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Among Developmental Regulators, StuA but Not BrlA Is Essential for Penicillin V Production in *Penicillium chrysogenum*▽†

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In filamentous fungi, secondary metabolism is often linked with developmental processes such as conidiation. In this study we analyzed the link between secondary metabolism and conidiation in the main industrial producer of the β-lactam antibiotic penicillin, the ascomycete *Penicillium chrysogenum*. Therefore, we generated mutants defective in two central regulators of conidiation, the transcription factors BrlA and StuA. Inactivation of either *brlA* or *stuA* blocked conidiation and altered hyphal morphology during growth on solid media, as shown by light and scanning electron microscopy, but did not affect biomass production during liquid-submerged growth. Genome-wide transcriptional profiling identified a complex StuA- and BrlA-dependent regulatory network, including genes previously shown to be involved in development and secondary metabolism. Remarkably, inactivation of *stuA*, but not *brlA*, drastically downregulated expression of the penicillin biosynthetic gene cluster during solid and liquid-submerged growth. In agreement, penicillin V production was wild-type-like in *brlA*-deficient strains but 99% decreased in *stuA*-deficient strains during liquid-submerged growth, as shown by high-performance liquid chromatography (HPLC) analysis. Thus, among identified regulators of penicillin V production StuA has the most severe influence. Overexpression of *stuA* increased the transcript levels of *brlA* and *abaA* (another developmental regulator) and derepressed conidiation during liquid-submerged growth but did not affect penicillin V productivity. Taken together, these data demonstrate an intimate but not exclusive link between regulation of development and secondary metabolism in *P. chrysogenum*.

The filamentous fungus *Penicillium chrysogenum* plays an important role in biotechnology as the main industrial producer of the β-lactam antibiotic penicillin, a secondary metabolite. A better understanding of parameters which directly or indirectly influence biosynthesis of penicillin is fundamental for optimizing industrial production of penicillin and possibly secondary metabolites in general. Previous studies revealed an association of fungal secondary metabolism with developmental processes such as asexual reproduction, termed conidiation (12). Both processes are associated with the stationary growth phase (3). In agreement, it recently has been shown that inactivation of either VelA or LaeA, both components of the *velvet*-like complex, decreases conidiation and penicillin production in *P. chrysogenum* (27). On the other hand, inactivation of the Gα subunit of a heterotrimeric G protein, Pga1, increases conidiation but decreases production of penicillin and the mycotoxin roquefortine, another secondary metabolite, in *P. chrysogenum* (19). Regulation of conidiation has been extensively studied in the ascomycete *Aspergillus nidulans*, but little is known about regulation of conidiation in *P. chrysogenum*. In response to nutrient sensing, *A. nidulans* conidia (spores) swell and form germ tubes and subsequently septated hyphae. After gaining so-called "developmental competence" and exposure to air, hyphae initiate conidiation, which starts with the growth of an aerial stalk followed by the formation of metulae and phialides. These phialides give rise to chains of uninucleate conidia (2). Notably, nutritionally sufficient submerged culture allows vegetative growth but represses conidiation in *A. nidulans* and *P. chrysogenum*.

Conidiogenesis is under complex genetic control. In *A. nidulans*, FluG (assumed to be associated with the production of a small diffusible molecule) and FlbA (regulator of G-protein signaling) are key upstream activators of conidiation, both acting via inhibition of the repressive Zn_2C_6-type zinc finger transcription factor SfgA and FadA (ortholog of *P. chrysogenum* Pga1), respectively (36–38, 57). Four additional downstream transcription factors, FlbB, FlbC, FlbD, and FlbE, individually are required for transcriptional activation of *brlA* and subsequent conidiation (20, 21, 75, 76). Furthermore, a Pcl-like cyclin (54) and the cyclin-dependent kinase encoded by $nimX^{Cdc2}$ of *A. nidulans* seem to mediate cell cycle events during developmental cell type formation (54, 55). BrlA together with AbaA and WetA forms a regulatory cascade, required for the formation of conidiophores and conidia. The transcription factors StuA and MedA are developmental modifiers required for correct conidiophore morphogenesis through spatial and temporal regulation of *brlA* and *abaA* expression (5, 39, 44). BrlA is a C_2H_2-type zinc finger transcription factor, which begins to accumulate shortly after induction of development (52). Two overlapping transcripts orig-

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TABLE 1. *P. chrysogenum* orthologs of genes previously implicated in conidiogenesis of *A. nidulans* or *A. fumigatus*

Gene	ID	Species	*P. chrysogenum* gene	ID	e value[a]	Reference
abaA	AAA33286	*A. nidulans*	*abaA*	Pc16g09610	0.0	5
abr1	XP_756089.2	*A. fumigatus*	*abrA*	Pc21g16380	0.0	68
abr2	ACJ13064.1	*A. fumigatus*	*yA*	Pc22g08420	0.0	68
alb1	EAL94057	*A. fumigatus*	*wA*	Pc21g16000	0.0	68
arp1	XP_756093.1	*A. fumigatus*	*arpA*	Pc21g16420	4e–65	68
arp2	XP_756091.1	*A. fumigatus*	*arpB*	Pc21g16430	7e–100	68
asp f4	XP_749515	*A. fumigatus*	*asp* f4	Pc16g11310	6e–77	16
ayg1	EDP55259.1	*A. fumigatus*	*aygA*	Pc21g16440	6e–162	68
brlA	AAB48671	*A. nidulans*	*brlA*	Pc06g00470	2e–147	1
catA	AAC49254	*A. nidulans*	*catA*	Pc20g06360	0.0	45
dewA	CBF73692	*A. nidulans*	*dewA*	Pc16g06690	3e–17	64
fadA	AAC49476	*A. nidulans*	*pga1*	Pc13g12240	0.0	19
flbA	AAA73955	*A. nidulans*	*flbA*	Pc15g00600	0.0	36
flbB	CAM35586.1	*A. nidulans*	*flbB*	Pc12g01640	7e–138	18
flbC	ACP28867	*A. nidulans*	*flbC*	Pc12g12190	5e–124	75
flbD	AAA61913	*A. nidulans*	*flbD*	Pc13g03170	5e–93	21
flbE	ACP28868	*A. nidulans*	*flbE*	Pc16g12460	2e–47	20
fluG	AAC37414	*A. nidulans*	*fluG*	Pc20g02420	1e–120	37
laeA	AAQ95166	*A. nidulans*	*laeA*	Pc16g14010	9e–134	7
medA	AAC31205	*A. nidulans*	*medA*	Pc22g13450	0.0	10
nimX	AAA20597	*A. nidulans*	*nimX*	Pc20g10270	7e–145	54
pclA	CAC06384	*A. nidulans*	*pclA1*	Pc16g08700	1e–118	55
			pclA2	Pc03g00090	8e–63	
			pclA3	Pc22g26190	2e–69	
phiA	CAA09585	*A. nidulans*	*phiA*	Pc22g00190	3e–52	40
ppoC	AAT36614	*A. nidulans*	*ppoC*	Pc18g00240	0.0	69
rodA	AAA33321	*A. nidulans*	*rodA*	Pc22g14290	5e–52	63
rodB	XP_753093.1	*A. fumigatus*	*rodB*	Pc21g18350	2e–19	51
sfgA	AAY99779	*A. nidulans*	*sfgA*	Pc21g18560	3e–177	57
stuA	CBF70741	*A. nidulans*	*stuA*	Pc13g04920	9e–180	41
tmpA	AAP13095	*A. nidulans*	*tmpA*	Pc21g07830	2e–170	38
veA	AAD42946	*A. nidulans*	*velA*	Pc13g13200	2e–112	61
velB	ABQ17967	*A. nidulans*	*velB*	Pc22g22320	2e–77	11
vosA	ABI51618	*A. nidulans*	*vosA*	Pc22g06890	3e–104	46
wetA	AAA33330	*A. nidulans*	*wetA*	Pc22g03220	3e–138	39

[a] Homology (at the amino acid level) of the *P. chrysogenum* gene product to the corresponding *Aspergillus nidulans* or *Aspergillus fumigatus* gene product.

inate from the *brlA* locus, *brlA*α and *brlA*β, both having essential functions for normal development (25). *brlA*-deficient mutants are blocked in development at an early stage in the transition from polar growth of the conidiophore stalk to the swelling of the vesicle. The stalks somehow elongate indeterminately and fail to differentiate, which gives the mutants a "bristle" appearance (14). Furthermore, the mutants fail to accumulate other developmentally regulated transcripts like *abaA* and *wetA* (9). Forced expression of *brlA* in submerged cultures is sufficient to transcriptionally activate *abaA* and *wetA* and to induce conidiation (1). StuA is a basic helix-loop-helix-like transcription factor with an APSES DNA-binding motif (17). Expression of *stuA* is upregulated with the onset of developmental competence and remains at that level during conidiation (41). Similarly to *brlA* expression, *stuA* expression results in two overlapping and differentially regulated transcripts. Conidiophores of *stuA* null mutants have shortened stalks and reduced vesicles and lack metulae and phialides. In contrast to *brlA* mutants, *stuA* mutants still produce few conidia, which directly bud from the vesicle as shown in *A. nidulans* and *A. fumigatus* (42, 58). In *A. nidulans*, deficiency in StuA, but not BrlA, also impairs sexual development (35, 78).

BrlA orthologs are present only in *Aspergillus*, *Neosartorya*, *Penicillium*, and *Talaromyces* species. In contrast, StuA is evolutionary widely conserved and orthologs have been shown to regulate development in *A. fumigatus*, *Penicillium marneffei*, *Neurospora crassa*, *Fusarium oxysporum*, *Glomerella cingulata*, *Saccharomyces cerevisiae*, and *Candida albicans* (6, 8, 22, 50, 58, 62, 67, 72). In *Aspergillus fumigatus*, 6 of 22 putative secondary metabolite-encoding gene clusters were found to be StuA dependent (15, 70), including clusters for biosynthesis of gliotoxin, pseurotin A, and the alkaloids fumigaclavin and fumitremorgin, whereas BrlA affects expression of only 2 of these gene clusters: fumigaclavin positively and fumitremorgin negatively. In *A. nidulans*, the synthesis of the mycotoxin sterigmatocystin is influenced positively by FluG and FlbA but negatively by FadA. In contrast, penicillin production is positively affected by FadA (26, 57, 65).

The genetic program controlling development in *P. chrysogenum* appears to be highly similar to that of aspergilli, as the *P. chrysogenum* genome encodes orthologs of all regulators identified in *A. nidulans* (Table 1). In this study, we demonstrate that in *P. chrysogenum* inactivation of either *brlA* or *stuA* blocks conidiation but affects expression of genes involved in development and secondary metabolism partially differently. In particular, penicillin production was found to be absolutely dependent on *stuA* but not *brlA*.

MATERIALS AND METHODS

Strains and culture conditions. *Escherichia coli* strain K-12 Fusion-Blue (*recA endA*) was used for plasmid construction (In-Fusion Dry-Down PCR cloning kit;

Clontech). Reproduction and maintenance of plasmids were done according to standard protocols (53). *P. chrysogenum* carrying Δ*Pcku70* (herein referred to as the Δ*ku70* mutant) (28), derived from P2*niaD18* (herein referred to as the *niaD18* mutant) carrying a mutated *niaD* gene (29), served as the parental strain in all transformation experiments (see Table S1 in the supplemental material). Additionally, most assays included the *niaD18* mutant as an additional control. Fungal strains were cultivated in liquid complete CCM (43) or minimal MM1 medium for 48 to 120 h at 25°C and 230 rpm or on solid minimal medium MM2 as already described (23). For sporulation assays, strains were grown on solid complete medium CM2 [3.5 g/liter $(NH_4)_2SO_4$, 2 g/liter K_2SO_4, 0.2 g/liter KH_2PO_4, 1 g/liter of soy flour, 5 g/liter limestone powder, 50 g/liter lactose, 20 g/liter agar] for transcript analysis on CM2 on sterile membranes (Supor-200; Pall Life Sciences, USA) or in liquid production medium (28). Cultures were inoculated with 5×10^4 spores for point inoculations on plates, with 2×10^6 spores for inoculation of whole plates (sporulation assay and microarrays) and 1×10^7 spores for liquid cultures. In studies including nonsporulating mutants, cultures were inoculated with 5 μl, 200 μl, and 500 μl of liquid 72-h CCM cultures for point inoculation, whole plate assays, and liquid cultures, respectively.

Transformation of *P. chrysogenum*. Transformation was performed as described by Windhofer et al. (77) with some modifications. Liquid CCM medium cultures were incubated for 48 h at 25°C and 230 rpm. Protoplasts were transformed by linear PCR, restriction fragments, or circular plasmids 4.103, 4.104, 4.122, 4.127, 4.128, 4.196, 4.181, 4.182, and 4.177 (see Table S2 in the supplemental material). Transformants were selected on either terbinafine (0.9 μg/ml) or phleomycin (45 μg/ml) containing MM2 supplemented with 0.5 g/liter arginine and either 2.5% xylose or 2.5% glucose depending on the promoter used for the selection marker gene. Screening of positive clones was performed by PCR (data not shown). To obtain homokaryotic transformants, colonies from single homokaryotic spores were picked and homologous integration of the constructs was verified by Southern blot analysis.

Identification of *P. chrysogenum* genes *stuA* and *brlA*. The genomic sequence of *P. chrysogenum* ATCC 28089 (71) served as a source for the sequences used here. The sequences for the *P. chrysogenum* genes *brlA* (CAP79040.1) and *stuA* (CAP91561.1) were obtained from the public database NCBI Entrez (http://www.ncbi.nlm.nih.gov/sites/gquery). Protein sequence alignments were performed with the ClustalW program (66).

Preparation of nucleic acids, hybridization protocol, and PCR. Fungal genomic DNA for Southern blot analysis was isolated from hyphal cells, grown for 3 days at 25°C and 230 rpm in liquid CCM medium, based on protoplasting as previously described (80). RNA extraction for microarray and quantitative real-time PCR (qRT-PCR) analysis from mycelia grown on solid complete CM2 or in liquid production medium was done using Trizol reagent (no. 15596-018; Invitrogen), the RNeasy midikit (no. 75144; Qiagen) and the DNase I kit (no. 79254; Qiagen) for DNase I treatment according to manufacturer's protocols. For PCR analysis, transformants were grown on solid complete CM2 medium for 6 days at 25°C and mycelia or spores were washed off with H_2O. A 5-μl portion of this suspension was used for DNA extraction with the REDExtract-N-Amp seed PCR kit (no. 086K6825; Sigma).

For clonings, PCR amplifications were performed with the Accu Prime Pfx DNA polymerase (no. 12344-024; Invitrogen) with "proofreading" activity, for screening approaches with the REDTaq DNA polymerase (no. R4775; Sigma), according to the manufacturer's instructions.

Southern hybridization was carried out with Duralon-UV membranes (no. 420101; Stratagene) and digoxigenin-labeled probes (PCR DIG probe synthesis kit, no. 11636090910; Roche). The attached probes were detected with the DIG luminescence detection kit (no. 11363514910; Roche). All techniques were used according to the method of Sambrook and Russell (53).

Quantification of penicillin (penicillin V) and biomass production. For quantification of penicillin productivity, 5 g culture broth from shake flask cultures grown for 144 h at 25°C and 230 rpm in a production medium were analyzed by high-performance liquid chromatography (HPLC) (56). For quantification of biomass production, 5-ml portions of culture broth (grown as mentioned above) from each sample were filtered and dried for 18 h at 100°C.

Quantification of conidiation. Spores (2×10^6) were spread on four independent CM2 plates per strain and incubated for 168 h at 25°C. Areas of 1 cm in diameter were cut out from each plate, pooled, and vortexed together with five small glass balls in 5 ml M3 (8.5 g/liter NaCl, 1 ml/liter Tween 80) for 1.5 min. Spores of each sample were counted three times in the Thoma chamber.

Microarray analysis. The custom-designed Affymetrix GeneChip DSM_PENa520255F, representing the genome of *P. chrysogenum* Wisconsin54-1255, was used for gene expression quantification (71). RNA was isolated from the strains carrying the Δ*ku70*, Δ*brlA*, and Δ*stuA* mutations after 36, 48, 60, 72, and 96 h of growth on solid complete CM2 plates separated from the medium by dialysis membranes. RNA of three biological replicates was pooled for sample preparation and microarray hybridization (28). Differentially expressed genes were determined using the R/Bioconductor packages limma and affylmGUI (60, 74); raw data were preprocessed using the implemented RMA algorithm (31, 32). Correlation analysis was performed using GeneSpring 7.2 (Silicon Genetics) and a correlation coefficient of ≥0.9. Heat maps were generated with the program TreeView by Michael Eisen (version 1.60; Howard Hughes Medical Institute, University of California at Berkeley [http://rana.lbl.gov/EisenSoftware.htm]).

Quantitative real-time PCR (qRT-PCR). RNA was extracted from three biological replicates of the *niaD18*, Δ*ku70*, Δ*brlA* and Δ*stuA* strains in a time course experiment on solid CM2 plates (see microarray analysis). Furthermore, from three biological replicates of the Δ*ku70* and *stuA-OE* strains, RNA was isolated in a time course experiment in liquid production medium after 48, 72, 96, 120 and 144 h. From all RNA samples, cDNA was synthesized with the High-Capacity cDNA archive kit (no. 4322171; ABI). qRT-PCR was carried out with 4 to 8 ng/μl cDNA. Detection was based on the SYBR green I dye detection system (no. 4309155; ABI) and measured with a 7900HT Fast-Realtime PCR system (Applied Biosystems). qRT-PCR assays were performed in triplicate for each sample. Data analysis was performed according to the method of Nowrousian et al. (48). All values were normalized to the housekeeping gene *actA* (Pc20g11630) encoding γ-actin.

Scanning electron microscopy. Strains were grown on solid complete CM2 plates at 25°C for 48 h and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3). A brief washing in the same buffer was followed by 1 h of postfixation with 1% aqueous OsO_4, gradual dehydration with ethanol, and critical point drying with liquid CO_2 (Bal-Tec CPD 030; Balzers, Liechtenstein). Specimens were mounted on aluminum stubs with Leit C (Göcke, Plano GmbH), sputtered with 10 nm Au/Pd (MED 020; Balzers, Liechtenstein), and examined with a Zeiss field emission scanning electron microscope (FESEM, Gemini 982).

RESULTS AND DISCUSSION

Identification of the *P. chrysogenum stuA* and *brlA* orthologs. *P. chrysogenum brlA* and *stuA* were identified by homology searches as described in Materials and Methods. *P. chrysogenum brlA* encodes a protein of 433 amino acids showing 63% and 62% identity to the orthologs of *A. nidulans* and *A. fumigatus*, respectively (see Fig. S1 in the supplemental material). *P. chrysogenum stuA* encodes a protein of 818 amino acids. Alignment of the predicted *P. chrysogenum* StuA protein shows 62% to 67% identity with the corresponding published amino acid sequences of *A. flavus*, *Neosartorya fischeri*, *A. nidulans*, and *A. fumigatus*, with *A. flavus* StuA being the most similar ortholog. Interestingly, compared to the *A. nidulans* and *A. fumigatus* orthologs, which are annotated as proteins 622 and 635 amino acids in length, respectively, the annotated StuA orthologs of *P. chrysogenum*, *A. flavus*, and *N. fischeri* contain an "N-terminal extension" of about 200 amino acids. TBLASTN searches revealed that the amino acid sequence of the N-terminal extension also is highly conserved in the *A. nidulans* and *A. fumigatus* orthologs, indicating misannotation of these two StuA-encoding orthologs (see Fig. S2 in the supplemental material).

Generation of *brlA* and *stuA* deletion strains. Deletion of either the *brlA* or the *stuA* locus was performed by replacement of the respective coding region with the terbinafine resistance gene *ergA* under the control of the xylose-inducible *xylP* promoter (59, 80) using the bipartite marker technique (47) in a Δ*ku70* mutant (28), as described in Fig. S3A and B and "Generation of transformation fragments" in the supplemental materials. A total of 42 *brlA* knockout strains out of 221 transformants and 27 *stuA* knockout strains out of 50 transformants were verified by Southern blot analysis after reisolation. South-

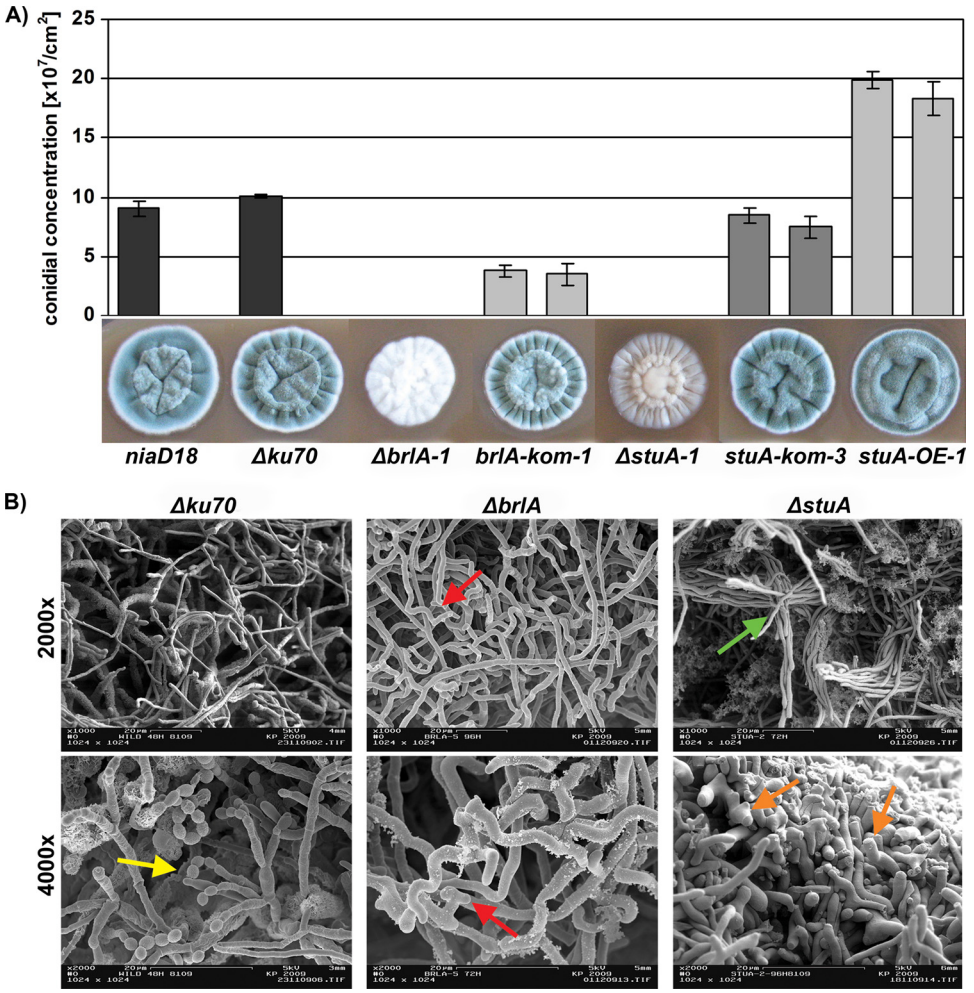

FIG. 1. Characterization of Δ*brlA* and Δ*stuA* strains compared to the recipient Δ*ku70* and *niaD18* mutant strains. (A) Colony phenotypes on solid complete CM2 after growth for 168 h at 25°C. Quantification of conidial formation in mutant and complemented strains was performed in triplicate and repeated twice with two independent primary transformants of each strain; bars show standard deviations. (B) Scanning electron microscopy of Δ*brlA* and Δ*stuA* mutants. Both mutants lack conidia and conidiophores (yellow arrow indicates conidia on conidiophores in the Δ*ku70* mutant); the Δ*brlA* strain displayed zigzag growth and increased and abnormal branching (red arrows); Δ*stuA* strain hyphae were bundled at the surface of the cultures (green arrow) and showed increased fragmentation (orange arrow).

ern blot analysis confirmed the homologous integration of the selection marker as well as the loss of the gene to be deleted (see Fig. S3A and B in the supplemental material). In conclusion, the homologous *brlA* and *stuA* recombination frequencies correspond to about 19 and 54%, respectively, clearly reinforcing the qualification of the *P. chrysogenum* Δ*ku70* mutant as a valuable recipient for gene deletion experiments. For simplicity, only three deletants each of *brlA* and *stuA* were used for further analysis.

For reconstitution of the Δ*brlA* mutant, a functional copy of the *brlA* gene was inserted ectopically. This was achieved by transforming the Δ*brlA* mutant with plasmid p4.122 containing the *brlA* gene and the *ergA* selection marker gene driven by the *P. chrysogenum* gene γ-actin promoter (Pc20g11630). This promoter exchange enables a second selection on terbinafine with glucose instead of xylose as a carbon source in the selection medium, which represses the *xylP*-driven expression of *ergA* (80). Four transformants, designated *brlA-kom*, were proved to carry the *brlA* gene after single-spore reisolation and Southern blot analysis (see Fig. S3C in the supplemental material). For reconstitution, the Δ*stuA* strain was transformed with plasmid p4.196 carrying a functional copy of the *stuA* gene and the Tn*5* phleomycin selection marker gene of *E. coli* driven by the *ipnA* promoter of *P. chrysogenum*. Three transformants were identified as positive complementation strains (designated *stuA-kom*), after single-spore reisolation and Southern blot analysis (see Fig. S3D). Interestingly, further PCR and Southern blot analyses (data not shown) demonstrated that in all three *stuA-kom* strains the entire plasmid p4.196 integrated homologously at the *stuA* locus by single crossover.

Inactivation of either *brlA* or *stuA* blocks conidiation and causes aberrant hyphal morphology. Primary transformants lacking *brlA* or *stuA* did not produce conidia and therefore had to be propagated in hyphal form. Growth tests starting with point inoculation of defined amounts of vegetative hyphae on CM2 plates confirmed that both Δ*brlA* and Δ*stuA* strains are completely blocked in conidiation, as evident from visual (Fig. 1A) and light microscopic inspection (data not shown). In

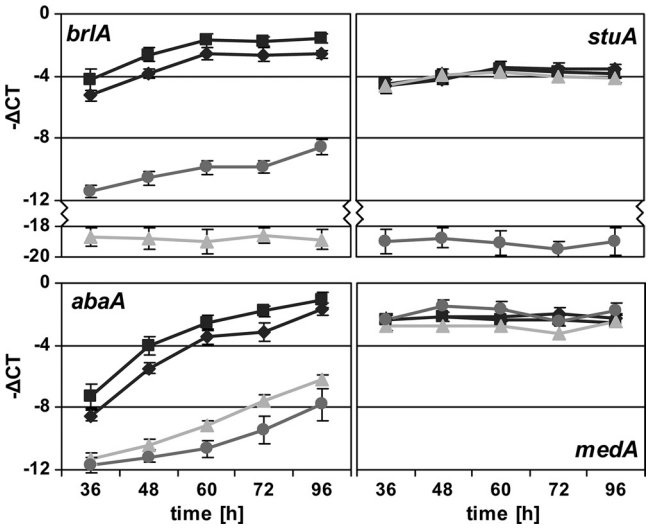

FIG. 2. Expression of *brlA*, *stuA*, *abaA*, and *medA* in *niaD18*, Δ*ku70*, Δ*brlA*, and Δ*stuA* strains in a time course experiment measured by qRT-PCR. The results are given as means ± standard deviations of results for independent biological triplicates with technical triplicates. Black diamonds, *niaD18*; black squares, Δ*ku70*; light gray triangles, Δ*brlA*; medium gray circles, Δ*stuA*.

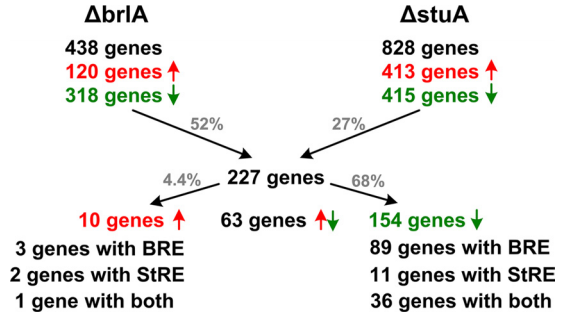

FIG. 3. Schematic summary of the genome-wide comparative expression profiling of Δ*brlA* and Δ*stuA* strains compared to the Δ*ku70* strain. Genes were scored as deregulated when their expression was ≥3-fold changed at ≥3 consecutive time points compared to the Δ*ku70* strain. BRE and StRE elements in forward, reverse, or both orientations within 1,000 bp upstream of the start codons were considered.

contrast, *stuA* deficiency in both *A. nidulans* and *A. fumigatus* results in the production of extremely shortened conidiophores that lack metulae and phialides but produce conidia directly from buds formed on the conidiophore vesicle (2, 58).

The complemented *brlA-kom* and *stuA-kom* strains showed restored conidiation, demonstrating that the conidiation defect was indeed caused by the deletion of the respective gene. Interestingly, the Δ*brlA* strain formed wild-type-like white mycelia whereas the Δ*stuA* mycelia were brown pigmented, indicating deregulation of pigment biosynthesis (see below).

Scanning electron microscopy of 48-h surface cultures visualized formation of conidiophores and conidia in the Δ*ku70* mutant, while the Δ*brlA* and Δ*stuA* mutants lacked these structures (Fig. 1B). Moreover, hyphae of the Δ*brlA* mutant showed zigzag growth and hyperbranching; hyphae of the Δ*stuA* mutant congregated to thick bundles and displayed numerous stumps, possibly indicating increased susceptibility to fragmentation (Fig. 1B). Measurement of the biomass production after 144 h in submerged culture indicated that inactivation of either *brlA* or *stuA* has no significant effect on the vegetative growth rate (see Fig. S4 in the supplemental material).

Inactivation of either *brlA* or *stuA* decreases expression of developmental regulators. In a time course experiment, samples were taken after 36 h to 96 h of growth on solid medium. qRT-PCR measurements revealed an increase of *brlA* and *abaA* transcript levels, whereas *stuA* and *medA* levels remained largely constant in both Δ*ku70* and *niaD18* strains (Fig. 2). A difference in threshold cycle values ($-\Delta C_T$) of <-18 (i.e., a decrease of <18 in C_T) confirmed the deletion of the respective genes in Δ*brlA* and Δ*stuA* mutants (Fig. 2). Remarkably, *brlA* was severely downregulated in the Δ*stuA* mutant ($-\Delta C_T$ of -8, equaling about 250-fold), while *stuA* transcript levels remained unaltered in the Δ*brlA* mutant, which indicates that StuA regulates the *brlA* transcript level but not vice versa. Moreover, inactivation of either *brlA* or *stuA* decreased expression of *abaA* ($-\Delta C_T$ of up to -6, equaling about 60-fold) but did not affect the *medA* transcript level. Interestingly, the *brlA* transcript level was still upregulated during the time course in the Δ*stuA* mutant and the *abaA* transcript level was still upregulated during the time course in the Δ*stuA* and Δ*brlA* mutants, demonstrating that expression of both *brlA* and *abaA* is responsive to more than one regulatory mechanism. Taken together, these data reveal a regulatory cascade with BrlA activating expression of *abaA* and StuA activating expression of *brlA* and *abaA* during conidiogenesis, whereby StuA might influence *abaA* indirectly via BrlA.

Microarray analysis of Δ*brlA* and Δ*stuA* mutants reveals a complex regulatory network. In order to gain more insight into molecular changes associated with the gene deletions of *brlA* and *stuA*, the Δ*ku70*, Δ*brlA* and Δ*stuA* mutant strains were compared by genome-wide transcriptome analysis. For a time course study, RNA was isolated from all strains grown on solid CM2 for 36, 48, 60, 72 and 96 h. Formation of conidia was observed after 48 h of growth for the Δ*ku70* mutant but not the Δ*brlA* and Δ*stuA* mutants. All strains showed similar radial growth rates, and therefore differences in the expression profiles of the two mutant strains are not expected to be caused by differences in growth rate.

In the Δ*ku70* strain, orthologs of 17 genes previously found to encode regulatory and structural genes upregulated during conidiogenesis in *A. nidulans* or *A. fumigatus* (2, 51, 68) were upregulated ≥3-fold at three or more time points compared to their status at the 36-h time point, e.g., the hydrophobin-encoding genes and the pigment biosynthetic genes (Table 1; see Fig. 4A to D). In contrast, transcript levels of *stuA* and *medA* did not significantly change during the time course. The microarray-based expression profiles of *brlA*, *abaA*, *stuA*, and *medA* nicely fit to the qRT-PCR analyses shown here (Fig. 2). A schematic summary of the transcription profiling of the Δ*ku70*, Δ*brlA*, and Δ*stuA* mutants is shown in Fig. 3. Compared to the results for the Δ*ku70* mutant, 438 genes were ≥3-fold deregulated at three or more consecutive time points in the Δ*brlA* mutant, with 27% of these genes being upregulated and 73% being downregulated. In the Δ*stuA* mutant, 828 genes were deregulated, with 50% being upregulated and 50% being downregulated. A total of 227 deregulated genes are common

to both Δ*brlA* and Δ*stuA* mutants, a majority, 68%, of which were downregulated (see Table S4 in the supplemental material). Within 1 kb of the 5′ upstream region, 58% of these genes contain at least one BrlA response element (BRE) (M RAGGGR) (13), 7% contain at least one StuA response element (StRE) (WCGCGWNM) (17), and 23% contain both elements. Taking into account the downregulation of *brlA* by inactivation of *stuA* (see above), these data indicate that StuA might regulate the majority of these genes rather indirectly via BrlA. The genes downregulated in both Δ*brlA* and Δ*stuA* mutants enclosed orthologs of nine genes, including *abaA*, previously found to be involved in conidiogenesis in *A. nidulans* (Table 1; Fig. 4B). Gene expression correlation analysis indicated that 93 genes are coregulated with *abaA* (see Table S5 in the supplemental material), 85 of which are downregulated in both Δ*brlA* and Δ*stuA* mutants, including seven of the nine orthologs previously identified to be involved in conidiogenesis in *A. nidulans* (Fig. 4B). Of the 154 genes downregulated in both Δ*brlA* and Δ*stuA* mutants and of the 93 genes coregulated with *abaA*, 93.5% and 94.6%, respectively, contain at least one AbaA-responsive element (CATTCY, identified in *A. nidulans* [5]) within 1 kb of the 5′ upstream region. These data indicate that the majority of genes downregulated in the Δ*brlA* and Δ*stuA* mutants and coregulated with *abaA* are regulated via AbaA. Interestingly, this gene set also includes an ortholog of Asp F4 (Table 1), an allergen of *A. fumigatus* (16), which indicates developmental regulation of allergen formation. Orthologs of seven other genes previously found to be involved in conidiogenesis in *A. nidulans*, including *wetA*, were downregulated in the Δ*brlA* mutant but upregulated at early time points in the Δ*stuA* mutant (Table 1, Fig. 4C). Analysis of *wetA* coregulated genes identified six of these seven genes and additional 38 genes (Fig. 4C; see Table S6 in the supplemental material). This suggests that these genes are activated by BrlA (possibly via AbaA) but are temporally repressed by StuA, in both cases most likely via WetA. A proposed regulatory scheme is summarized in Fig. S5 in the supplemental material.

The derepression of genes encoding early steps of conidial melanin synthesis (*wA*, *arpA*, *arpB*, and *aygA*) combined with the repression of genes encoding late steps (*abrA* and *yA*) might explain the brownish pigmentation of the Δ*stuA* hyphae (Fig. 1A and 4B and C), in particular as deletion of *abrA* and *yA* changes the conidial color from green to brown in *A. fumigatus* (34, 68). In agreement, in the Δ*brlA* mutant, all these pigment genes were repressed, resulting in white hyphae (Fig. 1A and 4B and C).

The 212 genes downregulated in the Δ*stuA* mutant but not the Δ*brlA* mutant included *pclA1* and *ppoC* (Fig. 4D). Interestingly, *P. chrysogenum* possesses three genes (*pclA1*, *pclA2*, and *pclA3*) homologous to the single *pcl*-like cyclin-encoding *pclA* of *A. nidulans* (55), whereas the three paralogs display different expression patterns (Fig. 4B and D). *ppoC* encodes a fatty acid oxygenase, contributing to the production of a secreted lipogenic signal molecule, that together with other factors (*ppoA*, *ppoB*) governs timing and balance of sexual and asexual development in *A. nidulans* (69).

Other genes implicated in conidiogenesis in *A. nidulans* were largely unaffected or affected ≤3-fold by inactivation of *brlA* and *stuA*, e.g., *medA*, *phiA*, *sfgA*, *pga1*, *velA*, *laeA*, and the "fluffy" genes *fluG*, *tmpA*, *flbA*, *flbB*, *flbC*, *flbD*, and *flbE* (Table 1 and data not shown).

Similarly to *P. chrysogenum*, a complex regulatory network involving BrlA, StuA, AbaA, and WetA with several feedback loops controlling conidiogenesis has been characterized previously in *A. nidulans* and, in a more fragmentary form, in other fungal species (1, 8, 49, 58). The numerous genes identified in this study to be affected by deletion of *brlA*, *stuA*, or both, as well as the genes found to be coregulated with *abaA* and *wetA*, will now aid further elucidation of conidiogenesis of *P. chrysogenum* and other fungal species.

Inactivation of *stuA* or *brlA* affects expression of secondary metabolite-encoding genes. van den Berg et al. (71) identified 51 genes encoding putative nonribosomal peptide synthases (NRPS), polyketide synthases (PKS), or hybrid enzymes postulated to be part of 39 gene clusters. This indicates that *P. chrysogenum* has the potential to produce a variety of secondary metabolites. The genome-wide expression profiling revealed that inactivation of either BrlA or StuA deregulates expression ≥3-fold of six of these putative secondary metabolite-encoding clusters (for the cluster definition, see the legend to Fig. 4) and nine single genes encoding NRPS or PKS (Fig. 4E and F). Of these gene clusters, only one has been functionally characterized and shown to be responsible for biosynthesis of the β-lactam antibiotic penicillin. The cluster containing the putative NRPS Pc21g15480 and the tryptophan dimethylallyl transferase Pc21g15430 (*dmaW*) most likely encodes the biosynthesis of the mycotoxin roquefortine C (33, 71). Roquefortine C, a prenylated indole derivative containing tryptophan and histidine moieties, was isolated first from *Penicillium roqueforti* and identified later in many *Penicillium* strains, including *P. chrysogenum*. Roquefortine C is also the precursor for further prenylated indole alkaloids such as meleagrine, oxaline, and glandicolines (79). Among all StuA- or BrlA-regulated gene clusters, the pencillin and roquefortine C biosynthetic gene clusters were affected most significantly. Expression of both clusters was largely unaffected in the Δ*brlA* strain but drastically downregulated in the Δ*stuA* strain (Fig. 4E). qRT-PCR analysis confirmed downregulation of the penicillin biosynthetic genes *pcbAB* ($-\Delta C_T$ of −7, equaling about 130-fold), *pcbC* ($-\Delta C_T$ of −7, equaling about 130-fold), and *penDE* ($-\Delta C_T$ of −9, equaling about 500-fold) in the Δ*stuA* mutant but not the Δ*brlA* mutant at all time points (Fig. 5A).

***stuA* but not *brlA* is essential for PENV production.** To further analyze the involvement of *stuA* and *brlA* in secondary metabolism, penicillin V (PENV) production was measured in liquid cultures. In agreement with expression patterns of PENV biosynthesis genes scored on solid medium, PENV production was wild-type-like in the Δ*brlA* mutant and 99% reduced in the Δ*stuA* mutant (Fig. 5B). Complementation of Δ*stuA* with a functional *stuA* copy restored productivity to wild-type levels, reinforcing the crucial role of *stuA* in penicillin biosynthesis. qRT-PCR analysis confirmed that inactivation of *stuA* but not *brlA* blocks PENV production by transcriptional downregulation of the penicillin biosynthetic genes *pcbAB*, *pcbC*, and *penDE* in liquid medium cultures (Fig. 5C) as well as in solid medium cultures as shown above (Fig. 5A). These data also show that the developmental regulator StuA, which is essential for conidiation, also plays a role during submerged

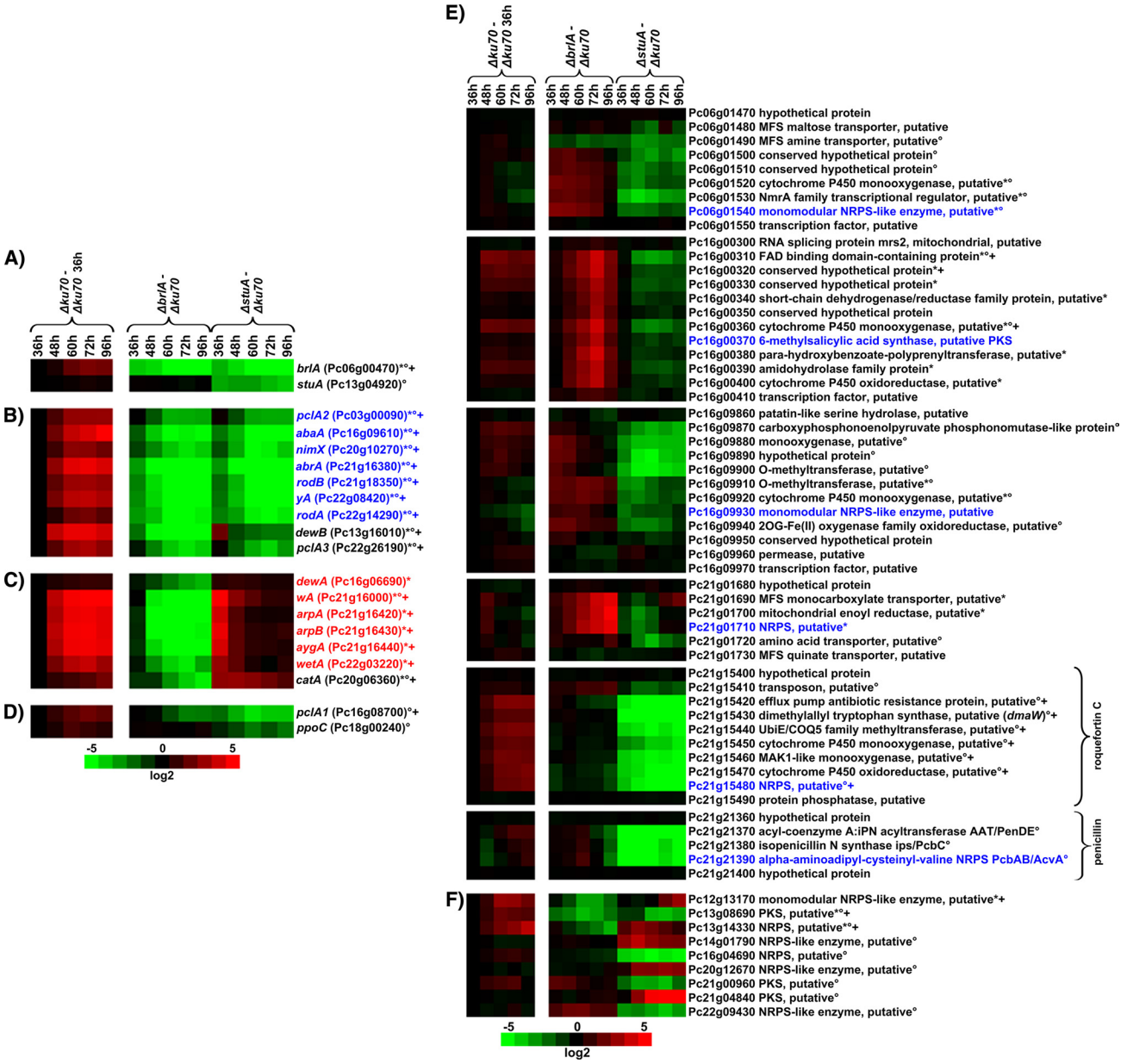

FIG. 4. Heat map representation of the microarray analysis of Δ*brlA* and Δ*stuA* mutants compared to the Δ*ku70* mutant, including orthologs of genes previously implicated in conidiogenesis in *A. nidulans* or *A. fumigatus* and genes potentially involved in secondary metabolism. (A) *brlA* and *stuA* mutants. (B) Conidiogenesis-related genes downregulated in both Δ*brlA* and Δ*stuA* mutants. (C) Conidiogenesis-related genes downregulated in the Δ*brlA* mutant. (D) Conidiogenesis-related genes downregulated in Δ*stuA* mutant. (E) Gene clusters containing NRPS- or PKS-encoding genes deregulated in either the Δ*brlA* or the Δ*stuA* mutant or both. (F) Individual NRPS- or PKS-encoding genes deregulated in either the Δ*brlA* or Δ*stuA* mutant. The left column displays the time course expression in the Δ*ku70* mutant, with all values normalized to the respective 36-h value; +, genes upregulated ≥3-fold at three or more consecutive time points compared to results at the 36-h time. The right column displays the change in transcript levels in Δ*brlA* and Δ*stuA* mutants, respectively, normalized to the same time point as the Δ*ku70* mutant. Values are shown in log 2 scale in green for downregulation and red for upregulation. * and °, genes deregulated ≥3-fold at three or more consecutive time points compared to Δ*ku70* in Δ*brlA* and Δ*stuA* strains, respectively. *abaA* coregulated and *wetA* coregulated genes are shown in blue and red, respectively. Gene clusters were regarded as deregulated when three or more genomically neighboring genes were ≥3-fold deregulated at three or more consecutive time points; gaps of single less-regulated genes were allowed for three or more neighboring genes; the heat map representation of regulated gene clusters includes the flanking unregulated genes. NRPS and PKS in panel E are shown in blue.

growth. StuA has the most severe influence on PENV production among all identified regulators yet. Recently, deficiency in LaeA and VelA was shown to decrease PENV production in the same *P. chrysogenum* strain grown under identical conditions by approximately 80% (27). In *Aspergillus fumigatus*, 6 of 22 putative secondary metabolite-encoding gene clusters were found to be StuA dependent (15, 70), including those for synthesis of gliotoxin, pseurotin A, and the alkaloids fumigaclavin and fumitremogen. Moreover, the StuA ortholog was found to play a crucial role in production of the mycotoxin

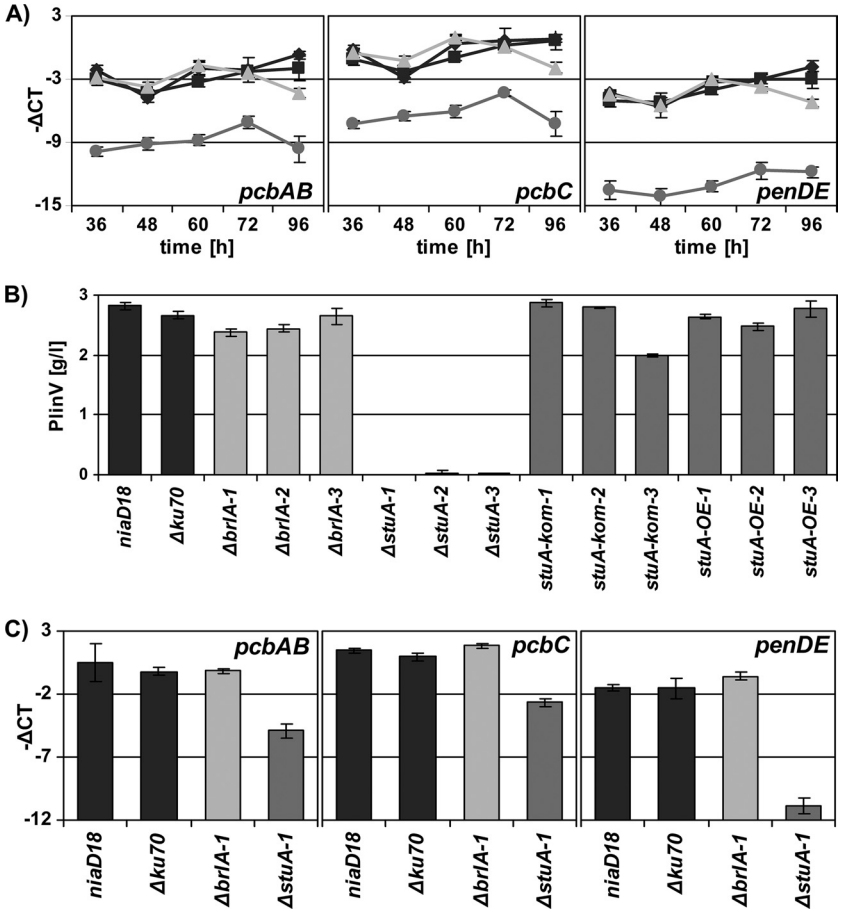

FIG. 5. Penicillin biosynthesis by Δ*brlA* or Δ*stuA*. (A) qRT-PCR analysis of the expression of penicillin biosynthetic genes in a time course experiment. (B) Quantification of PENV production by HPLC analysis. Mean values ± standard deviations are derived from biological sextuplicates. (C) qRT-PCR analysis of the expression of the penicillin biosynthetic genes in panel B. The results are given as means ± standard deviations of three independent biological with three technical triplicates. Black diamonds, *niaD1*; black squares, Δ*ku70*; light gray triangles, Δ*brlA*; medium gray circles, Δ*stuA*.

alternariol in *Stagnospora nodorum* (30), underlining the impact of StuA orthologs on secondary metabolism.

Overexpression of *stuA* derepresses conidiation during submerged growth but does not affect PENV production. To further analyze the role of *stuA* in conidiogenesis and PENV production, *stuA* was overexpressed by replacement of its native promoter by the *Pc22g-P* promoter, which drives the constitutively highly expressed gene Pc22g10010 (28) in the Δ*ku70* strain as described in "Generation of transformation fragments" in the supplemental material. Six of 13 transformants, termed *stuA-OE*, were proved, by single-spore reisolation and Southern blot analysis, to carry the promoter replacement (see Fig. S3E in the supplemental material). *stuA-OE* colonies appeared phenotypically identical on CM2 plates compared to the recipient strain but displayed an approximately doubled production of conidia (Fig. 1A). Remarkably, overexpression of *stuA* resulted in heavy conidiation during submerged growth (Fig. 6A), causing dark green staining of the culture broth. qRT-PCR demonstrated that the promoter replacement indeed caused overexpression of *stuA*, up to 4 $-\Delta C_T$ units, equaling about 16-fold (Fig. 6B). Moreover, qRT-PCR analyses proved that *stuA* overexpression causes upregulation of *brlA* ($-\Delta C_T$ of up to 3, equaling about 8-fold) and *abaA* ($-\Delta C_T$ of up to 6, equaling about 60-fold), which underlines the positive role of *stuA* in regulation of *brlA* and *abaA* identified in the *stuA* deletion analysis (see above). Consequently, the induction of conidiation during submerged growth might be due to upregulation of *stuA*, *brlA*, *abaA*, or a combination. In *A. nidulans*, overexpression of either *fluG*, *flbA*, *tmpA*, *flbC*, or *flbD* but not *flbB* was shown to induce *brlA* expression and conidiation in submerged cultures (2, 18, 38). Moreover, overexpression of *brlA* induces the expression of *abaA* and *wetA* and conidiation during submerged growth in *A. nidulans* (1, 24), while overexpression of *abaA*, *stuA*, and *wetA* does not result in conidiation (17, 24, 39). In further contrast to results with *P. chrysogenum*, forced expression of *stuA* in submerged cultures leads to repression of *abaA* in *A. nidulans*, indicating differences despite the general conservation of the regulatory system in these two species.

In contrast to conidiation, *stuA* overexpression did not influence PENV production (Fig. 5B). In agreement, the transcript levels of the penicillin biosynthesis genes *pcbAB*, *pcbC*, and *penDE* were similar in *stuA-OE* and Δ*ku70* strains (Fig. 6B).

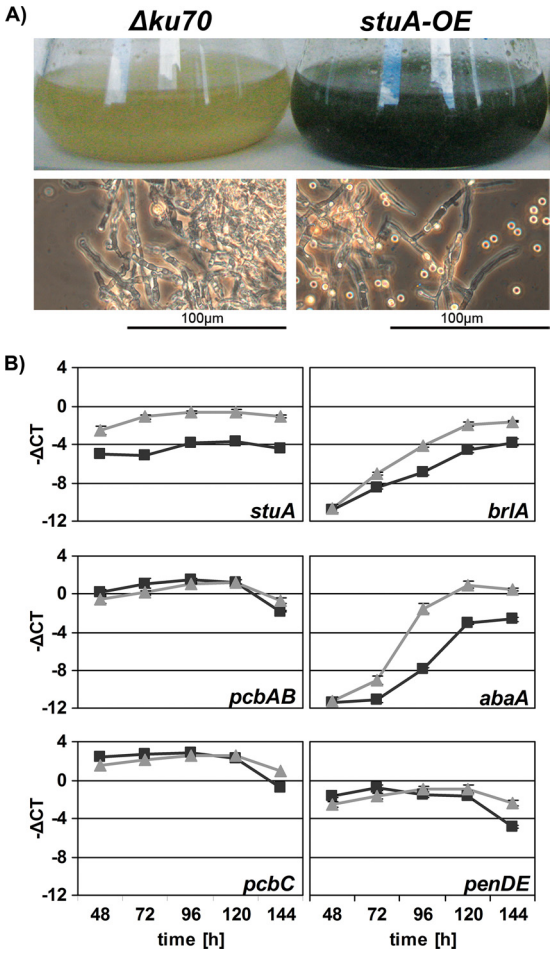

FIG. 6. (A) *stuA* overexpression derepresses conidiation in submerged growth in minimal medium. MM1-containing flasks were inoculated with Δ*ku70* and *stuA-OE-1* strains and incubated for 120 h at 25°C. (B) Expression of penicillin biosynthetic genes, *stuA*, *brlA*, and *abaA* in *stuA-OE-1* and Δ*ku70* strains, measured by qRT-PCR. The results are given as means ± standard deviations of independent biological triplicates with technical triplicates. Black squares, Δ*ku70*; light gray triangles, *stuA-OE-1*.

Analysis of a putative StuA-binding site in the *penDE* promoter. A search in the promoter regions of *pcbAB*, *pcbC*, and *penDE* strains for a putative StuA response element (StRE) (WCGCGWNM) identified in *A. nidulans* (17) revealed a single putative StRE 50 bp upstream of the *penDE* start codon. In order to analyze its functionality, StuA-binding activity was inactivated by mutation of two nucleotides within the core sequence, as described previously in *A. nidulans* (17). Native and mutated *penDE* promoters were fused to the *E. coli uidA* reporter gene and integrated at the *Pcku70* locus using the terbinafine resistance gene *ergA* as a selection marker, driven by the *acnP* promoter, as described in "Generation of transformation fragments" in the supplemental material. After single-spore reisolation, Southern blot analysis demonstrated that 3 out of 9 transformants carried the reporter constructs with the native promoter (strain *GUS*) and 9 out of 27 transformants with the mutated promoter (strain *GUS-m*) (see Fig. S3F in the supplemental material). Notably, growth rate, conidiation, and penicillin production were wild-type-like in all reporter strains, indicating that the transformation procedure did not result in side effects (data not shown). qRT-PCR analyses did not reveal differences in the expression of *uidA* in the reporter strains carrying the native and mutated *penDE* promoter (see Fig. S6 in the supplemental material), indicating either that the putative StuA-binding site is not functional or that the performed mutation did not inactivate StuA binding.

Conclusion. Taken together, these data indicate an intimate but not exclusive link between regulation of conidiation and secondary metabolism in *P. chrysogenum.* Inactivation of *stuA* was demonstrated to block both conidiation and PENV production. Consistently, deficiency in LaeA and VelA was recently shown to decrease conidiation and PENV production in the same *P. chrysogenum* strain grown under identical conditions by 80% (27), underlining the link between development and secondary metabolism. On the other hand, inactivation of the Gα subunit of a heterotrimeric G protein, Pga1, increases conidiation but decreases production of penicillin and roquefortine C (19), and inactivation of *brlA* blocks conidiation but does not affect PENV biosynthesis in *P. chrysogenum* (see above).

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REFERENCES

1. **Adams, T. H., M. T. Boylan, and W. E. Timberlake.** 1988. *brlA* is necessary and sufficient to direct conidiophore development in *Aspergillus nidulans*. Cell **54:**353–362.
2. **Adams, T. H., J. K. Wieser, and J. H. Yu.** 1998. Asexual sporulation in *Aspergillus nidulans*. Microbiol. Mol. Biol. Rev. **62:**35–54.
3. **Adams, T. H., and J. H. Yu.** 1998. Coordinate control of secondary metabolite production and asexual sporulation in *Aspergillus nidulans*. Curr. Opin. Microbiol. **1:**674–677.
4. Reference deleted.
5. **Andrianopoulos, A., and W. E. Timberlake.** 1994. The *Aspergillus nidulans abaA* gene encodes a transcriptional activator that acts as a genetic switch to control development. Mol. Cell. Biol. **14:**2503–2515.
6. **Aramayo, R., Y. Peleg, R. Addison, and R. Metzenberg.** 1996. Asm-1+, a *Neurospora crassa* gene related to transcriptional regulators of fungal development. Genetics **144:**991–1003.
7. **Bayram, O., et al.** 2008. VelB/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. Science **320:**1504–1506.
8. **Borneman, A. R., M. J. Hynes, and A. Andrianopoulos.** 2002. A basic helix-loop-helix protein with similarity to the fungal morphological regulators, Phd1p, Efg1p and StuA, controls conidiation but not dimorphic growth in *Penicillium marneffei*. Mol. Microbiol. **44:**621–631.
9. **Boylan, M. T., P. M. Mirabito, C. E. Willett, C. R. Zimmerman, and W. E. Timberlake.** 1987. Isolation and physical characterization of three essential conidiation genes from *Aspergillus nidulans*. Mol. Cell. Biol. **7:**3113–3118.
10. **Busby, T. M., K. Y. Miller, and B. L. Miller.** 1996. Suppression and enhancement of the *Aspergillus nidulans* medusa mutation by altered dosage of the bristle and stunted genes. Genetics **143:**155–163.
11. **Calvo, A. M.** 2008. The VeA regulatory system and its role in morphological and chemical development in fungi. Fungal Genet. Biol. **45:**1053–1061.
12. **Calvo, A. M., R. A. Wilson, J. W. Bok, and N. P. Keller.** 2002. Relationship between secondary metabolism and fungal development. Microbiol. Mol. Biol. Rev. **66:**447–459.
13. **Chang, Y. C., and W. E. Timberlake.** 1993. Identification of *Aspergillus* BrlA response elements (BREs) by genetic selection in yeast. Genetics **133:**29–38.
14. **Clutterbuck, A. J.** 1969. A mutational analysis of conidial development in *Aspergillus nidulans*. Genetics **63:**317–327.
15. **Coyle, C. M., S. C. Kenaley, W. R. Rittenour, and D. G. Panaccione.** 2007. Association of ergot alkaloids with conidiation in *Aspergillus fumigatus*. Mycologia **99:**804–811.
16. **Crameri, R., and K. Blaser.** 1996. Cloning *Aspergillus fumigatus* allergens by

the pJuFo filamentous phage display system. Int. Arch. Allergy Immunol. **110:**41–45.
17. **Dutton, J. R., S. Johns, and B. L. Miller.** 1997. StuAp is a sequence-specific transcription factor that regulates developmental complexity in *Aspergillus nidulans*. EMBO J. **16:**5710–5721.
18. **Etxebeste, O., et al.** 2008. Basic-zipper-type transcription factor FlbB controls asexual development in *Aspergillus nidulans*. Eukaryot. Cell **7:**38–48.
19. **Garcia-Rico, R. O., et al.** 2008. The heterotrimeric G alpha protein Pga1 regulates biosynthesis of penicillin, chrysogenin and roquefortine in *Penicillium chrysogenum*. Microbiology **154:**3567–3578.
20. **Garzia, A., et al.** 2009. *Aspergillus nidulans* FlbE is an upstream developmental activator of conidiation functionally associated with the putative transcription factor FlbB. Mol. Microbiol. **71:**172–184.
21. **Garzia, A., O. Etxebeste, E. Herrero-Garcia, U. Ugalde, and E. A. Espeso.** 2010. The concerted action of bZip and cMyb transcription factors FlbB and FlbD induces *brlA* expression and asexual development in *Aspergillus nidulans*. Mol. Microbiol. **75:**1314–1324.
22. **Gimeno, C. J., and G. R. Fink.** 1994. Induction of pseudohyphal growth by overexpression of PHD1, a *Saccharomyces cerevisiae* gene related to transcriptional regulators of fungal development. Mol. Cell. Biol. **14:**2100–2112.
23. **Haas, H., K. Albermann, I. Zadra, and G. Stöffler.** 1997. Overexpression of *nreB*, a new GATA factor-encoding gene of *Penicillium chrysogenum*, leads to repression of the nitrate assimilatory gene cluster. J. Biol. Chem. **272:**22576–22582.
24. **Han, S., and T. H. Adams.** 2001. Complex control of the developmental regulatory locus *brlA* in *Aspergillus nidulans*. Mol. Genet. Genomics **266:**260–270.
25. **Han, S., J. Navarro, R. A. Greve, and T. H. Adams.** 1993. Translational repression of *brlA* expression prevents premature development in *Aspergillus*. EMBO J. **12:**2449–2457.
26. **Hicks, J. K., J. H. Yu, N. P. Keller, and T. H. Adams.** 1997. *Aspergillus* sporulation and mycotoxin production both require inactivation of the FadA G alpha protein-dependent signaling pathway. EMBO J. **16:**4916–4923.
27. **Hoff, B., et al.** 2010. Two components of a velvet-like complex control hyphal morphogenesis, conidiophore development and penicillin biosynthesis in *Penicillium chrysogenum*. Eukaryot. Cell **9:**1236–1250.
28. **Hoff, B., J. Kamerewerd, C. Sigl, I. Zadra, and U. Kück.** 2010. Homologous recombination in the antibiotic producer *Penicillium chrysogenum*: strain Delta Pcku70 shows up-regulation of genes from the HOG pathway. Appl. Microbiol. Biotechnol. **85:**1081–1094.
29. **Hoff, B., S. Pöggeler, and U. Kück.** 2008. Eighty years after its discovery, Fleming's *Penicillium* strain discloses the secret of its sex. Eukaryot. Cell **7:**465–470.
30. **IpCho, S. V. S., et al.** 2010. The transcription factor SnStuA regulates central carbon metabolism, mycotoxin production and effector gene expression in the wheat pathogen *Stagonospora nodorum*. Eukaryot. Cell **9:**1100–1108.
31. **Irizarry, R. A., et al.** 2003. Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Res. **31:**e15.
32. **Irizarry, R. A., et al.** 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics **4:**249–264.
33. **Kosalkova, K., et al.** 2009. The global regulator LaeA controls penicillin biosynthesis, pigmentation and sporulation, but not roquefortine C synthesis in *Penicillium chrysogenum*. Biochimie **91:**214–225.
34. **Langfelder, K., M. Streibel, B. Jahn, G. Haase, and A. A. Brakhage.** 2003. Biosynthesis of fungal melanins and their importance for human pathogenic fungi. Fungal Genet. Biol. **38:**143–158.
35. **Lara-Ortiz, T., H. Riveros-Rosas, and J. Aguirre.** 2003. Reactive oxygen species generated by microbial NADPH oxidase NoxA regulate sexual development in *Aspergillus nidulans*. Mol. Microbiol. **50:**1241–1255.
36. **Lee, B. N., and T. H. Adams.** 1994. Overexpression of *flbA*, an early regulator of *Aspergillus* asexual sporulation, leads to activation of *brlA* and premature initiation of development. Mol. Microbiol. **14:**323–334.
37. **Lee, B. N., and T. H. Adams.** 1996. *fluG* and *flbA* function interdependently to initiate conidiophore development in *Aspergillus nidulans* through *brlA*β activation. EMBO J. **15:**299–309.
38. **Mah, J. H., and J. H. Yu.** 2006. Upstream and downstream regulation of asexual development in *Aspergillus fumigatus*. Eukaryot. Cell **5:**1585–1595.
39. **Marshall, M. A., and W. E. Timberlake.** 1991. *Aspergillus nidulans* WetA activates spore-specific gene expression. Mol. Cell. Biol. **11:**55–62.
40. **Melin, P., J. Schnurer, and E. G. Wagner.** 2003. Characterization of *phiA*, a gene essential for phialide development in *Aspergillus nidulans*. Fungal Genet. Biol. **40:**234–241.
41. **Miller, K. Y., T. M. Toennis, T. H. Adams, and B. L. Miller.** 1991. Isolation and transcriptional characterization of a morphological modifier: the *Aspergillus nidulans* stunted (*stuA*) gene. Mol. Gen. Genet. **227:**285–292.
42. **Miller, K. Y., J. Wu, and B. L. Miller.** 1992. StuA is required for cell pattern formation in *Aspergillus*. Genes Dev. **6:**1770–1782.
43. **Minuth, W., P. Tudzynski, and K. Esser.** 1982. Extrachromosomal genetics of *Cephalosporium acremonium*. Curr. Genet. **25:**34–40.
44. **Mirabito, P. M., T. H. Adams, and W. E. Timberlake.** 1989. Interactions of three sequentially expressed genes control temporal and spatial specificity in *Aspergillus* development. Cell **57:**859–868.
45. **Navarro, R. E., M. A. Stringer, W. Hansberg, W. E. Timberlake, and J. Aguirre.** 1996. *catA*, a new *Aspergillus nidulans* gene encoding a developmentally regulated catalase. Curr. Genet. **29:**352–359.
46. **Ni, M., and J. H. Yu.** 2007. A novel regulator couples sporogenesis and trehalose biogenesis in *Aspergillus nidulans*. PLoS One **2:**e970.
47. **Nielsen, M. L., L. Albertsen, G. Lettier, J. B. Nielsen, and U. H. Mortensen.** 2006. Efficient PCR-based gene targeting with a recyclable marker for *Aspergillus nidulans*. Fungal Genet. Biol. **43:**54–64.
48. **Nowrousian, M., C. Ringelberg, J. C. Dunlap, J. L. Loros, and U. Kück.** 2005. Cross-species microarray hybridization to identify developmentally regulated genes in the filamentous fungus *Sordaria macrospora*. Mol. Genet. Genomics **273:**137–149.
49. **Ogawa, M., M. Tokuoka, F. J. Jin, T. Takahashi, and Y. Koyama.** 2010. Genetic analysis of conidiation regulatory pathways in koji-mold *Aspergillus oryzae*. Fungal Genet. Biol. **47:**10–18.
50. **Ohara, T., and T. Tsuge.** 2004. FoSTUA, encoding a basic helix-loop-helix protein, differentially regulates development of three kinds of asexual spores, macroconidia, microconidia, and chlamydospores, in the fungal plant pathogen *Fusarium oxysporum*. Eukaryot. Cell **3:**1412–1422.
51. **Paris, S., et al.** 2003. Conidial hydrophobins of *Aspergillus fumigatus*. Appl. Environ. Microbiol. **69:**1581–1588.
52. **Prade, R. A., and W. E. Timberlake.** 1993. The *Aspergillus nidulans brlA* regulatory locus consists of overlapping transcription units that are individually required for conidiophore development. EMBO J. **12:**2439–2447.
53. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
54. **Schier, N., and R. Fischer.** 2002. The *Aspergillus nidulans* cyclin PclA accumulates in the nucleus and interacts with the central cell cycle regulator $NimX^{Cdc2}$. FEBS Lett. **523:**143–146.
55. **Schier, N., R. Liese, and R. Fischer.** 2001. A Pcl-like cyclin of *Aspergillus nidulans* is transcriptionally activated by developmental regulators and is involved in sporulation. Mol. Cell. Biol. **21:**4075–4088.
56. **Schmitt, E. K., et al.** 2004. Winged helix transcription factor CPCR1 is involved in regulation of beta-lactam biosynthesis in the fungus *Acremonium chrysogenum*. Eukaryot. Cell **3:**121–134.
57. **Seo, J. A., Y. J. Guan, and J. H. Yu.** 2006. FluG-dependent asexual development in *Aspergillus nidulans* occurs via derepression. Genetics **172:**1535–1544.
58. **Sheppard, D. C., et al.** 2005. The *Aspergillus fumigatus* StuA protein governs the up-regulation of a discrete transcriptional program during the acquisition of developmental competence. Mol. Biol. Cell **16:**5866–5879.
59. **Sigl, C., M. Handler, G. Sprenger, H. Kürnsteiner, and I. Zadra.** 2010. A novel homologous dominant selection marker for genetic transformation of *Penicillium chrysogenum*: overexpression of squalene epoxidase-encoding *ergA*. J. Biotechnol. **150:**307–311.
60. **Smyth, G. K.** 2004. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. **3:**article 3.
61. **Spröte, P., and A. A. Brakhage.** 2007. The light-dependent regulator velvet A of *Aspergillus nidulans* acts as a repressor of the penicillin biosynthesis. Arch. Microbiol. **188:**69–79.
62. **Stoldt, V. R., A. Sonneborn, C. E. Leuker, and J. F. Ernst.** 1997. Efg1p, an essential regulator of morphogenesis of the human pathogen *Candida albicans*, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. EMBO J. **16:**1982–1991.
63. **Stringer, M. A., R. A. Dean, T. C. Sewall, and W. E. Timberlake.** 1991. Rodletless, a new *Aspergillus* developmental mutant induced by directed gene inactivation. Genes Dev. **5:**1161–1171.
64. **Stringer, M. A., and W. E. Timberlake.** 1995. *DewA* encodes a fungal hydrophobin component of the *Aspergillus* spore wall. Mol. Microbiol. **16:**33–44.
65. **Tag, A., et al.** 2000. G-protein signalling mediates differential production of toxic secondary metabolites. Mol. Microbiol. **38:**658–665.
66. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
67. **Tong, X. Z., et al.** 2007. GcSTUA, an APSES transcription factor, is required for generation of appressorial turgor pressure and full pathogenicity of *Glomerella cingulata*. Mol. Plant Microbe Interact. **20:**1102–1111.
68. **Tsai, H. F., M. H. Wheeler, Y. C. Chang, and K. J. Kwon-Chung.** 1999. A developmentally regulated gene cluster involved in conidial pigment biosynthesis in *Aspergillus fumigatus*. J. Bacteriol. **181:**6469–6477.
69. **Tsitsigiannis, D., T. M. Kowieski, R. Zarnowski, and N. P. Keller.** 2004. Endogenous lipogenic regulators of spore balance in *Aspergillus nidulans*. Eukaryot. Cell **3:**1398–1411.
70. **Twumasi-Boateng, K., et al.** 2009. Transcriptional profiling identifies a role for BrlA in the response to nitrogen depletion and for StuA in the regulation of secondary metabolite clusters in *Aspergillus fumigatus*. Eukaryot. Cell **8:**104–115.
71. **van den Berg, M. A., et al.** 2008. Genome sequencing and analysis of the filamentous fungus *Penicillium chrysogenum*. Nat. Biotechnol. **26:**1161–1168.

72. **Ward, M. P., C. J. Gimeno, G. R. Fink, and S. Garrett.** 1995. SOK2 may regulate cyclic AMP-dependent protein kinase-stimulated growth and pseudohyphal development by repressing transcription. Mol. Cell. Biol. **15:** 6854–6863.
73. Reference deleted.
74. **Wettenhall, J. M., K. M. Simpson, K. Satterley, and G. K. Smyth.** 2006. affylmGUI: a graphical user interface for linear modeling of single channel microarray data. Bioinformatics **22:**897–899.
75. **Wieser, J., and T. H. Adams.** 1995. *flbD* encodes a Myb-like DNA-binding protein that coordinates initiation of *Aspergillus nidulans* conidiophore development. Genes Dev. **9:**491–502.
76. **Wieser, J., B. N. Lee, J. Fondon III, and T. H. Adams.** 1994. Genetic requirements for initiating asexual development in Aspergillus nidulans. Curr. Genet. **27:**62–69.
77. **Windhofer, F., K. Hauck, D. E. Catcheside, U. Kück, and F. Kempken.** 2002. Ds-like restless deletion derivates occur in *Tolypocladium inflatum* and two foreign hosts, *Neurospora crassa* and *Penicillium chrysogenum*. Fungal Genet. Biol. **35:**171–182.
78. **Wu, J. G., and B. L. Miller.** 1997. *Aspergillus* asexual reproduction and sexual reproduction are differentially affected by transcriptional and translational mechanisms regulating stunted gene expression. Mol. Cell. Biol. **17:**6191–6201.
79. **Yin, W.-B., A. Grundmann, J. Cheng, and S.-M. Li.** 2009. Acetylaszonalenin biosynthesis in *Neosartorya fischeri*: identification of the biosynthetic gene cluster by genomic mining and functional proof of the genes by biochemical investigation. J. Biol. Chem. **284:**100–109.
80. **Zadra, I., B. Abt, W. Parson, and H. Haas.** 2000. *xylP* promoter-based expression system and its use for antisense downregulation of the *Penicillium chrysogenum* nitrogen regulator NRE. Appl. Environ. Microbiol. **66:**4810–4816.