FlbD, a Myb Transcription Factor of Aspergillus nidulans, Is Uniquely Involved in both Asexual and Sexual Differentiation

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In the fungus Aspergillus nidulans, inactivation of the flbA to -E, fluG, fluF, and tmpA genes results in similar phenotypes, characterized by a delay in conidiophore and asexual spore production. flbD to -D encode transcription factors needed for proper expression of the brlA gene, which is essential for asexual development. However, recent evidence indicates that FlbB and FlbE also have nontranscriptional functions. Here we show that fluF1 is an allele of flbD which results in an R47P substitution. Amino acids C46 and R47 are highly conserved in FlbD and many other Myb proteins, and C46 has been proposed to mediate redox regulation. Comparison of ΔflbD and flbDR47P mutants uncovered a new and specific role for flbD during sexual development. While flbDR47P mutants retain partial function during conidiation, both ΔflbD and flbDR47P mutants are unable to develop the peridium, a specialized external tissue that differentiates during fruiting body formation and ends up surrounding the sexual spores. This function, unique among other fluffy genes, does not affect the viability of the naked ascospores produced by mutant strains. Notably, asexual development in these mutants is still dependent on the NADPH oxidase NoxA. We generated R47K, C46D, C46S, and C46A mutant alleles and evaluated their effects on asexual and sexual development. Conidiation defects were most severe in ΔflbD mutants and stronger in R47P, C46D, and C46S strains than in R47K strains. In contrast, mutants carrying the flbDC46A allele exhibited conidiation defects in liquid culture only under nitrogen starvation conditions. The R47K, R47P, C46D, and C46S mutants failed to develop any peridial tissue, while the flbDC46A strain showed normal peridium development and increased cleistothecium formation. Our results show that FlbD regulates both asexual and sexual differentiation, suggesting that both processes require FlbD DNA binding activity and that FlbD is involved in the response to nitrogen starvation.

All living organisms require sensing and integration of environmental signals in appropriate ways to generate responses that determine transitions between cell growth, programmed cell death, and differentiation. The filamentous fungus Aspergillus nidulans is an excellent model for studying these processes, as it presents complex but defined cell cycle transitions between growth (mycelia) and asexual (conidiation) and sexual (cleistothecium and ascospore development) differentiation and is amenable to genetic analysis.

A. nidulans asexual reproduction is induced by environmental signals such as exposure to air (2, 70) or nutrient starvation (63). It involves the production of chemical signals (9, 25, 38, 40, 44, 64, 72) and depends on activation of the brlA gene (10), which encodes a transcription factor (TF) of the Zn finger family (1, 29, 48, 54). Normally, conidiation involves the production of a mycelial cell compartment, from which a conidiophore stalk develops. After the stalk reaches a determined length, polar growth stops for development of a multineucleated vesicle, from which two successive uninucleate cell types are produced by budding (metulae and phialides). The phialides are conidiogenic cells that produce uninucleate spores through several rounds of consecutive mitosis (reviewed in references 2, 5, 16, and 81).

fluffy mutants, which show a cotton-like morphology and a notable delay in asexual development, were proposed to define genes upstream of brlA that are required for conidiation (13, 64, 68, 77). Indeed, extensive genetic analysis led to the identification of developmental regulators acting upstream of brlA (fluG and flhA) to -E (flhA-E) genes and required for their proper expression (77). FlhB is a regulator of a heterotrimeric G protein signaling pathway that stimulates vegetative growth and inhibits conidiation (83). In contrast, FluG is responsible for the production of an extracellular signaling factor required for activation of the flbD-E genes in vegetative cells and to induce conidiation, along with other unidentified compounds (38, 39, 44, 56, 64). FlbB is a TF of the bZIP type that unexpectedly also localizes at the hyphal tips of vegetative hyphae, forming a complex with FlbE (17, 18, 21). flbE encodes a small protein with two conserved but uncharacterized domains, and FlbE and FlbB are functionally interdependent and proposed to regulate the transition from vegetative growth to conidiation (20, 34). FlbB is found in nuclei, but notably, it is detected only at the most apical nucleus in vegetative hyphae (18). flbD encodes a TF of the Myb family (76) which, together with FlbB, is required to jointly activate brlA expression and conidiation (21). FlbC is a zinc finger TF that binds the brlA promoter in vitro, and the highest expression level of flbC correlates with cessation of apical extension and swelling of the conidiophore vesicle, which are phenotypes also observed for flbC overexpression (33). Two other mutants with delayed asexual development defined the tmpA and fluF genes. tmpA encodes a flavoenzyme required for the production of a sporulation chemical signal independent of the FluG factor (64). A fluF1 mutant was isolated after treatment with 5-azacytidine (68), but the identity of the gene remained unknown.
Frequently, sexual development (formation of fruiting bodies containing ascospores) occurs after conidiation, usually under low-nutrient and oxygen limitation conditions and in the absence of light (9). The first sign of sexual development is the formation of Hülle cells, which later surround the mature fruiting body, or cleistothecium. Cleistothecium formation involves the differentiation of a central ascomogenous tissue that gives rise to asc and ascospores and the development of a network of sterile hyphae surrounding the ascomogenous tissue, which finally develops into a melanized cleistothecial wall, or peridium (9, 62). Signaling involved in initiation of fruiting body development is mediated by membrane-bound G-protein-coupled receptors (GprA, GprB, and GprD) (23, 61). Besides signaling through the G protein (FadA, SfaD, and GpgA) (57, 61, 83), the mitogen-activated protein kinase (MAPK) module SkaA-AtfA (31, 36), the COP9 signalosome (7), VeA (5, 32, 65, 81), and several transcription factors, including SteA and several other factors, peridium (9, 62). 

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**FibD Myb in Asexual and Sexual Development**

**Disruption of fibD, fibD point mutations, and GAP tagging.** Genomic DNA was used as the template to produce an fibD gene replacement construct by double-joint PCR (82). The 5’ fibD fragment was obtained with primers 5FORfibD and 5REV+TAILfibD (see Table 2 for primer sequences). The 3’ fibD fragment was amplified with primers 3FOR+TAILfibD and 3REVibD. An Aspergillus fumigatus pyrG marker was amplified with primers pyrGforward and pyrGreverse, using plasmid pFN03 (50) as the template. The three fragments were purified, mixed, and used in a fusion PCR with primers FORNestfibD and REVnestfibD. The final, 4,000-bp fibD-AfpyrG-fibD cassette was purified and used to transform A. nidulans strain CFL3 by comiddium electroporation (59). Point mutations in C46 and R47 in the fibD Myb domain were introduced by overlap extension PCR. A 5’ fibD fragment including the mutation was amplified with primers MutFORfibD and RevfibD(C-D), RevfibD(C-S), RevfibD(C-A), or RevfibD(R-K). A 3’ fibD fragment including the mutation was amplified with primers MutRevfibD and ForfibD(C-D), ForfibD(C-S), ForfibD(C-A), or ForfibD(R-K). These fragments were combined in overlap extension reaction mixtures with primers ForfibDp ENTER and RevfibDpENTERs/stop to clone these PCR fragments as reported previously (71), placing the fibD open reading frame (ORF) (wild-type or mutant alleles) under the control of the alcA promoter and tagging the fibD C terminus with monomeric red fluorescent protein (mRFP). To obtain the fibD-AFPYR allele, the fibD ORF was amplified with the primers ForfibDpENTER and RevfibDpENTERs/stop, using genomic DNA from the fibD mutant as the template. Plasmids obtained in this way were designated pFlbDwtENTR, pFlbDC46DENTR, pFlbDC46SENTR, pFlbD46AENTR, pFlbD47KENTR, and pFlbD47PENTR. After confirming the fibD ORF sequence in each plasmid, the pFlbDENTR plasmids were used for recombination with the destination vector pM-ORF (71), using Gateway LR Clonase enzyme mix (Invitrogen, Carlsbad, CA) as indicated by the manufacturer. The resulting plasmids, palCA-fibD-mRFP plasmids (pIAQ9 to -14), were used to transform strain CIAQ25 by protoplast fusion. Three PCR products were used to generate an fibD C-terminal green fluorescent protein (GFP) wild-type or mutant construct according to the method of Yang et al. (79). First, a 5’ fragment upstream of the stop codon, including the entire fibD ORF, was amplified with primers fibDGSP1b and fibDGFP2 (for the wild-type construct) or with primers fibDGSp1b and RevfibDpENTERs/stop, using palCA-fibD-mRFP plasmids as the templates. Second, a 3’ fibD fragment was amplified with primers fibDGSP3 and fibDGSp4. Third, the GFP gene and the A. fumigatus pyrG marker were amplified with primers fibDGFP1 and fibDGFP2, using plasmid pFN03 as the template (50). Purified fragments were mixed and used in a fusion PCR with primers fibDGSp4 and fibDGSp1 or fibDGSp1b. The 4,630-bp fibD-gfp-AfpyrG cassette was used to transform A. nidulans strain A1155 by comiddium electroporation or protoplast fusion (59, 60, 80).
TABLE 1  Aspergillus nidulans strains used in this work

| Strain    | Genotype                        | Source or reference |
|-----------|---------------------------------|---------------------|
| CLK43     | pabaA1 yA2; veA1                | 31                  |
| CFL3      | pabaA1 yA2; pyrG89; veA1        | 36                  |
| FGSC26    | biA1; veA1                      | FGSC                |
| FGSCA4    | biA1                            | FGSC                |
| PW-1      | biA1; argB2; metG1; veA1        | P. Weglenksi        |
| GRS       | pyrG89; veA3; pabaA4; veA1       | FGSC                |
| A1155     | pyrG89; pyroA4 ∆hkuA2; bar; veA1 | M. Hynes; T. Adams  |
| RIF009    | biA1; metG1; veA1; fblC8         | J. Fondon and T. Adams |
| RIF120    | yA2; fblE58; metG1; veA1         | 77                  |
| TJW30.1   | pabaA1 yA2; fblD::trpC trpC801 veA1 | 76                  |
| CJ16A     | biA1; metG1; brlA::lacZ; ∆fblB; veA1 | 3                  |
| G1059A3   | addF17 pabaA1 yA2; fblF1 veA1   | M. Tamame           |
| TJAQ10    | Diploid CAF2/TJW30.1            | This work; progeny from G1059A3 × CS2902 cross |
| TJAQ18    | Diploid FGSC26/TJW30.1          | C. Scaccocio        |
| TJAQ21    | Diploid PW-1/G1059A3            | This work           |
| TJAQ24    | pabaA1 yA2; fblF1 veA1          | This work           |
| TJAQ25    | pabaA1 yA2; argB2; ∆fblD::AfpyrG veA1 | This work; progeny from TJAQ15 × TAH1 cross |
| TJAQ40    | pabaA1 yA2; ∆NoxA::AfpyrG; ∆fblD::AfpyrG veA1 | This work; progeny from TJAQ15 × GR5 cross |
| TJAQ51    | pyroA4; veA1                    | This work           |
| TJAQ52    | pyroA4; fblD::AfpyrG veA1        | This work           |
| TJAQ15    | pabaA1 yA2 pyrG89; ∆fblD::AfpyrG veA1 | This work; progeny from CAF2 × FGSC26 cross |
| TJAQ20    | pabaA1 yA2; alcA::fblDÆ<sup>+</sup>•mRFP•argB; ∆fblD::AfpyrG veA1 | This work; progeny from CAF2 × FGSC26 cross |
| TJAQ24    | pabaA1 yA2; alcA::fblDÆ<sup>+</sup>•mRFP•argB; ∆fblD::AfpyrG veA1 | This work; progeny from CAF2 × FGSC26 cross |
| TJAQ30    | pabaA1 yA2; alcA::fblDÆ<sup>+</sup>•mRFP•argB; ∆fblD::AfpyrG veA1 | This work; progeny from CAF2 × FGSC26 cross |
| TJAQ35    | pabaA1 yA2; alcA::fblDÆ<sup>+</sup>•mRFP•argB; ∆fblD::AfpyrG veA1 | This work; progeny from CAF2 × FGSC26 cross |
| TJAQ41    | pabaA1 yA2; alcA::fblDÆ<sup>+</sup>•mRFP•argB; ∆fblD::AfpyrG veA1 | This work; progeny from CAF2 × FGSC26 cross |
| TJAQ46    | pabaA1 yA2; alcA::fblDÆ<sup>+</sup>•mRFP•argB; ∆fblD::AfpyrG veA1 | This work; progeny from CAF2 × FGSC26 cross |
| TJAQ48    | pyrG89; pyroA4; fblF1::AfpyrG veA1 | This work; progeny from CAF2 × FGSC26 cross |
| TJAQ49    | pyrG89; pyroA4; fblF1::gfp::AfpyrG veA1 | This work; progeny from CAF2 × FGSC26 cross |
| TJAQ50    | pyrG89; pyroA4; fblF1::gfp::AfpyrG veA1 | This work; progeny from CAF2 × FGSC26 cross |
| TJAQ51    | pyrG89; pyroA4; fblF1::gfp::AfpyrG veA1 | This work; progeny from CAF2 × FGSC26 cross |
| COS400    | pabaA1 yA2; ∆fblD::AfpyrG        | This work; progeny from TJAQ15 × FGSCA4 cross |

Transformants with point mutations were confirmed by Southern blot analysis and DNA sequencing.

**RNA extraction and Northern blot analysis.** Samples were frozen in liquid nitrogen and stored at −70°C until use, at which time they were ground with a mortar and pestle under liquid nitrogen. Total RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Ten to 15 μg of RNA was separated in a 1% agarose gel containing formaldehyde, transferred to a Hybond N+ membrane, and hybridized using specific probes.

**RESULTS**

**fluF1 is an fblD allele.** A fluffy mutant isolated after treatment with 5-azacytidine defines the *fluF1* gene, which was assigned to chromosome VIII (68). *fluF1* is located in the standard genetic map of *A. nidulans* as the most centromere-proximal identified locus in the long arm of chromosome VIII (http://www.gla.ac.uk/ibls/molgen/aspergillus/viiicountigs.html; our unpublished observations) or with *sonA* (ANID_1379) (78), which is centromere proximal to *choC*. We thus investigated whether *fluF1* is allelic with other mutations in chromosome VIII that result in a fluffy phenotype. To identify the affected gene, we carried out complementation tests on diploids with mutations in other genes located in chromosome VIII whose mutation results in a fluffy phenotype map (fblD [76] and tempA [64]). Diploid strains obtained by crosses between *fluF1* mutants and wild-type (Fig. 1A) or *ΔtmpA* (not shown) strains showed wild-type conidiation. In contrast, diploids obtained by crosses between *fluF1* and *fblD1* mutants maintained a fluffy phenotype, indicating that *fluF1* and *fblD1* are alleles (Fig. 1A). Standard genetic crosses between *fluF1* and *fblD1* strains failed to yield wild-type recombinants among 329 progeny analyzed (Fig. 1B), confirming the allelism of *fluF1* and *fblD1*. We used *fluF1* strain genomic DNA as the template to amplify the cognate *fblD9* gene. Direct sequencing of two independent PCR products showed a single G-to-C substitution in codon 47 (CCG → CCG), which
In performing sexual crosses between \textit{flbD1} and \textit{flbD^{R47P}} (\textit{fluF1}) strains, we noticed that it was very difficult to detect and isolate cleistothecia, as putative fruiting bodies appeared very soft and wet. To follow sexual development more closely in isolated fruiting bodies, we successfully adapted a method developed for \textit{Neurospora crassa} (6, 12). Under these conditions, we observed that all \textit{flbD} mutants produced atypical fruiting structures (Fig. 2B), characterized by the presence of many Hülle cells and naked ascospores, which lacked the external tissue (peridium) that normally surrounds and protects the ascospores (9, 62). Ascospores produced in this way presented normal viability (not shown), and their production was denoted by the deposition of a reddish purple pigment on the plate surface (Fig. 2B). Having shown previously that the NADPH oxidase NoxA is required for the production of ROS at the peridial tissue and for cleistothecium development (35), we asked if the NoxA requirement could be bypassed in \textit{flbD} mutants, which can develop ascospores in the absence of peridial tissue. We generated \(\Delta flbD \Delta noxA\) double mutants and followed sexual development as described before. As shown in Fig. 2C, a \(\Delta flbD \Delta noxA\) double mutant was arrested at a very early stage of development (initial cleistothecia) and, similarly to a \(\Delta noxA\) single mutant, was unable to produce any ascospores. These results demonstrate that peridium and ascospore development represents two separable developmental processes and that in addition to its role in asexual development, \textit{flbD} is required for peridium formation during sexual development but dispensable for ascospore production, while the absence of NoxA results in a blockage of sexual development at a very early stage and prevents both developmental outcomes.

\textbf{flbD, but not other fluffy genes, has a \textit{veA}}^{-}\textit{independent role in sexual development.} As indicated before, inactivation of the \textit{flbB}, \textit{flbC}, \textit{flbD}, \textit{flbE}, and \textit{tmpA} genes results in \textit{fluffy} phenotypes related to a delay in asexual development and less expression of the \textit{brlA} gene (\textit{fluffy} low \textit{brlA}) (64, 77). Furthermore, \textit{FlbB} and \textit{FlbD} act together to regulate asexual sporulation as well as polarized growth (21). We therefore asked if these or other types of \textit{fluffy} gene interactions were also required for peridium development or general sexual differentiation. In contrast to the \(\Delta flbD\) mutant, \textit{flbB}, \textit{flbC}, \textit{flbE}, and \textit{tmpA} mutants were all able to differentiate normal fruiting bodies (Fig. 3), which produced normal and viable ascospores like those of a wild-type strain. These results indicate that \textit{FlbD}'s role in peridium development is specific and independent of the \textit{FlbD} partner \textit{FlbB} and the \textit{flbC}, \textit{flbE}, and \textit{tmpA} genes.

The \textit{A. nidulans} \textit{veA} gene encodes a 573-amino-acid protein required for cleistothecium formation (32). Because our laboratory strains contain a defective \textit{veA1} allele that results in more production of conidia and smaller numbers of cleistothecia (30), we also evaluated the \textit{flbD} mutant’s sexual phenotype in the presence of a wild-type \textit{veA} allele. As shown in Fig. S4 in the supplemental material, a \(\Delta flbD veA^{-}\) strain developed fruiting structures which also lacked the peridium and produced naked ascospores, indicating that \textit{FlbD}’s role in peridium development is independent of the \textit{veA} gene.

\textbf{FlbD Myb domain residue C46 plays critical roles in both asexual and sexual development.} Many reports indicate that Myb transcription factors can be regulated by redox modification. This mechanism involves a cysteine conserved in all R1R2R3 and R2R3 Myb family members from plants, fungi, and animals (see Fig. S1 in the supplemental material). This cysteine can act as a redox

\textbf{TABLE 2 DNA primers used in this work}

| Primer name   | Sequence                                                                 |
|---------------|--------------------------------------------------------------------------|
| 3REV+TAILflbD | AGG AGG GGG ATT GAG AGC ATT GAG TTA GGG CTC CTC TGG CTC AGG ACC GAG ACC |
sensor, and in fact, its oxidation impairs DNA binding in vitro (49). Adjacent to this cysteine in these Myb proteins is a highly conserved arginine residue whose positive charge affects cysteine’s pKa and therefore its redox properties (11). In FlbD, these residues correspond to C46 and R47, and the fact that the R47P substitution results in a virtually complete lack of function (Fig. 4 and 5) suggests that this change might affect C46’s redox properties. However, the role of C46 in FlbD’s function has not been evaluated. To test this, we generated mutant alleles with mutations in C46 and R47. C46D and C46S changes were expected to impair DNA binding (49). These results highlight the regulatory role of C46 in FlbD during conidiation. The expression of the flbDR47P and flbDC46D alleles resulted in only a modest increase in conidiation. The flbDR47K allele showed improved conidiation in comparison to the flbDR47P allele, but without reaching wild-type levels. This indicates that a positive charge at position 47 is important for FlbD function in conidiation but that K is not functionally equivalent to R in FlbD. Strains carrying an flbDC46S allele showed about half of wild-type conidiation levels, similar to the level exhibited by the flbDR47K strain. Notably, the flbDC46A allele resulted in conidiation levels that were indistinguishable from those found in the wild type, consistent with the expectation that a C46A change should improve or not affect FlbD DNA binding (49). These results highlight the regulatory role of C46 in FlbD during conidiation.

To test the functionality of these flbD alleles during sexual differentiation, we evaluated the strain phenotypes by using a standard assay for inducing sexual development in which conidiation occurs first and is then followed by fruiting body development. The results in Fig. S6 in the supplemental material show that under these conditions, alcA-driven expression of wild-type and flbDC46A alleles was also enough for conidiation (see condioshores and yellow conidia) but not enough to rescue normal sexual development (note that water droplets were formed around clumps of naked ascospores).

As an alternative approach to evaluate the effects of point mutations within the FlbD Myb domain on sexual development, we generated a new set of constructs to express wild-type and mutant alleles fused to GFP from the endogenous flbD promoter. These constructs were used to transform A. nidulans strain A1155 (nkuA), and integration was directed to the flbD locus. Each point mutation was confirmed by DNA sequence analysis of the entire flbD ORF (data not shown). The results in Fig. 5A show that strains containing wild-type and flbDC46A alleles showed wild-type

FIG 1 fluF1 is an flbD allele in which arginine 46 codon CGG is replaced by proline codon CCG. (A) A diploid between fluF1-1 and flbD1 fluffy gene mutants shows a fluffy mutant phenotype. Strains FGS26 (WT; top panel), PW-1 (WT; middle panel), CAF2 (fluF1-1), G1059A3 (fluF1-2), TJW30.1 (flbD1), CJAQ10 (fluF1-1/flbD1), CJAQ18 (WT/fluF1), and CJAQ21 (WT/fluF1-2) were point inoculated onto supplemented minimal medium (MM) plates and incubated at 37°C for 3 days. (B) fluF1 (CJAQ8) and flbD1 (TJW30.1) strains show very little recombination in sexual crosses. Ascospores from a hybrid cleistothecium were inoculated onto supplemented MM plates containing yeast extract and incubated at 37°C for 3 days, and then the fluffy phenotype was scored. (C) FlbD, a transcription factor of the Myb domain family, contains two repeats with three alpha helices, indicated as H1 to H3. The second repeat contains the highly conserved amino acids cysteine (C46) and arginine (R47). (D) fluF1 mutation results in replacement of arginine 47 by proline (R47P). Genomic DNAs from wild-type (CLK43) and fluF1 mutant (G1059A3) strains were amplified by PCR and sequenced as shown in the pherogram. See Table 1 for full strain genotypes.
conidiation, indicating that the GFP tag did not affect FlbD’s function under these conditions. However, the GFP fluorescence signal was not detectable during growth or asexual or sexual differentiation, likely due to low FlbD protein levels in vivo. flbDC46D and flbDR47P strains showed delayed conidiation, again confirming the results in Fig. 4. Regarding sexual differentiation, flbDC46A and flbDR47P strains produced only naked ascospores without a peridium (Fig. 5B). In contrast, the flbDC46A strain was able to

**FIG 2** flbD is required for peridium but not noxA-dependent ascospore development during sexual differentiation. (A) Strains CLK43 (WT), CJAQ24 (fluF1; referred to as flbDR47P), TJW30.1 (flbD1), and TJAQ15 (ΔflbD) were point inoculated onto supplemented MM plates and incubated at 37°C for 5 days. Total conidiospores were harvested and counted for each colony. Error bars indicate standard deviations for three independent experiments. (B) Schematic representation of early stages of cleistothecium development (upper left). Strains TJAQ15 (ΔflbD) and CJAQ24 (fluF1; referred as flbDR47P) were induced to undergo sexual development by use of a method adapted from an N. crassa method (see Materials and Methods), and samples were observed using a stereoscopic microscope (magnification, ×8) after 7 days of development (lower left) or processed for scanning electron microscopy after 8 days of development (right panels). Red aggregates of ascospores lacking the surrounding peridial tissue are indicated with black arrows. Scanning electron micrographs show a wild-type cleistothecium and ΔflbD ascospore aggregates next to some filamentous cells (hyphae). (C) Strains CAH4 (ΔnoxA) and CJAQ40 (ΔflbD ΔnoxA) were grown as described above. Images were taken after 8 days of sexual development by use of scanning electron microscopy. For scanning electron microscopy images, white bars indicate the magnification. PT, peridial tissue; AT, ascogenic tissue; As, ascospores; Cl, cleistothecia; H, Hülle cells; Pr, primordium. See Table 1 for full strain genotypes.

**FIG 3** flbD is unique among other fluffy genes with regard to its specific role in sexual development. Strains CLK43 (WT), TJAQ15 (ΔflbD), CJA16 (flbB), RJF009 (flbC), RIW120 (flbE), and TG6 (ΔtmpA) were point inoculated onto supplemented agar plates and incubated at 37°C for 5 days. Sexual induction was carried out as indicated. Images were taken with a stereoscopic microscope. Magnification, ×8. Some heterogeneity in fruiting body size was observed in all strains. See Table 1 for full genotypes.
differentiate fully developed fruiting bodies, which produced viable ascospores just like those of the wild-type strain, also showing that the GFP tag did not interfere with FlbD’s function in sexual development. Moreover, the flbD<sup>C46A</sup> strain produced larger numbers of cleistothecia than the wild-type strain, although the process occurred within the wild-type time frame (Fig. 5C). Our result indicates that amino acids C46 and R47 play an essential role for FlbD, as C46 can be replaced by A, which presumably does not affect FlbD binding to DNA.

We have previously shown that carbon starvation during liquid culture induces the differentiation of minimal conidiophores, while nitrogen starvation induces the formation of fully differentiated conidiophores (63). We found that under these conditions, fluffy mutants such as tmpA, flbB, flbC, flbD, and flbE mutants conidiated well under carbon starvation conditions but failed to conidiate under nitrogen starvation conditions (not shown). Having shown that the flbD<sup>C46A</sup> allele is fully functional in asexual and sexual differentiation, we asked whether it was also functional for nitrogen starvation-induced conidiation. The results in Fig. 6 show that under nitrogen starvation conditions, the wild-type and flbD<sup>C46A</sup> strains formed fully developed conidiophores, in sharp contrast to the ΔflbD and flbD<sup>C46A</sup> mutants, which failed to differentiate any conidiophore structures. These results clearly indicate that the C46A mutant cannot replace wild-type FlbD regulation of conidiation induced by nitrogen starvation and that FlbD plays multiple roles during A. nidulans development.

**DISCUSSION**

In this report, we have shown that fluf is an allele of the flbD gene, whose only known role was to activate brlA, a gene essential for asexual sporulation. Indeed, FlbD is a Myb TF whose expression depends on the bZIP TF FlbB, and both FlbD and FlbB are jointly required to activate brlA expression (21). In this study, we found that FlbD is also essential for the differentiation of the peridium, a tissue that constitutes the fruiting body external wall protecting the sexual spores. Furthermore, we showed that this role is specific for FlbD, as fluffy genes flbB, flbC, flbE, and tmpA are not required for peridium development. This is particularly important in the case of FlbB, as it indicates that in contrast to what occurs during conidiation, FlbB is not necessary for flbD expression during sexual differentiation, and that FlbD does not require interaction with FlbB to carry out its functions in peridial development.

Recently, it was found that when fluffy mutants affected in flbC or flbE were induced to undergo sexual development, they showed increased cleistothecium formation and decreased conidiation, suggesting that these genes repress sexual development and maintain the balance between asexual and sexual differentiation (33, 34). Whether or not this relates to FlbD’s role in sexual development requires further investigation.

In the plant pathogen Gibberella zeae, the inactivation of MTY1, a different Myb transcription factor highly conserved in the ascomycetes, results in decreased female fertility and peritheciun production and, in some cases, the production of immature ascospores (41). This suggests that different Myb transcription factors might be involved in different aspects of fungal sexual development. In plants, members of the Myb family regulate flavonoid biosynthesis, cell fate and identity, the cell cycle, and responses to biotic and abiotic stresses. In these roles, Myb TFs have been described as coactivators or positive regulators, sometimes carrying out partially redundant functions (15, 19, 37). Myb TFs have been described as targets for redox regulation.
through reduction/oxidation of the cysteine C46 conserved in FlbD and in many other R1R2R3 and R2R3 family members from plants, fungi, and animals. In fact, the oxidation of this cysteine impairs DNA binding \textit{in vitro}. Our results show that in FlbD, C46S and C46D substitutions impair function during asexual and sexual development. In contrast, a C46A substitution resulted in wild-type conidiation and fully developed fruiting bodies. Presumably, FlbDC46A can bind its DNA targets constitutively or with wild-type affinity (Fig. 4A and B and 5), as the same replacement in the Myb TF Bas1p resulted in a functional TF \textit{in vitro} and \textit{in vivo} (22, 49, 52). Since flbB is necessary to express flbD during conidiation but not during sexual development, putative constitutive DNA binding by FlbDC46A would not interfere with FlbD’s normal function during conidiation but could explain the larger numbers of fully differentiated fruiting bodies than those of a wild-type strain.

ROS produced by the NADPH oxidase NoxA are required for fruiting body development in \textit{A. nidulans} and other fungi (4, 8, 35, 42). \textit{ΔnoxA} and \textit{ΔflbD ΔnoxA} mutants were arrested at a similar early stage of development (cleistothecia initial) (Fig. 2C). This
indicates that ROS are required for both peridium and ascospore development and that these stages are separable differentiation processes, with FlbD playing a role only in peridium formation. Although flbD mRNA levels were very low during sexual development, slightly increased levels were detected at the time of cleisothecium production (not shown). This and the fact that alcA-driven expression of flbD was enough for normal conidiation but not for peridium differentiation under conditions of drastically reduced expression (see Fig. S6 in the supplemental material) suggest that high and/or localized flbD mRNA levels are needed to develop the peridial tissue. Further research is needed to determine if localized NoxA-generated ROS can regulate FlbD function during sexual development.

Besides air contact, nutritional stress such as carbon or nitrogen starvation induces conidiation (63, 64). We have found that when fluffy mutants affected in the flbB, flbC, flbD, flbE, or tmpA gene (64) are starved for nitrogen, they fail to fully induce the brlA gene and to conidiate. Surprisingly, the flbDC46A allele, which was fully functional in sexual differentiation and conidiation induced by an air interface, was not able to induce conidiation under nitrogen starvation conditions in submerged cultures. Under these conditions, FlbD might perform additional functions in nitrogen signaling or utilization. In Schizosaccharomyces pombe, the Myb-type DNA binding protein Reb1 regulates G1 cell cycle arrest and sexual differentiation in response to nitrogen starvation (55), while in the unicellular red alga Cyanidioschyzon merolae Cm-MYB1, an R2R3-type Myb TF activates expression of key nitrogen assimilation genes in response to nitrogen status (28).

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