LaeA, a Regulator of Secondary Metabolism in *Aspergillus* spp.

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Secondary metabolites, or biochemical indicators of fungal development, are of intense interest to human-kind due to their pharmaceutical and/or toxic properties. We present here a novel *Aspergillus* nuclear protein, LaeA, as a global regulator of secondary metabolism in this genus. Deletion of *laeA* (∆*laeA*) blocks the expression of metabolic gene clusters, including the sterigmatocystin (carcinogen), penicillin (antibiotic), and lovastatin (antihypercholesterolemic agent) gene clusters. Conversely, overexpression of *laeA* triggers increased penicillin and lovastatin gene transcription and subsequent product formation. *laeA* expression is negatively regulated by AflR, a sterigmatocystin Zn₂Cys₆ transcription factor, in a unique feedback loop, as well as by two signal transduction elements, protein kinase A and RasA. Although these last two proteins also negatively regulate sporulation, ∆*laeA* strains show little difference in spore production compared to the wild type, indicating that the primary role of LaeA is to regulate metabolic gene clusters.

A complex and fascinating aspect of fungal development is the production of secondary metabolites. These compounds, frequently associated with sporulation processes, are considered part of the chemical arsenal required for niche specialization and have garnered intense interest by virtue of their biotechnological and pharmaceutical applications (9, 11). Many of them display a broad range of useful antibiotic, antiviral, antitumor, antihypercholesterolemic, and immunosuppressant activities as well as less desirable phytotoxic activities. Hawksworth’s studies of fungal biodiversity led to the conclusion that nearly 1.5 million fungal species exist on Earth, with only 5% identified thus far (16). Thus, the potential to the conclusion that nearly 1.5 million fungal species exist on Earth, with only 5% identified thus far (16). Thus, the potential for fungal secondary metabolite discovery is vast. Furthermore, the discovery of global regulators for fungal secondary metabolite production is critical, as it would allow for universal manipulation of secondary metabolite production.

A large number of known fungal secondary metabolites have been ascribed to the Ascomycete genus *Aspergillus*. Studies of *Aspergillus* nidulans have demonstrated the power of using a model system to elucidate the biochemical and molecular genetics of fungal secondary metabolism, principally penicillin (PN, an antibiotic) and sterigmatocystin (ST, a carcinogen biochemically related to the agricultural contaminant aflatoxin) biosynthesis (6, 17). These studies have established several characteristics of fungal secondary metabolism, including the clustering of biosynthetic and regulatory genes and a genetic connection linking secondary metabolite biosynthesis with sporulation through a shared signal transduction pathway (9).

The discovery of the G protein-cyclic AMP (cAMP)-protein kinase A regulation of ST, aflatoxin, and other fungal secondary metabolites (4, 17, 29, 34) has been helpful for establishing a model of global regulation of secondary metabolism. However, all of the signal transduction mutants described in the literature have pleiotropic effects on fungi, the most notable effect being the gross impact on spore production and vegetative hyphal growth (1, 9, 18, 29). Similarly, mutations in other major regulators of ST biosynthesis, such as RcoA, a WD protein (19), and SmpA, or spermidine synthase (20), also have gross effects on fungal morphology. Thus, currently available *Aspergillus* mutants are so impaired in fungal development that further elucidation of genes that are specific for the regulation of secondary metabolism is difficult.

In a previous mutagenesis hunt, 23 *A. nidulans* mutants that displayed a phenotype of loss of ST production but had normal sporulation were isolated (8). Here we describe the identification of a gene called *laeA* that complements one of these mutants. *laeA* encodes a nuclear protein that is required for the expression of secondary metabolite genes. We propose that *laeA* is a regulator of secondary metabolism in *Aspergillus*, as it is required not only for ST biosynthesis but also for PN biosynthesis and the biosynthesis of mycelial pigments in *A. nidulans* and gliotoxin and mycelial pigments in *Aspergillus* fumigatus. Furthermore, this protein is required for expression of the heterologous lovastatin (LOV) gene cluster in *A. nidulans* as well as for native LOV expression in *Aspergillus terreus*. Interestingly, the protein appears to be conserved in filamentous fungi, but it is not present in *Saccharomyces cerevisiae*, a fungus devoid of secondary metabolites. Unlike other genes that regulate secondary metabolism, the loss of *laeA* has a negligible impact on morphological developmental processes.

**MATERIALS AND METHODS**

Fungal strains and growth conditions. Table 1 lists all of the fungal strains used for this study. Some strains are not discussed in the text but were used for sexual crosses to obtain the strains of interest. Sexual crosses of *A. nidulans* strains were conducted according to the method of Pontecorvo et al. (26). All strains were maintained as glycerol stocks and were grown at 37°C for *A. nidulans* and *A. fumigatus* or 32°C for *A. terreus* on glucose minimal medium (GMM) (29), threonine minimal medium (TMM) (29), or lactose minimal medium (LMM) (21) amended with 30 mM cyclopentanone. Threonine and cyclopentanone both induce alcA, which was used to promote *laeA* expression. All media contained appropriate supplements to maintain auxotrophs (2).

Cloning and sequence of *A. nidulans* and *A. fumigatus* *laeA* genes. The *A. nidulans* aflR expression mutant, BYJ8 (derived from strain MR800), was transformed with an *A. nidulans* genomic cosmid library. Norsolorinic acid-producing transformants were purified, and a cosmid, pCOSJW3, that comple-
mented the mutation was rescued from one transformant. Norsolorinic acid is an orange pigmented precursor in the ST biosynthetic pathway and is commonly used as an indicator of ST production (8). pJW15, a 4.5-kb KpnI-EcoRI subclone of pCOSJW3, also complemented the mutation and was sequenced by use of synthetic primers and an ABI PRISM DNA sequencing kit (Perkin-Elmer Life Sciences). The mutant allele *laeA* was sequenced after subcloning of a 3-kb PCR fragment from RYJ8 genomic DNA amplification with primers LAE1 and LAE2 (Table 2) into the Zero Blunt TOPO vector (Invitrogen) to produce pJW31. Rapid amplification of cDNA ends technology using a Gene Racer kit (Invitrogen) was employed to clone *laeA* cDNA according to the manufacturer's instructions. The cloned cDNA was then sequenced. The Institute for Genomic Research (TIGR) database contains a partial *A. fumigatus* genome data with the *laeA* gene. The expression of cDNA according to the manufacturer's instructions with the *laeA* gene was employed to clone *laeA* cDNA according to the manufacturer's instructions. The cloned cDNA was then sequenced. The Institute for Genomic Research (TIGR) database contains a partial *A. fumigatus* genome data with the *laeA* gene. The expression of cDNA according to the manufacturer's instructions with the *laeA* gene was employed to clone *laeA* cDNA according to the manufacturer's instructions.

### Table 1. Fungal strains used for this study

| Strains or category | Genotype | Source or reference |
|---------------------|----------|---------------------|
| **Wild type and controls** |  |  |
| *A. nidulans* strains |  |  |
| FGSC 26 | *bla1 veA1* Fungal Genetics Stock Center |
| RDIT 2.1 | *methG1* D. Tsitsigiannis |
| RAMB38 | *bla1 methG1 Δflfr:*argB trpC801 veA1 A. M. Bergh |
| RDIT 2.3 | veA1 D. Tsitsigiannis |
| RDIT 7.24 | *methG1 veA1* D. Tsitsigiannis |
| RDIT 30.34 | *methG1 trpC801 pyrGS9 veA1* D. Tsitsigiannis |
| RJH26 | *bla1 wa3 argB2 ΔstcE:*argB veA1 trpC801 This study |
| RJW3 | *pyrGS9 wa3 argB2 pyrA4 ΔstcE:*argB veA1 trpC801 This study |
| RJW51 | *alcAp:*lovB:*pyr4 hgyB:*lov gene cluster This study |
| MKS 1 | *pbaA1 ya2 veA1* 29 |
| RMFV2 | *pbaA1 ya2 veA1 argB2 Δsofr:*argB 29 |
| TJH3.40 | *bla1 wa3 argB2 methG1 ΔstcE:argB2 veA1* 8 |
| TJH3.41 | *pbaA1 ya2 veA1 alca p:sofr::trpC 29 |
| TPK1.1 | *bla1 methG1 veA1* N. Keller |
| WMH1739 | *pbaA1 ya2 alca p:*lovB:*pyr4 hgyB:*lov gene cluster 21 |
| **A. fumigatus strains** |  |  |
| AF293 | *pyrG* G. May |
| AF293.1 | *pyrG* , *A. parasiticus pyrG* G. May |
| TJW55.2 | *pyrG* , *A. parasiticus pyrG* This study |
| **A. terreus strains** |  |  |
| ATCC 20542 | *hygB* American Type Culture Collection |
| TJW58.9 | *hygB* This study |
| **laeA mutants** |  |  |
| *A. nidulans* strains |  |  |
| MRB300 | *bla1 wa3 methG1 ΔstcE:*argB2 veA1 laeA1* 8 |
| RJW32 | *bla1 wa3 argB2 methG1 ΔstcE:*argB veA1 trpC801 ΔlaeA:*methG This study |
| RJW33.2 | *wa3 argB2 methG1 pyro4 ΔstcE:*argB veA1 trpC801 ΔlaeA:*methG This study |
| RJW 40.4 | *bla1 methG1 veA1 ΔlaeA:*methG This study |
| RJW42.2 | *bla1 methG1 alca p:*lovB:*pyr4 hgyB:*lov gene cluster This study |
| RJW 46.4 | *methG1 veA1 ΔlaeA:*methG This study |
| RJY 8 | *bla1 wa3ΔstcE:*argB veA1 trpC801 laeA1 This study |
| RJW 52 | *alcAp:*laeA:*trpC alca p:*lovB:*pyr4 hgyB:*lov gene cluster This study |
| RJW 53 | *ΔlaeA::methG alca p:*lovB:*pyr4 hgyB:*lov gene cluster This study |
| RJW57.9 | *ΔlaeA::methG alca p:*lovB:*pyr4 hgyB:*lov gene cluster This study |
| TJW 46.16 | *bla1 wa3 argB2 methG1 ΔstcE:argB veA1 alca p:*lovB:*pyr4 hgyB:*lov gene cluster This study |
| **A. fumigatus strains** |  |  |
| TJW 54.2 | *ΔlaeA:*A. parasiticus pyrG pyrG* This study |
| **A. terreus strains** |  |  |
| TJW 58.2 | *hygB alca p:*laeA This study |
| TJW 58.4 | *hygB alca p:*laeA This study |
| TJW 58.7 | *hygB alca p:*laeA This study |
| TJW 58.8 | *hygB alca p:*laeA This study |
| TJW 58.14 | *hygB alca p:*laeA This study |
| **Signal transduction mutants** |  |  |
| HIFAD4 | *bla1 veA1 fad4* This study |
| RKIS 11.1 | *pbaA1 ya2 veA1 argB2 ΔfadA::argB 29 |
| RKIS 28.5 | *pbaA1 ya2 veA1 alca p:*ras17A:*argB 29 |
| TBN 39.5 | *bla1 methG1 argB2 ΔflfbA::argB veA1 23 |
| TJI 34.10 | *pbaA1 ya2 trpC801 trpC::alcA::sofr veA1 J. Hicks |
| TKS 18.11 | *pbaA1 ya2 ΔargB::trpC trpC801 veA1 ΔpkaA::argB 29 |
| TKS 20.1 | *pbaA1 ya2 veA1 alca p:*kaA:*trpC 29 |
| TSRR 1.38 | *bla1 methG1 argB2 Δsofr:*argB veA1 27 |
A. terreus were used for cotransformation to introduce the overexpression of the laeA gene. The amount of the overexpressed laeA gene was determined by comparison to standard spots on thin-layer chromatography (TLC) plates by dilution spotting. All cultures with 30 mM cyclopentanone for 36 h at 32 °C were used as probes. pL11C09 contains most of the ST gene cluster, whereas cosmids pW07H03, pL11C09, and pL24B03 were used as probes. pL11C09 contains most of the ST gene cluster, whereas cosmids pW07H03, pL11C09, and pL24B03 primarily contain genes located upstream and downstream of the ST gene cluster, respectively (7).

Construction of transformation vectors and strains. Plasmids were generated by standard techniques, and the primers used for this study are listed in Table 2. Primers were used as probes. pL11C09 contains most of the ST gene cluster, whereas cosmids pW07H03, pL11C09, and pL24B03 were used as probes. pL11C09 contains most of the ST gene cluster, whereas cosmids pW07H03, pL11C09, and pL24B03 primarily contain genes located upstream and downstream of the ST gene cluster, respectively (7).

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TABLE 2. Primers

| Primer | Sequence | Restriction site |
|--------|----------|-----------------|
| LAE1   | AGTCACTTCTCTGCGCCTCGTG | HindIII |
| LA2    | CGTCTGAAGCCTGGGAACAGTGGAAGGAAGGGAAC | SacI |
| LA3    | GCAGGCGTATGACGGGATACGCAGCAA | HindIII |
| EOE    | GCTGATATCGCTTGGTCTCCTTGCGCCTGCG | HindIII |
| EOE    | GTTTGAGCTTTGCGTGCAGTTGCAGAACGCG | HindIII |
| MT1    | ATGCTGAAAGCTTGGAAAAGGGGTTC | HindIII |
| GFP2   | TGACGAGCTCGTGGAACAGTGGAAGGAAC | EcoRI |
| GFP3   | TCCGAATCCATCGAAGGGGAGAAAAA | EcoRI |
| GFP4   | GGAATGGCCCTCGAATGGTTCGGTCTCTATCGC | EcoRI |
| GFP5   | AACGCACTGAGAGAAGAAAGCCAGGACCAA | EcoRI |
| GFP6   | GTACGAGCTCGTGGAACAGTGGAAGGAAC | HindIII |
| GF2    | CGCGCAATCCATCTGACGCTCTGCTACAG | HindIII |
| GF3    | TTGGGATTTCCGCGTGCTGTTGTTTGG | EcoRI |
| FUM1   | GCCCACTCTTCTGTTGTTTTCCCT | EcoRI |
| FUM2   | CATGCAATCTTCTCGTGGGCC | EcoRI |
| FUM3   | TACCAGGATCACAATTCTTCGCGA | BamHI |
| FUM4   | CATGCAATCTAATTCTGATGTTG | EcoRI |

* Underlined sequences show the placement of restriction sites.

extracted from Aspergillus strains by use of Triozol reagent (Invertogen) according to the manufacturer’s instructions. RNA blots were hybridized with a 0.7 kb SacII-KpnI fragment from pKB7 containing the stcU coding region (18), a 1.3 kb EcoRV-XhoI fragment from pW19 containing the gfp coding region, a 3 kb HindIII fragment from pWJ45.4 containing the laeA coding region, a 1.1 kb EcoRI-HindIII fragment from pUCH1(48) containing the gfp coding region (36), a 5 kb BamHI fragment from pWHM410 containing the lovC coding region (21), and a 1.3 kb PCR product from pWH1263 containing the lovC coding region (21). Also, A. nidulans cosmids pW07H03, pL11C09, and pL24B03 were used as probes. Plasmids containing most of the ST gene cluster, whereas cosmids pW07H03 and pL24B03 primarily contain genes located upstream and downstream of the ST gene cluster, respectively (7).

RESULTS

Cloning and characterization of A. nidulans and A. fumigatus laeA. A mutagenesis screen previously led to the isolation of 23 mutants displaying a loss of ST production but normal sporulation. Three of the mutants were unable to express aflR, which encodes an ST cluster Zn2Cys6 transcription factor regulating ST biosynthetic gene expression (12). We were able to complement one of these three mutants, RYJ8, with an A. nidulans trpC genomic cosmid library. Sequencing of...
a 4.5-kb subclone (pJW15) of the complementing cosmid pCOSJW3 revealed a 3-kb open reading frame designated laeA (for loss of afl expression). Sequencing of the mutant allele, laeA1, from RYJ8 showed that it has a base pair transversion (at position 1455; C3G) and a 1-bp deletion (at position 1453) in the gene. The deletion resulted in a premature stop codon. An examination of genomic and cDNA sequences revealed that laeA has one intron and three putative Afl binding sites (12), one in the promoter (H11002607) and two in the encoding region (positions 607 and 1487) (Fig. 1A). cDNA analysis showed that laeA possesses a 5'-untranslated region (642 bp) (Fig. 1A).

Analyses of available genomic databases indicated that only filamentous fungi, including Aspergillus fumigatus, Neurospora crassa, Magnaporthe grisea, C. immitis, and F. sporotrichioides LaeA proteins showing conserved protein methyltransferase SAM binding sites in red. (C) A. nidulans LaeA protein localizes to the nucleus. GFP was fused to the N-terminal end of LaeA. Nuclei were stained with the DNA-specific dye 4,6-diamidino-2-phenylindole (DAPI).
Transcriptional regulation of secondary metabolism gene clusters by LaeA. (i) Native cluster regulation. To confirm our initial observation that laeA is required for ST gene regulation, we assessed aflR and stcU (a gene encoding a biosynthetic enzyme required for ST production) (17) expression in the ∆laeA background. Neither gene was expressed (Fig. 3A). A transcriptional profile of the entire ST gene cluster, which covers ca. 60 kb and contains ca. 26 genes (7), suggested that LaeA transcriptional control is ST cluster specific, as transcription of the upstream and downstream genes of the ST cluster was unaffected (data not shown). Because many uncharacterized metabolites were reduced in the ∆laeA strains (Fig. 2B and C), we thought it possible that LaeA is a global regulator for secondary metabolite gene expression. To address this hypothesis, we examined PN gene expression in the A. nidulans ∆laeA strain. Figure 3A shows that ipnA (encoding isopenicillin N synthetase, a biosynthetic enzyme required for PN biosynthesis) (6) expression was greatly reduced in the ∆laeA strain.

(ii) Heterologous cluster regulation. Our results for ST and PN gene expression suggested a role for LaeA in secondary metabolite gene cluster regulation. To further address this potential role, we examined the expression of the heterologous LOV gene cluster in the A. nidulans ∆laeA background. The partial LOV cluster, derived from A. terreus, was originally transformed into A. nidulans to study aspects of LOV biosynthesis (21). This strain was used to cross the LOV cluster (LOV−) into appropriate mutant laeA backgrounds. Figure 3B shows that the ∆laeA/LOV+ strain displayed very diminished levels of both lovE (encoding a LOV-specific Zn2Cy5 biosynthetic enzyme) and lovC (a LOV biosynthetic gene) transcripts. Chloroform extracts of this strain also showed diminished production of MONJ (Fig. 3B), the LOV intermediate produced by the partial LOV cluster (21).

Overexpression of laeA upregulates PN and LOV gene expression but not ST gene expression. We next constructed laeA overexpression strains (OE::laeA) of both A. nidulans and A. terreus to examine secondary metabolite gene expression and product formation. As shown in Fig. 3C, ipnA, lovE, and lovC, but not stcU, expression levels were remarkably elevated in the A. nidulans OE::laeA background. Secondary metabolite production was correlated with transcript levels. MONJ production was increased ~400%, and high levels of PN were produced during times when the wild type showed no PN activity (Fig. 3D). ST levels remained the same as that of the wild type in the OE::laeA background (Fig. 3C). Overexpression of the A. nidulans laeA gene in the LOV-producing fungus A. terreus led to 400 to 700% increases in LOV (Fig. 3E).

Feedback regulation of laeA by aflR. An examination of the laeA transcript in wild-type strains showed that it is an inducible, low-expression-level gene that is observed in Northern blots before and after aflR transcripts are observed (data not shown). Due to the presence of three AflR binding sites in the gene (Fig. 1A), we thought it possible that AflR regulates laeA expression. As shown in Fig. 4, overexpression of aflR (OE::aflR) downregulates laeA expression, although elimination of aflR (∆aflR) does not affect the laeA transcript level (Fig. 5). This indicates that there are both negative (laeA) and positive (stc genes) regulatory effects of AflR on gene transcription.

Protein kinase A and RasA negatively regulate laeA expression. ST biosynthesis is regulated in A. nidulans via a signal transduction pathway, and many of the genes involved in this signaling pathway are known (9). Therefore, we looked at the possible interactions with laeA of five signaling genes, encoding two members of a heterotrimeric G protein (fadA and sfA) (27, 40), a regulator of G-protein signaling protein regulating FadA activity (flbA) (23), a cAMP-dependent kinase (pkaA) (29), and a Ras protein (rasA) (33). laeA expression was examined in the wild type and in strains carrying the following alleles: flbA, fadA<sup>G42R</sup>, ∆fadA, ∆sfA, ∆pkaA, OE::pkaA, and OE::rasA<sup>G12V</sup> (Table 1). mRNA analyses of these mutants showed that OE::pkaA and OE::rasA<sup>G12V</sup> completely inhibited laeA expression (Fig. 4), whereas laeA transcription was not repressed in any of the other strains (Fig. 5 and data not shown). Interestingly, the laeA transcript level was elevated in the ∆fadA strain (Fig. 5). The presence of laeA transcripts in these mutants (Fig. 5 and data not shown) shows that laeA is not sufficient for aflR expression, as aflR was not expressed in these strains (18). Figure 6 depicts our current understanding of LaeA involvement in secondary metabolite regulation.
FIG. 3. laeA regulation of secondary metabolism. (A) aflR, stcU, and ipnA gene expression in A. nidulans wild-type (WT) (RDIT2.3) and ΔlaeA (RJW46.4) strains grown in liquid shaking GMM for 12, 24, 48, and 72 h at 37°C. Ethidium bromide-stained rRNA is indicated as a loading control. (B) laeA, lovE, and lovC gene expression in A. nidulans WT:lov+ (RJW51) and ΔlaeA:lov+ (RJW53) strains grown in liquid shaking GMM for 12, 24, 48, and 72 h at 37°C. Ethidium bromide-stained rRNA is indicated as a loading control. MONJ was extracted from WT:lov+ (RJW51) and ΔlaeA:lov+ (RJW53) A. nidulans strains grown in liquid shaking GMM for 3 days. The experiment was performed in triplicate. (C) laeA, ipnA, stcU, lovE, and lovC gene expression in A. nidulans wild-type (WT) (RDIT2.3), OE::laeA (RJW47.3), WT:lov+ (RJW51), and OE::laeA:lov+ (RJW52) strains grown in liquid shaking GMM for 14 h at 37°C and then transferred to liquid shaking TMM for the induction of laeA expression. Time points were 0, 6, 12, and 24 h after transfer. ST and MONJ were extracted from A. nidulans WT (RDIT2.3), OE::laeA (RJW47.3), WT:lov+ (RJW51), and OE::laeA:lov+ (RJW52) strains grown in liquid shaking GMM for 14 h at 37°C and then transferred to liquid shaking TMM for 24 h. ST, ST standard. The MONJ standard was extracted from A. nidulans strain WMH1739. The experiment was performed in triplicate. (D) PN bioassay. Wild-type (FGSC26), ΔlaeA (RJW40.4), and OE::laeA (RJW44.2) strains were grown in liquid shaking GMM for 14 h at 37°C and then transferred to LMM amended with 30 mM cyclopentanone for the induction of laeA for 24 h at 37°C. (E) TLC examination of LOV production in A. terreus laeA overexpression strains. The wild type (ATCC 20542; lane 1), TJW58.9 (hygB resistance gene-containing transformant used as a control; lane 2), and OE::lae4 strains containing hygB (TJW58.2, TJW58.4, TJW58.7, TJW58.8, and TJW58.14, in lanes 3 to 7, respectively) were grown in liquid shaking GMM for 18 h at 32°C and then transferred to LMM with 30 mM cyclopentanone for the induction of laeA for 36 h at 32°C. Std, LOV standard. The experiment was performed in duplicate.
Multiple fungi (9) and advent of sporulation and cellular development in fungi and that at 37°C/H9004 VOL. 3, 2004 REGULATION OF FUNGAL SECONDARY METABOLITE PRODUCTION 533

Secondary metabolite biosynthesis is often associated with the advent of sporulation and cellular development in filamentous fungi. These developmental processes reflect the need to access multiple nutrients and to optimize cellular morphology and metabolic differentiation for effective competition in complex environments. We are interested in the identification of secondary metabolism-specific global regulators that can uncouple sporulation and secondary metabolism. Such regulatory elements are extremely desirable because they would possess broad specificity for the activation and/or repression of entire families of secondary metabolite gene clusters while providing strains that are capable of otherwise normal or near-normal development and growth. The identification of such regulatory elements would enable the increased production of secondary metabolites by providing improved strains of engineered organisms and would also contribute to a broader understanding of molecular mechanisms by which secondary metabolites are produced. Through complementation of an ST developmental mutant, we have identified such a protein, called LaeA, which is an archetypal global regulator of secondary metabolism in fungi.

LaeA regulation of metabolite production is transcriptional, as assessed by the effects of \( \Delta \text{laeA} \) and OE:\l aeA alleles on ST, PN, and LOV gene expression in A. nidulans and A. terreus. In all cases, gene transcripts were reduced or eliminated in \( \Delta \text{laeA} \) strains. However, although overexpression of \( \text{laeA} \) increased PN and LOV gene transcript and concomitant production formation (Fig. 3C, D, and E), this was not the case for ST gene transcription or production (Fig. 3A). The steady-state level of ST transcripts and product formation in the OE:\l aeA background, in contrast to the increased PN and LOV transcripts and product formation, suggested a unique interaction between \( \text{laeA} \) and ST gene regulation.

Due to the presence of three potential AflR binding sites in the A. nidulans gene (Fig. 1A) and the lack of ST cluster gene upregulation in the OE:\l aeA background, we thought it possible that AflR negatively regulates \( \text{laeA} \) expression. As shown in Fig. 4, overexpression of aflR (OE:aflR) downregulates \( \text{laeA} \) expression, although elimination of aflR (\( \Delta \text{aflR} \)) does not affect \( \text{laeA} \) transcript levels (Fig. 5). This indicates that there are both negative (\( \text{laeA} \)) and positive (\( \text{stc} \)) (12) regulatory effects of AflR on gene transcription. To our knowledge, this is the first description of a putative secondary metabolite feedback mechanism. As overproduction of ST negatively affects fungal growth (N. P. Keller, unpublished data), we speculate that this feedback loop may have evolved as a fitness trait. In contrast, neither the promoter nor the encoding region of A. fumigatus \( \text{laeA} \) contained AflR binding sites, and no aflR ortholog was found in the genome. Some A. fumigatus strains are reported to produce ST (14); it would be interesting to see if \( \text{laeA} \) genes from these isolates contained AflR binding sites. Initial examinations of the Aspergillus \( \text{laeA} \) strains showed them to be more susceptible to killing by neutrophils in vitro than the wild type (S. Balajee, L. Delbridge, J. Bok, N. Keller, and K. Marr, unpublished data). Presumably this is due to a loss of toxin secondary metabolites or melanins, known virulence factors in several fungal systems (5, 22).

\( \text{laeA} \) expression is also negatively regulated by two signal transduction molecules, PkaA and RasA. Both proteins have been shown to transcriptionally and posttranscriptionally regulate aflR (29, 30). It appears that LaeA mediates PkaA transcriptional regulation of aflR, since overexpression of \( \text{laeA} \) in a pkaA overexpression background, a condition that normally suppresses \( \text{stc} \) expression, partially restored \( \text{stc} \) gene expression.

**DISCUSSION**

**FIG. 4.** Regulation of \( \text{laeA} \). Effects of overexpression of aflR, pkaA, and ras\(^{G17V}\) on \( \text{laeA} \) expression. Wild-type (RKIS1), OE:aflR (TJH34.10), OE:pkaA (TKIS20.1), and OE:ras\(^{G17V}\) (RKIS28.5) strains were grown in liquid shaking GMM for 14 h at 37°C and then transferred to TMM. Time points were 0, 6, 12, and 24 h after transfer.

**FIG. 5.** \( \text{laeA} \) expression is not affected in \( \Delta \text{aflB} \), \( \Delta \text{asfA} \), and \( \Delta \text{aflR} \) strains. \( \text{laeA} \), aflR, and \( \text{stc} \) gene expression was examined in A. nidulans wild-type (TPK1.1 and RKIS1), \( \Delta \text{aflB} \) (TBN39.5), \( \Delta \text{asfA} \) (TSR1.38), and \( \Delta \text{aflR} \) (RMFV2) strains grown in liquid shaking GMM for 12, 24, 48, and 72 h at 37°C. Ethidium bromide-stained rRNA is indicated as a loading control.
With regard to PkaA regulation of laeA, the lack of conventional PkaA phosphorylation consensus sequences in LaeA indicates that PkaA regulation of LaeA is not direct. Alternatively, LaeA may contain unconventional PkaA phosphorylation sites. RasA regulation of laeA gene expression may occur through PkaA and/or another pathway(s).

The requirement of a kinase for laeA function is reminiscent—to a degree—of a Streptomyces global regulatory system involving the protein AfsR. AfsR is a transcription factor that regulates secondary metabolism in Streptomyces coelicolor but regulates morphogenesis in Streptomyces griseus (contrast this to the similar role LaeA has in the three Aspergillus spp. examined here). Phosphorylation of AfsR enhances its activity (37). Like the case for AfsR, LaeA regulation occurs at the transcriptional level, but it shows no homology to transcription factors. Its nuclear location, its role in transcriptional regulation, and the presence of a SAM motif in LaeA suggest that it may be a protein methyltransferase. Well-known protein methyltransferases include histone and arginine methyltransferases that play important roles in the regulation of gene expression in eukaryotes, in part through modification of the chromatin structure (15, 25, 35).

Regardless of the mechanism, these findings with LaeA present an advance toward understanding the complex regulation of secondary metabolite production and provide a means for the discovery of new metabolites. Indeed, initial comparative microarray studies between ΔlaeA and the wild type have identified putative secondary metabolism gene clusters in the Aspergillus genome (L. Maggio-Hall, J. Bok, and N. Keller, unpublished data). The manipulation of LaeA in filamentous fungi may enable the increased production of pharmaceuticals or the elimination of fungal toxins by providing improved strains of engineered organisms and may also contribute to the broader understanding of molecular mechanisms by which secondary metabolites are produced.

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