TNJ38), and *vosA* (THS15.1) deletion mutants were used to generate double-deletion mutants with $\Delta nsdD$, using the *pyroA*⁺ marker, by subsequent transformation. To generate $\Delta flbA \Delta nsdD$ and $\Delta rgsA \Delta nsdD$ double-deletion mutants, the *flbA* or *rgsA* gene was deleted from TNJ108, using the *pyroA*⁺ marker (*pyrG*; *pyroA4*; $\Delta nsdD::AfupyrG⁺) strain.$

Determination of alamarBlue reduction and dry weight

Fungal cell viability was determined by the percentage of reduction of alamarBlue (AB) (AbD Serotec). Conidia (10^6 ml⁻¹) of each strain were inoculated into 100 ml MMG and cultured from 1 to 6 days at 37°, 240 rpm. Then, 0.5 ml of mycelial aggregates was aliquoted into 1 ml of fresh liquid medium containing 150 μ l of the AB reagent. The samples were incubated for 6 hr at 37° in the dark as described in Shin *et al.* (2009). The supernatant was placed into the 96-well plates excluding mycelial aggregates for analysis of absorbance at A₅₇₀ and A₆₀₀. The percentage of AB reduction was detected by Synergy HT (BIO-TEK), using KC4 v3.1 software, and was calculated by a formula, $(117,216 \times A_{570} \text{ of sample} - 80,586 \times A_{600} \text{ of sample}) / (155,677 \times A_{600} \text{ of media} - 14,652 \times A_{570} \text{ of media}) \times 100$ (Shin *et al.* 2009). After getting the sample for the AB reduction, the remaining cultures were collected by filtering to determine the mycelial mass. The samples were dried in a 75° oven for 4 hr and subjected to dry weight determination.

Figure 1 Genetic model of conidiation and multicopy screening. (A) A genetic model for upstream and downstream developmental regulators of conidiation in *A. nidulans*. (B) Strategy and summary for multicopy screening. The pRG3-AMA1-based *A. nidulans* WT (FGSC4) genomic DNA library was introduced into $\Delta sfgA$ strains (veA⁺: TNJ30 and veA1: TNJ134). Six genes with the number of transformants are indicated. (C) Phenotypes of multicopy mutants. WT-veA⁺ (TNJ36.1), WT-veA1 (TNJ36.4), $\Delta sfgA$ M-AN1652 (TM1652), $\Delta sfgA$ M-AN2009 (TM2009), $\Delta sfgA$ M-AN7507 (TM7507), $\Delta sfgA$ M-AN3152 (TM3152), $\Delta sfgA$ M-AN5833 (TM5833), and $\Delta sfgA$ M-AN9141 (TM9141) strains were point inoculated on solid MMG and incubated at 37° for 3 days. For the colony morphologies of $\Delta sfgA$ with veA⁺ and $\Delta sfgA$ with veA1 strains, please see Figure 3B.

Sterigmatocystin extraction and TLC analysis

Briefly, 10^6 ml⁻¹ conidia of each strain were inoculated into 2 ml liquid complete medium (CM) and cultured at 37° for 3–5 days as described (Yu and Leonard 1995). ST was extracted by adding 2 ml of CHCl₃, and the organic phase was transferred to 1.5-ml microcentrifuge tubes and centrifuged at $700 \times g$ for 5 min. The CHCl₃ layer was collected, dried, and resuspended in 50 μ l of CHCl₃. Approximately 5 μ l of each sample was loaded onto a thin-layer chromatography (TLC) silica plate including a fluorescence indicator (Kiesel gel 60, 0.25 mm thick; Merck). ST standard (5 μ g; Sigma, St. Louis) was applied onto the TLC plate. The plate was then developed with toluene:ethyl acetate:acetic acid (80:10:10, v/v/v), where the *R_f* value of ST was ~0.65. Aluminum chloride (20% w/v in 95% ethanol) was sprayed on the TLC plate to enhance the detection of ST and the plate was baked at 70° for 5 min (Stack and Rodricks 1971). The TLC plate was exposed to UV of 320 nm, and ST levels were measured. This experiment was performed in triplicate.

Light and fluorescence microscopy

The colony photographs were taken using a Sony (Parkridge, NJ) DSC-F828 camera. Photomicrographs were taken using a Zeiss (Thornwood, NY) M2Bio microscope equipped with AxioCam and AxioVision (Rel. 4.8) digital imaging software.

Results

Strategy and results of multicopy screening for negative regulators of conidiation

A Δ sfgA strain with the *veA*⁺ or *veA1* allele was transformed with the pRG3-AMA1 (*NotI*)-based *A. nidulans* WT genomic DNA library (Osherov *et al.* 2000), and of >100,000 transformants, 61 candidate colonies that clearly exhibited reduced conidiation were isolated. All transformants were subjected to genomic DNA isolation followed by plasmid rescue through electroporation to *E. coli*. Of 61 candidates, genomic DNA samples of 31 transformants led to the successful rescue of plasmids in *E. coli*. Direct sequencing of each plasmid insert using the oMN33 and oMN35 primers (Park and Yu 2012b) followed by genome search identified 13 different genes, where 5 genes were identified ~2–10 times, and 8 genes were present in one transformant each. Rescued plasmids with varying inserts were then reintroduced into the recipient strains and 6 clones/genes are confirmed to cause changes in conidiation (summarized in Figure 1B).

From the Δ sfgA *veA*⁺ screen AN1652, AN2009, AN7507, and AN3152 were present in six, three, two, and eight transformants, respectively. From the Δ sfgA *veA1* screen AN3152, AN5833, and AN9141 were identified by two, three, and one transformants, respectively. Only AN3152 was identified in both screens, suggesting that the genetic screens did not reach a saturation, or visual screening based on the colony morphologies of transformants would have missed some of the candidates. The AN7507 locus encodes a GAL4-like Zn₂Cys₆ binuclear cluster DNA-binding domain protein. AN2009 encodes a homeodomain protein, and AN1652 encodes the C₂H₂-type zinc-finger protein MsnA (Han and Prade 2002). AN5833 encodes a protein with an acyl-CoA synthetase domain, and AN9141 encodes a protein with the GAL4-like Zn₂Cys₆ binuclear cluster DNA-binding domain. Finally, the most represented AN3152 locus encodes the GATA-type TF (NsdD) that is known to activate sexual development (Han *et al.* 2001). In summary, five genes encode putative TFs, whereas one (AN5833) encodes a putative metabolic enzyme.

To verify the repressive role of these genes in conidiation, each gene region (5'-flanking-ORF-3' flanking) was cloned into the pRG3-AMA1 multicopy vector and introduced into Δ sfgA *veA*⁺ or Δ sfgA *veA1* strains. As shown in Figure 1C, multicopy (M) of AN1652 and AN9141 resulted in the fluffy phenotypes in the absence of *sfgA*, suggesting these putative TFs might be associated with stimulating hyphal growth while inhibiting development. Both vegetative growth and development of the fungus were restricted by M-AN2009, suggesting that proper expression of this homeodomain protein is important for the

normal life cycle of *A. nidulans*. Multicopy of AN7507 and AN5833 caused reduced conidiation without distinctly enhanced vegetative growth. Finally, M-AN3152 (NsdD) resulted in the near absence of conidiation with the elevated accumulation of hyphal mass and enhanced formation of Hülle cells (specialized cells assisting sexual fruiting body formation), similar to those caused by enhanced expression of NsdD reported in the previous study (Han *et al.* 2001).

Overexpression and expression analyses of the six candidate genes

To further check their potential roles in repressing conidiation, the ORF of each of six genes was cloned under the inducible *niiA*(p) and then OE strains of individual genes were constructed by transformation. As shown in Figure 2B, when induced (NO₃ [I]), OE of AN1652 and AN3152 resulted in reduced conidiation and enhanced hyphal proliferation. Similar to the multicopy, OE of AN2009 caused restricted colony growth and inhibited conidiation. On the contrary, OE of AN7507, AN5833, and AN9141 did not affect conidiation or growth greatly. Somewhat surprisingly, OE9141 did not cause distinct changes in conidiation, which is very different from results for M-AN9141 (see Figure 1C), suggesting that the fluffy phenotype might be caused by the multicopy presence of the AN9141 *cis*-acting elements.

We further examined messenger (m)RNA accumulation patterns of the six genes throughout the life cycle of WT by Northern blot (Figure 2B). Both AN1652 and AN2009 appeared to be expressed at relatively constant levels, whereas AN7507 is expressed at a high level during sexual development (sexual 72–96 hr) and in sexual spores (ascospores, AS). AN3152 (*nsdD*) appeared to encode two transcripts where the lower one specifically accumulated in conidia, and the upper one accumulated at somewhat constant levels throughout the life cycle. Both AN5833 and AN9141 are expressed at low levels during vegetative growth and longer transcripts appeared to accumulate during development. These results suggest that mRNA accumulation of the six genes is not specifically coupled with *brlA* expression and/or conidiation.

Finally, we asked whether expression of any of the six genes is under the regulatory control of SfgA and/or FluG and checked mRNA accumulation patterns of each gene in vegetative cells of WT, Δ fluG, Δ sfgA, OEfluG, and OEsfgA strains (Figure 2C). We envisioned that if a given negative regulator is positively controlled by SfgA and repressed by FluG, Δ fluG and Δ sfgA would lead to enhanced and lowered expression of it, respectively. Conversely, OEfluG and OEsfgA would lead to inhibited and elevated mRNA levels of the tested gene. Quantitative analyses of each mRNA band calculated based on the loading control [ribosomal (r)RNA] indicate that mRNA levels the six genes were not distinctly elevated or diminished by the deletion of *fluG* or *sfgA*. Moreover, while mRNA levels of AN1652, AN7507, AN3152, and AN9141 appeared to be enhanced by OEsfgA, these were not affected by OEfluG. Interestingly, expression of AN5833 was greatly induced by threonine, but this was abolished by OEfluG. As vegetatively grown cells (liquid submerged culture) were used,

Figure 2 Overexpression and expression analyses of the six genes. (A) Phenotypes caused by OE of six genes. Colony photographs of WT (TNJ36.1), OE1652 (TNJ98), OE2009 (TNJ104), OE7507 (TNJ122), OE3152 (TNJ110), OE5833 (TNJ116), and OE9141 (TNJ128) strains are shown. Strains were point inoculated on noninducing medium (MMG containing 0.2% ammonium tartrate as a nitrogen source) or inducing medium (MMG containing 0.6% sodium nitrate as a nitrogen source) and incubated at 37° for 3 days. (B) Northern blots for AN1652, AN2009, AN7507, AN3152, AN5833, and AN9141 mRNA levels during the life cycle of *A. nidulans* WT. Conidia (C) and ascospores (AS) are indicated. The numbers indicate the time (hr) of incubation in liquid MMG (vegetative), postshift to solid MMG with air exposure (asexual), or limited air (sexual). Equal loading of total RNA was confirmed by ethidium bromide staining of rRNA. (C) Northern blots for AN1652, AN2009, AN7507, AN3152, AN5833, and AN9141 mRNA levels. WT (TNJ36.1), $\Delta fluG$ (TNJ79), and $\Delta sfgA$ (TNJ57) strains were grown in stationary culture in MMG with 0.5% YE. WT (TNJ36.1), OE $fluG$ (TNJ208), and OE $sfgA$ (TNJ70) under the *alcA*(p) were grown in stationary culture in inducing liquid medium (MM with 1.1% threonine plus 0.5% YE) (MMT) at 37° for 3 days. Equal loading of total RNA was confirmed by ethidium bromide staining of rRNA.

mRNA accumulation of AN7507 was almost undetectable. These results indicate that none of the six genes are likely subject to direct regulatory control by SfgA and, if positioned in the FluG pathway, the repressor(s) does not likely act between SfgA and FLB genes.

Deletion analyses and suppression of $\Delta fluG$ by $\Delta nsdD$

As we specifically looked for negative regulators of conidiation in the FluG-initiated developmental pathway, we then asked whether the deletion of each gene could suppress the conidiation defects caused by the lack of *fluG*. To do this, we first generated individual knockout mutants of AN7507, AN2009, AN1652, AN5833, AN9141, and AN3152, using *A. fumigatus pyrG*⁺. Then, to generate double mutants, individual deletion strains were used to further knock out the *fluG* gene, using *A. nidulans pyroA*⁺ as the marker (see Table 1). Sporulation and growth phenotypes of individual single- and double-deletion mutants were then compared. As shown in Figure 3A, as AN1652 (*msnA*) was speculated to be associated with vegetative growth, the deletion of *msnA* caused extremely restricted colony growth and could not suppress the conidiation defects caused by

$\Delta fluG$. The lack of AN2009 or AN5833 did not alter the $\Delta fluG$ phenotype either. Furthermore, the deletion of AN7507 or AN9141 even further enhanced the conidiation defects of the $\Delta fluG$ mutant. However, the deletion of AN3152 (*NsdD*) was able to restore conidiation in the $\Delta fluG$ mutant, and vegetative growth of the double mutant was similar to that of the $\Delta nsdD$ single mutant, suggesting that *nsdD* is epistatic to *fluG*. We also tested whether each double-deletion mutant regained the ability to produce ST and found that only $\Delta nsdD$ partially restored ST production in the $\Delta fluG$ mutant (data not shown, but see Figure 5A for $\Delta nsdD$). In summary, the presence of *nsdD* in 10 independent multicopy transformants and the suppression of $\Delta fluG$ by $\Delta nsdD$ strongly suggest that *NsdD* is a key negative regulator of conidiation. These findings led us to focus on further characterizing *NsdD*'s role in conidiation.

NsdD functions downstream of FluG and represses *brlA*

VeA is a founding member of the velvet regulators, and it plays a key role in activating sexual development, while repressing conidiation (Kim *et al.* 2002). The VeA1 mutant protein lacks the N-terminal Nuclear Localization Signal and

Table 1 A. *nidulans* strains used in this study

Strain	Genotype	Source
FGSC4	<i>A. nidulans</i> WT	FGSC ^a
FGSC26	<i>biA1</i> ; <i>veA1</i>	FGSC
RJMP1.59 ^b	<i>pyrG89</i> ; <i>pyroA4</i>	Shaaban <i>et al.</i> (2010)
RNIW3	<i>pyrG89</i> ; <i>pyroA4</i> ; <i>veA1</i>	Kwon <i>et al.</i> (2012)
TNJ36.1	<i>pyrG89</i> ; <i>AfupyrG</i> ⁺ ; <i>pyroA4</i>	Kwon <i>et al.</i> (2010b)
TNJ36.4	<i>pyrG89</i> ; <i>AfupyrG</i> ⁺ ; <i>pyroA4</i> ; <i>veA1</i>	This study
TNJ30	<i>pyrG89</i> ; Δ <i>sfgA::pyroA</i> ⁺ ; <i>pyroA4</i>	This study
TNJ57	<i>pyrG89</i> ; Δ <i>sfgA::AfupyrG</i> ⁺ ; <i>pyroA4</i>	Kwon <i>et al.</i> (2012)
TNJ134	<i>pyrG89</i> ; Δ <i>sfgA::pyroA</i> ⁺ ; <i>pyroA4</i> ; <i>veA1</i>	This study
TNJ135	<i>pyrG89</i> ; Δ <i>sfgA::AfupyrG</i> ⁺ ; <i>pyroA4</i> ; <i>veA1</i>	This study
TM7507	<i>pyrG89</i> ; Δ <i>sfgA::pyroA</i> ⁺ ; <i>pyroA4</i> ; pRG-AMA1::AN7507	This study
TM2009	<i>pyrG89</i> ; Δ <i>sfgA::pyroA</i> ⁺ ; <i>pyroA4</i> ; pRG-AMA1::AN2009	This study
TM1652	<i>pyrG89</i> ; Δ <i>sfgA::pyroA</i> ⁺ ; <i>pyroA4</i> ; pRG-AMA1::AN1652	This study
TM3152	<i>pyrG89</i> ; Δ <i>sfgA::pyroA</i> ⁺ ; <i>pyroA4</i> ; <i>veA1</i> ; pRG-AMA1::AN3152	This study
TM5833	<i>pyrG89</i> ; Δ <i>sfgA::pyroA</i> ⁺ ; <i>pyroA4</i> ; <i>veA1</i> ; pRG-AMA1::AN5833	This study
TM9141	<i>pyrG89</i> ; Δ <i>sfgA::pyroA</i> ⁺ ; <i>pyroA4</i> ; <i>veA1</i> ; pRG-AMA1::AN9141	This study
TNJ160	<i>pyrG89</i> ; Δ <i>sfgA::pyroA</i> ⁺ ; <i>pyroA4</i> ; pRG-AMA1::AN3152	This study
TNJ173	<i>pyrG89</i> ; <i>AfupyrG</i> ⁺ ; <i>pyroA4</i> ; pRG-AMA1::AN3152	This study
TNJ174	<i>pyrG89</i> ; <i>AfupyrG</i> ⁺ ; <i>pyroA4</i> ; <i>veA1</i> ; pRG-AMA1::AN3152	This study
TNJ98 ^c	<i>pyrG89</i> ; <i>AfupyrG</i> ⁺ ; <i>pyroA4</i> ; <i>niiA</i> (p)::AN1652::FLAG::3/4 <i>pyroA</i> ⁺	This study
TNJ104 ^c	<i>pyrG89</i> ; <i>AfupyrG</i> ⁺ ; <i>pyroA4</i> ; <i>niiA</i> (p)::AN2009::FLAG::3/4 <i>pyroA</i> ⁺	This study
TNJ122 ^c	<i>pyrG89</i> ; <i>AfupyrG</i> ⁺ ; <i>pyroA4</i> ; <i>niiA</i> (p)::AN7507::FLAG::3/4 <i>pyroA</i> ⁺	This study
TNJ110 ^c	<i>pyrG89</i> ; <i>AfupyrG</i> ⁺ ; <i>pyroA4</i> ; <i>niiA</i> (p)::AN3152::FLAG::3/4 <i>pyroA</i> ⁺	This study
TNJ116 ^c	<i>pyrG89</i> ; <i>AfupyrG</i> ⁺ ; <i>pyroA4</i> ; <i>niiA</i> (p)::AN5833::FLAG::3/4 <i>pyroA</i> ⁺	This study
TNJ128 ^c	<i>pyrG89</i> ; <i>AfupyrG</i> ⁺ ; <i>pyroA4</i> ; <i>niiA</i> (p)::AN9141::FLAG::3/4 <i>pyroA</i> ⁺	This study
TNJ79	<i>pyrG89</i> ; Δ <i>fluG::AfupyrG</i> ⁺ ; <i>pyroA4</i>	This study
TNJ133	<i>pyrG89</i> ; Δ <i>fluG::AfupyrG</i> ⁺ ; <i>pyroA4</i> ; <i>veA1</i>	This study
TNJ70 ^c	<i>pyrG89</i> ; <i>pyroA4</i> ; <i>alcA</i> (p):: <i>sfgA</i> ::FLAG::3/4 <i>pyroA</i> ⁺	Kwon <i>et al.</i> (2012)
TNJ208 ^c	<i>pyrG89</i> ; <i>pyroA4</i> ; <i>alcA</i> (p):: <i>fluG</i> ::FLAG::3/4 <i>pyroA</i> ⁺	This study
TNJ96	<i>pyrG89</i> ; <i>pyroA4</i> ; Δ AN1652:: <i>AfupyrG</i> ⁺	This study
TNJ97	<i>pyrG89</i> ; Δ <i>fluG::pyroA</i> ⁺ ; <i>pyroA4</i> ; Δ AN1652:: <i>AfupyrG</i> ⁺	This study
TNJ102	<i>pyrG89</i> ; <i>pyroA4</i> ; Δ AN2009:: <i>AfupyrG</i> ⁺	This study
TNJ103	<i>pyrG89</i> ; Δ <i>fluG::pyroA</i> ⁺ ; <i>pyroA4</i> ; Δ AN2009:: <i>AfupyrG</i> ⁺	This study
TNJ108	<i>pyrG89</i> ; <i>pyroA4</i> ; Δ <i>nsdD::AfupyrG</i> ⁺	This study
TNJ109	<i>pyrG89</i> ; Δ <i>fluG::pyroA</i> ⁺ ; <i>pyroA4</i> ; Δ <i>nsdD::AfupyrG</i> ⁺	This study
TNJ111	<i>pyrG89</i> ; <i>pyroA4</i> ; Δ <i>nsdD::AfupyrG</i> ⁺ ; <i>veA1</i>	This study
TNJ112	<i>pyrG89</i> ; Δ <i>fluG::pyroA</i> ⁺ ; <i>pyroA4</i> ; Δ <i>nsdD::AfupyrG</i> ⁺ ; <i>veA1</i>	This study
TNJ114	<i>pyrG89</i> ; Δ AN5833:: <i>AfupyrG</i> ⁺ ; <i>pyroA4</i>	This study
TNJ115	<i>pyrG89</i> ; Δ AN5833:: <i>AfupyrG</i> ⁺ ; Δ <i>fluG::pyroA</i> ⁺ ; <i>pyroA4</i>	This study
TNJ120	<i>pyrG89</i> ; Δ AN7507:: <i>AfupyrG</i> ⁺ ; <i>pyroA4</i>	This study
TNJ121	<i>pyrG89</i> ; Δ <i>fluG::pyroA</i> ⁺ ; Δ AN7507:: <i>AfupyrG</i> ⁺ ; <i>pyroA4</i>	This study
TNJ126	<i>pyrG89</i> ; <i>pyroA4</i> ; Δ AN9141:: <i>AfupyrG</i> ⁺	This study
TNJ127	<i>pyrG89</i> ; Δ <i>fluG::pyroA</i> ⁺ ; <i>pyroA4</i> ; Δ AN9141:: <i>AfupyrG</i> ⁺	This study
TNJ32	<i>pyrG89</i> ; <i>pyroA4</i> ; Δ <i>flbE::AfupyrG</i> ⁺	Kwon <i>et al.</i> (2010b)
TNJ179	<i>pyrG89</i> ; <i>pyroA4</i> ; Δ <i>nsdD::pyroA</i> ⁺ ; Δ <i>flbE::AfupyrG</i> ⁺	This study
TNJ45	<i>pyrG89</i> ; <i>pyroA4</i> ; Δ <i>flbB::AfupyrG</i> ⁺	This study
TNJ175	<i>pyrG89</i> ; <i>pyroA4</i> ; Δ <i>flbB::AfupyrG</i> ⁺ ; Δ <i>nsdD::pyroA</i> ⁺	This study
TNJ177	<i>pyrG89</i> ; <i>pyroA4</i> ; Δ <i>flbD::AfupyrG</i> ⁺	This study
TNJ178	<i>pyrG89</i> ; <i>pyroA4</i> ; Δ <i>nsdD::pyroA</i> ⁺ ; Δ <i>flbD::AfupyrG</i> ⁺	This study
TNJ31	<i>pyrG89</i> ; <i>pyroA4</i> ; Δ <i>flbC::AfupyrG</i> ⁺	Kwon <i>et al.</i> (2010a)
TNJ176	<i>pyrG89</i> ; <i>pyroA4</i> ; Δ <i>nsdD::pyroA</i> ⁺ ; Δ <i>flbC::AfupyrG</i> ⁺	This study
TNJ182	<i>pyrG89</i> ; Δ <i>flbA::AfupyrG</i> ⁺ ; <i>pyroA4</i>	This study
TNJ183	<i>pyrG89</i> ; Δ <i>flbA::pyroA</i> ⁺ ; <i>pyroA4</i> ; Δ <i>nsdD::AfupyrG</i> ⁺	This study
TNJ37	<i>pyrG89</i> ; <i>pyroA4</i> ; Δ <i>abaA::AfupyrG</i> ⁺	Kwon <i>et al.</i> (2010a)
TNJ187	<i>pyrG89</i> ; <i>pyroA4</i> ; Δ <i>nsdD::pyroA</i> ⁺ ; Δ <i>abaA::AfupyrG</i> ⁺	This study
TNJ38	<i>pyrG89</i> ; <i>pyroA4</i> ; Δ <i>brlA::AfupyrG</i> ⁺	Kwon <i>et al.</i> (2010a)
TNJ186	<i>pyrG89</i> ; <i>pyroA4</i> ; Δ <i>nsdD::pyroA</i> ⁺ ; Δ <i>brlA::AfupyrG</i> ⁺	This study
TNJ63	<i>pyrG89</i> ; <i>pyroA4</i> ; Δ <i>rgsA::AfupyrG</i> ⁺	This study
TNJ185	<i>pyrG89</i> ; <i>pyroA4</i> ; Δ <i>rgsA::pyroA</i> ⁺ ; Δ <i>nsdD::AfupyrG</i> ⁺	This study
THS15.1	<i>pyrG89</i> ; <i>pyroA4</i> ; Δ <i>vosA::AfupyrG</i> ⁺	Park <i>et al.</i> (2012)
TNJ181	<i>pyrG89</i> ; <i>pyroA4</i> ; Δ <i>nsdD::pyroA</i> ⁺ ; Δ <i>vosA::AfupyrG</i> ⁺	This study

^a Fungal Genetics Stock Center (University of Missouri, Kansas City).

^b Strains contain the *veA*⁺ allele unless otherwise indicated as *veA1*.

^c The 3/4 *pyroA* marker in pH511 and pH53 causes the targeted integration at the *pyroA4* locus.

Figure 3 Deletion analyses and the role of *nsdD* in the FluG pathway. (A) Phenotypes of double mutants. WT (TNJ36.1), $\Delta AN1652$ (TNJ96), $\Delta fluG \Delta AN1652$ (TNJ97), $\Delta AN2009$ (TNJ102), $\Delta fluG \Delta AN2009$ (TNJ103), $\Delta AN7507$ (TNJ120), $\Delta fluG \Delta AN7507$ (TNJ121), $\Delta AN3152$ (TNJ108), $\Delta fluG \Delta AN3152$ (TNJ109), $\Delta AN5833$ (TNJ114), $\Delta fluG \Delta AN5833$ (TNJ115), $\Delta AN9141$ (TNJ126), and $\Delta fluG \Delta AN9141$ (TNJ127) strains were point inoculated on MMG and incubated at 37° for 3 days. (B) Colonies of WT (*veA*⁺: TNJ36.1 and *veA1*: TNJ36.4), $\Delta sfgA$ (*veA*⁺: TNJ57 and *veA1*: TNJ135), *M-nsdD* (*veA*⁺: TNJ173 and *veA1*: TNJ174), and $\Delta sfgA$ *M-nsdD* (*veA*⁺: TNJ160 and *veA1*: TNJ152) strains grown on solid MMG at 37° for 3 days. (C) Colony photographs of WT (*veA*⁺: TNJ36.1 and *veA1*: TNJ36.4), $\Delta fluG$ (*veA*⁺: TNJ79 and *veA1*: TNJ133), $\Delta nsdD$ (*veA*⁺: TNJ108 and *veA1*: TNJ111), and $\Delta fluG \Delta nsdD$ (*veA*⁺: TNJ109 and *veA1*: TNJ112) strains grown on solid MMG at 37° for 3 days. (D) Quantitative analysis of conidiation of strains shown in C. Conidia in 1-cm² area of the colonies were collected and counted (****P* < 0.005). (E) Plates of WT (TNJ36.1), $\Delta fluG$ (TNJ79), $\Delta nsdD$ (TNJ108), and $\Delta fluG \Delta nsdD$ (TNJ109) strains. Spores were spread on solid MMG and incubated at 37° for 3 days. (F) Northern blot for *brlA*, *abaA*, *wetA*, and *vosA* mRNA levels in WT (TNJ36.1) and $\Delta nsdD$ (TNJ108) strains during the life cycle. C, conidia. The numbers indicate the time (hr) of incubation in liquid MMG (vegetative) or on solid MMG (postasexual induction). Transcript levels of γ -actin are shown as a control.

the ability to interact with another sex-activating velvet regulator VelB, and *veA1* mutant strains show highly restricted sexual fruiting with elevated conidiation (Mooney *et al.* 1990; Stinnett *et al.* 2007; Bayram *et al.* 2008). Thus, comparison of the phenotypes resulting from the multicopy or the deletion of NsdD in combination with *veA*⁺ and *veA1* along with *sfgA*⁺ and $\Delta sfgA$ was carried out to better understand the role of NsdD in growth and developmental control. As shown in Figure 3B, *M-nsdD* was sufficient to inhibit conidiation with the *veA*⁺ or *veA1* allele, yet the greatest reduction of conidiation was observable with the *veA*⁺ and *sfgA*⁺ alleles (*M-nsdD* in Figure 3B, left). These results suggest that repression by

M-nsdD is maximized when other negative regulators of asexual development, *e.g.*, VeA and SfgA, are functional.

We then checked whether suppression of $\Delta fluG$ by deletion of *nsdD* is affected by the *veA* allele and found that, when point inoculated, the $\Delta fluG \Delta nsdD$ double-mutant colonies exhibited high levels of conidiation regardless of *veA*⁺ or *veA1* (Figure 3C). We then quantified the levels of conidiation by measuring the number of conidia from the WT, $\Delta fluG$, $\Delta nsdD$, and $\Delta fluG \Delta nsdD$ colonies (all with *veA*⁺; Figure 3D). The $\Delta nsdD$ mutant formed more conidia than WT with *veA*⁺ (*P* < 0.005). The $\Delta fluG \Delta nsdD$ double mutant formed ~10-fold more conidia than the $\Delta fluG$ mutant

($P < 0.005$), but not to WT levels. We then further quantified the levels of conidiation by spreading conidia ($\sim 10^5$ per plate) of WT, $\Delta fluG$, $\Delta nsdD$, and $\Delta fluG \Delta nsdD$ strains onto solid medium and measuring the number of conidia produced upon 2 and 3 days of incubation (Figure 3E). The $\Delta nsdD$ mutant formed ~ 1.7 - to 1.5 -fold more conidia than WT with veA^+ and $veA1$ ($P < 0.001$). The $\Delta fluG \Delta nsdD$ double mutant formed ~ 2 - to 3 -fold more conidia than the $\Delta fluG$ mutant under these experimental conditions ($P < 0.001$). However, $\Delta fluG \Delta nsdD$ strains could not produce conidia to WT levels. These results indicate that NsdD plays an important role in inhibiting conidiation downstream of FluG, yet the removal of $nsdD$ alone is not sufficient to cause full activation of conidiation.

We then checked whether the absence of NsdD altered the patterns of *brlA*, *abaA*, *wetA*, and *vosA* mRNA accumulation during vegetative growth and asexual development. As shown in Figure 3F, the deletion of $nsdD$ caused accumulation of *brlA* mRNA at 24, 36, and 48 hr in submerged shake cultures (vegetative), whereas no *brlA* accumulation was observable in WT vegetative cells. Overall mRNA levels of *brlA*, *abaA*, and *wetA* in the $\Delta nsdD$ mutant during asexual developmental induction were much higher than those of WT. Moreover, elevated *brlA* mRNA accumulation in the $\Delta nsdD$ mutant at 10, 12, and 18 hr postdevelopmental induction led to precocious and enhanced accumulation of *abaA* and *wetA* mRNAs compared to WT. Levels of *vosA* mRNA were not much different between the $\Delta nsdD$ mutant and WT.

Genetic position of NsdD in the FluG-mediated conidiation pathway

The above genetic and expression data suggest that NsdD functions downstream of *fluG* but upstream of *brlA*. This is consistent with the finding that expression of $nsdD$ is not directly regulated by SfgA or FluG, and NsdD does not likely act between SfgA and FLBs. To determine the genetic position of $nsdD$ in the FluG-initiated conidiation control cascade, a series of double mutants were generated. As shown in Figure 4, the deletion of $nsdD$ could restore conidiation in the null mutants of *flbE*, *flbB*, *flbD*, and *flbC*. In contrast, $\Delta nsdD$ could not suppress $\Delta brlA$ or $\Delta abaA$. These results indicate that NsdD functions downstream of FlbE/B/D/C and upstream of *brlA*. Moreover, nullifying $nsdD$ in the $\Delta flbA$ mutant partially rescued the conidiation defects and suppressed autolysis caused by $\Delta flbA$, suggesting that NsdD might also be associated with vegetative growth signaling mediated by FadA ($G\alpha$) $\rightarrow$ PkaA (Shimizu and Keller 2001). In the $\Delta rgsA$ mutant, the deletion of $nsdD$ restored conidiation and enhanced growth restriction, suggesting that NsdD and RgsA play an additive role in colony growth.

Roles of NsdD in ST production, autolysis, and colony growth

Our previous studies demonstrated that the absence of *fluG* or *flbA* resulted in the lack of ST production and that biosynthesis of ST required the removal of repressive effects imposed by the heterotrimeric G protein composed of FadA

and SfaD::GpgA (Hicks *et al.* 1997). We also showed that the $\Delta fluG \Delta sfgA$ mutant regained the ability to produce ST and restored the expression of ST-specific genes to WT levels (Seo *et al.* 2006). We envisioned that if NsdD functions downstream of most of the developmental activators, the deletion of $nsdD$ might also restore ST biosynthesis in the defective mutants. As shown in Figure 5A, the removal of $nsdD$ partially restored ST production in the $\Delta fluG$, $\Delta flbB$, $\Delta flbA$, and $\Delta rgsA$ mutants. However, mRNA accumulation of *afIR* (encoding an activating TF) and *stcU* was much reduced in the $\Delta nsdD$ mutant compared to WT. These results indicate that NsdD negatively affects ST production acting downstream of FluG, FlbB, FlbA, and RgsA, likely at a post-transcriptional level.

The deletion of *flbA* causes accelerated and enhanced cell death and autolysis (Lee and Adams 1994b; Shin *et al.* 2009). As $\Delta nsdD$ partially restored conidiation and suppressed autolysis of the $\Delta flbA$ mutant in solid culture, we further quantified its effects on suppressing cell death and hyphal disintegration in the $\Delta flbA$ mutant in liquid culture. As shown in Figure 5, B and C, WT and $\Delta nsdD$ strains maintain cell viability and hyphal integrity at approximately days 3–6, whereas the $\Delta flbA$ mutant began to show cell death at day 3 and autolysis at day 4. The deletion of $nsdD$ suppressed both cell death and autolysis caused by $\Delta flbA$. When WT and $\Delta nsdD$ strains were compared at approximately days 1–3, the $\Delta nsdD$ mutant exhibited reduced AB reduction rates and delayed vegetative proliferation measured by dry weight in liquid submerged culture ($P < 0.005$; Figure 5, B and C).

We also observed that the deletion of $nsdD$ was epistatic to other developmental mutations in colony radial growth, resulting in restricted colony growth of double mutants similar to that of the single $nsdD$ null mutant (see Figure 4). To quantify the radial growth progression, WT, $\Delta nsdD$, $\Delta fluG$, and $\Delta fluG \Delta nsdD$ strains were point inoculated and their radial growth was measured at approximately days 1–4. As shown in Figure 5, D and E, $\Delta nsdD$, and $\Delta fluG \Delta nsdD$ strains showed a statically significant growth reduction ($P < 0.005$) at days 3 and 4 in both the veA^+ and the $veA1$ genetic background. These results collectively suggest that NsdD might function downstream of the FadA-PkaA-controlled vegetative growth regulatory network, which is attenuated by FlbA (Shimizu and Keller 2001).

Additive role of NsdD and VosA in repressing conidiation

The above results indicate that NsdD plays its repressive role likely by repressing expression of *brlA*. We previously showed that VosA is a feedback negative regulator of *brlA*, and its velvet domain directly binds to the *brlA* β promoter (Ahmed *et al.* 2013). The deletion of either *vosA* or $nsdD$ caused elevated expression of *brlA* and formation of conidiophores in liquid submerged culture, where WT strains do not develop (Ni and Yu 2007) (Figure 6B). Based on these observations, we hypothesized that the deletion of two key repressors would have additive effects on expression of *brlA*,

Figure 4 Genetic position of *nsdD*. Phenotypes of various single- and double-deletion mutants are shown. WT (TNJ36.1), $\Delta nsdD$ (TNJ108), $\Delta flbE$ (TNJ32), $\Delta nsdD \Delta flbE$ (TNJ179), $\Delta flbB$ (TNJ45), $\Delta nsdD \Delta flbB$ (TNJ175), $\Delta flbD$ (TNJ177), $\Delta nsdD \Delta flbD$ (TNJ178), $\Delta flbC$ (TNJ31), $\Delta nsdD \Delta flbC$ (TNJ176), $\Delta flbA$ (TNJ182), $\Delta flbA \Delta nsdD$ (TNJ183), $\Delta brlA$ (TNJ38), $\Delta nsdD \Delta brlA$ (TNJ186), $\Delta abaA$ (TNJ37), $\Delta nsdD \Delta abaA$ (TNJ187), $\Delta rgsA$ (TNJ63), and $\Delta nsdD \Delta rgsA$ (TNJ185) strains were point inoculated on solid MMG and incubated at 37° for 3 days. Entire colonies and close-up views of the center of individual colonies are shown. Bar, 100 μ m.

leading to even more enhanced hyperactive conidiation. To test this, we generated the $\Delta nsdD \Delta vosA$ double mutant in *veA*⁺ and examined its developmental phenotypes. The double mutant showed both $\Delta nsdD$ and $\Delta vosA$ phenotypes, i.e., restricted colony growth and light-green conidia with rapid loss of viability on solid medium (Figure 6A). In liquid submerged culture, while both $\Delta nsdD$ and $\Delta nsdD \Delta vosA$ mutant strains produced conidiophores, the double mutant elaborated conidiophores much more abundantly than the $\Delta nsdD$ single mutant (Figure 6B). Moreover, the double mutant exhibited extremely high levels of accumulation of *brlA* mRNA even at 16 hr of vegetative growth in liquid submerged culture (Figure 6C). These results indicate NsdD and VosA play a negative regulatory role on *brlA* expression in an additive manner, and both are required for proper control of *brlA* expression and conidiation during vegetative growth of *A. nidulans*.

Discussion

In this study, to further investigate the regulatory mechanisms of asexual sporulation in *Aspergillus*, we designed and carried out a unique high-copy repressor screen employing the *sfgA* deletion mutant strains. Among the six genes verified to inhibit conidiation when present in multiple copies and/or OE, only NsdD, previously identified as an activator

of sexual development, has been shown to function in the FluG-mediated conidiation regulatory pathway. Analyses of *nsdD* function and genetic position reveal that NsdD negatively controls expression of *brlA*, conidiophore development, and ST production.

The homothallic ascomycete *A. nidulans* can also reproduce sexually by forming sexual spores (ascospores) in the fruiting bodies called cleistothecia. Sexual development is a complex multistep process that requires special environmental conditions and activities of various regulators, including the mating-type genes *matA* (HMG-box) and *matB* (α -box) that transcriptionally coordinate expression of sex-specific genes (Kronstad and Staben 1997; Fraser and Heitman 2005). Sexual fruiting begins with the formation of primordia from ascogenous hyphae in the nest-like structure made of a number of specialized Hülle cells that support fruiting body formation. The primordia mature to cleistothecia in which many mycelia grow and develop into croziers, where a transient nuclear fusion and subsequent meiosis occur (Sohn and Yoon 2002; Han *et al.* 2007), leading to the formation of macroscopic fruiting bodies (cleistothecia) containing a number of ascospores in asci. The *nsdD* gene was identified as one of the never in sexual development (NSD) loci (*nsdA*–*nsdD*) (Han *et al.* 1990). Later studies also identified the *nsdC* gene encoding a putative TF with a novel zinc-finger DNA-binding domain

Figure 5 Multiple roles of *nsdD*. (A) ST levels (top panels) and Northern blots for *aflR* and *stcU* mRNA levels (bottom panels). WT (TNJ36.1), $\Delta fluG$ (TNJ79), $\Delta nsdD$ (TNJ108), $\Delta fluG \Delta nsdD$ (TNJ109), $\Delta flbB$ (TNJ45), $\Delta nsdD \Delta flbB$ (TNJ175), $\Delta flbA$ (TNJ182), $\Delta flbA \Delta nsdD$ (TNJ183), $\Delta rgsA$ (TNJ63), and $\Delta nsdD \Delta rgsA$ (TNJ185) strains were stationary cultured in liquid MMG at 37° for 3 days. ST standard was loaded as a positive control. For Northern blot analyses, WT (TNJ36.1) and $\Delta nsdD$ (TNJ108) strains were cultured throughout the life cycle. C, conidia. Numbers indicate time (hr) of liquid-submerged culture (vegetative) and developmental induction conditions (postasexual induction). Equal loading of total RNA was confirmed by ethidium bromide staining of rRNA. (B) Cell death of WT (TNJ36.1), $\Delta nsdD$ (TNJ108), $\Delta flbA$ (TNJ182), and $\Delta flbA \Delta nsdD$ (TNJ183) strains determined by AB reduction rates for 6 days. (C) Dry weight of WT (TNJ36.1), $\Delta nsdD$ (TNJ108), $\Delta flbA$ (TNJ182) and $\Delta flbA \Delta nsdD$ (TNJ183) strains through liquid-submerged culture in MMG were quantified for 6 days. (D) Colony diameter of WT (*veA*⁺: TNJ36.1 and *veA1*: TNJ111), $\Delta fluG$ (*veA*⁺: TNJ79 and *veA1*: TNJ133), and $\Delta fluG \Delta nsdD$ (*veA*⁺: TNJ109 and *veA1*: TNJ112) strains was measured on MMG grown at 37° for ~1–4 days. Note *veA*⁺ (left) and *veA1* alleles (right) in *A. nidulans*. Error bars indicate standard deviations calculated from biological triplicates. ****P* < 0.005; ***P* < 0.05.

consisting of two C₂H₂ and one C₂HC motifs (Kim *et al.* 2009). Loss-of-function *nsdD* or *nsdC* results in the complete blockage in sexual development under all conditions favoring sexual development. Conversely, overexpression of *nsdD* or *nsdC* not only enhances the formation of fruiting bodies but also partially overcomes the inhibitory effects of certain stresses on sexual fruiting (Han *et al.* 2001; Kim *et al.* 2009). These results lead to the conclusion that NsdD and NsdC play an essential role in sexual development of *A. nidulans*.

However, previous studies also have suggested that NsdD might be a potential repressor of conidiation (Han *et al.* 2001; Cary *et al.* 2012). For instance, overexpression of

nsdD resulted in the near total absence of conidiation with formation of elongated aerial hyphae. Moreover, the conidial yield was reduced by ~1000-fold by overexpression of *nsdD*. Furthermore, forced expression of *nsdD* inhibited conidiation and caused a coiled-hyphal structure in *A. fumigatus* (Grosse and Krappmann 2008). Importantly, most NSD mutants developed conidiophores earlier than WT by several hours, regardless of environmental conditions (Han *et al.* 1998). For instance, when WT was cultured on solid medium with limited air and in the dark for >30 hr, the mycelia did not produce any differentiated cells, but were irreversibly determined to undergo sexual development with the formation of a few asexual spores after air was

Figure 6 An additive role of *nsdD* and *vosA* in repressing conidiation. (A) Phenotypes of WT (TNJ36.1), $\Delta nsdD$ (TNJ108), $\Delta vosA$ (THS15.1), and $\Delta nsdD \Delta vosA$ (TNJ181). Strains were point inoculated on solid MMG and incubated at 37° for 3 days. Entire colonies (top) and close-up views of the middle of individual colonies (bottom) are shown. Bar, 100 μ m. (B) Conidiophore formation in liquid submerged culture. Strains were grown in liquid-submerged MMG culture at 37° for 48 hr (vegetative), and the mycelial aggregates were observed under a microscope. Bar, 50 μ m. (C) Northern blot for *brlA* mRNA levels in strains grown in liquid-submerged MMG culture (vegetative) at 37° for 16 hr. Equal loading of total RNA was confirmed by ethidium bromide staining of rRNA.

introduced (Han *et al.* 1990). The NSD mutants, however, began to produce conidiophores instantaneously after air exposure, indicating that the NSD mutants have not been genetically determined to develop sexually. However, as enhanced expression of *nsdD* also resulted in elevated sexual development even under unfavorable conditions for sexual fruiting, it has been hypothesized that NsdD primarily functions in positively regulating sexual development rather than repressing conidiation. Additional studies in *A. flavus* demonstrated that the removal of *nsdD* resulted in elevated expression of *brlA* (Cary *et al.* 2012). They found that both NsdC [another sexual activator (Kim *et al.* 2009)] and NsdD are required for production of asexual sclerotia, normal aflatoxin biosynthesis, and conidiophore development. Along with these important observations, our forward genetic screen clearly supports the idea that NsdD plays an equally important role in repressing conidiation by acting at the *brlA* level.

The deletion of *nsdD* suppressed all upstream developmental mutations, but not $\Delta brlA$. A previous study identified and characterized two suppressors of FlbD (*sfdA* and *sfdB*) (Kellner and Adams 2002) that phenocopy *nsdD*. These *sfd* mutant alleles restored developmental timing and *brlA* expression to strains with *flbD* deletion. Importantly, *sfd* mutations suppressed the developmental defects of the *fluG*, *flbA*,

flbB, *flbC*, and *flbE* null mutants. All isolated alleles of *sfdA* and *sfdB* were recessive to their WT alleles in diploids, suggesting that the mutations were likely loss-of-function ones. Moreover, *sfd* mutant strains with WT upstream activators exhibited normal conidiation with restricted colony growth. Additionally, they developed conidiophores in liquid submerged culture. These data indicated that *sfdA* and *sfdB* activities are required for proper control of conidiation downstream of FLBs and that the absence of *sfdA* or *sfdB* can cause hyperactive conidiation. While no *sfd* mutant alleles were cloned and identified, these phenotypic and genetic characteristics suggested that one might be an allele of *nsdD*.

NsdD appears to affect other biological processes including ST production, cell death, autolysis, and colony growth. The deletion of *nsdD* suppressed the enhanced cell death/autolysis of the $\Delta flbA$ mutant. Moreover, the absence of *nsdD* could restore ST production in *fluG*, *flbA*, and *flbB* null mutants, not through enhanced transcription of the ST genes. These observations led us to position NsdD in a multiple-control point that activates sexual development and stimulates vegetative growth, but inhibits conidiation and ST production (Figure 7). Previously, we demonstrated that two antagonistic signaling pathways control *A. nidulans* growth, conidiation, and ST production. Growth signaling

is primarily mediated by FadA and SfaD::GpgA (heterodimer), the α and $\beta\gamma$ subunits for a heterotrimeric G protein, respectively. When FadA ($G\alpha$) is active (GTP bound), FadA and SfaD::GpgA signal to enhance vegetative growth and repress both asexual sporulation and ST production (Yu *et al.* 1996; Hicks *et al.* 1997; Adams *et al.* 1998; Rosen *et al.* 1999; Seo *et al.* 2005). This FadA-dependent growth-signaling pathway is in part transduced through PkaA [a catalytic subunit of cAMP-dependent protein kinase A (Shimizu and Keller 2001)]. FlbA is an RGS domain protein that negatively regulates FadA-mediated growth signaling (Berman *et al.* 1996; Koelle and Horvitz 1996; Yu *et al.* 1996). Integrating the previous and present findings, we propose to place NsdD under the control of FadA-PkaA, downstream of FLBs, and upstream of BrlA (Figure 7; see below).

Our recent studies demonstrated that VosA and VelB control expression of various genes in conidia via directly binding to the promoters of their target genes (Ahmed *et al.* 2013). The removal of *vosA* causes abnormal accumulation of *brlA* in conidia and vegetative cells (Ni and Yu 2007; Ahmed *et al.* 2013). The *nsdD* gene was one of the target genes that are directly controlled by the VosA-VelB heterodimer in conidia, and the lower transcript of *nsdD* was not detectable in the $\Delta vosA$ or $\Delta velB$ mutant conidia (Ahmed *et al.* 2013). These results suggest that the VosA-VelB heterodimer activates expression of NsdD in conidia, which confers proper downregulation of *brlA* during the initial period of vegetative growth. It has been shown that conidiation does not typically occur in *A. nidulans* until cells have gone through a defined period (~ 18 hr) of vegetative growth (Axelrod *et al.* 1973; Champe *et al.* 1981); *i.e.*, *A. nidulans* cells require ~ 18 hr of growth before they are competent to conidiate. It is important to note that the deletion of both *nsdD* and *vosA* might have reduced this developmental competence period to 16 hr or earlier (Figure 6, B and C). These results imply that developmental competence might be determined by the time and physiological status required to remove the repressive effects imposed by multiple negative regulators, *e.g.*, SfgA, NsdD, and VosA.

Taken together, we propose a working model depicting regulation of conidiation in *A. nidulans* (Figure 7). In this model, NsdD and VosA are expressed in conidia, preoccupying the *brlA* promoter in spores, which allows proper vegetative growth to occur after germination of conidia. Upon a certain period of growth (~ 18 hr), the FluG factor accumulates in the cells, which then removes the upstream negative controller SfgA (Seo *et al.* 2006). Such derepression triggers the transition from vegetative growth to development, involving the sequential activation of FlbE-B $\rightarrow$ D and FlbC. Activated FlbB-FlbD (Garzia *et al.* 2010) and FlbC then displace NsdD from the *brlA* promoter and further activates *brlA*. VosA is likely removed from the *brlA* promoter by VelB (Park *et al.* 2012, not indicated in the model). Furthermore, FLBs and VelB activate *brlA* expression, which collec-

Figure 7 Genetic model for growth and developmental control in *A. nidulans*.

tively leads to (full) activation of conidiation-specific genes and the development of conidiophores. Expression of AbaA and WetA also leads to high-level accumulation of *vosA* in phialides and conidia, which in turn shuts off expression of *brlA* at the later phase of conidiation and turns on expression of trehalose biogenesis genes and *nsdD* in conidia. Stimulation of vegetative proliferation and inhibition of ST biosynthesis mediated by FadA $\rightarrow$ PkaA might occur in part through NsdD, possibly involving phosphorylation of NsdD by PkaA (indicated by a dashed arrow with a ? in Figure 7). There are six predicted PKA-mediated serine phosphorylation sites in NsdD (<http://kinasephos2.mbc.nctu.edu.tw/>).

The *nsdD* gene is predicted to encode a 461-aa polypeptide (49,250 kDa), rich in proline (11.3%) and serine (13.4%), which contains the conserved amino acid sequence of type IVb C-X₂-C-X₁₈-C-X₂-C zinc-finger domain typically found in the GATA-type TFs (Teakle and Gilmartin 1998; Han *et al.* 2001). The NsdD polypeptide(s) is highly conserved in most *Aspergillus* species and many other important fungi including *Penicillium*, *Coccidioides*, *Ajellomyces*, *Fusarium*, and more. GATA TFs (GATA-1 to -6) bind to a DNA sequence called a GATA motif [(A/T)GATA(A/G)] in the regulatory regions of their target genes through two zinc-finger domains (Urnov 2002), and many can bind to DNA as homodimers or heterodimers. Typically, homodimers recognize inverted repeats within the target nucleotide sequence, whereas heterodimers bind to direct repeats (Laity *et al.* 2001; MacPherson *et al.* 2006). There are multiple (at least four) potential GATA TF binding sites in the promoter region (~ 1000 nt) of *brlA*. Identification and verification of NsdD binding sites in the *brlA* promoter and its target genes in the genome and investigation of NsdD interacting partners are in progress to further comprehend the molecular mechanisms of NsdD-mediated developmental regulation in *A. nidulans*.

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NsdD Is a Key Repressor of Asexual Development in *Aspergillus nidulans*

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Table S1 Oligonucleotides used in this study

Name	Sequence (5' → 3')	Purpose
oNK1006	ATATGCGGCCGCAACATTGCGTGGTTGTTGCTGAAC	5' AN1652 with <i>NotI</i> for multi copy
oNK881	ATATGCGGCCGCATGCAGGATTGAGTAGAGGGAATC	3' AN1652 with <i>NotI</i> for multi copy
oNK1007	ATATGCGGCCGCTGACTCCATTACTGAATCAGGAAAC	5' AN2009 with <i>NotI</i> for multi copy
oNK883	ATATGCGGCCGCTCCCATCGAAGATCTACGCGCAC	3' AN2009 with <i>NotI</i> for multi copy
oNK1008	ATATGCGGCCGCACCTTGACTCTTGATGATGCTGG	5' AN7507 with <i>NotI</i> for multi copy
oNK885	ATATGCGGCCGCACTCAAGTTCCAACAACCTTCTGAC	3' AN7507 with <i>NotI</i> for multi copy
oNK1013	ATATGCGGCCGCAGTTCGCAGCCTGTAAAGCTTCTG	5' AN3152 with <i>NotI</i> for multi copy
oNK1014	ATATGCGGCCGCACGGTATGGAGAGGAAACACGGATG	3' AN3152 with <i>NotI</i> for multi copy
oNK1011	ATATGCGGCCGCTGTCTGGACATGTGAGGATTCTCG	5' AN5833 with <i>NotI</i> for multi copy
oNK1012	ATATGCGGCCGCATCAGAACCAGTCATTCTCTTTAC	3' AN5833 with <i>NotI</i> for multi copy
oNK1009	ATATGCGGCCGCTGGTGACGATGAGGCGAGAAACAG	5' AN9141 with <i>NotI</i> for multi copy
oNK1010	ATATGCGGCCGCTTGGTCGGTGGTAAGGAGGTAGAG	3' AN9141 with <i>NotI</i> for multi copy
oMN33	AAATAAGCTTGCATGCGC	5' pRG3-AMA1 <i>NotI</i>
oMN35	GCCAGTGAATTCGAGCTC	3' pRG3-AMA1 <i>NotI</i>
oJH84	GCTGAAGTCATGATACAGGCCAAA	5' <i>AfupyrG</i> marker
oJH85	ATCGTCGGGAGGTATTGTGCTCAC	3' <i>AfupyrG</i> marker
oBS08	GCAATGTAAAGCTAACGTGCGTG	5' <i>AnipyrG</i> marker
oBS09	TGCCTTTAAGCTTCGGGTAGAG	3' <i>AnipyrG</i> marker
oNK395	ATCTCATGGGTGCTGTGCGAAAGG	5' <i>pyroA</i> marker
oNK396	TTGCATCGCATAGCATTGCATTGC	3' <i>pyroA</i> marker
oNK397	AACGCAACGCAAAGGATAGCGAGC	5' flanking region of <i>sfgA</i>
oNK398	ACTTCTGCAGTCGGAATTGGCCTGAGAGAAAGATCGACGGTGGGAGAG	5' <i>sfgA</i> with <i>pyroA</i> tail
oNK399	TGGTGAGAACACATGCACAACCTGTTGACCGGACCATACACCTCGCAC	3' <i>sfgA</i> with <i>pyroA</i> tail
oNK400	AGCAGACGTTCACTCAGAGCCACC	3' flanking region of <i>sfgA</i>
oNK401	AATTTGGTAGTTGCGCCTACGGC	5' nested of <i>sfgA</i>
oNK402	GTCGCAGAGGCGTAGCTCTACAG	3' nested of <i>sfgA</i>
oNK612	GCTTGGCCTGTATCATGACTTCAAGAGAAAGATCGACGGTGGGAGAG	5' <i>sfgA</i> with <i>AfupyrG</i> tail
oNK613	ATCGACCGAACCTAGGTAGGGTATTGACCGGACCATACACCTCGCAC	3' <i>sfgA</i> with <i>AfupyrG</i> tail
oNK788	AGATTCGACCGATGGAGCGAGCTC	5' flanking region of <i>fluG</i>
oNK789	ACTTCTGCAGTCGGAATTGGCCTGTGGCGATGAACCAGCAAACCTAAAG	5' <i>fluG</i> with <i>pyroA</i> tail
oNK790	TGGTGAGAACACATGCACAACCTGTTGAGTCTAATCTTCTCCTGAGC	3' <i>fluG</i> with <i>pyroA</i> tail
oNK791	ATCGCCGAATCCTATGGCGAGATC	3' flanking region of <i>fluG</i>
oNK792	TCCAAGGGCTCAGACAGGTCAACG	5' nested of <i>fluG</i>
oNK793	ACGTATACAAGGGTCAATCGCCGTG	3' nested of <i>fluG</i>
oWS7	GCTTGGCCTGTATCATGACTTCAAGAGAAAGATCGACGGTGGGAGAG	5' <i>fluG</i> with <i>AfupyrG</i> tail

oWS8	ATCGACCGAACCTAGGTAGGGTATTGAGTCTAATCTTCTCCTGAGC	3' <i>fluG</i> with <i>AfupyrG</i> tail
oNK748	AAGCGATCTGGCTGCGCTGATGG	5' flanking region of AN1652
oNK749	GCTTTGGCCTGTATCATGACTTCATTGTGTATGCGTCCATTGTCCTAG	5' AN1652 with <i>AfupyrG</i> tail
oNK750	ATCGACCGAACCTAGGTAGGGTAAGCTATCACGAACGCTATGCGACC	3' AN1652 with <i>AfupyrG</i> tail
oNK751	TGTATCGAATCATTGTCGCTATTG	3' flanking region of AN1652
oNK752	AACATTGCGTGGTTGTTGCTGAAC	5' nested of AN1652
oNK753	ATGCAGGATTGAGTAGAGGGAATC	3' nested of AN1652
oNK740	TGACTCCATTACTGAATCAGGAAAC	5' flanking region of AN2009
oNK741	GCTTTGGCCTGTATCATGACTTCATCGAGTGAAGAGTGAAAGTGGAG	5' AN2009 with <i>AfupyrG</i> tail
oNK742	ATCGACCGAACCTAGGTAGGGTATGAGTGGCTGATTGAGCTCATTC	3' AN2009 with <i>AfupyrG</i> tail
oNK743	TCCCATCGAAGATCTACGCGCAC	3' flanking region of AN2009
oNK744	TGGAGGTCTGGAGGTTGGAGATGC	5' nested of AN2009
oNK745	ATCCGAGGCTCTCGAGATTGAAGAG	3' nested of AN2009
oNK912	AGTTCGCAGCCTGTAAAGCTTCTG	5' flanking region of AN3152
oNK913	GCTTTGGCCTGTATCATGACTTCAAGTGATCCCATAGCGCAGGCAATC	5' AN3152 with <i>AfupyrG</i> tail
oNK914	ATCGACCGAACCTAGGTAGGGTAACCGAGGAGTCATTAACACGCTAG	3' AN3152 with <i>AfupyrG</i> tail
oNK915	ACGGTATGGAGAGGAAACACGGATG	3' flanking region of AN3152
oNK916	TAAGCTGATCGATCGTCCGCTCTC	5' nested of AN3152
oNK917	TCACTTGAGCTGGTTATGTGGTGG	3' nested of AN3152
oNK914	ACTTCTGCAGTCGGAATTGGCCTGAGTGATCCCATAGCGCAGGCAATC	5' AN3152-IR with <i>AnipyroA</i>
oNK1023	TGGTGAGAACACATGCACAACCTTGACCGAGGAGTCATTAACACGCTAG	3' AN3152-IF with <i>AnipyroA</i>
oNK906	TGTCTGGACATGTGAGGATTCTCG	5' flanking region of AN5833
oNK907	GCTTTGGCCTGTATCATGACTTCATGTGTCATAGTGGCAAGTGTGTAC	5' AN5833 with <i>AfupyrG</i> tail
oNK908	ATCGACCGAACCTAGGTAGGGTAAGGCGAAGCTCTAAATGTCTAGAC	3' AN5833 with <i>AfupyrG</i> tail
oNK909	ATCAGAACCAGTCATTCTCTCTTAC	3' flanking region of AN5833
oNK910	AGATTGGAGTGGCCAGGTGTCAG	5' nested of AN5833
oNK911	AGGTCACTGAGTACACCACCTAC	3' nested of AN5833
oNK732	ACTTGACTCTTGTGATGATGCTGG	5' flanking region of AN7507
oNK733	GCTTTGGCCTGTATCATGACTTCATATCATTGAGGATGCGGAGCAGC	5' AN7507 with <i>AfupyrG</i> tail
oNK734	ATCGACCGAACCTAGGTAGGGTAAGTTCGCGGGACTCAATCTG	3' AN7507 with <i>AfupyrG</i> tail
oNK735	ACTCAAGTTCCAACAACCTTCTGAC	3' flanking region of AN7507
oNK736	TGAATCTCGAAGCTGAGTTGCAGG	5' nested of AN7507
oNK737	ACTAGCTCTAGACACGCTGAGAC	3' nested of AN7507
oNK896	TGGTGACGATGAGGCGAGAAACAG	5' flanking region of AN9141
oNK897	GCTTTGGCCTGTATCATGACTTCATCATAACGTTATCGGCAGAGCTGTC	5' AN9141 with <i>AfupyrG</i> tail
oNK898	ATCGACCGAACCTAGGTAGGGTATCGCTGCAAGCTGATCCAGCATTTTC	3' AN9141 with <i>AfupyrG</i> tail
oNK899	TTGGTCGGTGGTAAGGAGGTAGAG	3' flanking region of AN9141
oNK900	TCAGCATCTGCATTGCTTCCAAGC	5' nested of AN9141
oNK901	AGACGATCGACGGTGACGAAGTTG	3' nested of AN9141
oNK106	ACCACAGAGTTAGCGCATTGTAC	5' flanking region of <i>flbE</i>
oNK456	GCTTTGGCCTGTATCATGACTTCAAGCATGTAGACTGGCATGGTAAG	5' <i>flbE</i> with <i>AfupyrG</i> tail
oNK457	ATCGACCGAACCTAGGTAGGGTATGCGTTGAGTATGTACTAATCGTTG	3' <i>flbE</i> with <i>AfupyrG</i> tail

oNK107	ACTAGTGAGACCTACCAGCAATAC	3' flanking region of <i>flbE</i>
oNK110	TGAATTGCCTCGAGGTATGCTAG	5' nested of <i>flbE</i>
oNK111	TGGTCGCTTCAGTAGGAAGCATT	3' nested of <i>flbE</i>
oNK522	AGAGGCTAGAGTCGAGGCTAGAGG	5' flanking region of <i>flbB</i>
oNK523	GCTTTGGCCTGTATCATGACTTCATCATGGTGGTCGAGCTGTGAATAG	5' <i>flbB</i> with <i>AfiupyrG</i> tail
oNK524	ATCGACCGAACCTAGGTAGGGTAACGATGTATTCATGACCTGACAGC	3' <i>flbB</i> with <i>AfiupyrG</i> tail
oNK525	TGTACATTCCAGAGATGCCGACAG	3' flanking region of <i>flbB</i>
oNK526	CTGCAAAGGCCATCGACAAGCCAG	5' nested of <i>flbB</i>
oNK527	AGCCTATCAGACATAGGATGGTGG	3' nested of <i>flbB</i>
oNK1017	TGGTCGATGCTCTAACAGCTTCGTG	5' flanking region of <i>flbD</i>
oNK1018	GCTTTGGCCTGTATCATGACTTCATGTGTTGGTGATGGAGGGTGCAAG	5' <i>flbD</i> with <i>AfiupyrG</i> tail
oNK1019	ATCGACCGAACCTAGGTAGGGTATGAACGAACGATCACACGACTCTC	3' <i>flbD</i> with <i>AfiupyrG</i> tail
oNK1020	AGGAACACAATACCTCAATACGTC	3' flanking region of <i>flbD</i>
oNK1021	TCGTCCTATCCAGACTGATGGGAC	5' nested of <i>flbD</i>
oNK1022	TAGCCTTACGGTGTACGGCGTACG	3' nested of <i>flbD</i>
oNK142	TCACATCTCGATGATTGGTTGAATG	5' flanking region of <i>flbA</i>
oNK1032	GCTTTGGCCTGTATCATGACTTCATGGCATTGAAGAGTGCAGGTCGGAG	5' <i>flbA</i> with <i>AfiupyrG</i> tail
oNK1033	ATCGACCGAACCTAGGTAGGGTAACAGTAATTATCTACACGCGTGATG	3' <i>flbA</i> with <i>AfiupyrG</i> tail
oNK145	ACTACTCACTACCTAACTTGACTG	3' flanking region of <i>flbA</i>
oNK146	TGGTTGAATGGTGTATGGGTCAGC	5' nested of <i>flbA</i>
oNK147	TGTAGCTTTCGTTTCAGGCGATAGTG	3' nested of <i>flbA</i>
oNK143	ACTTCTGCAGTCGGAAITGGCCTGTGGCATTGAAGAGTGCAGGTCGGA	5' <i>flbA</i> with <i>pyroA</i> tail
oNK144	TGGTGAGAACACATGCACAACITGACAGTAATTATCTACACGCGTGATG	3' <i>flbA</i> with <i>pyroA</i> tail
oNK540	TGTTCTGGATGATAGTCTTCTAG	5' flanking region of <i>rgsA</i>
oNK1030	GCTTTGGCCTGTATCATGACTTCATTGACTGAACGTATCTTAAGAGTG	5' <i>rgsA</i> with <i>AfiupyrG</i> tail
oNK1031	ATCGACCGAACCTAGGTAGGGTAACGTCGTGGCACAATCTTCCGGTG	3' <i>rgsA</i> with <i>AfiupyrG</i> tail
oNK543	AGTCCAATACACTGCATGTGGAG	3' flanking region of <i>rgsA</i>
oNK544	TGTCGCCGACCACTACTACTACC	5' nested of <i>rgsA</i>
oNK545	TTGAACTATCTTAACGAGGGTGAC	3' nested of <i>rgsA</i>
oNK541	ACTTCTGCAGTCGGAAITGGCCTGTGACTGAACGTATCTTAAGAGTG	5' <i>rgsA</i> with <i>pyroA</i> tail
oNK542	TGGTGAGAACACATGCACAACITGACGTCGTGGCACAATCTTCCGGTG	3' <i>rgsA</i> with <i>pyroA</i> tail
oJA142	CTGGCAGGTGAACAAGTC	5' <i>brlA</i> probe
oJA143	AGAAGTTAACACCGTAGA	3' <i>brlA</i> probe
oJA154	AGCTCTTCAGAATACGTC	5' <i>abaA</i> probe
oJA155	GTTGTGAGATGCCTCCAT	3' <i>abaA</i> probe
oMN66	TTTCCAGATCCTTCGCAG	5' <i>vosA</i> probe
oMN63	ATAGAAACAGCCACCCAG	3' <i>vosA</i> probe
oJA150	CAGTACGTCAATATGGAC	5' <i>wetA</i> probe
oJA151	GTGAAGTTGACAAACGAC	3' <i>wetA</i> probe
oJA144	TTCCTATGTCATGGTAGC	5' <i>stcU</i> probe
oJA145	GTACGGTGTGACTAACTG	3' <i>stcU</i> probe
oJA244	CCTGTATCATATGCAAATATC	5' <i>aflR</i> probe

oJA245	ATCATTCCAGATTATTCAACC	3' <i>aflR</i> probe
oJA174	CATTGAGCACGGTGTGTG	5' <i>r-actin</i> probe
oJA175	ATCCCTTGATCTCGTTG	3' <i>r-actin</i> probe

^aTail sequence is in italic.

^bRestriction enzyme site is in bold.