Suppressor Mutagenesis Identifies a Velvet Complex Remediator of *Aspergillus nidulans* Secondary Metabolism

Mona I. Shaaban,1,2 Jin Woo Bok,1 Carrie Lauer,1 and Nancy P. Keller1,3*

Department of Medical Microbiology and Immunology, University of Wisconsin-Madison, Madison, Wisconsin1; Department of Microbiology and Immunology, Faculty of Pharmacy, Mansoura University, Egypt2; and Department of Bacteriology, University of Wisconsin-Madison, Madison, Wisconsin3

Received 30 July 2010/Accepted 29 September 2010

Fungal secondary metabolites (SM) are bioactive compounds that are important in fungal ecology and, moreover, both harmful and useful in human endeavors (e.g., as toxins and pharmaceuticals). Recently a nuclear heterocomplex termed the Velvet complex, characterized in the model ascomycete *Aspergillus nidulans*, was found to be critical for SM production. Deletion of two members of the Velvet complex, *laeA* and *veA*, results in near loss of SM and defective sexual spore production in *A. nidulans* and other species. Using a multicopy-suppressor genetics approach, we have isolated an *Aspergillus nidulans* gene named *rsmA* (remediation of secondary metabolism) based upon its ability to remediate secondary metabolism in *ΔlaeA* and *ΔveA* backgrounds. Overexpression of *rsmA* (OE::*rsmA*) restores production of sterigmatocystin (ST) (a carcinogenic SM) via transcriptional activation of ST biosynthetic genes. However, defects in sexual reproduction in either *ΔlaeA* or *ΔveA* strains cannot be overcome by OE::*rsmA*. An intact Velvet complex coupled with an OE::*rsmA* allele increases SM many fold over the wild-type level, but loss of *rsmA* does not decrease SM. *RsmA* encodes a putative bZIP basic leucine zipper-type transcription factor.

Secondary metabolites are biologically active compounds that are diverse in structure and often possess either pharmacological or toxic properties. To customize and control the biosynthesis of these natural products, we must understand how secondary metabolites (SM) are regulated. In fungi, SM production is controlled by global transcription factors encoded by genes unlinked to the SM biosynthetic gene clusters. Such genes regulate multiple physiological processes and generally respond to environmental cues such as pH, temperature, and nutrition (27, 45). Regulation of many clustered genes is also largely dependent on pathway-specific transcription (18, 22, 42) and signal transduction pathways that link secondary metabolism with sporulation (13).

LaeA is a global regulator of SM in the aspergillii. LaeA, a putative methyltransferase, was first identified in *Aspergillus nidulans* (5), *A. fumigatus* (6), and, subsequently, *A. flavus* (25). Overexpression (OE) of *laeA* increases the production of multiple metabolites in the aspergilli, whereas deletion of *laeA* silences expression of SM genes (1, 5, 8, 20). LaeA is thus used as a genomic mining tool, providing a novel method for identifying new SM through microarray and chemical analyses (8, 20, 34). Moreover, deletion of *laeA* in *A. fumigatus* decreases the virulence of this human pathogen (4, 6); similarly, *A. flavus laeA* deletants are also reduced in virulence on host seed (1, 25). LaeA homologs are also found in other filamentous fungi such as *Penicillium chrysogenum* and *Fusarium fujikuroi*, where they control SM, pigmentation, sporulation, and virulence (29, 43).

Another important SM regulatory protein identified in *A. nidulans* is encoded by *veA*, otherwise known as *velvet*. VeA is a light-dependent regulator governing both development and SM production in several aspergilli (*A. flavus*, *A. parasiticus*, and *A. nidulans*) (12, 14, 16, 26) as well as in other genera, including *Fusarium* (30, 31, 43), *Neurospora* (2), and *Acremonium* (15). VeA is also involved in activation of sexual development and inhibition of asexual development (reviewed in references 11 and 28). In fungi, developmental processes are commonly associated with secondary metabolism (13), and *VeA* has been found to function as a key global metabolic regulator in the biosynthesis of many SM concomitantly with sexual development (11). Disruptions of *veA* result in decreased production of numerous SM (15, 16, 26, 31). The VeA mechanism of action still remains elusive; however, recent studies at the protein level have provided important insight into the molecular machinery involved in this regulatory system, including a light-regulated VeA nuclear heteromeric complex formed with LaeA and another protein termed VelB (3, 36). This complex is conserved in genera as diverse as *Fusarium* (43).

The characterization of the VelB/VeA/LaeA transcriptional complex (known as the Velvet complex) provides a foundation for mechanistic studies linking SM with fungal development (3). Sexual development and SM production are both repressed in light. VeA is hypothesized to act as a scaffold protein that integrates external stimuli (e.g., light through the interaction with light-sensing proteins [11, 36]) with a nuclear response through an orchestrated action with other proteins, including LaeA and VelB, to regulate both processes. In the dark, the VelB/VeA/LaeA complex interaction controls the activity of LaeA, which subsequently controls the expression of SM gene clusters. In the light, this interaction is diminished as the entrance of the VelB LaeA-bridging factor VeA to the nucleus is decreased. As yet,
how the Velvet complex identifies, binds to, and/or activates SM gene clusters (or sexual development pathways) is unknown, although biochemical analysis of laeA and heterochromatin mutants (e.g., histone deacetylase and histone methyltransferase mutants) of A. nidulans reveal that SM gene expression is associated with removal of heterochromatin marks which, in part, require LaeA activity (7, 37, 40).

We have been able to further a mechanistic understanding of Velvet complex activity through examination of ∆laeA suppressor mutants. Here we show that overexpression of rsmA, encoding a putative bZIP transcription factor, results in remediation of secondary metabolism in ∆laeA and ∆veA backgrounds. This remediation retains in part the light regulation associated with VeA activity. In contrast, sexual development defects associated with both ∆laeA and ∆veA alleles cannot be rescued by RsmA, implicating this protein as functioning primarily in chemical development in A. nidulans.

MATERIALS AND METHODS

Fungal strains and culture conditions. The strains used in the study are listed in Table S1 in the supplemental material. All strains were propagated at 37°C on glucose minimal medium (GMM) (39) with appropriate supplements corresponding to the auxotrophic markers and were maintained as glycerol stocks at −80°C. Three veA alleles were examined in this study: either the wild type (veA), an N-terminally truncated version commonly used in A. nidulans studies (veA1), or a veA deletant (∆veA) (3).

Transformation with AMA1 cDNA library. The recipient strain RJW34.1 (pyrG89 wA3 Δate::arg8 veA1; trpC801; ΔlaeA;ΔveA) (see Table S1 in the supplemental material) was transformed with the pRCS-AMA1-based A. nidulans wild-type genomic DNA plasmid library with pyrG of Neurospora crassa as the selective marker (32), and transformants restoring norsolorinic acid (NOR) (an orange pigment intermediate in the sterigmatocystin pathway) on oatmeal solid medium (10) were isolated. Total DNA from individual transformants was used to amplify the insert by PCR with primers flanking the DNA insert in the AMA1 plasmid (AMAFWD and AMAREV). The PCR amplicons were sequenced, and the assembled sequences were used for BLASTn search of the A. nidulans genome (www.broad.harvard.edu/annotation/genome/aspergillus_group/). The AMA1 plasmid pMS65 was rescued from one of the NOR-producing transformants. Total DNA was extracted (38), and 1 μl was electroporated into Top10 Escherichia coli competent cells. Miniinprep preparation was performed on the bacterial transformants to yield pMS65. The sequence of the insert in pMS65 was reconfirmed by BLAST searching of the A. nidulans genome (www.broad.harvard.edu/annotation/genome/aspergillus_group/). The AMA1 plasmid pMS65 was rescued from one of the NOR-producing transformants. Total DNA was extracted (38), and 1 μl was electroporated into Top10 Escherichia coli competent cells. Miniinprep preparation was performed on the bacterial transformants to yield pMS65. The sequence of the insert in pMS65 was reconfirmed by BLAST searching of the A. nidulans genome (www.broad.harvard.edu/annotation/genome/aspergillus_group/). pMS65 was reintroduced into A. nidulans to confirm NOR production in transformation.

Generation of transformation cassettes and genetic manipulations. Plasmids pMS11, pMS29, pMS33, and pMS50 for overexpression (OE) of the four genes on plasmid pMS65 (AN4559.3, AN4560.3, AN4561.3, and AN4562.3) were constructed using a single-joint PCR strategy (46). For plasmid construction, pMS11, pMS29, pMS33, and pMS50 was used to overexpress AN4559.3, AN4560.3, AN4561.3, and AN4562.3, respectively. Five micrograms of the double-joint cassette was used to delete rsmA, and 10 μg of plasmid pMS6 was used for complementation of ∆rsmA. Overexpression, deletion, and complementation strains were verified by PCR and Southern blot analysis as described in Results. Sexual crosses of A. nidulans strains were conducted according to standard methods (35). Strain genotypes were identified by PCR amplification of the correct allele using primers sets described in Table S2 in the supplemental material.

Nucleic acid analysis. DNA extraction, digestion with restriction enzyme, gel electrophoresis, blotting, hybridization, and probe preparation were performed by standard methods (38).

Sterigmatocystin (ST) and norsolorinic acid (NOR) analyses. Ten microliters containing 10^4 spores of A. nidulans strains was point inoculated onto solid glucose minimal medium (GMM) (39) in 10-cm-diameter petri dishes and incubated for 3, 4, or 5 days at 37°C. A 15-mm-diameter agar plug was removed from the center of each plate culture, homogenized in 3 ml of double-distilled water, and extracted with an equal amount of chloroform by agitation for 30 min at room temperature. The chloroform extract was then dried completely at room temperature and resuspended in 100 μl of chloroform. Metabolites were separated in the developing solvent toluene-ethyl acetate-acetic acid (TEA) (8:1:1) on silica-coated thin-layer chromatography (TLC) plates (40), and photographs were taken following exposure to UV radiation at 254- and 366-nm wavelengths.

Analysis of sexual spore production. Five × 10^5 conidia of A. nidulans strains were inoculated onto a 5-mm overlay of Champes medium (21) or GMM in 10-cm-diameter petri dishes and incubated for 5 or 7 days at 37°C. An equal-size core was removed from each plate culture and homogenized in 3 ml of double-distilled water. Serial dilutions were carried out in double-distilled water, and sexual spores were quantified with a hemocytometer prior to overlay.

mRNA studies. Extractions were made from mycelia of cultures where 10^6 spores/ml were grown in 50 ml liquid GMM at 37°C with shaking at 250 rpm. The veA1 strains were incubated in light for 24 and 48 h; the veA strains were incubated in dark and light for 24, 48, and 72 h; and the ∆veA strains were incubated in light for 24, 48, and 72 h. In each case, the mycelium was subsequently harvested and lyophilized. RNA was extracted using TriZol (Invitrogen) according to the manufacturer’s instructions with approximately 30 μg of total RNA for RNA blot analysis. RNA blots were hybridized with stcE and rsmA DNA fragments which were generated by PCR using gene-specific primers as shown in Table S2 in the supplemental material. All experiments were performed in duplicate or triplicate.

Statistical analysis. For statistical analyses, the Excel data analysis package was used to calculate the mean, standard deviation of the mean, and standard error. Data were analyzed using the GraphPad Instate software package (version 3.05) according to the Tukey-Kramer multiple-comparison test at a P value of ≤0.05. In Fig. 6, mean values with different letters are significantly different.

RESULTS

Remediation of NOR phenotype by suppressor mutagenesis of ∆laeA. In order to identify genes which might remediate secondary metabolism in a null laeA background, the strain RJW34.1 (∆laeA Δate::pyrG89 veA1; see Table S1 in the supplemental material) was transformed with the autoreplicative AMA1 plasmid library (19, 32) and transformants assessed for the production of the orange pigment norsolorinic acid (NOR) on oatmeal medium. Screening of over 31,600 transformants identified 65 NOR-producing transformants. Four-
teen transformants turned out to harbor the laeA gene itself and one the NOR-specific transcriptional activator aflR (18). Of the remaining transformants, one, no. 65, showing exceptionally high NOR production was chosen for this study. The AMAI plasmid was rescued from this transformant, sequenced, and, by using the Aspergillus comparative database (www.broad.harvard.edu/annotation/genome/aspergillus_group/), found to contain four genes annotated as AN4559.3, AN4560.3, AN4561.3, and AN4562.3 with homologies to a DNAJ/histone deacetylase-interacting protein, a ribosomal protein, a phosducin homolog, and a bZIP transcription factor, respectively.

Overexpression of AN4562.3, encoding a bZIP protein, restores NOR production. To determine which of the four genes was responsible for NOR remediation in the ΔlaeA strain, overexpression (OE) constructs were made for each ORF and placed in the pyroA locus of strain RJW33.2 (%H9004; see Table S1 in the supplemental material). Transforms from all four OE constructs were screened on oatmeal to look for NOR production, and only transforms of OE::AN4562.3 produced the bright orange NOR pigment (Fig. 1A). Genomic DNA was extracted from all transformants and analyzed by PCR followed by Southern blot analysis (Fig. 1C).
Correct transformants containing OE alleles of AN4559.3, AN4560.3, AN4561.3, and AN4562.3 were called TMS1.2, TMS2.6, TMS3.26, and TMS4.2, respectively (Fig. 1C).

TMS1.2, TMS2.6, and TMS3.26 were retested for remediation of NOR production on oatmeal medium and TLC plates (Fig. 1B). TMS1.2, TMS2.6, and TMS3.26 showed NOR production equivalent to that of the control isogenic strain TMS5.4 (a strain deleted for \textit{laeA}; LaeA deletants can produce some NOR when point inoculated on oatmeal agar, but this is visible only by TLC and not by eye). Only TMS4.2 produced visible NOR on oatmeal medium, which was well reflected upon TLC examination. AN4562.3 was then designated \textit{rsmA} (remediation of secondary metabolism).

Bioinformatic analysis indicated that the \textit{rsmA} ORF, consisting of 1,098 bp with two introns (68 and 76 bp), encodes a predicted protein of 317 amino acids (aa). Sequence analysis of \textit{RsmA} (www.ebi.ac.uk/Tools/ppsearch/index.html) highlighted a basic region mediating sequence-specific DNA binding (KR KAQNRAAQRAFRER), followed by an adjacent C terminus bZIP domain with four leucine zippers (Fig. 2A). These motifs are typical of known bZIP transcription factors, and RsmA indeed shows high homology to many known and putative...
bZIP proteins from several fungi as well as higher eukaryotes. A BLASTp search of the protein database (National Center for Biotechnology Information, Bethesda, MD) showed the existence of putative homologs in other *Aspergillus* species such as *A. fumigatus*, *A. flavus*, *A. terreus*, and *A. oryzae*, as well as other filamentous fungi, such as *P. chrysogenum*, and higher eukaryotes. The amino acid sequence alignments of *Aspergillus* homologs were analyzed using the ClustalW multiple-sequence alignment software program (version 1.83; European Bioinformatics Institute, Cambridge, United Kingdom) (Fig. 2B). (B) *rsmA* and *stcU* gene expression in *A. nidulans* wild-type (RTD2.3), OE:*rsmA* veA1 (RMS11.1), ΔlaeA veA1 (RJW46.4), and OE:*rsmA* ΔlaeA veA1 (RMS31.1) strains. RNA was extracted after 24, 48, and/or 72 h of growth in liquid GMM shake cultures in duplicate at 37°C. Ethidium bromide-stained rRNA is indicated as a loading control.

**FIG. 3.** Overexpression of *rsmA* restores the laeA phenotype and regulates ST production. (A) TLC plates of ST extracts from the wild-type (WT), OE:*rsmA* veA1, ΔlaeA veA1, and OE:*rsmA* ΔlaeA veA1 strains (RDIT2.3, RMS11.1, RJW46.4, and RMS53.1, respectively) in dark and light. OE:*rsmA* veA1 has a high impact on ST production, with remediation of ST production of the ΔlaeA mutant in either dark or light. ST, sterigmatocystin standard. (B) *rsmA* and *stcU* gene expression in *A. nidulans* wild-type (RTD2.3), OE:*rsmA* veA1 (RMS11.1), ΔlaeA veA1 (RJW46.4), and OE:*rsmA* ΔlaeA veA1 (RMS31.1) strains. RNA was extracted after 24, 48, and/or 72 (not shown) h of growth in liquid GMM shake cultures in duplicate at 37°C. Ethidium bromide-stained rRNA is indicated as a loading control.

RsmA mediates the ΔlaeA phenotype and globally regulates SM production. Verification that OE:*rsmA* remediated SM production in the ΔlaeA background was obtained by crossing the OE:*rsmA* allele into a stcE wild-type background (RMS53.1). TLC examination of chloroform extracts of *A. nidulans* OE:*rsmA* ΔlaeA veA1 strains showed that ST production was restored similarly to NOR production in the ΔstcE background (compare Fig. 1B to Fig. 3A). Additional uncharacterized metabolites not observed in the ΔlaeA veA1 control were also visualized in extracts from the OE:*rsmA* ΔlaeA veA1 strain (see Fig. S1 in the supplemental material). Restoration appeared to be transcriptional, as *stcU* (ST gene) was expressed in the OE:*rsmA* ΔlaeA veA1 strain but not in the ΔlaeA veA1 strain (Fig. 3B).

Although SM was restored to a degree in the OE:*rsmA* ΔlaeA veA1 background, the combination of an OE:*rsmA* allele with wild-type *laeA* was considerably more striking. Expression levels of *stcU* were elevated in the OE:*rsmA* ΔlaeA veA1 strain, as shown in Fig. 3B, and ST production was severalfold higher than in the wild type (Fig. 3A). Additionally, the production of other, uncharacterized SM was increased in the OE:*rsmA* veA1 strain (see Fig. S1 in the supplemental material).

RsmA interactions with the veA allele. veA1 is recognized as a mutant veA allele, and so to obtain more insight into the role of OE:*rsmA* in ΔlaeA remediation, we created an isogenic set of strains in the veA genetic background. Here, in contrast to the case for OE:*rsmA* ΔlaeA veA1, OE:*rsmA* ΔlaeA veA remediates SM production only in the dark, with little or no remediation in the light (Fig. 4A and B; see Fig. S2 in the supplemental material). Expression of *rsmA* itself also was light regulated in the veA background (Fig. 4A). Although *rsmA* was not expressed as highly under light as under dark conditions, its enhanced expression still resulted in greatly increased ST production in the *laeA* wild-type strain regardless of illumination. The ST levels were largely consistent with the level of *stcU* expression (Fig. 4A).

For further analysis of RsmA interaction with the Velvet complex, we next examined SM in an OE:*rsmA* ΔveA strain. Figure 4D shows that OE:*rsmA* remediates ST production under both light and dark conditions; again, metabolite expression largely parallels *stcU* expression (Fig. 4C). Other, uncharacterized metabolites were also remediated in these double mutants (see Fig. S3 in the supplemental material).

**An RsmA deletant produces SM.** RsmA deletants were created by replacing *rsmA* with the *A. parasiticus* pyrG gene, as schematically represented in Fig. 5A. The RsmA complement strain TMS2.17 was created by transforming the ΔrsmA mutant (TMS6.30) with pMS6 such that an *rsmA* allele was integrated at the *pyrA* locus. The correct gene replacement and complementation transformants were verified by diagnostic PCR and Southern blot hybridization analysis (Fig. 5A). A preliminary examination of five correct deletants indicated no discernible effect on ST production (data not shown).

To more accurately assess if loss of *rsmA* affected SM production, TMS6.30 was crossed to obtain the prototrophic strain RMS8.1 (see Table S1 in the supplemental material). Both deletant (RMS8.1) and complement TMS2.17 produced a level of ST similar to or slightly higher than that produced by the wild type (Fig. 5C). The *stcU* transcript was delayed in RMS8.1 and TMS2.17 compared to in the wild type (Fig. 5B); however, this was not reflected in formation of product (ST) in the time frame chosen.

**Sexual defects of *ΔlaeA* and *ΔveA* strains are not remediated by OE:*rsmA*.** Enhanced SM production under dark conditions is coupled with sexual development, which is also regulated by the Velvet complex (3). VeA is absolutely required
for cleistothecial formation; however, no information about the effects of LaeA on sexual development is available. Here an examination of the /H9004 laeA strains shows that it has a negative impact on sexual development associated with misshapen and fewer cleistothecia, resulting in decreased ascospore production (Fig. 6). Unexpectedly, when grown on Champes medium but not on GMM (Fig. 6B; see Fig. S4 in the supplemental material), /H9004 laeA strains appeared blind to light, as ascospore production was not diminished in the light as it is in the wild type.

As OE::rsmA partially or wholly remediated the SM loss associated with ΔveA and ΔlaeA, we next inquired if sexual development was also rescued in these backgrounds. However, our results indicate that the sexual defect was not remediated in the double mutants, as the ΔlaeA OE::rsmA mutant generated a similar number of fewer ascospores than the mutant with ΔlaeA alone and neither the ΔveA or the ΔveA OE::rsmA mutant produced any ascospores (Fig. 6B; see Fig. S4 in the supplemental material). The OE::rsmA allele presented a mild diminishment in ascospore number when present in a wild-type laeA or veA background, which was possibly a reflection of a delay in development.

DISCUSSION
Filamentous fungi produce a variety of SM with diverse activities both beneficial and detrimental to humankind. Identification of SM regulatory elements could potentially provide a means of increasing production of beneficial metabolites, aid in the identification of “silent” natural products, and also contribute to a broader understanding of the molecular mechanisms by which SM are produced. Recent breakthroughs in fungal biology have led to the discovery of a nuclear complex, known as the Velvet complex, that is required for coordinating SM biosynthesis with fungal development (3). Two critical members of the Velvet complex are LaeA, a global regulator of SM (1, 5, 8, 29), and VeA, or velvet, involved in the regulation of diverse cellular processes, including asexual and sexual development (11, 28) as well as secondary metabolism (26, 31). Deletion of either gene leads to a loss or decrease of SM
production, and overexpression leads to enhanced production (1, 5, 8, 20, 26, 43).

Currently, the manner in which Velvet controls SM production is unknown, although LaeA activity is increasingly linked to chromatin modifications (37, 40). To obtain insight into possible mechanisms by which this complex operates, we employed a multicopy suppressor mutagenesis using a well-described autonomously replicating \( A. \) nidulans library (19, 32) to identify \( A. \) nidulans genes that could remediate \( /H9004 \) laeA loss of SM. By taking advantage of a known LaeA-regulated orange pigment, NOR (10), we were able to visually identify several \( /H9004 \) laeA suppressors that restored NOR production. Identification of both laeA and aflR (a NOR/ST transcription factor previously shown able to complement laeA loss dependent on chromosome location [9]) in the transformant pool provided strong support for the soundness of this approach. Other transformants yielded DNA sequences carrying a variety of putative genes involved in diverse aspects of fungal biology. One transformant contained an AMAI plasmid carrying four ORFs, AN4559.3, AN4560.3, AN4561.3, and AN4562.3, encoding a putative DNAJ/histone deacetylase interacting protein, a ribosomal protein, a phosducin homolog, and a bZIP transcription factor, respectively. As several of these putative functions appeared to logically fit into our current model of LaeA and Velvet SM regulation, efforts were directed to identify which, if any, of these genes was able to remediate laeA function.

As shown here, AN4562.3, henceforth designated RsmA, was the protein responsible for laeA remediation. Overexpression of this putative bZIP-encoding gene restored ST production in \( /H9004 \) backgrounds under most conditions, with the exception of there being minimal to no remediation in light in the \( /H9004 \) veA strain (Fig. 4B). This nonrestoration could be due to several factors. Decreased expression of \( rsmA \) in light, perhaps indicative of the known gpdA response to light and circadian rhythms (21), could have contributed to the absence of ST production under this condition; however, we note that this decreased expression did not seem to affect ST production in the isogenic laeA wild-type strain (Fig. 4A). Thus, we cannot rule out VeA/light mediation of RsmA functionality in this genetic background. However, the fact that OE::rsmA rescued ST production under both light and dark conditions in the \( /H9004 \) veA background indicates that VeA is not required for RsmA regulation of ST biosynthesis.

An intact Velvet complex is also required for normal light mediation of asexual/sexual development in \( A. \) nidulans. VeA appears to be part of a light-sensing apparatus resulting in asexual spore development in light and sexual spore development in the dark (2). Deletion of veA results in complete blockage of sexual development; however, no information exists on any effect of LaeA on sexual development in \( A. \) nidulans. Because \( \Delta laeA \) in \( A. \) flavus results in sclerotial loss similarly to \( \Delta veA \) in \( A. \) flavus (1) and sclerotia have recently been shown to house ascospore development in this species (23), we took a closer look at sexual development in \( \Delta laeA \) strains. Although not as severe as the null cleistothecial \( \Delta veA \) phenotype, \( \Delta laeA \) strains demonstrate distinct cleistothecial morphology aberrancies coupled with
decreased ascospore numbers (Fig. 6). Interestingly, ascospore production in the ΔlaeA mutant on Champes medium did not vary with light regimen, in contrast to the case for the wild type, thus suggesting that LaeA plays some role in the light response in this fungus.

Considering that both VeA and LaeA were critical not only for SM synthesis but also for sexual development, it seemed reasonable to query if OE::rsmA could also remediate sexual defects in either ΔlaeA or ΔveA backgrounds. Our results showed that RsmA has little impact on sexual development, as ascospore numbers and overexpression of the allele were unable to remediate sexual defects of ΔveA or ΔlaeA strains under the conditions tested. Thus, we conclude that the main role of RsmA may be in SM cluster activation.

Both metabolite analysis and transcriptional data strongly indicate RsmA to be a positive regulator of many secondary metabolite pathways. Often the deletion of positive regulatory factors leads to loss of phenotype (e.g., with ΔlaeA or ΔveA); however, in this case the rsmA deletant had relatively little effect on SM synthesis. This could indicate a distinct RsmA mechanism separate from Velvet complex regulation of SM and/or may suggest that RsmA works in concert with another bZIPs. bZIP proteins are dimerizing transcription factors found in all eukaryotes. They are characterized by the highly conserved bZIP domain comprised of a basic region (BR) and a leucine zipper (LZ) (24); both regions are well conserved in RsmA (Fig. 2A). The LZ is a short amphipathic coiled-coil protein domain responsible for recognition and dimerization specificity between two bZIPs (17). It is possible that RsmA binds with itself and at least one other bZIP and that the forming dimers interact with the Velvet complex to direct the necessary transcriptional activation machinery to SM clusters. Thus, when overexpressed, RsmA homodimers are preferentially formed and greatly activate SM production. However, if RsmA is absent in the cell, its other dimerization partner can still function to direct the Velvet complex to the necessary DNA sites, a theory to be tested in future studies in our lab.

Regardless of direct or indirect interaction with the Velvet complex, we propose RsmA to be a direct conduit to transcriptional regulation of multiple secondary metabolite clusters,
and ongoing metabolomic efforts in our laboratory are directed toward characterization of all RsmA-regulated SM.

ACKNOWLEDGMENTS

We acknowledge Mitchell Elgin, whose assistance with the suppressor screen was much appreciated. Special thanks and appreciation also go to Jon Palmer, Saori Amaike, and all Keller lab members for help and support.

This work was funded in part by the Egyptian government, Man- soura University (M.I.S.), and NIH grant GM084077 to N.P.K.

REFERENCES

1. Amaike, S., and N. P. Keller. 2009. Distinct roles for VeA and LaeA in development and pathogenesis of Aspergillus flavus. Eukaryot. Cell 8:1051–1061.
2. Bayram, Ö., S. Krappmann, S. Seiler, N. Vogt, and G. H. Braus. 2008. Neurospora crassa ve-1 affects axon bud formation. Fungal Genet. Biol. 45: 127–138.
3. Bayram, Ö., S. Krappmann, M. Ni, J. W. Bok, K. Helmstaedt, O. Valerius, S. Braus-Stromeyer, N. Kwon, N. P. Keller, J. Yu, and G. H. Braus. 2008. VeIB/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. Science 320:1504–1508.
4. Ben-Ami, R., R. E. Lewis, K. Leventakos, and D. P. Kontoyiannis. 2009. Aspergillus fumigatus inhibits angiogenesis through the production of glotoxin and other secondary metabolites. Blood 114:5393–5399.
5. Bok, J. W., and N. P. Keller. 2004. LaeA, a regulator of secondary metabolism in Aspergillus spp. Eukaryot. Cell 3:527–535.
6. Bolms, D., S. L., Johnsson, K. A. Marr, D. Andes, N. F. Nielsen, J. C. Frisvad, and N. P. Keller. 2005. LaeA, a regulator of morphogenetic fungal virulence factors. Eukaryot. Cell 4:1574–1582.
7. Bok, J. W., Y. M. Chiang, E. V. Szewczyk, Y. Reyes-Dominguez, A. D. Davidson, J. F. Sanchez, H. C. Lo, A. Watanabe, J. Strauss, B. R. Oakley, C. C. Wang, and N. P. Keller. 2009. Chromatin-level regulation of biosynthetic gene clusters. Nat. Chem. Biol. 5:174–182.
8. Bok, J. W., D. Hoffmeister, L. A. Maggio-Hall, R. Murillo, D. J. Glassner, and N. P. Keller. 2006. Genomic mining for Aspergillus natural products. Chem. Biol. 13:37–37.
9. Bok, J. W., D. Noordermeer, S. Kale, and N. P. Keller. 2006. Metabolic gene cluster silencing in Aspergillus nidulans. Mol. Microbiol. 61:1636–1645.
10. Butchko, R. A. E., T. H. Adams, and N. P. Keller. 1999. Aspergillus nidulans mutants defective in stc gene cluster regulation. Genetics 153:715–720.
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., J. W. Bok, W. Brooks, and N. P. Keller. 2004. VeA is required for toxin and sclerotial production in Aspergillus parasiticus. Appl. Environ. Microbiol. 70:4733–4739.
13. 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.
14. Cary, J. W., G. R. Obrian, D. M. Nielsen, W. Nierman, P. Harris-Coward, J. Dreyer, J., H. Eichhorn, E. Friedlin, H. Kurnsteiner, and U. Kück. 2008. Requirement of LaeA for secondary metabolism and sclerotial production in Aspergillus flavus. Fungal Genet. Biol. 45:1422–1429.
15. Kale, S. P., L. Milde, M. K. Trapp, J. C. Frisvad, N. P. Keller, and J. W. Bok. 2008. Further characterization of VeA for secondary metabolism and sclerotial production in Aspergillus flavus. Mycologia 100:433–429.
16. Hurst, H. 1996. Leucine zipcodes, transcription factors, p. 105–168. In P. Sheterline (ed.). Protein profile. Academic Press, New York, NY.
17. Horn, B. W., G. G. Moore, and I. Carbone. 2009. Sexual reproduction in Aspergillus flavus. Mycologia 101:133–143.
18. Hohn, T. M., R. Krishna, and R. H. Proctor. 1999. Characterization of a transcriptional activator controlling trichothecene toxin biosynthesis. Fungal Genet. Biol. 26:224–235.
19. Huang, L., W. C. Nierman, and N. P. Keller. 2006. FvVE1 regulates filamentous growth, the ratio of microconidia to macroconidia and cell wall formation in Fusarium verticillioides. Mol. Microbiol. 62:1418–1433.
20. Hymen, K. S., L. I. Butchko, M. Busman, R. H. Proctor, H. K. Abbas, and A. M. Calvo. 2009. FvVE1 regulates biosynthesis of the mycotoxins fumonisins and fusarins in Fusarium verticillioides. J. Agric. Food. Chem. 57:657–664.
21. Oshevo, N., and G. S. May, 2000. Conidial germination in Aspergillus nida- luns requires RAS signaling and protein synthesis. Genetics 155:647–656.
22. Otsani, A. H., G. S. May, and S. A. Otsani. 1999. The extremely conserved pyroA gene of Aspergillus nidulans is required for pyridoxine synthesis and is required indirectly for resistance to photosensitizers. J. Biol. Chem. 274:23565–23569.
23. Perrin, R. M., N. D. Fedorova, J. W. Bok, R. A. Cramer, J. R. Wortman, H. S. Kim, W. C. Nierman, and N. P. Keller. 2007. Transcriptional regulation of chemical diversity in Aspergillus fumigatus by LaeA. PLoS Pathog. 3:508–517.
24. Pontecorvo, G., J. A. Roper, L. M. Hemmons, K. P. Macdonald, and A. W. J. Button. 1953. The genetics of Aspergillus nidulans. Adv. Genet. 5:141–238.
25. Purschwitz, S., M. Müller, C. Kastner, M. Schöser, H. Haas, E. A. Espeso, A. Atoui, A. M. Calvo, and R. Fischer. 2008. Functional and physical interaction of blue and red-light sensors in Aspergillus nidulans. Curr. Biol. 18:1–5.
26. Reyes-Dominguez, Y., J. W. Bok, H. Berger, E. K. Shwab, A. Basheer, A. Gallmetzer, C. Sczaczochez, N. P. Keller, and J. Strauss. 2010. Heterochromatic markers are associated with the repression of secondary metabolism gene clusters in Aspergillus nidulans. Mol. Microbiol. 76:1376–1386.
27. Sambrook, J., and D. W. Russell. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
28. Shima, K., and N. P. Keller. 2001. Genetic involvement of a cAMP-dependent protein kinase in A gene protein signaling pathway regulating morphological and chemical transitions in Aspergillus nidulans. Genetics 157:591–600.
29. Shwab, E. K., J. W. Bok, M. Tribus, J. Galehr, S. Graesse, and N. P. Keller. 2007. Histone deacetylase activity regulates chemical diversity in Aspergillus. Eukaryot. Cell 6:1655–1664.
30. Tsetsiugi, D., L. R. Zarnowski, and N. P. Keller. 2004. The lipid body protein, PyroA, coordinates sexual and asexual sporulation in Aspergillus nidulans. J. Biol. Chem. 279:11344–11353.
31. Tsugi, G., Y. Kenmochi, Y. Takano, J. Swiegard, L. Farrall, I. Furusawa, O. Horino, and Y. Kubo. 2000. Novel fungal transcriptional activators, Cm rIp of Colletotrichum lagenarium and pigIp of Magnaporthe grisea, contain Cys2His2 zinc finger and Zn(H)Cys finger cluster motifs, including typical Cys2His2 zinc finger motifs and regulate transcription of melamin biosynthesis genes in a developmentally specific manner. Mol. Microbiol. 38:940–954.
32. Wiersmann, P., D. W. Brown, K. Kleigrewe, J. W. Bok, N. P. Keller, H.-U. Humpf, and B. Todayski. 2010. FvVE1 and Fvlace, components of a velvet-like complex in Fusarium fulva, affect differentiation, secondary metabolism and virulence. Mol. Microbiol. 77:992–914.
33. Yang, X., D. Tallihi, S. Weber, G. Poisson, and M. Raymond. 2001. Functional isolation of the Candida albicans FCR1 gene encoding a bZIP transcription factor homologous to Saccharomyces cerevisiae Yap1p. Yeast 18:1227–1225.
34. Yu, J. H., and N. Keller. 2005. Regulation of secondary metabolism in filamentous fungi. Annu. Rev. Phytopathol. 43:437–458.
35. Yu, J. H., Z. Hamari, and Y. Reyes-Dominguez. 2004. Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. Fungal Genet. Biol. 41:973–981.