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Activation of a Silent Fungal Polyketide Biosynthesis Pathway through Regulatory Cross Talk with a Cryptic Nonribosomal Peptide Synthetase Gene Cluster▽†

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Filamentous fungi produce numerous natural products that constitute a consistent source of potential drug leads, yet it seems that the majority of natural products are overlooked since most biosynthesis gene clusters are silent under standard cultivation conditions. Screening secondary metabolite genes of the model fungus *Aspergillus nidulans*, we noted a silent gene cluster on chromosome II comprising two nonribosomal peptide synthetase (NRPS) genes, *inpA* and *inpB*, flanked by a regulatory gene that we named *scpR* for secondary metabolism cross-pathway regulator. The induced expression of the *scpR* gene using the promoter of the alcohol dehydrogenase AlcA led to the transcriptional activation of both the endogenous *scpR* gene and the NRPS genes. Surprisingly, metabolic profiling of the supernatant of mycelia overexpressing *scpR* revealed the production of the polyketide asperfuranone. Through transcriptome analysis we found that another silent secondary metabolite gene cluster located on chromosome VIII coding for asperfuranone biosynthesis was specifically induced. Quantitative reverse transcription-PCR proved the transcription not only of the corresponding polyketide synthase (PKS) biosynthesis genes, *afoE* and *afoG*, but also of their activator, *afoA*, under *alcAp-scpR*-inducing conditions. To exclude the possibility that the product of the *inp* cluster induced the asperfuranone gene cluster, a strain carrying a deletion of the NRPS gene *inpB* and, in addition, the *alcAp-scpR* overexpression cassette was generated. In this strain, under inducing conditions, transcripts of the biosynthesis genes of both the NRPS-containing gene cluster *inp* and the asperfuranone gene cluster except gene *inpB* were detected. Moreover, the existence of the polyketide product asperfuranone indicates that the transcription factor ScpR controls the expression of the asperfuranone biosynthesis gene cluster. This expression as well as the biosynthesis of asperfuranone was abolished after the deletion of the asperfuranone activator gene *afoA*, indicating that ScpR binds to the *afoA* promoter. To the best of our knowledge, this is the first report of regulatory cross talk between two biosynthesis gene clusters located on different chromosomes.

Fungi are prolific producers of low-molecular-weight molecules with various biological activities, such as antibiotic, immunosuppressive, and antitumor activities (5, 12, 15). However, bioinformatic analyses of the sequenced fungal genomes indicate that their potential to produce secondary metabolites is greatly underestimated, and it appears that many cryptic natural products await discovery (6, 9, 11). For example, mining the published *Aspergillus nidulans* genome sequence led to the identification of 53 putative secondary metabolite gene clusters (21). This number does not reflect the much lower number of secondary metabolites identified from the fungus, until today. Since most of the fungal biosynthesis gene clusters are silent under laboratory conditions, one of the major challenges is to understand the physiological role of these genes in the field (5, 6). Recently, we have shown that the intimate interaction of the fungus *Aspergillus nidulans* with a distinct soil-dwelling bacterium, i.e., *Streptomyces hygroscopicus*, led to activation of a so far silent polyketide biosynthesis gene cluster, indicating that communication between microorganisms might play a key role in activating silent gene clusters (18). It is also known that particular stress conditions, variations of culture conditions, and epigenetic modulation may induce significant changes in the metabolome (3, 17, 23). In most cases, however, the physiological conditions inducing such gene clusters are not understood, and it is often not possible to predict the complex regulatory circuits involved in pathway regulation (6, 16). Despite this limitation, combining genomic data, genetic engineering, and analytical techniques is a promising avenue to discover novel and potentially bioactive natural products (19). Nevertheless, more targeted approaches to induce selected biosynthetic pathways are desirable. Recently, we demon-

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† Supplemental material for this article may be found at http://aem.asm.org/.

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TABLE 1. Fungal strains used in this study

Strain	Description	Reference or source
AXB4A2	*pyrG89 pabaA1 fwA1 bga0 argB2*::pAXB4A *argB*$^+$	22a
AXB4A	*biA1 bga0 argB2*::pAXB4A *argB*$^+$	3a
TN02A7	*pyrG89 pyroA4 nkuA*::*argB riboB2*	20
SB3.5	*pabaA1 fwA1 bga0 argB2*::*pAXB4A argB*$^+$ *pyrG89*::pAL4scpR	This study
SB2.3	*pyrG89 pyroA4 nkuA*::*argB riboB2*::*inpB riboB2*	This study
SB2.7	*fwA1 pyrG89*::*pAL4scpR riboB2*::*inpB* prototroph progeny Recovered from meiotic cross of strains SB3.5 and SB2.3	This study
ALF1.1	*fwA1 bga0 argB2*::*pAXB4A argB*$^+$ *pyrG89*::pAL4scpR *pabaA1*::*afoA*	This study

strated that it is possible to activate a specific silent fungal gene cluster by the controlled expression of a gene coding for a pathway-specific transcription factor (2). Since various biosynthesis gene clusters contain such putative activator genes, we postulated that this is a generally applicable method for the activation of silent gene clusters.

Here we report another successful activation of a silent gene cluster using a potentially pathway-specific transcription factor. Strikingly, however, expression of this activator gene also led to the activation of yet another silent gene cluster located on a different chromosome. To the best of our knowledge, this is the first report showing regulatory cross talk between two different fungal biosynthesis gene clusters.

MATERIALS AND METHODS

Strains and plasmids. The *scpR* overexpression strain SB3.5 (Table 1) was obtained by transformation of *A. nidulans* AXB4A2 with the expression vector pAL4 (22) containing *scpR* in fusion with the *alcA* promoter and the *pyr-4* gene of *Neurospora crassa* (pAL4scpR) complementing *pyrG* mutants of *A. nidulans* to uracil prototrophy.

The *scpR* gene was amplified by PCR from *A. nidulans* genomic DNA with the oligonucleotides 3492 XmaI for and 3492 XmaI rev (Table 2). Mutant strain SB2.3 with the *inpB* deletion was obtained by transformation of *A. nidulans* TN02A7 (14) using a linear DNA fragment that encoded 1-kb sequences homologous to the regions upstream and downstream of gene *inpB* (AN3496) flanking the *riboB2* gene of *A. nidulans* as a selectable marker gene (20). DNA fragments encoding the upstream and downstream sequences of *inpB* were obtained by PCR with *A. nidulans* genomic DNA as the template and the oligonucleotide pairs 3496 up SspI for and 3496 up SspI rev and 3496 dwn XhoI for and 3496 dwn XhoI rev, respectively (Table 2).

The obtained recombinant plasmid was designated pKOinpB. *A. nidulans* strain SB2.7, encoding the deletion of *inpB* and the *alcAp-scpR* gene fusion in multiple copies ectopically integrated into the genome, was created by crossing strain SB3.5 with strain SB2.3. Plasmids were propagated in *Escherichia coli* DH5α (Invitrogen).

Mutant strain ALF1.1 with the *afoA* deletion was obtained by transformation of *A. nidulans* SB3.5 using a linear DNA fragment that encoded 1.3-kb sequences homologous to the regions upstream and downstream of gene *afoA* (AN1029) flanking the *pabaA1* gene of *A. nidulans* as a selectable marker gene (see Fig. S1 in the supplemental material). This DNA fragment was created by fusion PCR of three precursor DNA fragments, namely, ko1029up, ko1029down, and the *pabaA1* cassette amplified from *A. nidulans* DNA with primer pairs 1029 up for and 1029 up rev, 1029 dwn for and 1029 dwn rev, and pabaA1 for and pabaA1 rev, respectively. Fusion PCR was carried out with primers 1029 ko for and 1029 ko rev (Table 2).

Media and cultivation of strains. *E. coli* was grown at 37°C in LB medium supplemented with ampicillin (100 μg ml^{-1}). *A. nidulans* strains were cultivated in *Aspergillus* minimal medium (AMM [4]). Required supplements were added as follows: uridine (1 mg ml^{-1}), *p*-aminobenzoic acid (3 μg ml^{-1}), or biotin (0.04 mg ml^{-1}). As a preculture, a 50-ml AMM overnight culture inoculated with 5×10^6 conidia ml^{-1} was used. The biomass was separated from the medium using Miracloth material (Calbiochem), washed in 0.9% (wt vol^{-1}) NaCl, and inoculated into 50 ml of fresh AMM. For growth under *alcAp*-repressing conditions, glucose (2%, wt vol^{-1}) was used as the carbon source. For growth under *alcAp*-inducing conditions, lactose (2%, wt vol^{-1}) was used as the carbon source and 10 mM cyclopentanone was added to the medium. After 24 h, samples were taken for Northern blot analysis, quantitative reverse transcription-PCR (qRT-PCR), transcriptome analysis, and high-pressure liquid chromatography (HPLC) analysis. For structure elucidation, a total of 8 liters fermentation broth divided into 250-ml portions in 1-liter flasks was incubated for 48 h under inducing conditions. Wild-type strain AXB4A was incubated in 250 ml AMM in a 1-liter flask as a control. Fungal cultures were homogenized and extracted with ethyl acetate.

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′ → 3′)
3492 XmaI for	CCCGGGGTATGAACACCATGAACAACATG CATCTC
3492 XmaI rev	CCCGGGTCAATCAAACACCACCCCAGAAGC CGCG
3496 up SspI for	AATATTCCTTGTTGAAGTGGTGTACAGGGG
3496 up SspI rev	AATATTAATGATGGTGATGATGATGCTTGT
3496 dwn XhoI for	CTCGAGGCTATGTTATACATCTCTGACACG
3496 dwn XhoI rev	CTCGAGTTTTGCGTCCATTGATCGGACCCA
1029 up for	GGAATCAGACCTTTAATTCCTCAGGCCGAG
1029 up rev	TTTCTACAGATCTGGCAATGCCTCTGTCCG GACCGGAAAC
pabaA1for	TCCGGACACAGGCATTGCCAGATCTGTAGA AAGGTCTGC
pabaA1rev	GCCAGCTAATCAGTCTATCTGGACATGCGA CGGAGATCG
1029 dwn for	GTCGCATGTCCAGATAGACTGATTAGCTGG CCGATAGC
1029 dwn rev	GCTTCAATTCCTCAACCTGGACCAGC
1029 ko for	CAGGCCGAGTCGGGATCATTGAAG
1029 ko rev	GTGACCGTGCAGATGGCCTGATAC
3490 for	CCCCTCCAGTATGGGGCCCTGAGATGATGG
3490 rev	CCCCGCCAGGCCGTCAAGTGTGCCAGGATC
3491 for	GGAAAAGACTCAGACCCCGTCGCTCACGCC
3491 rev	CACCAGCATAAAAGCGAGCATGATAA CGCC
3492 for	GAACACCATGAACAACATGCATCTCTACGC
3492 rev	CCATCTGACTGGGCTCGCTCCAATCGGCC
3495 for	GTCTCACTCAATGTCATCATCATCATCATC
3495 rev	CCAACCCCCAGGCCGCATTGGCGATGGTCG
3496 for	CCTGGCGCTATAGAATCCTCCCCATCGGAG
3496 rev	GAGAGACCTGGACCAGCTGTAGTCGG TGTG
3497 for	GAACGCCGTCGACAGTTTACTTGGAG TAGG
3497 rev	CGGTAGCTTGAGCGCTTCCATACGTGCATG
1029 for	CAGTTATTTTTTATTCTAACCCTGCTAG
1029 rev	CTATGTACAATACCACCTCGTATCCTGTC
1036 for	GTACAGCTGGCCTGACGATTCCATCCATCG
1036 rev	GGGATGAACCCCTTCTCCATGGCCAGCACG
3492 for qRT-PCR	GACGATGAACGAGATCGAGAC
3492 rev qRT-PCR	GTGGATGGTGCAATATGCTAGG
3495 for qRT-PCR	ACAATGCAGGACCACATCAG
3495 rev qRT-PCR	GTCAATGAGAGGGCGAACAG
3496 for qRT-PCR	TCAGTGGATCACACCAGGAG
3496 rev qRT-PCR	CGAGACATCAACACCGAGAG
1029 for qRT-PCR	ACGAGTGTTAAGCGAGCTG
1029 rev qRT-PCR	CAATACCACCTCGTATCCTGTC
1034 for qRT-PCR	GCAACCAGTTTACGCAAGTTC
1034 rev qRT-PCR	GCTCTTCTCTTCGTCTTCGTC
1036 for qRT-PCR	TTGAGAAGCCGAATCCGAAG
1036 rev qRT-PCR	AAAGTTGTTCACGCTCGCC
6542 for qRT-PCR	CACCCTTGTTCTTGTTTTGCTC
6542 rev qRT-PCR	CAAGTTCGCTTTGGCAACG

Preparation of RNA, reverse transcription, labeling, ASMA, array hybridization, and data processing. The hybridization of the *Aspergillus* secondary metabolism array (ASMA) using cDNA of reverse-transcribed RNA from *A. nidulans* and the data processing were performed as described by Schroeckh et al. (18).

Northern blot analyses. Northern blot probes were labeled with digoxigenin-11-dUTP (DIG; Roche). The primers used for the amplification of the probes are listed in Table 2. TriSURE reagent (Bioline)-isolated total RNA samples from *A. nidulans* were separated on formaldehyde-containing agarose gels and blotted on Hybond-N^+ positively charged nylon membranes (GE Healthcare). Hybridization was carried out in DIG-easy Hyb buffer, followed by binding of antidigoxigenin-alkaline phosphatase Fab fragments and application of CDP-Star ready-to-use solution (Roche). Thereby, fluorescence signals were generated and visualized on Super RX X-ray films (Fuji).

qRT-PCR. To quantify transcript levels by qRT-PCR, total RNA was purified and its quality was controlled as described previously (18). Ten micrograms of DNase I-treated RNA was utilized as a template for cDNA synthesis for 3 h at 48°C using Superscript III reverse transcriptase (Invitrogen). qRT-PCR was performed on an Applied Biosystems StepOnePlus real-time PCR system in triplicate for each sample. The *A. nidulans* β-actin gene AN6542 was used as an internal standard for calculation of expression levels. The cDNA samples were diluted 10 times for amplification to obtain EvaGreen reagent (Biotium)-labeled PCR fragments (GeneAmp Fast PCR master mix; Applied Biosystems) by using primers specific for *scpR*, *inpA*, *inpB*, *afoA*, *afoE*, *afoG*, and the β-actin gene (Table 2), resulting in amplicons of 83, 84, 86, 111, 101, 116, and 105 bp, respectively. The cycling parameters included an initial DNA denaturation step at 95°C for 2 min, followed by 40 cycles with DNA denaturation at 95°C for 10 s and primer annealing and extension at 62°C for 15 s. Controls with no added template were included for each primer pair to exclude primer dimers from interfering with amplification detection. qRT-PCR results were analyzed as described previously (18).

Preparation of chromosomal DNA and Southern blot analysis. Genomic DNA from *A. nidulans* mycelia was isolated using a MasterPure yeast DNA purification kit (Epicentre Biotechnologies), according to a modified isolation protocol (13). Cells were disrupted in a Precellys24 homogenizer (Peqlab) for 3 cycles (30 s grinding/30 s pause) at a rotation speed of 6,500 rpm in an Innuspeed lysis tube A (Analytik Jena). Southern blot analysis was carried out by using a nonradioactively labeled DNA probe as described for Northern blot analysis.

Extraction and isolation of compounds. NMR spectra were recorded on a Bruker Avance DRX 500 instrument. Spectra were referenced to the residual solvent signals. Analytical HPLC was performed on a Shimadzu HPLC system consisting of an autosampler, high-pressure pumps, a column oven, and a photodiode array detector (PDA), using a C_{18} column (250 by 4.6 mm; Eurospher 100-5) and gradient elution with acetonitrile (MeCN)–0.1% (vol/vol) trifluoroacetic acid (TFA) from (0.5/99.5 in 30 min to MeCN–0.1% (vol/vol) TFA at 100/0 and then 100% MeCN for 10 min, and the flow rate was 1 ml min^{-1}. Preparative HPLC was performed using a Shimadzu HPLC system (LC-8A) with PDA and a C_{18} column (250 by 20 mm; Eurospher 100-5).

The entire fermentation broth was extracted with ethyl acetate, and the combined extracts were concentrated under reduced pressure. The crude extract was separated by size-exclusion chromatography on a Sephadex LH-20 column using methanol as an eluent. Metabolite-containing fractions were further purified by preparative HPLC (gradient mode with MeCN–H_2O at 2% MeCN to 83% MeCN in 30 min and then with 83% MeCN for 10 min; flow rate, 10 ml min^{-1}). Additional physicochemical data for asperfuranone are shown in Table S1 in the supplemental material.

Identification of putative binding site for C2H2 transcription factor AN3492. The whole intergenic regions of the genes *inpA*/*inpB*, *inpC*/*inpD*, and *afoA*/AN1030 were submitted to the MEME tool for motif prediction (1). Only one motif was found in all three sequences with a *P* value of 10^{-7}. This motif was further submitted to the TOMTOM tool (10) (see Fig. S2 in the supplemental material) in the MEME suite of programs in order to find similarities to known transcription factor binding sites. The motif showed a significant similarity ($P = 10^{-3}$) to the matrix for RME1, a yeast zinc-finger transcription factor with a C2H2 domain.

RESULTS

Activation of silent *inp* gene cluster of *A. nidulans* by overexpression of its putative pathway-specific regulatory gene *scpR*. The secondary metabolite gene cluster *inp* (see Fig. 4A) of *A. nidulans* comprises two nonribosomal peptide synthetase (NRPS) genes, which were named as described by von Döhren (21): interacting NRPSs *inpA* (AN3495) and *inpB* (AN3496). The metabolic product of this gene cluster is unknown, and no related gene cluster can be found among the aspergilli (21). The product of the NRPSs InpA and InpB was proposed to be a tripeptide with a reduced C terminus (21). Since the NRPS genes are not transcribed under standard laboratory conditions (Fig. 1), we reasoned that this gene cluster is a typical silent gene cluster. The *inp* locus contains a putative activator gene, which we designated "secondary metabolism cross-pathway regulator" *scpR* (AN3492). Its deduced gene product resembles a typical C2H2-type zinc-finger transcription factor. For overexpression of this putative pathway-specific regulatory gene (*scpR*), we applied the inducible alcohol dehydrogenase promoter *alcAp* of *A. nidulans* (2). Transformation of *A. nidulans* strain AXB4A2 with the plasmid harboring the *scpR* gene under the control of *alcAp* resulted in several transformant strains that are chemically inducible. By Southern blot analysis, a transformant designated SB3.5 with multiple ectopic integrations of the *alcAp-scpR* gene fusion was identified (see Fig. S1A in the supplemental material). The transcription of the regulatory gene was unequivocally proven by Northern blot analysis (Fig. 1A). Under inducing conditions (with cyclopentanone), a significant amount of *scpR* mRNA was detected in strain SB3.5. This mRNA was absent under noninducing conditions, confirming that this gene is silent under standard laboratory conditions. As expected, the genes encoding both NRPS genes, *inpA* and *inpB*, were also transcribed under inducing conditions in strain SB3.5, indicating that the regulatory gene *scpR* controls the expression of these biosynthesis genes (Fig. 1A and B) and, moreover, that these two genes are also silent under standard laboratory conditions.

Asperfuranone is produced under *alcAp-scpR*-inducing conditions. The induced expression of the biosynthesis genes prompted us to monitor the metabolome of the transformant strain for new metabolites under inducing conditions. Comparison of the HPLC profiles of the extracts from the transformant strain SB3.5 under inducing conditions with the transformant strain under noninducing conditions revealed the formation of a new metabolite by the induced transformant (Fig. 2A). The compound was isolated from the extract of an upscaled culture (8 liters), and its structure was elucidated by one-dimensional and two-dimensional nuclear magnetic resonance (NMR) and mass spectrometry (MS) measurements. Compound 1 has a molecular formula of $C_{19}H_{24}O_5$, as established by high-resolution electron spray ionization mass spectrometry analysis and ^{13}C NMR analysis (Fig. 2B). The ^1H NMR spectrum displayed four methyl, two methine, two methylene, and three olefinic proton signals as well as one aromatic proton (δ 9.04 ppm). H,H-correlated spectroscopy (H,H-COSY) and heteronuclear multiple bond correlation (HMBC) couplings established the side chain. The substitution pattern of the ring system was deduced from the HMBC correlations, as shown in Fig. 2C. The HMBC coupling of H-16 with C-14, which could be observed only when d_6-acetone was used as the solvent, and the nuclear Overhauser effect (NOE) signal between H-16 and H-7 revealed the connectivity of both parts of the molecule. In the course of our studies, this very compound was described (7) and named asperfuranone (Fig. 2B).

Transcriptome analysis reveals activation of the physically unrelated silent asperfuranone gene cluster on chromosome VIII by overexpression of *scpR*. Asperfuranone is a polyketide metabolite and thus is clearly not the product of the predicted

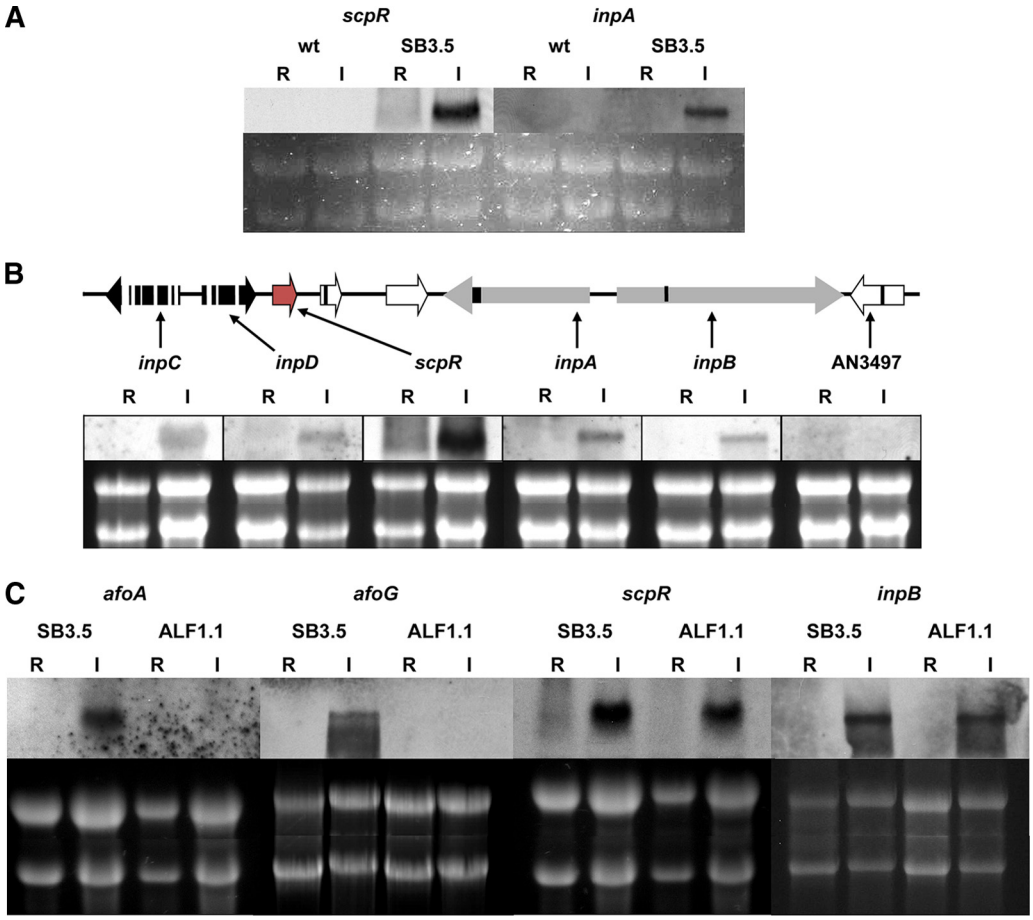

FIG. 1. Northern blot analysis. Total RNA (10 μg) from the *A. nidulans* wild-type and transformant strains was analyzed. All strains were incubated in AMM under *alcAp*-repressing (lanes R) and *alcAp*-inducing (lanes I) conditions. *scpR* (AN3492) blots were hybridized with an *scpR* full-length 834-bp DNA fragment obtained by PCR from *A. nidulans* chromosomal DNA with the oligonucleotides 3492 XmaI for and 3492 XmaI rev (Table 2). (A) *A. nidulans* wild-type (wt) strain AXB4A and strain SB3.5 *alcAp-scpR*. The 800-bp *inpA* (AN3495) probe was obtained by PCR from *A. nidulans* chromosomal DNA with the oligonucleotides 3495 for and 3495 rev (Table 2). (B) Strain SB3.5 *alcAp-scpR*. Probes of 800 bp for *inpA* (AN3495), *inpB* (AN3496), *inpC* (AN3490), *inpD* (AN3491), and AN3497 were obtained by PCR from *A. nidulans* chromosomal DNA with oligonucleotide pairs 3495 for and 3495 rev, 3496 for and 3496 rev, 3490 for and 3490 rev, 3491 for and 3491 rev, and 3497 for and 3497 rev, respectively (Table 2). (C) Strain SB3.5 *alcAp-scpR* and strain ALF1.1 *alcAp-scpR* carrying the *afoA* deletion. The probes for *afoA* (AN1029), *afoG* (AN1036), and *inpB* (AN3496) were obtained by PCR from *A. nidulans* chromosomal DNA with oligonucleotide pairs 1029 for and 1029 rev, 1036 for and 1036 rev, and 3496 for and 3496 rev, respectively (Table 2).

NRPS assembly line, thus pointing to an intriguing regulatory cross talk. To analyze which other genes are induced upon induction of the regulatory gene *scpR*, we employed ASMA, which contains probes representing predicted secondary metabolite genes of *A. nidulans* (18). mRNA of transformant strain SB3.5 was isolated after cultivation under inducing and repressing conditions and reverse transcribed to cDNA, and the cDNA was hybridized with ASMA. The expression profile monitored by ASMA confirmed that the gene cluster *inp*, which harbors the *scpR* gene, was induced in the overexpressing strain. Surprisingly, a putative silent polyketide biosynthesis gene cluster was also specifically induced (see Fig. 4B and Table S2 in the supplemental material). The genes *afoA* (AN1029), *afoB* (AN1031), *afoC* (AN1032), *afoD* (AN1033), *afoE* (AN1034), *afoF* (AN1035), and *afoG* (AN1036) are required for asperfuranone biosynthesis (8). The *afo* cluster is represented within ASMA by probes for the genes *afoA* (AN1029), *afoD* (AN1033), *afoE* (AN1034), and *afoG* (AN1036), all of which were upregulated under *scpR*-overexpressing conditions in comparison to the level of regulation under repressing conditions. Chiang et al. (8) suggested that the gene AN1030 is not part of the gene locus because a deletion mutant still produced asperfuranone. Here, we found that this gene was also induced in strain SB3.5, suggesting that it may also belong to the *afo* cluster. All of these genes were clearly upregulated when the transcription of the *alcAp-scpR* gene fusion was induced (Table 3). Quantitative RT-PCR further confirmed the specific induction of both gene clusters in strain SB3.5, i.e., cluster *inp* on chromosome II, including the *scpR* gene, and the *afo* gene cluster on chromosome VIII, as shown by analysis of the regulatory gene *afoA* (AN1029) and the two polyketide synthase (PKS)-encoding genes *afoE* (AN1034) and *afoG* (AN1036) (Fig. 3).

Induction of the asperfuranone gene cluster is mediated not by a product of the *inp* cluster but by the transcription factor *scpR*. Two alternative scenarios could explain the induction of

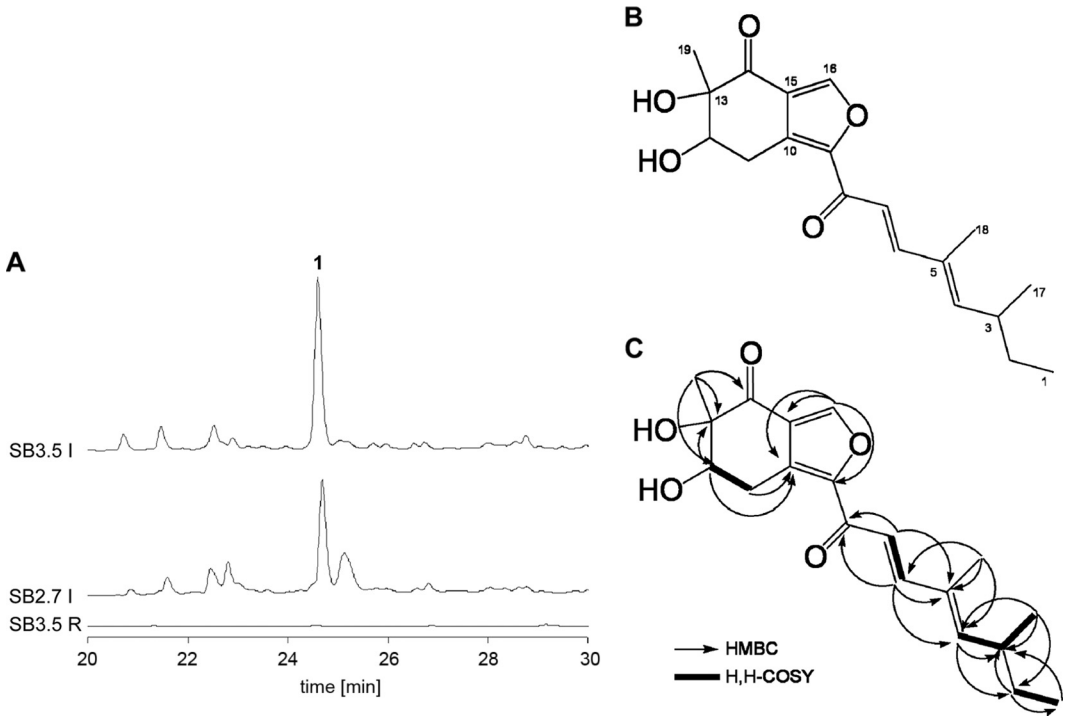

FIG. 2. (A) HPLC profiles of extracts from *A. nidulans* chemically inducible transformant strains SB3.5 and SB2.7 producing a novel compound. The strains were incubated in AMM under *alcAp*-inducing (I) and -repressing (R) conditions (the repressing condition profile of strain SB2.7 is identical to the one for SB3.5 [data not shown]). (B) Structure of asperfuranone (8). (C) H,H-COSY and HMBC couplings.

the asperfuranone gene cluster by the expression of the *scpR* gene. First, it is conceivable that ScpR induces the NRPS-encoding genes *inpA* and *inpB* of the respective gene cluster and that the product of this biosynthesis pathway leads to induction of the asperfuranone biosynthesis gene cluster. Alternatively, ScpR might directly or indirectly control both biosynthesis gene clusters, even though these loci are located on two different chromosomes. To address these questions, the formation of the yet unknown peptide had to be blocked by deletion of the NRPS gene *inpB* (AN3496). For this purpose, the *inpB* gene was replaced by the *riboB2* gene in the *A. nidulans* ΔKU70 strain TN02A7 (14). The deletion of *inpB* in the transformant strain obtained, strain SB2.3, was confirmed by Southern blot analysis (see Fig. S1B in the supplemental material). Strain SB2.3, which carries the deletion, was then crossed with strain SB3.5, which carries the inducible *alcAp-scpR* transcription factor fusion. The resulting progeny, carrying both the deletion of the *inpB* gene and, in addition, the inducible *alcAp-scpR* transcription factor fusion ectopically integrated in multiple copies into the genome, was named SB2.7 (see Fig. S1C and D in the supplemental material). Strain SB2.7 was cultivated under inducing and repressing conditions

TABLE 3. Changes in gene expression

Gene	Fold change in gene expression at 24 h		
	SB3.5	SB2.7	AXB4A
inpC	2.114	3.285	−1.191
scpR	1.064	1.278	1.099
inpA	4.825	9.963	−1.054
inpB	2.427	−1.092	−1.222
AN3497	1.567	−1.027	1.639
afoA	1.300	1.558	1.489
AN1030	3.859	4.969	−1.080
afoD	3.741	4.760	1.022
afoE	1.555	2.219	1.015
afoG	2.598	3.907	1.152

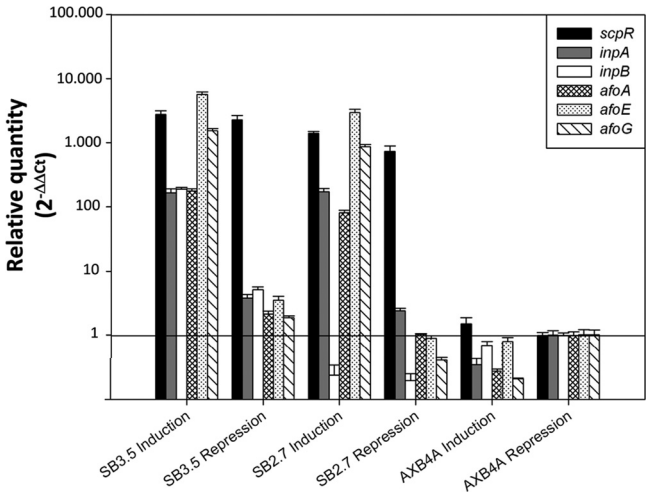

FIG. 3. Relative quantity of the mRNA steady-state level determined by qRT-PCR of *scpR*, *inpA*, *inpB*, *afoA*, *afoE*, and *afoG* in strains SB3.5, SB2.7, and AXB4A (wild type) under inducing and repressing conditions. Relative quantities are given as the $\log_{10}$ of $-\Delta\Delta C_T$, where C_T is threshold cycle. Data obtained by cultivation of *A. nidulans* AXB4A under repressing conditions were set as equal to 1.

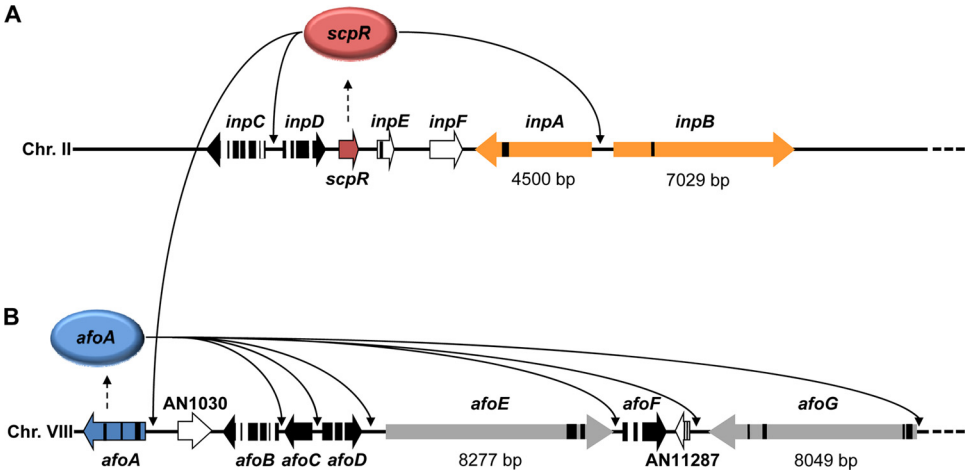

FIG. 4. Model of cross talk between the *A. nidulans* secondary metabolite gene cluster *inp* and *afo* in *A. nidulans* (modified as described elsewhere [8]). Each arrow indicates the direction of transcription, the relative sizes of the open reading frames, and the intron distribution deduced from analysis of the nucleotide sequences. (A) *inp* gene cluster on chromosome II. Orange arrows, NRPS genes; black arrows, additional putative cluster genes; red arrow, regulator gene *scpR*; white arrows, genes not characterized here; *inpC*, putative AMP binding enzyme; *inpD*, major facilitator superfamily; *inpE*, proteasome a type and b type; *inpF*, serine hydrolase (FSH1). (B) *afo* (asperfuranone) gene cluster on chromosome VIII. Gray arrows, PKS genes; black arrows, additional cluster genes; blue arrow, regulatory gene *afoA*; white arrows, genes whose involvement in asperfuranone biosynthesis is unclear.

and analyzed for both expression of biosynthesis genes by ASMA and its metabolic profile in comparison to the wild type. The Southern blot analysis indicating the deletion of *inpB* (see Fig. S1B in the supplemental material) was confirmed by data obtained with ASMA (Table 3) and qRT-PCR (Fig. 3). qRT-PCR analyses revealed that the relative quantities of mRNA steady-state levels of genes of both clusters increased upon overexpression of the *alcAp-scpR* gene fusion (Fig. 3). The only difference between transformant strains SB3.5 and SB2.7 is that SB2.7 lacks *inpB* expression due to the gene deletion. Furthermore, the production of asperfuranone was clearly detectable in both strains (Fig. 2). These data clearly rule out involvement of an NRPS product in the regulation. Instead, they indicate that the ScpR transcription factor plays a dual role in also inducing another biosynthesis gene cluster. This is even more remarkable, as *scpR* is part of one cluster and the two clusters are located on different chromosomes; cluster *inp* is located on chromosome II, whereas the *afo* gene cluster is located on chromosome VIII.

Deletion of the asperfuranone activator *afoA* abolishes asperfuranone biosynthesis under *alcAp-scpR*-inducing conditions. To demonstrate that upon *scpR* overexpression the asperfuranone gene cluster is activated by AfoA, as previously shown (7), and not directly by ScpR, we deleted the regulatory gene *afoA*. A DNA fragment encoding the cassette for the *p*-aminobenzoate synthase from *A. nidulans* flanked by upstream and downstream sequences of *afoA* was created by fusion PCR (see Materials and Methods). Transformation of strain SB3.5 yielded the strain ALF1.1, which is unable to produce AfoA. Northern blot analyses showed that under inducing conditions *scpR* mRNA was formed in both strains SB3.5 and ALF1.1, but only strain SB3.5 was able to produce *afoA* mRNA (Fig. 1C). Consistently, HPLC analysis provided evidence that the strain with the *afoA* deletion did not produce asperfuranone anymore. Hence, the expression of the *afo* gene cluster as well as the biosynthesis of asperfuranone under *alcAp-scpR* inducing conditions was abolished after the deletion of the asperfuranone activator gene *afoA*. Therefore, we concluded that for activation of the asperfuranone biosynthesis gene cluster via ScpR, the *afoA* regulatory gene is required (Fig. 4).

The promoters of genes of the *inp* cluster share a motif with the *afoA* promoter. Comparison of the intergenic regions of the genes *inpA*/*inpB*, *inpC*/*inpD*, and *afoA*/AN1030 revealed that all three sequences share one motif which is not present in other intergenic regions of the *inp* and *afo* gene clusters. This motif (CT/C/AAAAGGAT/AT/GG/CA) shows significant similarity to the binding site of RME1, a yeast zinc-finger transcription factor which also contains a C2H2 domain. These data strongly support the theory that ScpR binds only to the *afoA* promoter, leading to the transcription of the asperfuranone activator gene *afoA*, which then activates the other genes of the *afo* gene cluster (Fig. 4).

DISCUSSION

The genome of *A. nidulans* harbors 53 putative secondary metabolite gene clusters, most of which are silent under standardized cultivation conditions in the laboratory. In this study, we have focused on a silent gene cluster (*inp*) comprising two NRPS genes and a regulator gene. Induction of transcription factor ScpR using the regulatable *alcA* promoter triggered the expression of the *inp* pathway genes. Surprisingly, instead of the predicted NRPS product, we detected a novel polyketide, asperfuranone, in the broth of the induced transformant. By transcriptome analyses we found that the asperfuranone biosynthesis genes were significantly upregulated. This is remarkable, given the fact that the requisite gene cluster itself harbors a pathway-specific transcription factor, whose function was demonstrated (8). We thus concluded that either ScpR or the

NRPS product would interact with the regulation of the *afo* gene locus. Through deletion of one of the NRPS genes of the *inp* gene cluster, we could rule out that the predicted coproduct produced is responsible for activating the *afo* gene cluster. This finding is also supported by our notion that the addition of culture supernatant or whole-culture extract of the induced SB3.5 strain with the *afoE* promoter fused to a reporter gene did not trigger the expression of the reporter gene, whereas homologous overexpression of *scpR* did (data not shown). In this context, it should also be noted that although a small peak in the HPLC profile of the mutant with the NRPS gene deletion disappeared, the isolation of the product from the induced transformant was hampered, likely due to the minute amounts produced. Future studies will address the nature of this cryptic peptide metabolite.

Because the four genes which have been proven to be involved in emericellamide biosynthesis by functional analysis, i.e., *easA*, *easB*, *easC*, and *easD*, were also upregulated under *scpR*-overexpressing conditions, the implication is that they are also regulated by ScpR. However, because the expression of these genes in glucose mineral salt media was previously demonstrated (7), they clearly do not form a silent gene cluster. Thus, it remains to be shown whether the emericellamide biosynthesis is also affected by ScpR.

In conclusion, we could unequivocally show that ScpR regulates both the PKS-encoding *afo* gene cluster and the NRPS-encoding *inp* gene cluster. Notably, both clusters are silent under noninducing conditions and are even located on different chromosomes, i.e., chromosomes II and VIII. Deletion of the *afoA* regulatory gene abolished the possibility to induce the *afo* gene cluster by overexpression of the *scpR* gene. Therefore, we concluded that ScpR controls the *afo* gene clusters directly by binding to the promoter of the *afoA* gene. This finding was also supported by the notion that *afoA* as well as the genes belonging to the *inp* gene cluster encodes potential binding sites for ScpR.

By replacing the native promoter of *afoA* (AN1029) with an *alcA*-inducible promoter, it was shown previously that the entire asperfuranone gene cluster can be induced. However, when the second transcription factor present in the cluster (AN1028) was deleted, the pathway was still induced. These results indicate that induction of *afoA* alone is sufficient to turn on the whole asperfuranone biosynthetic pathway and that AN1028 is probably not involved in the regulation of the pathway (8).

To the best of our knowledge this is the first report of cross talk between gene clusters in fungi, yet the role of this unprecedented cross talk is enigmatic and remains to be elucidated. However, this unexpected level of complexity in the communication between gene clusters has important implications for future work in the field of fungal secondary metabolism. Not only is it important to consider a potential regulatory cross talk in genome-mining approaches but also one should be aware of this possibility when studying the functions of individual biosynthesis gene clusters.

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