Beyond Asexual Development: Modifications in the Gene Expression Profile Caused by the Absence of the *Aspergillus nidulans* Transcription Factor FlbB

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ABSTRACT In the model fungus *Aspergillus nidulans*, asexual development is induced from vegetative hyphae by a set of early regulators including the bZIP-type transcription factor FlbB. To determine the range of genes under the influence of the transcriptional activity of FlbB and to characterize their role in fungal development, we sequenced and compared the transcriptomes of a $\Delta flbB$ mutant and its isogenic wild-type strain at different developmental stages. Results confirmed the activating role of FlbB on downstream regulators of conidiation such as *flbD* and *brlA*. However, FlbB has additional functions beyond the induction of asexual development. Among the changes observed, absence of a functional FlbB caused induction of the *dba* cluster and synthesis of a secondary metabolite with bactericidal properties. In addition, a new transcriptional target of FlbB was unveiled, *urdA*, that codes for a putative transcription factor that represses premature sexual development. Taken together, our results indicate that the activators of asexual development simultaneously exert a role on other cellular functions, including an inhibitory effect on the sexual cycle, and reinforce the hypothesis that mutually exclusive metabolic and cellular patterns are associated with different morphogenetic programs.

KEYWORDS *Aspergillus nidulans*; mRNA sequencing; transcriptional regulation; development; secondary metabolite cluster

CELLULAR morphogenesis can be defined as programmed gene expression changes that lead to the development of specialized cell types. Basic eukaryotic morphogenetic mechanisms are commonly studied using fungi as models. *Aspergillus nidulans* is a nonpathogenic fungus that is phylogenetically related to clinically important species (*A. fumigatus*) as well as species with economical or industrial value such as *A. niger* and *A. oryzae*. In addition, *A. nidulans* is the main reference organism in basic studies of asexual development (Etxebeste *et al.* 2010a; Park and Yu 2012). The production of asexual spores (conidia) is induced in nonspecialized cells called *vegetative hyphae* and

involves the biogenesis of a succession of cell types that results in the generation of structures called *conidiophores* (Mims *et al.* 1988).

A significant number of genes involved in asexual development have been identified and characterized (Etxebeste *et al.* 2010a; Ni *et al.* 2010; Park and Yu 2012). Some of them are specific to the morphogenetic process and control the later stages leading to conidia production. *brlA*, the first development-specific transcription factor; *abaA*; and *wetA* constitute the backbone of the central developmental pathway (CDP) and regulate the expression of genes involved in the correct spatiotemporal formation of the conidiophore cell types (Park and Yu 2012).

The specialization of vegetative hyphae into asexual reproductive structures is induced by exogenous and endogenous stimuli (Fischer and Kües 2006). This requires the existence of an efficient genetic mechanism to guarantee that these cues are correctly transduced into signals that activate the CDP. This role is played by upstream developmental activators (UDAs) (Wieser *et al.* 1994; Etxebeste *et al.* 2010a),

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Figure 1 Model for the induction of conidiophore development through FlbB. The bZIP transcription factor is retained at the tip of vegetative hyphae through the interaction with FlbE (left). The complex may play a sensor role in the reception of inducing environmental signals, which would provoke a modification of FlbB sequence (FlbB*) and its transport to the nucleus (right). There FlbB activates the expression of *flbD*, and then both factors jointly induce the expression of *brlA*, the first conidiation-specific transcription factor. Updated from Etxebeste *et al.* (2010a).

a set of proteins that launch the initial morphogenetic transformations leading to vesicle formation. Loss-of-function mutations in UDA genes yield the “fluffy” phenotype, characterized by the absence of *brlA* activation and thus cell differentiation (Wieser *et al.* 1994).

A key UDA factor in the activation of *brlA* is FlbB, the first transcription factor in *A. nidulans* detected at the tip of vegetative hyphae (Etxebeste *et al.* 2010a). Apical FlbB is thought to act as a sensor mechanism involved in the transduction of environmental signals to nuclei (Figure 1). Nuclear FlbB first induces the expression of the cMyb-type UDA transcription factor–coding gene *flbD*, and then both factors jointly bind *brlA* promoter to trigger conidiophore development.

To gain a better understanding of FlbB activity and identify new transcriptional targets, we sequenced the transcriptomes of a $\Delta flbB$ mutant and its isogenic wild-type (WT) strain at specific stages of the *A. nidulans* life cycle. Results indicate that FlbB has an important role not only in the induction of the asexual process but also in the repression of a premature induction of sexual development. The inability to induce asexual development caused by the absence of FlbB also results in an important variation in the expression of secondary metabolite clusters and the production of antibacterial metabolites.

Materials and Methods

Fungal strains and culture conditions

We used for the RNA-seq experiment *A. nidulans* strain BD143 (Etxebeste *et al.* 2008) as an *flbB* null mutant and strain MAD2666, which was provided by Dr. A. Markina-Iñarrairaegui, as the isogenic WT reference (Garzia *et al.* 2013). Vegetative cell samples and those from the asexual stage were obtained as described previously (Garzia *et al.* 2013). Two biological replicates were processed for each culture condition and strain.

Genomic cassettes for the generation of *urda*, *dba*-cluster or *An2030/2038*-cluster mutants were amplified through the fusion-PCR technique (Yang *et al.* 2004). The oligonucleotides and strains used are shown in supporting information, File S1, Table S1 and Table S2, respectively. Transformation of *A. nidulans* protoplasts followed the protocol described by Tilburn *et al.* (1983). The revertant strain for the null *urda* mutant was generated by transforming protoplasts of this strain with a genomic cassette bearing the *urda* ORF plus its promoter and the 3'-UTR region. The selection of transformants was done using 5-fluoroorotic acid (FOA; 2 mg/ml; Apollo Scientific, Cheshire, UK), and the homologous recombination of the construct at the *urda* locus was confirmed by Southern blot. Northern and Southern blot experiments (oligonucleotides for probe generation are shown in Table S1) were done as described previously (Garzia *et al.* 2009). *Aspergillus* minimal medium (MMA) was prepared as described by Kafer (1965). Saline or osmotic stress conditions were induced by adding sodium dihydrogen phosphate (0.5 M), sucrose (1 M), sorbitol (1.2 M), or NaCl (0.8 M) to MMA. Cycloheximide (20 μ g/ml) was used as an inhibitor of mRNA translation.

Quantitative counts of conidia (in conidia/cm²) were obtained after culturing strains in MMA at 37° for 72 hr. Colony diameters were measured and spores collected and quantified using a hemocytometer. Cleistothecia counts were obtained after culturing strains in MMA for 156 hr. The protocol described by Baidya *et al.* (2011) was followed. Briefly, cores (8 mm in diameter) were collected from each plate, and ethanol (70%) was sprayed onto the core surface to facilitate visualization of cleistothecia under the microscope. Results provided are the mean values of three replicates per strain and condition with the standard error of the mean (SEM).

Cocultures of *A. nidulans* and *Micrococcus luteus* were prepared starting from solutions containing 10^7 *A. nidulans* conidia or 10^8 bacterial cells per milliliter. Briefly, fungal conidia and bacterial cells were independently cultured at 200 rpm for 4 hr at 37° or 1 hr at 30°, respectively. Fungal conidia were diluted in Eppendorf tubes to 10^6 per milliliter, and increasing concentrations of bacterial cells were added to each tube (0, 10^7 , 2.5×10^7 , and 5.0×10^7 cells/ml, respectively). Control tubes containing only bacterial cells were prepared using the same set of concentrations. Then 10 μ l of each mixture (2.0×10^4 conidia) was point inoculated on Luria-Bertani (LB) plates adequately supplemented to support growth of the fungal strains assayed. Photographs were acquired after 48 hr of culture. At this time point, 0.60-cm plugs from the cultures were excised. Cells were resuspended in 2.5 ml of LB medium by vortexing for 30 sec. Bacterial cells were counted using a hemocytometer. All results are the mean and SEM of three biological and nine technical replicates per strain.

RNA isolation, mRNA library construction, and Illumina sequencing

Total RNA extraction from cell samples was performed according to the Invitrogen protocol (Invitrogen, Carlsbad, CA) based on TRIzol reagent (Garzia *et al.* 2013). An RNeasy Mini Kit (QIAGEN, Valencia, CA) was used for RNA sample purification. The concentration and integrity of total RNA were checked using a Nanodrop (Thermo Fisher Scientific, Waltham, MA) and a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA).

mRNA libraries were prepared from *A. nidulans* total RNA samples following Illumina standard protocols (Illumina, San Diego, CA). Briefly, each total RNA sample (20–50 μ g) was treated with DNase and enriched for mRNA using oligo(dT) tags. Samples of poly(A) RNA (0.2–1 μ g) were fragmented into smaller pieces (200–500 bp; mean for all libraries is approximately 280 bp) and used to synthesize cDNA. The cDNA library construction involved end repair, A-tailing, adapter ligation, and library amplification followed by cluster generation and sequencing. Sequencing was performed in a pair-end-read, 2 $\times$ 76-base mode on a GAIIx Sequencer (Illumina) running four samples per lane (multiplexing).

Demultiplexing, mapping, assembling, and quantifying sequencing data

Sequences were demultiplexed with demultiplexing software by Brian J. Knaus (<http://brianknaus.com/>). Reads with quality values higher than Q30 in the fastQC program were introduced for mapping. They were mapped using Bowtie 2.0.0-beta5 (<http://bowtie-bio.sourceforge.net/index.shtml>) using parameters by default. The *Aspergillus* Genome Database (AspGD; <http://www.aspergillusgenome.org/>) provided version s07-m02-r07 of the annotated genome of *A. nidulans*, which was used as the template for mapping.

Figure 2 Northern blot analysis showing the hourly expression of selected UDA genes (*flbB*, *flbD*, *flbC*, and *fluG*) and CDP genes (*briA*, *abaA*, and *wetA*), both during vegetative growth (18–24 hr) and asexual development (1–13 hr after the induction). The orange rectangles demark time points with the maximum UDA expression. The red rectangles demark the expression peak for UDAs during conidiophore development. The blue rectangles demark the increase in the accumulation of CDP transcripts. rRNAs are shown as loading control.

Gene expression analysis

Open-source software such as TopHat and Cuffdiff were used for the mathematical analysis and comprehensive expression analysis of high-throughput RNA sequencing data. To associate with predicted genes, the reference for the gtp file generated in previous mapping steps was provided in a FASTA format by the AspGD. TopHat version 2.0.9 (<http://tophat.cbcb.umd.edu/>) provided the preformatted mapping files that were used as input files. The steps described followed the protocol by Trapnell and colleagues (Trapnell *et al.* 2012; Garzia *et al.* 2013).

Cufflinks version 2.1.1 was used for transcript assembly, estimation of transcript abundance, and testing of differential expression. The significance in the expression difference of a specific gene in two genetic backgrounds was determined, which calculates the *P*-value (the uncorrected *P*-value of the test statistic) and the *Q*-value [the false-discovery-rate (FDR)–adjusted *P*-value of the test statistic]. An expression difference will be significant if *P* is greater than the FDR after Benjamini-Hochberg correction (Benjamini *et al.* 2001) for multiple testing (in our case, *Q*-values between 0 and 0.05 indicate significant changes). Data were analyzed in the R statistical language based on CummeRbund and pheatmap packages.

Gene ontology analysis

Gene ontology terms for each *A. nidulans* gene were obtained from the AspGD (http://www.aspgd.org/download/go/gene_association.aspgd.gz) and were related with terms downloaded in OBO from the Gene Ontology (GO) project (http://www.geneontology.org/ontology/obo_format_1_2/gene_ontology_ext.obo). The GO project provided a standardized set of terms describing the molecular function of genes. We used

Figure 3 Experimental approach and clustering of significantly deregulated genes. (A) Experimental design and general transcriptomic results. Strains ($\Delta flbB$ and an isogenic WT strain) and time points selected [19 hr of vegetative growth (VG) and 5 hr of asexual development (AD)] are indicated. Black

the topGO package from the Bioconductor project (<http://www.bioconductor.org/packages/release/bioc/html/topGO.html>) to identify overrepresented GO terms from a set of differentially expressed genes. Python (<http://www.python.org/>) was used to prepare the data using rpy2 (<http://rpy.sourceforge.net/rpy2.html>) to call R for the statistical analysis.

Nucleotide sequence accession number

Sequence reads were submitted to the NCBI Sequence Read Archive (SRA) and deposited under Accession Numbers SRX206691 for WT (Garzia *et al.* 2013) and SRX286239 for $\Delta flbB$ strains.

Quantitative PCR (qPCR)

Total RNA samples from three independent biological replicates were treated with RNase-free DNase I and purified with the RNeasy Mini Kit (QIAGEN). First-strand cDNA was synthesized using the AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies) under the manufacturer's instructions. Each cDNA sample was tested in triplicate and included a control lacking reverse transcriptase.

Specific probes were designed and employed in qPCR assays (IDT primers and ZEN double-quenched probes; <http://eu.idtdna.com/site>) (Table S1). These probes were designed taking into consideration the sequences obtained from the RNA-seq experiment, which coincided with those obtained in a second, independent cDNA sequencing of *An7895*, *An7896*, and *An7901* (Table S1). qPCR assays were performed in an ABI 7900 HT system (Applied Biosystems, Foster, CA) by adding 5 μ l of 2 $\times$ Brilliant II QPCR Master Mix (Agilent Technologies) to each well containing 20 ng of the template cDNA, 500 nM of forward and reverse primers, and 250 nM of the probe (total reaction volume of 10 μ l). Thermal cycling conditions were set as follows: an initial step at 95° for 2 min and 40 cycles of 20 sec at 95° and 1 min at 60°. We included control reactions without template [no template controls (NTCs)]. Transcript levels were expressed in ΔCt values, which represent $Ct_{\text{target}} - Ct_{\text{reference}}$, the difference in the Ct of the target gene compared with reference genes (*actA*; *mipA*).

Results

flbB deletion results in a broad transcriptional down-regulation

To determine the appropriate time point to compare WT and $\Delta flbB$ transcriptomes, we performed Northern blot analyses

of the expression of selected UDA genes (*flbB*, *flbD*, *flbC*, and *fluG*) and CDP genes (*brlA*, *abaA*, and *wetA*) all during vegetative growth (18–24 hr in Figure 2) and asexual development (1–13 hr after induction). UDAs were most clearly expressed after 18–19 hr of vegetative growth (orange rectangles in Figure 2) and 4–5 hr after induction of conidiation (red rectangles). The UDA expression peak observed at early asexual development preceded the induction of CDP genes (blue rectangles in Figure 2). Taking these results into consideration, we performed two RNA-seq analyses separately (Figure 3A). The first one analyzed vegetative hyphae grown for 19 hr in supplemented liquid medium (the acronym VG will be used), whereas the second one compared the transcriptomes after filtering vegetative mycelia and culturing them for 5 hr in an aerial environment (early stages of asexual development; acronym AD). This meant that the AD stage compared a strain developed asexually for 5 hr (WT) with a second strain ($\Delta flbB$) that was incapable of initiating asexual development.

Two biological replicates were analyzed per strain (an *flbB* null and its isogenic WT strain) and condition (VG and AD). The density plot in Figure 3B shows a homogeneous distribution of gene density in the genome across all samples, whereas the dendrogram in Figure 3C shows paired VG and AD samples separately. The correlation values obtained ($r_{\text{VG}} = 0.90$ and $r_{\text{AD}} = 0.99$ for the $\Delta flbB$ mutant and $r_{\text{VG}} = 0.98$ and $r_{\text{AD}} = 0.92$ for the WT strain) (Figure S1) followed the general guidelines for biological duplicates (correlation must be equal to or higher than 0.90; <https://www.encodeproject.org/>) and thus validated our experimental approach.

Reads obtained for VG and AD conditions, corresponding to both WT and $\Delta flbB$ genetic backgrounds, mapped for 9564 and 9812 genes, respectively, representing 87.4 and 89.7% of a total of 10,943 genes predicted by the AspGD (File S1). Fragments per kilobase of exon per million fragments mapped (FPKM) values for all predicted *A. nidulans* genes in each strain and condition are shown in Table S3 (VG) and Table S4 (AD).

At VG, 1112 genes were up-regulated with FlbB activity (higher transcript levels in a WT strain than in a $\Delta flbB$ strain; $\log_2 FC > 0$), while only 56 were down-regulated ($\log_2 FC < 0$) (Figure 3A and red color in Figure 3D, Table S5). In contrast, AD samples showed 311 genes up-regulated and 75 down-regulated when FlbB was present (Figure 3, A and D, Table S6). Only 139 genes coincided in showing significant differences in expression in both conditions (listed in Figure S2;

arrows indicate the two transcriptional comparisons analyzed in this work. Dotted arrow indicates a previous transcriptional comparison described by Garzia *et al.* (2013). Purple indicates significantly deregulated genes, red is for FlbB up-regulated genes, and green is for FlbB down-regulated genes. (B) Density plot for the distribution of FPKM values for all genes across the four samples analyzed. (C) Dendrogram pairing VG and AD samples separately. (D) Volcano graphs representing transcriptional comparisons between $\Delta flbB$ and its isogenic WT strain at VG (left) and at AD (right), respectively. Genes (spots) with significant expression change are in red, and those with a nonsignificant expression change are in black. $\log_2 FC > 0$ values correspond to up-regulated genes, while $\log_2 FC < 0$ values correspond to down-regulated genes. (E) Heat-map illustration of a one-way hierarchical clustering analysis of differential expression based on a *t*-test and Benjamini-Hochberg correction and ranked by Z-score of the $\log_2$ RPKM, indicating genes that exhibited the most statistically significant changes in gene expression over the course of the experiment (205 genes in total). Several clusters of genes with similar expression profiles (EPs) were observed. Three of these EPs are indicated as F, G, and H and are presented in the corresponding panels, which show $\log_{10} (FPKM + 1)$ values in each strain and condition for genes from each EP.

Table 1 Up-regulated and down-regulated top 20 genes in VG and AD stages

Rank	Gene	Value of $\Delta flbB$ VG	Value of WT VG	Log ₂ FC	Description
Up-regulated					
1	AN2578	3.37	1307.97	8.60	Predicted peroxidase activity
2	AN8910	0.06	19.98	8.30	Putative polyketide synthase (PKS)
3	trxB	0.92	147.25	7.32	Putative thioredoxin reductase
4	AN2941	0.95	115.28	6.93	Unknown
5	AN7266	2.28	204.04	6.49	Unknown
6	AN0546	0.36	32.10	6.48	Unknown
7	AN11210	0.50	43.85	6.45	Unknown
8	AN10473	0.16	13.52	6.44	Unknown
9	AN10505	0.10	8.57	6.42	Putative unsaturated rhamnogalacturonan hydrolase
10	AN11990	0.76	63.25	6.37	Unknown
11	AN5664	0.47	35.93	6.26	Unknown
12	flbB	2.12	153.27	6.18	Transcription factor involved in regulation of conidiophore development
13	AN3557	0.98	62.70	6.00	Unknown
14	agnE	0.20	11.68	5.90	Putative α -1,3-glucanase
15	AN3228	0.12	6.93	5.87	Predicted prenyltransferase
16	AN11215	1.53	71.75	5.55	Predicted metal ion transmembrane transporter activity
17	AN2352	0.48	22.58	5.55	Unknown
18	AN8445	3.08	127.78	5.37	Putative aminopeptidase Y
19	AN11313	3.53	141.02	5.32	Unknown
20	AN4394	3.82	143.46	5.23	Putative positive regulation of asexual sporulation
Down-regulated					
1	AN2034	187.30	3.12	-5.91	Unknown
2	AN7898	18.23	0.31	-5.87	Predicted role in transmembrane transport
3	gelD	501.96	9.38	-5.74	Putative 1,3- β -transglycosidase
4	AN8308	530.99	12.35	-5.43	Unknown
5	AN2035	6.09	0.15	-5.33	Putative polyketide synthase
6	cipB	323.65	8.23	-5.30	Putative oxidoreductase
7	AN2032	10.68	0.40	-4.73	Putative polyketide synthase
8	AN2031	48.36	2.00	-4.60	α/β -Hydrolase
9	AN2033	66.93	3.66	-4.19	Predicted monooxygenase activity
10	AN2038	71.01	3.90	-4.19	Predicted oxidoreductase activity
11	AN2030	244.00	13.74	-4.15	Predicted oxidoreductase
12	AN2036	27.90	1.63	-4.09	Putative transcription factor; predicted role in secondary metabolism
13	AN7896	44.34	2.76	-4.01	Transcription factor with a role in secondary metabolism
14	AN2037	558.34	41.44	-3.75	Predicted NAD binding oxidoreductase
15	AN8099	70.91	6.03	-3.56	Putative oxalate decarboxylase
16	AN7796	116.56	13.19	-3.14	Predicted role in transmembrane transport
17	AN11702	268.14	30.67	-3.13	Unknown
18	AN7901	15.78	1.94	-3.02	Putative fungal transcription factor
19	furE	514.57	64.50	-3.00	Protein with homology to the <i>S. cerevisiae</i> uracil transporter Fur4p
20	AN6782	15.10	1.91	-2.99	Predicted sodium:dicarboxylate symporter activity
Rank	Gene	Value of $\Delta flbB$ AD	Value of WT AD	Log ₂ FC	Description
Up-regulated					
1	AN6401	1.97	929.35	8.89	Putative hydrophobin
2	agnE	0.12	32.10	8.08	Putative α -1,3-glucanase
3	AN1941	1.60	398.07	7.96	Predicted glycosylphosphatidylinositol (GPI)-anchored protein
4	AN3227	0.11	24.18	7.80	Predicted monooxygenase activity
5	AN5370	0.11	21.79	7.69	Predicted role in transmembrane transport
6	ivoB	1.07	214.46	7.64	Conidiophore-specific phenol oxidase
7	abnC	0.30	45.06	7.22	Protein with arabinan endo-1,5- α -L-arabinosidase activity
8	AN0587	0.10	12.16	6.92	Putative Hsp70 family chaperone
9	AN8890	0.14	16.95	6.90	Predicted carbohydrate binding and catalytic activity
10	AN2578	8.19	804.59	6.62	Predicted peroxidase activity

(continued)

Table 1, continued

Rank	Gene	Value of $\Delta flbB$ VG	Value of WT VG	Log ₂ FC	Description
11	<i>rodA</i>	0.95	93.00	6.62	Hydrophobin involved in conidium development
12	<i>easC</i>	0.46	45.18	6.61	Putative acyltransferase required for emericellamide biosynthesis
13	<i>AN8774</i>	0.11	9.16	6.41	Unknown
14	<i>brlA</i>	0.31	25.22	6.36	Transcription factor regulator of conidiophore development
15	<i>AN3247</i>	0.26	20.83	6.35	Predicted ATP binding, ATPase activity
16	<i>AN7385</i>	0.29	21.55	6.20	Putative phosphatidylserine decarboxylase
17	<i>AN3228</i>	0.86	56.93	6.05	Predicted prenyltransferase
18	<i>ivoC</i>	0.11	6.81	6.01	Putative cytochrome P450
19	<i>AN3339</i>	0.17	10.20	5.87	Predicted nucleotide binding, oxidoreductase activity, transferase activity
20	<i>AN3304</i>	0.11	6.22	5.85	Putative GABA transporter
Down-regulated					
1	<i>cipB</i>	6287.20	53.25	-6.88	Putative oxidoreductase
2	<i>AN7900</i>	69.54	1.98	-5.14	Predicted nucleotide binding and oxidoreductase activity
3	<i>AN7893</i>	284.06	10.12	-4.81	Predicted oxidoreductase activity
4	<i>AN0528</i>	381.75	14.08	-4.76	Predicted role in transmembrane transport
5	<i>AN7832</i>	208.22	8.63	-4.59	Predicted flavin adenine dinucleotide binding
6	<i>AN7898</i>	156.95	6.76	-4.54	Predicted role in transmembrane transport
7	<i>AN11584</i>	222.26	9.58	-4.54	Unknown
8	<i>AN7899</i>	103.42	4.99	-4.37	Esterase/lipase
9	<i>AN7894</i>	532.06	27.54	-4.27	Unknown
10	<i>AN7897</i>	71.03	3.79	-4.23	Predicted monooxygenase activity
11	<i>AN7903</i>	7.38	0.40	-4.20	Polyketide synthase
12	<i>AN8366</i>	1365.44	96.15	-3.83	Predicted role in transmembrane transport
13	<i>AN3567</i>	112.27	8.81	-3.67	Unknown
14	<i>AN8308</i>	3496.21	285.41	-3.61	Unknown
15	<i>AN2593</i>	4.51	0.40	-3.48	Predicted monooxygenase activity
16	<i>AN8593</i>	28.86	2.72	-3.40	Predicted hydrolase activity
17	<i>AN7333</i>	8.35	0.86	-3.28	Unknown
18	<i>AN7901</i>	18.75	2.01	-3.22	Putative fungal transcription factor
19	<i>AN7902</i>	38.96	4.22	-3.21	FAD-binding monooxygenase
20	<i>AN11702</i>	2563.91	283.34	-3.18	Unknown

see also File S1). Overall, these results suggest that FlbB activity influences the expression of differentiated groups of genes at each developmental stage and that the bZIP-type transcription factor potentially has a broader role as a transcriptional activator rather than as a suppressor.

Gene expression data point to a transcriptional activity of FlbB beyond conidiation

To interpret the significance of changes in gene expression occurring as a consequence of *flbB* deletion, we sorted significantly deregulated transcripts according to their expression profile (EP). Figure 3E shows a heat map corresponding to the expression levels of significantly deregulated genes included in Table S5 and Table S6. We focus here on three EPs. Figure 3F corresponds to EP1, comprising genes significantly up-regulated in a $\Delta flbB$ background in both stages but mainly in AD. EP1 includes some of the secondary metabolism genes that will be analyzed later. Classes EP2 and EP3 comprise transcripts strongly inhibited in the null *flbB* mutant (Figure 3, G and H). Those in Figure 3H belong to class EP2 and display in the WT higher expression levels in both VG and AD. Figure 3G shows class EP3, genes with low

or very low expression in VG that are induced at the beginning of conidiophore development. Thus it could be interpreted that genes of class EP3 encode protein activities required specifically during conidiation (see later).

We also analyzed the top 20 genes with the highest (up-regulated by FlbB; log₂ FC > 0) and lowest (down-regulated by FlbB; log₂ FC < 0) log₂ FC values at each developmental stage (Table 1). Predictions of a function for most of these genes were obtained from the AspGD and the *Aspergillus* Comparative Database. Those coding for proteins without predicted function were placed in a group designated as “Unknown” (Table 1). Among genes with a predicted/demonstrated function, we considered first those belonging to two clusters involved in the biosynthesis of secondary metabolites: the recently described *dba* cluster (*An7893–An7903*) (Gerke *et al.* 2012) (Figure 3F) and the *An2030–An2038* cluster (Ahuja *et al.* 2012). Finally, genes involved in development (*i.e.*, *brlA*, *ivoB*, *ivoC*, *rodA*, and *An4394*) (Figure 3, G and H) were placed in a third group. The existence of FlbB-regulated genes with low log₂ FC values but having an important role in the control of development and/or other cellular processes could not be discarded. Nevertheless, we continued focusing our work on the

Figure 4 Expression pattern of *A. nidulans* secondary metabolism gene clusters that are significantly regulated at the VG and early AD stages. The positions of PKSs or NRPSs in each cluster are indicated as well as the first and last gene names. Non-significantly regulated genes are in black, those down-regulated by FlbB activity are in green, and those up-regulated are in red. The extension of clusters defined by An2545/easA and An2547/easB or An3230/pkfA was delimited according to Chiang *et al.* (2008) and Khaldi *et al.* (2010). Results published by Gerke *et al.* (2012) and our *in silico* analyses were used as the reference for the *dba* cluster. The extension of the An2030–An2038 cluster was compared with that of Khaldi *et al.* (2010) and modified according to our *in silico* analyses (not shown).

analysis of the role of FlbB in the regulation of (1) secondary metabolite clusters and (2) developmental genes.

FlbB is required for the expression of four secondary metabolite clusters

The induction of asexual development requires the activity of specific secondary metabolites (SMs) (Ugalde and Rodriguez-Urra 2014). Genes and regulators of the biosynthesis of SMs are usually grouped in clusters within the genome [see annotation for clusters in different aspergilli in Inglis *et al.* (2013)]. Thus we analyzed our RNA-seq data to identify SM clusters with a significantly modified expression pattern in a null *flbB* background. Table S7 shows significantly regulated polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) genes (File S1). The information in Table S7 also indicates that there are four SM clusters with a significant expression change (Figure 4): (1) the emericellamide (EAS) cluster (An2545–An2549) (Chiang *et al.* 2008), which is significantly down-regulated in the null *flbB* mutant at both the VG and AD stages (Table S5 and Table S6 and Figure 3H and Figure 4); (2) the aspermidine A cluster, controlled by the PKS An3230/pkfA, in which a limited number of genes were significantly down-regulated in the absence of FlbB (Ahuja *et al.* 2012; Yaegashi *et al.* 2013); (3) the An2030–An2038 cluster, which was significantly down-regulated with a functional FlbB at VG but up-regulated at AD (Table S5 and Table S6 and Figure S3, Figure S4, and Figure S5); and (4) the recently characterized *dba* cluster (An7893–An7903) (Gerke *et al.* 2012), which is involved in the synthesis of the secondary metabolite 2,4-dihydroxy-3-methyl-6-(2-oxopropyl)benzaldehyde (DHMBA) and is strongly up-regulated in the absence of FlbB activity at both the VG and AD (see the top 20 FlbB down-regulated genes in Table 1; see also Figure 3F).

The expression behavior of the *dba* cluster was confirmed through qPCR analysis for the VG stage and the following cluster genes (Figure 5A): An7895/*cipB*, which is the top FlbB down-regulated gene in AD, and An7896/*dbaA* and An7901/*dbaG*, which code for the two transcriptional regulators that

control the expression of the remaining *dba* genes (Gerke *et al.* 2012). We included in the analysis total RNA samples from other UDA and CDP mutants, such as $\Delta fluG$ and $\Delta brlA$ null strains (Wieser *et al.* 1994; Adams *et al.* 1998). The significantly lower ΔCt values ($= Ct_{\text{target}} - Ct_{\text{reference}}$) compared with the WT strain confirmed the up-regulation of these three *dba* genes in all aconidial strains assayed ($P < 0.05$ in all cases).

Synteny and BLAST analyses showed that the *dba* cluster is not conserved in other aspergilli (Figure 5B and Figure S6). However, all genes from An7893 to An7903 matched the position and orientation of their orthologs in another gene cluster in *Talaromyces stipitatus* (Figure 5, B and C, and Figure S6), another soil fungus. We also found metabolic clusters in human pathogens such as *Coccidioides immitis* and *C. posadasii*, which contained orthologs of most of the *dba* genes. However, those genes displayed different relative position and orientation compared to the *dba* cluster (Figure 5B).

The metabolite linked to the activity of the *dba* cluster, DHMBA, has shown biological activity against the bacterium *M. luteus* (Gerke *et al.* 2012), an obligate aerobe found in soil, dust, water, and air and in a number of body tissues and secretions (Young *et al.* 2010). To verify the levels of activation and relevance of the *dba* cluster in the successful competition of *A. nidulans* with bacterial species, we cocultured $\Delta flbB$ strains (in which expression of *dba* cluster genes was induced as shown earlier) with *M. luteus* under experimental conditions suited to accurate quantitative analysis (bacteria/conidia = 25/1) (Figure 6A; see also *Materials and Methods*). After 48 hr of incubation, $\Delta flbB$ hyphae colonized the agar surface and formed aerial hyphae (black arrowhead in Figure 6A) in sharp contrast to those of the $\Delta flbB$; Δdba strains, which remained submerged in the co-cultured region. Bacterial load in the parental $\Delta flbB$ strain ($3.69 \times 10^8 \pm 1.23 \times 10^8$ cells/cm²) was 10-fold lower than in double-null ($\Delta flbB$; $\Delta dbaD$ and $\Delta flbB$; $\Delta dbaI$) mutants ($2.89 \times 10^9 \pm 0.51 \times 10^9$ and $2.75 \times 10^9 \pm 0.65 \times 10^9$ bacterial cells/cm², respectively; $P = 3.24 \times 10^{-7}$ and

Figure 5 Analysis of the *dba* cluster. (A) qPCR analysis of the expression of *An7895/cipB*, *An7896/dbaA*, and *An7901/dbaG* genes in the WT, $\Delta flbB$, $\Delta brlA$, and $\Delta fluG$ genetic backgrounds. ΔCt values ($= Ct_{gene} - Ct_{control}$) are given for VG samples of the selected strains as the mean values of three replicates together with the SEM. (B) Synteny analyses obtained from the *Aspergillus* Comparative Database (top) showed the absence of the *dba* cluster in other aspergilli such as *A. terreus*, *A. niger*, *A. oryzae*, *A. flavus*, and *A. fumigatus*. Dark gray squares designate regions conserved compared with the *A. nidulans* genome. BLAST analyses showed the total conservation of the *dba* cluster in the *T. stipitatus* genome and partial conservation in the *C. immitis* genome. (C) Phylogenetic tree (Mega software) linking *dba* cluster genes with *T. stipitatus* orthologs.

4.88×10^{-6} ; $n = 9$; significant change) (Figure 6B). A similar phenotype was observed when additional ($\Delta flbB$; Δdba) double-null mutants were tested with increasing bacterial concentrations (Figure S7).

Taken together, the results show that the loss of FlbB activity modified the expression pattern of specific SM clusters (File S1) and that up-regulation of the *dba* cluster resulted in the production of secondary metabolites with antibiotic activity.

Global analysis of FlbB activity in the regulation of genes involved in asexual development

Considering that *A. nidulans* conidiation genes can be grouped according to their participation in the upstream developmental activation (UDA) or as *brlA*-dependent (CDP) factors, we summarize the expression levels of these genes in Table 2. Within the UDA pathway, we identified three putative targets for FlbB, all of them up-regulated by the bZIP. The role of *An4394* will be analyzed separately later. We focus here, first, on the expression of the cMyb-type UDA transcription factor-coding gene *flbD*, which was, as expected, strongly inhibited in the null *flbB* background (Garzia *et al.* 2010) at both the VG and AD stages (Figure 3H). The second FlbB up-regulated UDA gene was *tmpA*, which encodes a transmembrane flavoenzyme and was postulated to act in a parallel pathway to that of FluG-FlbB (Soid-Raggi *et al.* 2006).

The expression of the first CDP gene, *brlA*, was completely inhibited in the absence of *flbB* at the AD stage (see class EP3 in Figure 3G), confirming the requirement of this transcription factor in the process of *brlA* activation on exposure to air. Another significantly regulated CDP gene is *rodA*, which codes for a hydrophobin (HPh) that is necessary for the generation of the rodlet layer that covers conidia (Stringer *et al.* 1991). The marked decrease in *rodA* expression observed in the AD comparison probably was a consequence of the inability to induce *brlA* in the null *flbB* mutant (Chang and Timberlake 1993). At this point, it is interesting to note that besides *rodA*, there were additional significantly deregulated genes that code for HPhs and CFEM hydrophobic proteins (Table S8 and File S1). All HPhs in *A. nidulans* have been shown recently to be involved in HPh rodlet formation on the surfaces of spores (Grünbacher *et al.* 2014). It is worth mentioning that *An6401/dewC* is the top FlbB up-regulated gene in AD, which suggests a BrlA-dependent role for it at this stage of asexual development.

Finally, the expression pattern observed in this work for *ivoB* and *ivoC*, which code for a phenol oxidase and a cytochrome P450 required for conidiophore pigmentation, respectively (Birse and Clutterbuck 1991), followed that described previously in the literature, where *ivoB* expression preceded that of *ivoC* (Birse and Clutterbuck 1991; Kelly *et al.* 2009; Garzia *et al.* 2013). The decrease in their transcription might reflect the altered *abaA* and *brlA* expression in the null *flbB* background (Birse and Clutterbuck 1991). Overall, these results support the previous model proposed by Garzia *et al.* (2010) that showed that FlbB bound the *brlA* promoter jointly with FlbD to induce the CDP pathway (see Discussion).

FlbB activity is required for the expression of *An4394*, coding for a putative helix-loop-helix (HLH)-type transcription factor that inhibits sexual development

The list of significantly regulated UDAs in Table 2 includes the gene *An4394*. According to the RNA-seq data, *An4394* displays an EP2 expression profile (Figure 3H), which means that its transcription is strongly inhibited in the $\Delta flbB$ background at

Figure 6 Cocultures of *A. nidulans* and *M. luteus*. (A) Phenotypic comparison between $\Delta flbB$ (line 1), $\Delta flbB \Delta dbaD$ (line 2), and $\Delta flbB \Delta dbal$ (line 3) strains after 48 hr of coculture with *M. luteus* in appropriately supplemented LB medium. Arrowheads indicate the cocultured region. The ratios between bacterial cell and fungal conidia concentrations are indicated. Control plates containing no bacterial cells or containing only the corresponding bacterial inoculum are shown as controls. Scale bar, 2 cm. (B) Average number of *M. luteus* cells after 48 hr of coculture with $\Delta flbB$ (blue), $\Delta flbB \Delta dbaD$ (red), and $\Delta flbB \Delta dbal$ (green) mutants. The graph illustrates the mean $\pm$ SEM of three biological and nine technical replicates.

both the VG and AD (Table 2). In addition, the AspGD (www.aspgd.org) indicated that its orthologs were involved in the positive regulation of asexual development (see also Table 1). Thus we decided to evaluate a putative regulation of *An4394* expression by factors from the UDA or CDP pathways

Table 2 FPKM (Fragments per kilobase of exon per million fragments mapped) values for genes involved in different aspects of conidiophore development

Locus	Type	Value of $\Delta flbB$ VG	Value of WT VG	$\log_2$ FC VG	Significant VG	Value of $\Delta flbB$ AD	Value of WT AD	$\log_2$ FC AD	Significant AD
UDA pathway									
<i>fadA/An0651</i>	Heterotrimeric G protein, subunit α	59.6	135.7	1.19	No	62.7	49.3	-0.35	No
<i>flbA/An5893</i>	Regulator of G-protein signaling	64.7	40.1	-0.69	No	32.7	29.9	-0.13	No
<i>flbB/An7542</i>	bZIP TF	2.1	153.3	6.18	Yes	3.0	107.6	5.18	Yes
<i>flbC/An2421</i>	C2H2 TF	69.8	223.9	1.68	No	365.4	213.5	-0.78	No
<i>flbD/An0279</i>	cMYB TF	4.0	103.1	4.70	Yes	6.6	61.3	3.22	Yes
<i>flbE/An0721</i>	Interactor of FlbB	80.2	101.6	0.34	No	37.1	42.5	0.20	No
<i>fluG/An4819</i>	Necessary for the synthesis of a inducer metabolite	16.2	36.3	1.17	No	22.0	25.4	0.21	No
<i>ganB/An1016</i>	Heterotrimeric G protein, subunit α	71.4	134.6	0.91	No	111.8	70.7	-0.66	No
<i>gpgA/An2742</i>	Heterotrimeric G protein, subunit γ	257.9	327.6	0.34	No	190.7	128.7	-0.57	No
<i>sfaD/An0081</i>	Heterotrimeric G protein, subunit β	148.2	118.6	-0.32	No	137.4	81.0	-0.76	No
<i>sfgA/An8129</i>	Gal4-type Zn(II)2Cys6 TF, negative regulator of conidiation	51.2	88.1	0.78	No	38.6	59.7	0.63	No
<i>tmpA/An0055</i>	Transmembrane flavoprotein	2.8	25.4	3.17	Yes	9.4	32.2	1.78	Yes
<i>urdA/An4394</i>	Positive regulation of asexual sporulation; regulation of transcription	3.8	143.5	5.23	Yes	2.6	66.1	4.68	Yes
CDP pathway									
<i>abaA/An0422</i>	TEA/ATTS TF	1.7	8.8	2.37	Yes	1.0	1.5	0.58	No
<i>brlA/An0973</i>	C2H2 TF	3.1	5.6	0.85	No	0.3	25.2	6.36	Yes
<i>dewA/An8006</i>	Hydrophobin	0.0	1.3	1.79769×10^{308}	No	0.3	1.1	1.96	No
<i>ivoA/An1057</i>	NRPS	0.4	1.1	1.64	No	1.0	0.8	-0.40	No
<i>ivoB/An0231</i>	Phenol oxidase	0.7	8.3	3.66	Yes	1.1	214.5	7.64	Yes
<i>ivoC/An10573</i>	Cytochrome P450	0.1	0.0	-1.79769×10^{308}	No	0.1	6.8	6.01	Yes
<i>medA/An6230</i>	Unknown	80.7	57.3	-0.49	No	52.2	36.2	-0.53	No
<i>rodA/An8803</i>	Hydrophobin	3.2	3.0	-0.09	No	0.9	93.0	6.62	Yes
<i>stuA/An5836</i>	APSES TF	143.7	190.5	0.41	No	122.3	157.8	0.37	No
<i>vosA/An1959</i>	Velvet-like TF	11.7	34.8	1.58	No	10.4	10.1	-0.04	No
<i>wA/An8209</i>	PKS	1.0	1.9	0.95	No	2.2	1.4	-0.68	No
<i>wetA/An1937</i>	Unknown	1.4	2.9	1.11	No	3.2	1.5	-1.10	No
<i>yA/An6635</i>	p-Diphenol oxidase	14.5	47.3	1.70	Yes	13.3	11.3	-0.24	No

This table has been updated from Etxebeste *et al.* (2010b). Genes have been classified according to their participation in the UDA or CDP pathways. Expression levels in each background and developmental stage, $\log_2$ FC values for VG and AD comparisons, and the significance of the expression differential are included. Bold signifies genes significantly up-regulated by FlbB activity

(Figure 7). Northern blots showed an increase in *An4394* expression after the induction of conidiation, followed by its inhibition after 24 and 48 hr (Figure 7A, top). In contrast to our RNA-seq results, the *An4394* transcript was not detected in the VG (0 hr) in these experiments. This observation is in agreement with results described by Sibthorp *et al.* (2013) that reported low reads per kilobase per million mapped reads (RPKM) levels for *An4394* in either minimal or complete *Aspergillus* culture medium. Furthermore, we were unable to

detect the *An4394::GFP* chimera in vegetative hyphae either by fluorescence microscopy or by Western blot. These results strongly support the view that *An4394* expression is low at the VG.

Northern blot experiments also confirmed the inhibition of *An4394* expression in a null *flbB* background (Figure 7A). The same effect was observed in null mutants of UDA genes such as *flbE*, *flbD*, and *gmcA* (Etxebeste *et al.* 2012). Interestingly, the *An4394* transcript was overexpressed in the

$\Delta brlA$ background (Figure 7A, bottom). This suggested that while the UDA pathway induced *An4394* expression, the CDP pathway inhibited it. The maintenance of the *flbB* expression peak in a $\Delta An4394$ strain compared with the parental WT strain (Figure 7B) confirmed that *An4394* is located downstream of known *flb* genes in the UDA pathway. Furthermore, we could confirm that the expression of two secondary metabolism genes from the *dba* cluster (*An7895/cipB* and *An7898/dbaD*; see earlier) was strongly up-regulated not only in the $\Delta flbB$ background but also in $\Delta flbD$ and $\Delta An4394$ strains. These results and those in previous sections of this paper strongly suggest that the up-regulation of the *dba* cluster initially described for the null *flbB* mutant is not specific to this strain.

To further understand the role of *An4394* in *A. nidulans* development, we characterized the phenotype of its deletion mutant. Colonies of the $\Delta An4394$ strain showed a marked inhibition of asexual development on MMA (Figure 8A; 48 and 72 hr). Conidia production in the $\Delta An4394$ strain ($3.6 \times 10^5 \pm 2.2 \times 10^5$ conidia/cm²) was 27 times lower than that in the parental WT strain ($9.6 \times 10^6 \pm 2.2 \times 10^6$ conidia/cm²) (Figure 8B, top). Premature onset of the sexual process also was observed, with profuse production of Hülle cells after only 4 days of culture at 37° (Figure 8A; 96 hr and the magnifications following). After 7 days, the null *An4394* mutant produced mature cleistothecia containing viable ascospores (2515 ± 157 cleistothecia/cm²) (Figure 8B, bottom) that germinated and developed mycelia normally (see the inset in Figure 8C corresponding to 48 hr of culture of ascospores in MMA at room temperature). The parental WT strain had initiated the development of cleistothecia after 7 days of culture (478 ± 67 cleistothecia/cm²) (Figure 8B, bottom), but these sexual structures were immature and contained no ascospores (not shown). The reversion of the $\Delta An4394$ phenotype in a revertant strain was accompanied by a recovery of WT expression levels of *An4394* and *An7895/cipB* strains (Figure S8; see also *Materials and Methods*). This confirmed that the phenotype of the $\Delta An4394$ strain was the consequence of the deletion of this gene. Based on the results shown, *An4394* was named *UrdA* because of its UDA-dependent role in the regulation of development in *A. nidulans* (see *Discussion*).

The *urdA* transcript sequence obtained from our RNA-seq approach matched the one predicted by the AspGD. Synteny analyses using this database showed that *urdA* orthologs are conserved at equivalent regions within the genomes of multiple aspergilli (not shown). The *A. oryzae* ortholog codes for the HLH-type transcription factor EcdR (early conidiophore development regulator) (Jin *et al.* 2011a). Sequence alignments for *UrdA* and EcdR, on the one hand [43.1% overall identity according to Jin *et al.* (2011a)] (Figure 8D), or *UrdA* and orthologs from closely related fungal species, on the other hand (Figure S9), demonstrate that the homology is not confined to the putative bHLH domain (black arrows in Figure 8D) [the extension of the EcdR bHLH domain, residues 150–211, was delimited by Jin *et al.* (2011a)].

Figure 7 Regulation of *urdA/An4394*. (A) Northern blot experiments showing the expression of *An4394*, coding for the *A. nidulans* ortholog of the HLH-type transcription factor EcdR from *A. oryzae*, in WT, $\Delta flbB$, $\Delta flbE$ (top), $\Delta flbD$, $\Delta gmcA$, and $\Delta brlA$ (bottom) genetic backgrounds. (B) Northern blot experiments showing the expression of *flbB*, *An7895/cipB*, and *An7898/dbaD* in WT, $\Delta flbB$, $\Delta flbD$, and $\Delta An4394$ strains at different time points of asexual development. rRNAs are shown as loading control in both panels. The graphs show the ratios between the average pixel intensity of each Northern blot band and the corresponding rRNA band. Graphs were obtained with Microsoft Excel, and the average pixel intensity for each band was calculated with Image Gauge (version 4.0; FujiFilm, Valhalla, NY).

Figure 8 Functional characterization of UrdA. (A) Phenotype of the $\Delta An4394$ mutant in MMA after 48, 72, and 96 hr of culture at 37°. Scale bar, 3 cm. Magnifications show the high number of Hülle cells produced by the null $An4394$ (black arrowhead, right) compared with the WT strain (left) after 96 hr of culture. Scale bar, 0.2 cm. (B) Conidia and cleistothecia production by the parental strains (WT and $\Delta flbB$) and $An4394$ null mutants (in both parental genetic backgrounds) in MMA plates after 72 and 156 hr of culture at 37°, respectively. Values are the mean of three replicates plus SEM. (C) (1) Phenotype of the $An4394$ null mutant after 7 days of culture in MMA. (2) Cleistothecia were isolated, (3) burst, and ascospores were cultivated in MMA for additional 48 hr at room temperature. (4) Phenotype of germinated $\Delta An4394$ colonies is shown. Scale bars, (1) 0.2 cm; (2) 100 μm ; (3) 0.3 mm; (4) 3 mm. (D) Sequence alignment of UrdA and EcdR, which was obtained using Genedoc software (version 2.7.000). The extension of the bHLH domain of EcdR is designated by black arrows and was delimited according to Jin *et al.* (2011a). (E) Northern blot experiments showing the expression of *urdA* during morphogenesis (12, 18, and 24 hr of VG) as well as asexual (6, 12, 24, and 48 hr of AD) and sexual (24, 48, 72, and 96 hr of SD) developmental cycles (left) or the expression of *brlA* and *nsdD* in WT, $\Delta flbB$, $\Delta flbD$, and $\Delta An4394/urdA$ strains at different time points of asexual development (right). rRNAs are shown as loading control in both panels. The graphs show the ratios between the average pixel intensity of each Northern blot band and the corresponding rRNA band.

Lee *et al.* (2014) have recently proposed that Flbs induce conidiation by simultaneously suppressing the *brlA* down-regulation caused by NsdD, a positive regulator of cleistothecia production (Han *et al.* 2001). To analyze whether the genetic relationship between Flbs and NsdD could be established through UrdA, we performed new Northern blot experiments. On the one hand, we could not detect the *urdA* transcript during the sexual development (SD in Figure 8E, left), suggesting that UrdA plays no role in sexual differentiation. On the other hand, we observed no remarkable differences in *nsdD*

expression levels in a $\Delta urdA$ background (Figure 8E, right) (a *brlA* probe was used as control), suggesting that the premature triggering of sexual development described for the null *urdA* mutant was not the consequence of an up-regulation of *nsdD*. These results strongly suggest that *nsdD* expression is not subject to UrdA control.

Overall, UrdA codes for a putative transcription factor located downstream of Flbs in the induction pathway and required not only for the correct development of conidiophores but also for the inhibition of sexual development (see later).

Discussion

According to the currently accepted model of the transcriptional activity of FlbB, it first induces the expression of *flbD*, and subsequently, both factors jointly activate *brlA* (Garzia *et al.* 2010). Results obtained in this work reinforce this model (Figure 9). On the one hand, RNA-seq confirms that the absence of FlbB results in a block of *brlA* expression after exposure of the hyphae to air (Wieser *et al.* 1994). On the other hand, *flbD* remains as the only known *flb* gene showing a transcriptional dependence on FlbB activity. Nevertheless, this work demonstrates that FlbB reaches beyond the induction of *brlA* expression and has a key role in modulating the timing of mutually exclusive morphogenetic programs such as asexual and sexual reproductive cycles (Figure 9). This function is exerted through UrdA because the absence of this hypothetical transcriptional regulator prematurely induces the sexual cycle and inhibits conidia production. Thus we propose that UrdA connects the UDA pathway with the control of sexual development.

A recent report provided experimental evidence of a functional link between the UDA pathway and the positive regulator of sexual development NsdD (Lee *et al.* 2014). Because *nsdD* deletion bypassed the need of UDAs (but not CDPs) for conidia production, the authors proposed that NsdD also down-regulated *brlA* expression to inhibit the asexual program. Our results show that UrdA does not control *nsdD* expression, suggesting that sexual and asexual developmental pathways are coordinated at different levels (Figure 9). Jin *et al.* (2011a) indicated that the *A. oryzae* ortholog of UrdA, EcdR, is required for early asexual development and proposed that it was repressed by its interaction with SclR, an additional HLH-type transcription factor that promotes sclerotial formation (Jin *et al.* 2011b). However, expression of the putative ortholog of *sclR*, *An7170*, is not significantly altered when *flbB* is deleted. This suggests that the UDA pathway inhibits sexual development through the activation of *urdA* but not the inhibition of *An7170*. Although identification of the genetic target(s) of UrdA activity remains elusive, the existence of proteins linking the UDA pathway with sexual reproduction fits with the idea that specific proteins and endogenous metabolites coordinate the morphogenetic programs to shape the fungal colony and suit the environmental conditions (Rodríguez-Romero *et al.* 2010; Park and Yu 2012; Ugalde and Rodríguez-Urra 2014).

It is known that development is tightly linked with the production of SMs. Activation of a particular developmental pathway entails the induction of specific SM clusters. In this work, we show experimental evidence of an up-regulation of the *dba* cluster resulting from deletion of UDA and CDP genes such as *flbB*, *flbD*, *fluG*, *urdA*, and *brlA* (Figure 9). An induction of the *dba* cluster also has been reported, on the one hand, when the signalosome, a multimeric complex involved in protein degradation, lacks the subunit CsnE (Gerke *et al.* 2012) and, on the other hand, on physical contact with the bacterium *Streptomyces hygroscopicus* (Schroeckh *et al.* 2009). In these two studies, the authors

Figure 9 Known roles of FlbB in the genetic control of asexual and sexual development as well as secondary metabolite production.

described a simultaneous up-regulation of *dba* genes and genes from the adjacent orsellinic acid cluster. Consequently, it was proposed that the regulation of both clusters might be linked and that they might cooperate in the production of SMs (Nielsen *et al.* 2011; Gerke *et al.* 2012). However, our RNA-seq data show that the deletion of *flbB* up-regulates the *dba* cluster but not the orsellinic acid cluster, suggesting an independent regulation for both gene clusters. Although the biological meaning of this observation remains unknown, recent studies on the role of SM clusters suggest that different metabolites can be generated by the same PKS (Zaehle *et al.* 2014). This has been defined as the promiscuity of PKSs and may contribute to the increase in SM diversity (Fischer 2014).

Conservation of the *dba* cluster between *A. nidulans* and *T. stipitatus* seems to be the consequence of a horizontal gene-transfer (HGT) process. Although we could not identify transposable elements, this hypothesis is supported by the absence of an equivalent cluster in other aspergilli and its location near a telomere on chromosome II (Milani *et al.* 2012). This hypothetical HGT process could have implications on the capability of these two fungal species to survive in an ecological niche in competition with other microorganisms. In fact, the metabolite DHMBA, produced by the *dba* cluster, inhibits growth of the bacterium *M. luteus* (Gerke *et al.* 2012). Therefore, it is reasonable to suggest that induction of the *dba* cluster could be a response to biotic stress conditions. In this context, the absence of an active signalosome (Gerke *et al.* 2012) could simulate specific effects caused by the competition with other microorganisms and thus activate the production of defense metabolites such as DHMBA. Similarly, the block in asexual development caused by the absence of UDA activity (Etxebeste *et al.* 2010a) could be construed by the fungus as a signal for the synthesis of a chemical compound with biological activity.

Overall, this work analyzed two remarkable examples on how developmental and metabolic processes are unbalanced when UDA activities are absent. However, the large number of purportedly deregulated genes identified in our RNA-seq comparisons points to the existence of multiple cellular processes influenced by FlbB activity. GO functional enrichment analyses (not shown) indicate that oxidation-reduction,

transmembrane transport, and metabolic processes, as well as the regulation of transcription and translation, are importantly affected. It can be inferred, therefore, that the direct or indirect influence of UDAs on all these processes falls within the wide scope of processes involved in the cellular transition from vegetative hypha to formation of the conidiophore. This opens a new holistic outlook to the study of regulatory networks during development.

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GENETICS

Supporting Information

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Beyond Asexual Development: Modifications in the Gene Expression Profile Caused by the Absence of the *Aspergillus nidulans* Transcription Factor FlbB

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Extended summary of the data set.

To identify transcripts up- or downregulated as a consequence of the loss of FlbB activity, we sequenced total RNA samples and compared the transcriptomes of a null *flbB* and an isogenic wild type strain during VG or AD (Figure 3). We obtained an average of 8,856,181 reads of 72bp per sample (70,849,449 reads for all 8 samples; each sample was duplicated), representing nearly 20.9 *A. nidulans* genome lengths per sample (~167 genome lengths with all 8 samples). The two biological replicates per strain and condition showed a correlation level equal to or higher than 0.9 ($r_{VG} = 0.98$ and $r_{AD} = 0.92$ for the isogenic wild type and $r_{VG} = 0.90$ and $r_{AD} = 0.99$ for the null *flbB* strain; Figure S1).

We observed that almost the whole set of genes encoded by the *A. nidulans* genome was expressed in all conditions and backgrounds tested. Out of 10,943 transcripts predicted by the Aspergillus Genome Database, 9,763 (89.2%) were expressed in a wild-type strain during VG and 10,059 (91.9%) during the early AD. For the $\Delta flbB$ strain, 9,866 (90.1%) transcripts were expressed in VG samples and 9,988 (91.3%) in early AD samples (Tables S3 and S4).

During VG, 10,065 (92%) genes were expressed in one or both strains, and 878 genes were not expressed in any strain. 199 were uniquely expressed in a wild type strain and 302 genes in a null mutant of *flbB*. Of a total of 9,564 genes expressed in both genetic backgrounds, 1,168 genes showed a significant differential expression. 1,112 genes showed higher transcript levels in a wild type strain than in a $\Delta flbB$ ($\log 2FC > 0$), and 56 showed lower transcript levels ($\log 2FC < 0$; Figure 3 and Table S5). In the AD samples, 10,255 (93.7%) genes were expressed in one or both strains, and 688 transcripts were not detected in any strain. Of the remaining 9,812 genes that were expressed in both genetic backgrounds, 386 showed a significant differential expression, being 311 activated and 75 repressed by FlbB (Figure 3 and Table S6). These data indicated that the number of upregulated genes in the presence of FlbB in VG is 3.6 times higher than those upregulated at early AD conditions. However, the rate of downregulated genes in VG versus AD is 0.75. This suggests that FlbB activity has a broader impact in the activation of subordinated cellular processes during VG while a putative repressor role for FlbB seems to be more important at AD conditions.

The analysis of the distribution of significantly regulated genes along *A. nidulans* chromosomes (not shown) confirmed that only chromosome III, and only in VG conditions, showed a significant decrease in downregulated genes, with a ratio (up- vs downregulated genes) of 76 in comparison to an average of 27.8.

Of a total of 1,168 (VG) and 386 (AD) significantly regulated genes, 139 were common to both analyses (Figure S2). 110 of these genes are upregulated in the presence of FlbB at both VG and AD, and 12 are downregulated. We also found genes with alternating expression profiles, 11 being downregulated at VG but upregulated at AD and 6 with the opposite behavior.

PKSs and NRPSs significantly regulated by FlbB.

Table S7 shows PKS- or NRPS-coding genes significantly up- or downregulated by FlbB. Six of them were upregulated by FlbB at both stages (Table S7, columns 7 and 8): *An2545/easA* and *An2547/easB*, coding for a NRPS and a PKS, respectively, from the emericellamide cluster (Chiang *et al.* 2008), *An2924* (NRPS), *An3230/pkfA*, coding for a PKS involved in the synthesis of Aspernidine A (Ahuja *et al.* 2012; Yaegashi *et al.* 2013), *An8910* (PKS) and *An10297* (NRPS). The PKS coding gene *An7903/pkeA/dbal*, from a cluster involved in the synthesis of violaceol (Nielsen *et al.* 2011) and the benzaldehyde derivative DHMBA (Ahuja *et al.* 2012; Gerke *et al.* 2012), was downregulated at AD. The PKS coding gene *An2032/pkhA* was downregulated by FlbB at VG but upregulated at AD, while *An2035/pkhB* (Ahuja *et al.* 2012), a second PKS-coding gene from the same cluster, was significantly downregulated by FlbB only at VG. Finally, *An6791* and *An9005*, coding for two PKSs, are upregulated by FlbB only at VG while *An2064* (NRPS) is upregulated only at AD.

According to Table S7, there are four SM clusters with a significant expression change in our RNA-seq analyses (Figure 4). Three of them will be analyzed in this section while the link between the UDA pathway and the transcriptional regulation of the *dba* cluster is deeply described in the main text.

Expression levels of the emericellamide (EAS) cluster (*An2545-An2549*; (Chiang *et al.* 2008)) fell to zero in the null *flbB* mutant at both VG and AD stages (see Tables S5 and S6; see also Figures 3H and 4). EASs are cyclic depsipeptides (peptides in which one or more of the amide bonds have been replaced by ester bonds) produced in the sexual cycle (Chiang *et al.* 2008; Oh *et al.* 2007). However, there is no information on their role in asexual development.

Aspernidine A is a prenylated isoindolinone alkaloid synthesized by the *An3230/pkfA* cluster in the null mutant of the MAPK-coding gene *mpkA* (Ahuja *et al.* 2012; Yaegashi *et al.* 2013), which shows severe defects in morphogenesis (De Souza *et al.* 2013). In the present analysis, the expression of specific genes within the Aspernidine A cluster was significantly downregulated as a consequence of *flbB* deletion (Tables S5 and S6; Figure 4).

Synteny analyses showed that the *An2030-An2038* cluster is not conserved in other *Aspergilli*, such as *A. fumigatus*, *A. terreus*, *A. oryzae*, *A. flavus* or *A. niger* (Figure S3 and S4). Additional bioinformatic analyses indicated a nearly perfect match with a cluster from the endophytic insect-parasitic fungus *Metharizium robertsii* (Figures S3 and S4). The *An2030-An2038* cluster is significantly downregulated with a functional FlbB at VG but upregulated at AD (Figure 4; Tables S5 and S6). This means that the expression pattern of this cluster in the null *flbB* background during the VG to AD transition is opposite to that shown by the isogenic wild-type strain (Garzia *et al.* 2013). Hence, FlbB might participate in the repression of this cluster in vegetative hyphae but induce it upon induction of conidiation. In an attempt to link the activity of this cluster with the induction of conidiation under different stress conditions, we generated a strain in which the entire cluster was deleted. Compared to the parental wild-type, we observed non-significant changes in conidia production in MMA, under osmotic or saline stress conditions, as well as in MMA

with cycloheximide, an inhibitor of mRNA translation produced by *Streptomyces griseus* (see the phenotypes in Figure S5). Taken these data into consideration, we cannot link the activity of the *An2030-An2038* cluster directly with the induction of conidiophore biogenesis or the response to the stress conditions analyzed in this work.

Significantly de-regulated genes coding for hydrophobic proteins.

Besides *rodA*, there were additional significantly de-regulated genes that code for hydrophobic proteins. HPhs and CFEM proteins contain 8-cysteine domains that, through disulfide bridge formation, create amphipathic monolayers on the surface of fungal cells, increasing hydrophobicity (de Groot *et al.* 2009; Kulkarni *et al.* 2003). The genome of *A. nidulans* contains six HPh-coding genes (de Groot *et al.* 2009) and three of them (*rodA*, *An6401* and *An7539*) are significantly regulated by FlbB (Table S8). From the two CFEM protein-coding genes, expression of *An5357* seems to be FlbB-dependent (Table S8). Transcription of *An6401* depends exclusively on FlbB activity at both stages but this hydrophobin activity seems to be mainly required at AD (Table S8), suggesting a role in controlling the hydrophobicity of conidiophore cell types. We observed a similar expression pattern of *An7539* and *An5357*, being significantly up-regulated with a functional FlbB at VG but not at AD (Table S8). Overall, these results indicate that FlbB influences, both at VG and AD, the expression of genes coding for HPh and CFEM proteins, which might be essential to guarantee a correct contact and communication between the fungus and its environment (Wessels 1997).

Figure S1 Individual pairwise comparisons of the biological replicates. Upper graphs represent the wild-type samples both in VG (left) and AD (right) conditions. Lower graphs are for $\Delta flbB$ samples. X and Y axes represent $\log_{10}(\text{FPKM} + 1)$.

Up in VG and AD

Gene	Chromosome	Coordinate	Gene	Chromosome	Coordinate
AN6413	Chromosome I	263852	AN9165	Chromosome VI	231317
AN6401	Chromosome I	298404	AN3230	Chromosome VI	1392710
AN6373	Chromosome I	381489	AN3228	Chromosome VI	1402956
AN5939	Chromosome I	1737725	AN3226	Chromosome VI	1407003
AN6774	Chromosome I	3120967	CYP631B1	Chromosome VI	1408873
AN6788	Chromosome I	3170948	AN3206	Chromosome VI	1480835
AN6812	Chromosome I	3260708	AN2955	Chromosome VI	2262553
AN6960	Chromosome I	3715298	AN2954	Chromosome VI	2273441
AN6963	Chromosome I	3730062	AN2941	Chromosome VI	2325927
pepJ	Chromosome II	333193	AN2924	Chromosome VI	2380864
AN8046	Chromosome II	595244	AN2912	Chromosome VI	2417460
AN8155	Chromosome II	937766	alcS	Chromosome VII	227576
trxB	Chromosome II	1181567	alcA	Chromosome VII	230912
AN4180	Chromosome II	1850398	AN8969	Chromosome VII	263224
AN4148	Chromosome II	1944776	AN11153	Chromosome VII	400280
AN3996	Chromosome II	2391335	AN8910	Chromosome VII	481319
AN10505	Chromosome II	2469691	AN1588	Chromosome VII	1265062
AN3781	Chromosome II	3073518	agnE	Chromosome VII	1324276
AN3526	Chromosome II	3886421	AN1621	Chromosome VII	1379569
ppoC	Chromosome III	237310	AN1734	Chromosome VII	1745931
AN5027	Chromosome III	244930	AN1920	Chromosome VII	2262137
AN4599	Chromosome III	1559647	AN1941	Chromosome VII	2332402
AN4394	Chromosome III	2165982	AN2025	Chromosome VII	2615524
AN8774	Chromosome III	2849632	AN2043	Chromosome VII	2682641
AN11120	Chromosome III	2864047	AN2044	Chromosome VII	2686178
AN8661	Chromosome III	3221381	AN11864	Chromosome VII	2811586
AN8628	Chromosome III	3326243	AN2228	Chromosome VII	3264627
AN7385	Chromosome IV	119375	AN2262	Chromosome VII	3365098
AN7269	Chromosome IV	482367	AN2263	Chromosome VII	3367219
AN7267	Chromosome IV	485028	AN2352	Chromosome VII	3656318
AN7266	Chromosome IV	486819	AN2387	Chromosome VII	3762207
AN7265	Chromosome IV	488659	AN2398	Chromosome VII	3799483
AN7264	Chromosome IV	489843	AN10297	Chromosome VII	3813028
AN7222	Chromosome IV	630008	AN2407	Chromosome VII	3825982
AN7181	Chromosome IV	757009	easA	Chromosome VII	4288452
flbB	Chromosome IV	1889783	easB	Chromosome VII	4313089
AN7594	Chromosome IV	2079203	easC	Chromosome VII	4321940
AN12088	Chromosome IV	2710082	easD	Chromosome VII	4323905
pgaC	Chromosome V	142833	AN2578	Chromosome VII	4414498
AN8367	Chromosome V	254351	AN9300	Chromosome VIII	220918
AN8368	Chromosome V	255598	AN11210	Chromosome VIII	224076
AN8369	Chromosome V	258021	AN9380	Chromosome VIII	460350
AN8439	Chromosome V	485591	AN1338	Chromosome VIII	791684
AN11079	Chromosome V	488126	AN1322	Chromosome VIII	832620
AN8445	Chromosome V	505695	AN1243	Chromosome VIII	1070769
AN8477	Chromosome V	607701	AN0903	Chromosome VIII	2117853
AN5245	Chromosome V	1616225	AN0546	Chromosome VIII	3223452
AN5664	Chromosome V	2042315	AN0500	Chromosome VIII	3359678
AN5550	Chromosome V	2441648	AN0457	Chromosome VIII	3495941
AN5487	Chromosome V	2648417	flbD	Chromosome VIII	4060949
AN5444	Chromosome V	2799910	ivoB	Chromosome VIII	4208565
AN5437	Chromosome V	2814273	AN0230	Chromosome VIII	4210291
AN5421	Chromosome V	2865556	tmpA	Chromosome VIII	4736178
AN5330	Chromosome V	3129243	AN0054	Chromosome VIII	4739984
AN5329	Chromosome V	3130527	AN0053	Chromosome VIII	4744983

VG and AD

Down in VG and AD

Down in VG/Up in AD

Gene	Chromosome	Coordinate	Gene	Chromosome	Coordinate
AN7893	Chromosome II	163722	AN8099	Chromosome II	752073
cipB	Chromosome II	167354	AN8608	Chromosome III	3381106
AN7896	Chromosome II	171086	gelD	Chromosome VII	348173
AN7898	Chromosome II	175939	AN2030	Chromosome VII	2638096
AN7899	Chromosome II	178094	AN2031	Chromosome VII	2639262
AN7901	Chromosome II	182132	AN2032	Chromosome VII	2640607
AN7832	Chromosome IV	2807782	AN2033	Chromosome VII	2647940
AN8308	Chromosome V	69779	AN2034	Chromosome VII	2649537
AN8366	Chromosome V	243479	AN2037	Chromosome VII	2663256
AN9141	Chromosome VI	322063	AN2038	Chromosome VII	2667020
AN11702	Chromosome VI	1977238	AN0493	Chromosome VIII	3382781
AN8894	Chromosome VII	528278			

Up in VG/Down in AD

Gene	Chromosome	Coordinate
AN6820	Chromosome I	3277884
AN3681	Chromosome II	3392941
AN5033	Chromosome III	223936
AN7524	Chromosome IV	1831427
AN1540	Chromosome VII	1104009
AN2207	Chromosome VII	3205096

Figure S2 Chromosomal distribution of significantly regulated genes common to both *ΔflbB* versus wild-type comparisons analyzed in this work. Tables show all significantly regulated genes common to both comparisons. In red and green are, respectively, genes up and downregulated by FlbB at both stages. In blue are genes downregulated by FlbB in VG while upregulated at early AD. Finally, in pink are represented genes that are upregulated by FlbB at VG but are downregulated at AD.

Figure S3 Conservation of the *An2030/An2038* metabolic cluster in fungal genomes. A) Synteny analyses obtained from the Aspergillus Comparative Database (Up) showed the absence of this cluster in other *Aspergilli* as *A. terreus*, *A. niger*, *A. oryzae*, *A. flavus* and *A. fumigatus*. Dark grey squares designate regions conserved comparing to the *A. nidulans* genome (for simplicity, we omit the degree of conservation). Blast analyses at the NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) showed the conservation of the *An2030-An2038* cluster in the *M. robertsii* genome (Down). B) Phylogenetic tree (obtained with Mega software) linking *An2030/2038* genes with *M. robertsii* orthologs.

Figure S4 Phylogenetic trees for each gene in the **An2030-An2038** cluster. The trees were obtained using Genedoc software (version 2.7.000). *A. nidulans* (ANID) and *M. robertsii* (MAA) orthologs are marked with dotted squares.

Figure S5 Phenotype of the $\Delta An2030-An2038$ strain (right) comparing to the parental wildtype (left) in MMA (using 2 % or 1 % glucose, Glc, as the main carbon source, respectively), MMA with 0.5 M $H_2PO_4^-$, 1 M sucrose, 1.2 M sorbitol, 0.8 M NaCl or 20 $\mu g/ml$ cycloheximide. Scale bar = 2 cm.

Figure S6 Phylogenetic trees for each gene within the *dba* cluster. The trees were obtained using Genedoc software (version 2.7.000). *A. nidulans* (ANID) and *T. stipitatus* (MAA) orthologs are marked with dotted squares.

Figure S7 Co-cultures of *Aspergillus nidulans* and *Micrococcus luteus*. Phenotype of $\Delta flbB$ and $\Delta flbB;\Delta cipB$, $\Delta flbB;\Delta dbaA$, $\Delta flbB;\Delta dbaD$, $\Delta flbB;\Delta dbaG$ or $\Delta flbB;\Delta dbaI$ strains after 48 hours of co-culture with increasing concentrations of *M. luteus* in appropriately supplemented LB medium. The ratios between bacterial cell and conidia concentrations are indicated (bacterial cell / fungal conidia). Arrowheads indicate the co-cultured regions. Scale bar = 2 cm. This Figure is summarized in Figure 6A.

Figure S8 Revertant strain for the null *urda* mutant. A) A diagram representing the recombination process induced for the generation of the revertant strain for the null *urda* mutant. Restriction sites as well as the probe used for the Southern-blot confirmation of the homologous recombination are indicated. B) Phenotype of transformants on regeneration medium containing FOA (left) or MMA without FOA (right). C) Southern-blot experiment for the wild-type, the null *urda* mutant and four revertant transformants. A BamHI restriction enzyme was used and the homologous recombination of the genomic cassette shown in panel A was confirmed after the detection of a 5.0 Kb band. D) Recovery of *urda* and *An7895/cipB* expression levels in one of the revertant strains. rRNAs are shown as loading control. The graph shows the ratios between the average pixel intensity of each Northern-blot band and the corresponding rRNA band.

Figure S9 Sequence alignment of UraA orthologs. The figure was obtained using Genedoc software (version 2.7.000). The extension of the bHLH domain of EcdR is designated by a black arrow and was delimited according to (Jin *et al.* 2011a). Nomenclature: Aver: *Aspergillus versicolor*; Asyd: *A. sydowii*; Afla: *A. flavus*; Ater: *A. terreus*; Anig: *A. niger*; Aaci: *A. acidus*; Aacu: *A. aculeatus*; Acar: *A. carbonarius*; Abra: *A. brasiliensis*; Acla: *A. clavatus*; Nfis: *Neosartorya fischeri*; Afum: *A. fumigatus*; Agla: *A. glaucus*; Atub: *A. tubigenensis*; Awen: *A. wentii*; Azon: *A. zonatus*.

Tables S1-S8

Available for download as PDF files at <http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.174342/-/DC1>

Table S1: Oligonucleotides used in this work.

Table S2: Strains used in this work.

Table S3: Transcriptomes of both *flbB* null and isogenic wild-type strains in VG conditions. Expression levels in each background are compared by the log2FC value. The last column shows the significance in the expression change for each gene (no: q values higher than 0.05; yes: q values lower than 0.05).

Table S4: Transcriptomes of both *flbB* null and isogenic wild-type strains in AD conditions. Expression levels in each background are compared by the log2FC value. The last column shows the significance in the expression change for each gene (no: q values higher than 0.05; yes: q values lower than 0.05).

Table S5: List of significantly de-regulated genes when comparing *flbB* null and wild-type transcriptomes in VG conditions.

Table S6: List of significantly de-regulated genes when comparing *flbB* null and wild-type transcriptomes in AD conditions.

Table S7: Summary of PKS and NRPS genes significantly regulated by FlbB at VG and/or AD stages. Log2FC values >0 (in red) correspond to PKS or NRPSs upregulated by FlbB while values <0 (in green) correspond to downregulated genes. The table also shows significantly regulated additional genes within each cluster.

Table S8: FPKM values for genes coding for hydrophobins or CFEM proteins significantly regulated by FlbB. The table shows the function as well as the FPKM and log 2FC values. Red color is for genes upregulated by FlbB.

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