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The Expression of Sterigmatocystin and Penicillin Genes in *Aspergillus nidulans* Is Controlled by *veA*, a Gene Required for Sexual Development

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Secondary metabolism is commonly associated with morphological development in microorganisms, including fungi. We found that *veA*, a gene previously shown to control the *Aspergillus nidulans* sexual/asexual developmental ratio in response to light, also controls secondary metabolism. Specifically, *veA* regulates the expression of genes implicated in the synthesis of the mycotoxin sterigmatocystin and the antibiotic penicillin. *veA* is necessary for the expression of the transcription factor *aflR*, which activates the gene cluster that leads to the production of sterigmatocystin. *veA* is also necessary for penicillin production. Our results indicated that although *veA* represses the transcription of the isopenicillin synthetase gene *ipnA*, it is necessary for the expression of *acvA*, the key gene in the first step of penicillin biosynthesis, encoding the delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase. With respect to the mechanism of *veA* in directing morphological development, *veA* has little effect on the expression of the known sexual transcription factors *nsdD* and *steA*. However, we found that *veA* regulates the expression of the asexual transcription factor *brlA* by modulating the α/β transcript ratio that controls conidiation.

Fungi of the genus *Aspergillus* are remarkable organisms that readily produce a wide range of natural products, also called secondary metabolites. These compounds are diverse in structure, and in many cases, the benefits the compounds confer on the organism are unknown. However, interest in these compounds is considerable, as some natural products are of medical, industrial, and/or agricultural importance. Some of these products are beneficial to humankind (e.g., antibiotics), whereas others are deleterious (e.g., mycotoxins) (13, 17).

For several years, the fungus *Aspergillus nidulans* has been used as a model system to investigate secondary metabolism in *Aspergillus* spp., including the study of mycotoxins. *A. nidulans* produces the polyketide sterigmatocystin (ST). ST and the aflatoxins (AF), which are related fungal secondary metabolites (29, 30), are among the most toxic, mutagenic, and carcinogenic natural products known (44, 47). The genes responsible for ST biosynthesis are located in a cluster (7). Among these genes, *aflR* encodes a pathway-specific transcription factor that simultaneously regulates the expression of other genes in the cluster (52). Hicks et al. (25) reported that *aflR* expression leading to ST biosynthesis is genetically linked with the production of asexual spores (conidia) in *A. nidulans* through a G-protein signaling pathway (Fig. 1). According to this model, FluG is involved in the synthesis of an unknown diffusible signal molecule that triggers FlbA (a homolog of GTPase-activating protein). FlbA negatively regulates FadA (an α subunit of a heterotrimeric G protein) by stimulating GTP hydrolysis. In its active form, FadA is GTP bound and transduces a growth-supporting signal to unidentified downstream targets. When FlbA represses FadA, it blocks G-protein signaling for growth and triggers initiation of conidiation and ST production. Knowledge of the mechanism by which FluG, FlbA, and FadA regulate conidiation and ST biosynthesis was recently extended by characterization of a cyclic-AMP-dependent protein kinase catalytic subunit, PkaA (43). Overexpression of *pkaA* inhibits transcription of *brlA* (a specific transcription factor that activates conidiation [2]) and *aflR* and, concomitantly, conidiation and mycotoxin production, respectively (43). Interestingly, certain mutations in genes involved in the G-protein signaling pathway also prevent the formation of spherical fruiting sexual bodies called cleistothecia, where the sexual spores, called ascospores, are formed. Specifically, these mutants are dominant activating mutations in *fadA* and loss-of-function mutations in *sfaD* (the β subunit of a heterotrimeric G protein [Fig. 1]) (42) and overexpression of *pkaA* (43). These findings suggest the existence of a genetic link, not only connecting conidiation and secondary metabolism, but also connecting with processes leading to the development of sexual stages in *A. nidulans*.

Studies of the molecular mechanism controlling sexual development are very limited. Only a few genes are known to be involved, and their possible roles in regulating secondary metabolism have never been investigated. One example is the velvet gene, *veA*, which in *A. nidulans* mediates a developmental light response (50). In *A. nidulans* strains containing a wild-type allele of the velvet gene (veA^+), light reduces and delays cleistothecial formation and the fungus develops asexually, whereas in the dark, fungal development is directed toward the sexual stage, forming cleistothecia. Under conditions inducing sexual development, the *veA* deletion (Δ*veA*) strain is unable to develop sexual structures (33), indicating that *veA* is required for cleistothecium and ascospore formation. However, the molecular mechanism by which *veA* regulates sexual development is still unknown, as VeA does not

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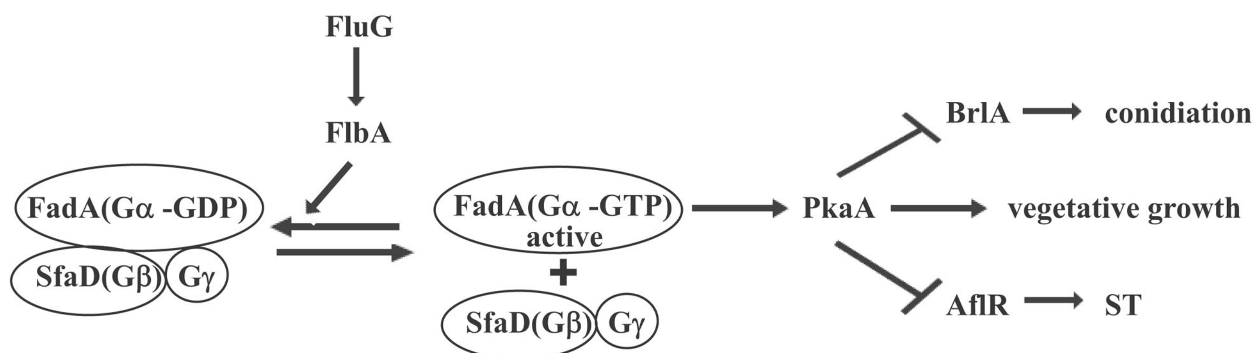

FIG. 1. Proposed model for the FadA (G-protein) signal transduction pathway regulating growth, ST production, and conidiation in *A. nidulans*. FluG is involved in the synthesis of a small signal molecule participating in the positive regulation of FlbA, a GTPase-activating protein that negatively regulates FadA (the α-subunit of the heterotrimeric G protein), forming a Gα-Gβ-Gγ complex. Active FadA (separated from Gβ-Gγ subunits) positively regulates PkaA, inducing vegetative growth and inhibiting conidiation and ST production. FadA inhibition allows development and secondary metabolism production to occur (25). AflR and BrlA are specific transcription factors activating ST production and conidiation, respectively.

present homology with any known protein that could indicate its functionality.

Other genes involved in *A. nidulans* sexual development include *lsdA*, *phoA*, *medA*, *stuA*, *tubB*, *nsdD*, and *steA* (9, 16, 21, 34, 35, 39, 48). Among these genes, *nsdD* and *steA* encode transcription factors that are required for sexual development (21, 48). The *nsdD* gene encodes a GATA-type transcription factor (21), whereas the *steA* gene (48) is a homolog of *Saccharomyces cerevisiae STE12* (38). Deletion of either *nsdD* or *steA* results in blockage of sexual development. Whether *veA* regulation of sexual development is mediated through *nsdD* and/or *steA* has not been investigated. As part of our study, we examined *nsdD* and *steA* expression in the Δ*veA* strain and in the wild-type control strain. Since *veA* regulates not only sexual development but also asexual development in *A. nidulans*, we also investigated the possible role of *veA* in controlling the expression of the asexual transcription factor *brlA*.

In this work, we report a connection between *veA* and secondary metabolism in *A. nidulans*. We have found that the *A. nidulans* Δ*veA* strain was unable to produce the mycotoxin ST and that such blockage is mediated by a pronounced reduction in or absence of *aflR* transcription. Concomitant with the effect of *veA* on ST production, our study revealed broader changes in the secondary-metabolite profile of the *A. nidulans* Δ*veA* strain. We examined the possible role of *veA* in regulating the penicillin (PN) genes. The PN pathway is well characterized in *A. nidulans* (5). PN production was lower in the Δ*veA* strain than in the wild-type control strain. Interestingly, we found that although *veA* negatively regulates the transcription of *ipnA*, the isopenicillin synthetase gene, *veA* is necessary for the expression of *acvA*, the key gene involved in the first step of the PN biosynthetic pathway encoding delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase (6, 41). Therefore, *veA* is necessary for both ST and PN production. This study contributes to the overall knowledge of the regulatory genes governing the production of secondary metabolites and to the development of control mechanisms for their detrimental and beneficial effects.

MATERIALS AND METHODS

Fungal strains and growth conditions. The *A. nidulans* strains used in this study are listed in Table 1. These strains were inoculated on YGT medium (0.5% [wt/vol] yeast extract, 2% [wt/vol] glucose, and 1 ml of trace element solution per liter of medium [27]), which has been used previously to promote sexual development in *A. nidulans* (11, 12, 14, 15), unless otherwise indicated. Appropriate supplements corresponding to the auxotrophic markers were added to the medium (27). Agar (15 g/liter) was added to produce solid medium.

Physiological studies. The studies of sexual development were performed with the *veA*$^+$ (FGSC4) and Δ*veA* (RNKT1) strains. RNKT1 was obtained by meiotic recombination of DVAR1 (provided by Keon-Sang Chae) and FGSC33 (Table 1). Plates containing 30 ml of solid YGT medium were spread with 100 μl of water containing 10^5 spores. The cultures were incubated at 37°C in the light or in the dark. After 7 days, a 16-mm-diameter core was removed from each spread plate culture and homogenized in water to release the spores. Sexual and asexual spores were counted using a hemacytometer. The number of Hülle cells, which are involved in the formation of the cleistothecial wall (50), was estimated in the same manner. Identical cores were taken to examine cleistothecial production. The cores were spread with 95% ethanol to enhance visualization of cleistothecia. Cleistothecium production was examined under a stereo-zoom microscope. Conidial production was estimated in cultures grown on glucose minimum medium (GMM), commonly used to study conidiation in *A. nidulans* (12, 13, 25, 43, 52), as well as on YGT medium. As in the case of ascospores, conidia were also counted using a hemacytometer. Colony growth was recorded as the colony diameters in point inoculation cultures on GMM. Experiments were carried out with four replicates.

Mycotoxin analysis. Four cores (16-mm diameter) from each replicate of *veA*$^+$ and Δ*veA* cultures were collected in a 50-ml Falcon tube, and ST was extracted

TABLE 1. *A. nidulans* strains used in this study

Strain	Genotype[a]	Source
FGSC4	*veA*$^+$	FGSC[b]
FGSC33	*biA1 pyroA4 veA1*	FGSC
DVAR1	*pabaA1 yA2* Δ*argB*::*trpC trpC801* Δ*veA*::*argB*	K.-S. Chae
RNKT1	Δ*veA*::*argB*	This study
RJH079	*biA1 argB2 alcA*(p)::*aflR*::*trpC veA1*	N. P. Keller
WIM126	*pabaA1 yA2 veA*$^+$	L. Yager
RNKT4	*alcA*(p)::*aflR*::*trpC* Δ*veA*::*argB*	This study
RNKT6	*alcA*(p)::*aflR*::*trpC veA*$^+$	This study

[a] The nomenclature Δ*veA*::*argB* indicates that *veA* has been deleted through gene replacement by the selectable marker gene *argB*.
[b] FGSC, Fungal Genetics Stock Center.

by adding 5 ml of $CHCl_3$ three consecutive times. The extracts were allowed to dry and then resuspended in 500 μl of $CHCl_3$ before 15 μl of each extract was fractionated on a silica gel thin-layer chromatography (TLC) plate using a toluene-ethyl acetate-acetic acid (80:10:10 [vol/vol/vol]) solvent system. The TLC plates were sprayed with aluminum chloride (15% in ethanol) to intensify ST fluorescence upon exposure to long-wave (365-nm) UV light and baked for 10 min at 80°C prior to being viewed. The approximate sensitivity of the assay was 25 ng. ST purchased from Sigma was used as a standard.

PN analysis. The PN bioassay was performed essentially as described by Brakhage et al. (6), using *Bacillus calidolactis* C953 (provided by Geoffrey Turner) as a test organism. First, one core (16-mm diameter) from each replicate of veA^+ and Δ*veA* cultures was homogenized and centrifuged at 15,000 × *g* for 10 min at 4°C. The resultant supernatants were evaporated in a vacuum centrifuge and resuspended in 50 μl of water, and their PN contents were evaluated as follows. Twenty-five milliliters of the *B. calidolactis* culture (optical density, 1) was added to 250 ml of melted tryptone-soya agar medium, mixed, and poured into four large petri dishes (150-mm diameter). Ten microliters of each extract, as well as commercial PN G (Sigma), at different concentrations (0.1, 0.25, 0.5, 1, 2.5, and 5 μg/ml) was applied to 2-mm-diameter wells and then incubated at 56°C for 24 h to allow bacterial growth and the visualization of inhibition halos. Inhibition halos were clearly detected at the lower standard amount used (1 ng). Additional controls containing penicillinase from *Bacillus cereus* (5 U per sample; purchased from Sigma) were used to confirm that the antibacterial activity observed was due to the presence of PN. This allowed us to distinguish the antibiotic activity of PN from those of other compounds that could have been produced. Experiments were carried out with four replicates.

mRNA studies. Five milliliters of melted 0.7% agar-YGT containing 10^6 conidia of either the veA^+ or Δ*veA* strain was poured on plates containing 25 ml of solid 1.5% agar-YGT and incubated at 37°C during a time course experiment performed in the dark, a condition that induces sexual development. Samples were harvested for RNA extraction 30, 45, and 60 h after inoculation. In order to evaluate the effect of light on fungal differentiation in the Δ*veA* strain, a similar experiment was carried out in which the veA^+ and Δ*veA* strains were incubated in the light (25 microeinsteins/m^2/s) or in the dark. After 60 h, the samples were harvested and processed for mRNA analysis. To determine the effect of overexpressing *aflR* on ST production in veA^+ and Δ*veA* strains, 100 ml of GMM liquid cultures were inoculated with 10^8 conidia of the following strains: FGSC4 (veA^+), RNKT6 [*alcA*(p)::*aflR* veA^+], RNKT1 (Δ*veA*), and RNKT4 [*alcA*(p)::*aflR* Δ*veA*]. RNKT6 and RNKT4 were generated by meiotic recombination of RJH079 (provided by Nancy Keller) with WIN126 and DVAR1, respectively (Table 1). After 14 h of incubation at 37°C, equal amounts of mycelia were harvested by filtration, washed, and transferred to 20-ml cultures of threonine minimum medium (TMM), which induces the *alcA* promoter. Mycelia for RNA extraction were collected at the 0- and 24-h points after shift into TMM, where the 0 h represents the moment of shift from GMM to TMM.

Total RNA was isolated from mycelia by using Trizol (Invitrogen) as described by the supplier. Approximately 20 μg of total RNA was used for RNA blot analysis. The probes used in the mRNA analysis were *brlA*, a 4.3-kb *Sal*I fragment from pTA111 (2); *aflR*, a 1.3-kb *Eco*RV-*Xho*I fragment of pAHK25 (7); *stcU*, a 0.75-kb *Sst*II-*Sma*I fragment of pRB7 (52); and *ipnA*, a 1.1-kb *Hin*dIII-*Eco*RI fragment of pUCHH(458) (46). PCR products amplified with the following primer pairs were used as *nsdD*- and *steA*-specific probes, respectively: *nsdD*, 5′-GATATGAATTCGCTGAC-3′ and 5′-TGCTCTTGAATTCCTCC-3′; *steA*, 5′-TCCACATCTCAGGTACCG-3′ and 5′-TCGCTCCTGAGTGTGAGT-3′. The identities of the *nsdD* and *steA* probes were confirmed by sequencing. RNA loading was normalized in each experiment to rRNA, which remains at constant levels. Densitometry data were acquired and analyzed with the NIH Image J version 1.30 program.

RT-PCR. *A. nidulans acvA*, encoding the first enzyme of the PN biosynthetic pathway, presents a transcript of 11,313 nucleotides. Detection of such large transcripts by Northern analysis is difficult to achieve. For this reason, reverse transcription-PCR (RT-PCR) was the method of choice to evaluate the presence or absence of *acvA* expression. The veA^+ and Δ*veA* strains were incubated on YGT medium in the light or in the dark for 60 h. At that time, the samples were harvested and processed for mRNA analysis. Total RNA samples were treated with a DNA-free kit (Ambion), according to the instructions of the supplier, to eliminate possible trace amounts of contaminant genomic DNA. Moloney murine leukemia virus reverse transcriptase (Promega) and random primers (Pharmacia) were used to synthesize the first-strand cDNAs (0.1 μg of RNA per 20-μl reaction mixture). One microliter from the previous reaction was added to the PCR mixture. The *acvA* primers used were 5′-AGACGGCCCTGGCTACAG-3′ and 5′-GCGAAACAGTTGGCCTGC-3′. The rRNA primers 5′-TTCTGCCCT ATCAACT-3′ and 5′-GGCTGAAACTTAAAGGAATTG-3′ (provided by Melvin Duvall) were used as internal controls. PCR results were analyzed by electrophoresis in a 1% agarose gel.

RESULTS

Velvet, or *veA*, is a major light-dependent regulatory gene governing development in *A. nidulans* (33, 50). As part of our preliminary studies, we reproduced the results supporting *veA* involvement in conidiation and cleistothecial formation. After this verification, we further investigated the role of *veA* in the regulation of transcription factors necessary for sexual and asexual development. Our group also investigated, for the first time, the involvement of *veA* in regulating secondary metabolism, particularly the role of *veA* in the synthesis of the mycotoxin ST and the antibiotic PN.

Roles of *veA* and light in *A. nidulans* morphological development. Several parameters, including sexual development, conidiation, and fungal growth, were analyzed to verify the effect of *veA* deletion on morphological development in *A. nidulans*. Since light is important in *veA* regulation of *A. nidulans* development (50), the study of colony growth, conidiation, cleistothecial formation, and mycotoxin production in veA^+ and Δ*veA* strains was done in white light or in the dark. The colony size of the veA^+ strain was slightly larger than that of the Δ*veA* strain after 7 days of incubation (data not shown; also confirmed by analysis of variance [$P < 0.05$]). As predicted, the Δ*veA* strain was unable to form sexual structures either in the light or in the dark. Our culture conditions were slightly different from those used by Kim et al. (33); however, our results were in agreement for dark conditions (light was not assayed by Kim et al.). These data verified that the *veA* gene is necessary for sexual development. The higher production of ascospores and lower production of conidia in the veA^+ strain grown in the dark were also consistent with previous observations (11, 50). In this experiment, we also noted that the veA^+ strain produced approximately two-fold more conidia than the Δ*veA* strain under both light and dark conditions when the fungus was cultured on GMM (data not shown).

Deletion of *veA* alters the expression of the transcription factors *nsdD*, *steA*, and *brlA*. In order to gain further insight into the mechanism through which *veA* governs morphological differentiation in *A. nidulans*, in this study we analyzed for the first time the possible role of *veA* in regulating the expression of key transcription factor genes directing sexual development, such as *steA* and *nsdD*, or asexual development, such as *brlA*. Initially, we investigated whether the blockage in cleistothecial formation was due to an effect of *veA* deletion on the transcription of the *nsdD* and/or *steA* gene, as it is known that inactivation of *nsdD* or *steA* expression blocks cleistothecial formation (21, 48). Total RNA was extracted from the veA^+ and Δ*veA* strains after growing them for 30, 45, and 60 h during morphological development (YGT solid cultures) in the dark. The *nsdD* and *steA* transcripts were detected in both the veA^+ and Δ*veA* strains (Fig. 2). Deletion of *veA* had little effect on *nsdD* and *steA* expression. The densitometry analysis indicated a decline in *nsdD* transcription levels at 60 h. At the earliest time point examined (30 h), *steA* transcription levels were slightly lower than in the wild type (times prior to 30 h were not examined due to the limitation of fungal biomass for analysis), reaching wild-type levels at the 60-h time point (Fig. 2).

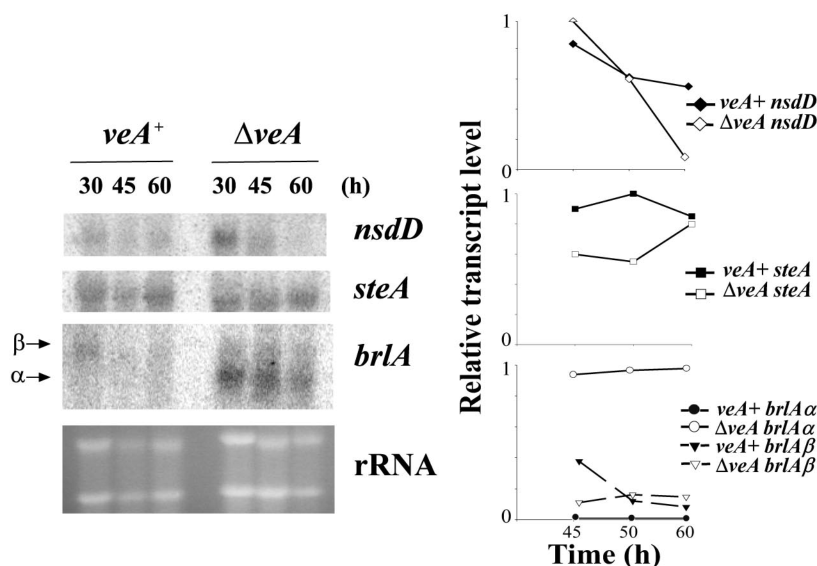

FIG. 2. Effects of the *veA* deletion on the transcription of genes implicated in morphological development in *A. nidulans*. The transcriptional patterns of the developmental genes *nsdD*, *steA*, and *brlA* in the *veA*$^+$ and Δ*veA* strains were evaluated by Northern analysis. (Left) Total RNAs of the *veA*$^+$ and Δ*veA* strains were isolated 30, 45, and 60 h after the strains were cultured on solid YGT medium in the dark. rRNA stained with ethidium bromide is shown to indicate RNA loading. (Right) mRNA was quantitated by densitometry and plotted as relative band intensity normalized to rRNA and to the highest band intensity in each graph (considered as 1 U). Two separate repetitions of these experiments yielded similar results.

Although the asexual-development transcription factor, *brlA*, is not required for ST biosynthesis (20), both *brlA* and the ST transcription factor *aflR* are coregulated by the *fadA* signaling pathway genes (Fig. 1) (25). We investigated the possible role of *veA* in regulating *brlA*. The *brlA* gene produces two overlapping transcripts, *brlA*α and *brlA*β (22, 23). *brlA*β transcripts were detected in the *veA*$^+$ and Δ*veA* strains; however, *brlA*α was clearly the predominant transcript in the Δ*veA* strain (Fig. 2), particularly in the Δ*veA* cultures incubated in the light (Fig. 3A). The increase in *brlA*α in Δ*veA* cultures coincided with an increase in conidial production (Fig. 3B). Physiological studies showing that *veA* also regulates asexual development were previously reported (33, 50); however, this is the first report of the control of *veA* over the expression of the conidiation-regulatory gene *brlA*.

Deletion of *veA* and light affects ST biosynthesis in *A. nidulans*. The possible role of *veA* in regulating secondary metabolism has never been investigated, and therefore the implications that *veA* could have for the production of mycotoxin and other secondary metabolites remain unknown. We studied the effect of *veA* deletion on ST production in cultures in the dark and in the light by TLC. As shown in Fig. 3C and D, the *A. nidulans veA*$^+$ strain produced the mycotoxin ST, while the Δ*veA* strain failed to produce ST in both light and dark cultures on YGT and GMM media. This result was also confirmed by liquid chromatography coupled with mass spectrometry (data not shown). In the *A. nidulans veA*$^+$ strain, illumination had an effect on ST biosynthesis that was medium dependent. Nutritional factors can affect not only development but also mycotoxin production. For example, it has been established that certain carbon and nitrogen sources stimulate (i.e., glucose [1, 49]) or inhibit (i.e., nitrate [4, 26]) AF biosynthesis in *Aspergillus parasiticus* and *Aspergillus flavus*. Less is known about carbon and nitrogen effects on ST production in *A. nidulans*. As in the case of AF in *A. parasiticus* and *A. flavus*, glucose does not repress but sustains ST production in *A. nidulans* (8, 25, 31, 43). As for the nitrogen source, Feng and Leonard (18) reported that nitrate GMM supports gene expression for ST biosynthesis in *A. nidulans* while ammonium GMM does not, showing a transcript expression pattern opposite to that of AF. On the other hand, expression of ST genes and ST production has been shown in ammonium complete medium (containing yeast extract) by Keller et al. (31). Therefore the nitrate-ammonium effect on ST production seems to interact with other medium components, and possibly with other environmental factors. In our experiments, we looked at the effect of light on ST production on two different media. In the *veA*$^+$ strain, more ST was produced in cultures growing on YGT (medium containing yeast extract) in the light than in the dark (Fig. 3C), whereas more ST production was observed in the dark when the fungus was grown on GMM (containing nitrate) (Fig. 3D). The higher ST production in the light on YGT medium coincides with higher levels of *aflR* and *stcU* (one of the enzymatic genes in the ST cluster used as an indicator of cluster activation by *aflR*) mRNAs in light cultures on YGT (Fig. 3A).

Deletion of *veA* represses transcription of the ST-specific regulatory gene, *aflR*. Our mRNA analysis showed that *aflR* transcript accumulation was notably lower in the Δ*veA* strain than the normal *aflR* transcript levels observed in the wild-type strain under the same experimental conditions (Fig. 3A and 4A). Inactivation of *aflR* expression is known to block ST biosynthesis (10). ST production was also evaluated in Δ*veA* and wild-type strains by TLC during a time course experiment (Fig. 4B). The Δ*veA* strain produced no ST, while ST produc-

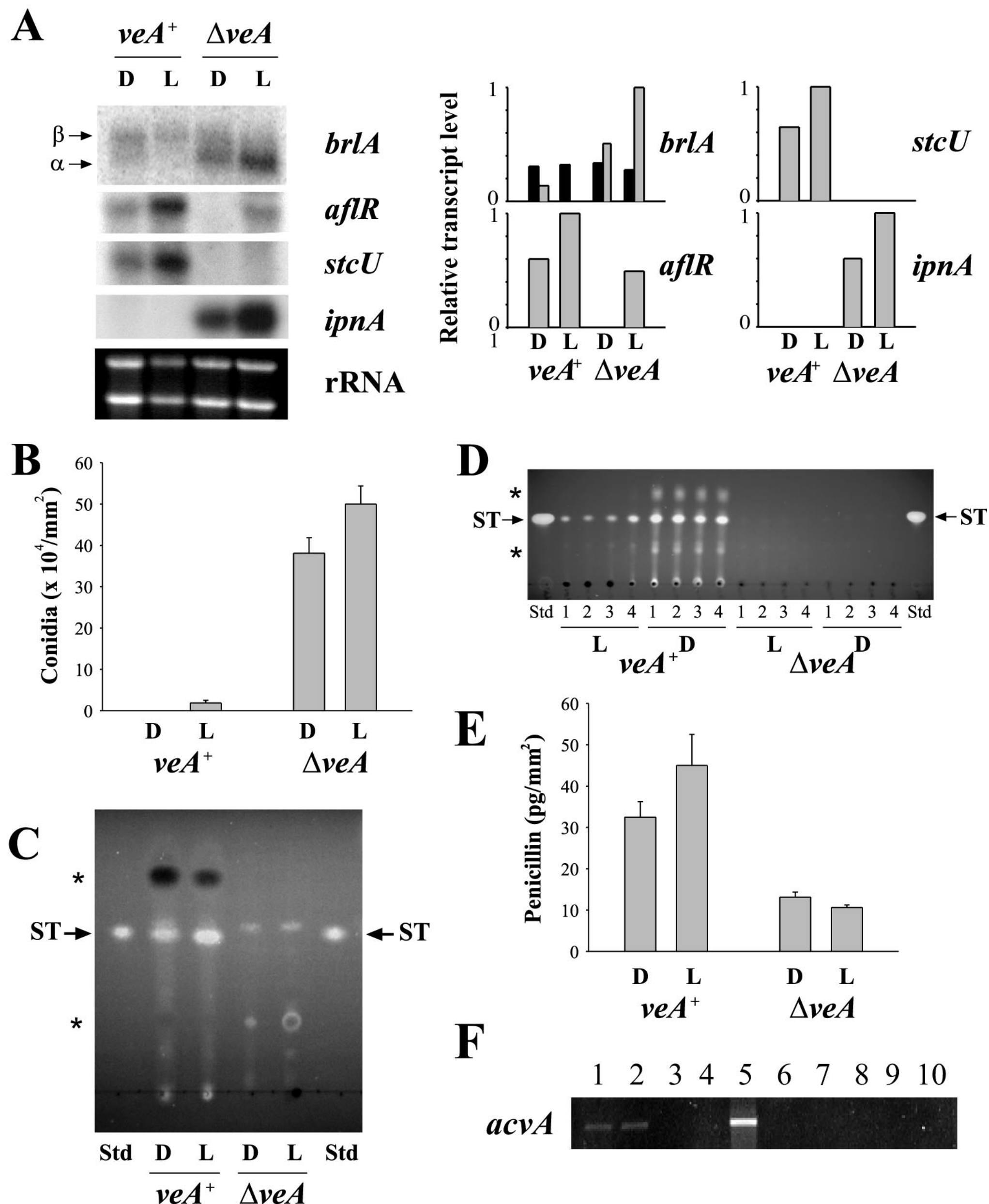

FIG. 3. Combined effects of *veA* deletion and light on the transcription of genes implicated in morphological and chemical differentiation in *A. nidulans*. (A) The expression of genes involved in conidiation (*brlA*) and secondary metabolism (*aflR*, *stcU*, and *ipnA*) was evaluated by Northern analysis in the veA^+ and Δ*veA* strains (left). Total RNAs were isolated from the cultures grown on solid YGT medium after 60 h in the light or in the dark. rRNA stained with ethidium bromide is shown to indicate RNA loading. mRNA was quantitated by densitometry and plotted as relative band intensity normalized to rRNA and to the highest band intensity in each graph (considered as 1 U) (right). Two separate repetitions of these experiments yielded similar results. Under the same experimental conditions, other parameters were analyzed as follows. (B) Conidial production per surface area. The values are means of four replicates. (C) ST analysis by TLC. The uncharacterized compound observed in Δ*veA* near the ST retention factor (R_f) is not ST (in this assay, ST fluoresces bright yellow while the unknown compound fluoresced blue). (D) ST analysis of samples extracted from GMM cultures by TLC. The uncharacterized compound observed in Δ*veA* near the ST R_f is not ST (in this assay, ST fluoresces bright yellow while the unknown compound fluoresced blue). (E) Effects of the *veA* deletion on PN production in *A. nidulans* on solid YGT medium in the light or in the dark. The values are means of four replicates. (F) Detection of *acvA* expression on YGT medium by RT-PCR. Lanes: 1, veA^+ in the dark; 2, veA^+ in the light; 3, Δ*veA* in the dark; 4, Δ*veA* in the light; 5, positive control containing genomic DNA as a template; 6, negative control without DNA template; 7 to 10, PCR results of RNA samples (veA^+ in the dark, veA^+ in the light, Δ*veA* in the dark, and Δ*veA* in the light, respectively) before RT, showing the absence of contaminant genomic DNA in the samples. L, light; D, dark; Std, standard; *, other uncharacterized metabolites absent or present in different amounts in Δ*veA* with respect to veA^+. The error bars indicate standard error.

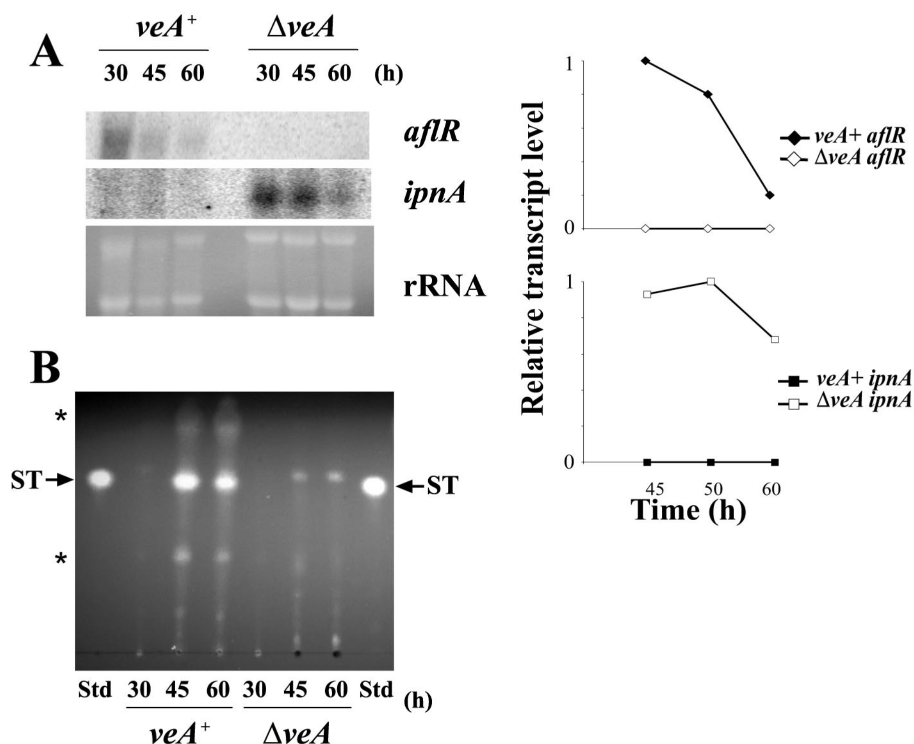

FIG. 4. Effects of *veA* deletion on transcription of genes implicated in production of secondary metabolites during morphological development in *A. nidulans*. (A) Transcriptional patterns of genes involved in secondary metabolism, *aflR* and *ipnA*, were evaluated by Northern analysis in the *veA*$^+$ and Δ*veA* strains. Total RNAs of the *veA*$^+$ and Δ*veA* strains were isolated 30, 45, and 60 h after inoculation on solid YGT medium (left). rRNA stained with ethidium bromide is shown to indicate RNA loading. mRNA was quantitated by densitometry and plotted as relative band intensity normalized to rRNA and to the highest band intensity in each graph (considered as 1 U) (right). Two separate repetitions of these experiments yielded similar results. (B) ST analysis by TLC. The uncharacterized compound observed in Δ*veA* near the ST R_f is not ST (in this assay, ST fluoresces bright yellow while the unknown compound fluoresced blue). Std, standard; *, other uncharacterized metabolites absent or present in different amounts in Δ*veA* with respect to *veA*$^+$.

tion was detected at 45 and 60 h in the *veA*$^+$ strain (Fig. 4B). Although *aflR* was detected at 60 h in the light cultures in the the Δ*veA* strain (the *aflR* transcription level was still lower than that of the wild type under the same experimental conditions [Fig. 3A]), *stcU* expression was completely absent (Fig. 3A).

Overexpression of *aflR* remediates the defect in ST production caused by *veA* deletion. Since v*eA* deletion results in a drastic reduction or absence of *aflR* transcription, and consequently an absence of ST production, we tested the effect of the forced expression of *aflR* on ST production in the Δ*veA* background. We generated isogenic *alcA*(p)::*aflR* strains differing only in the presence or absence of *veA* and cultivated them in liquid GMM followed by transfer to TMM, inducing conditions for the *alcA* promoter. Neither *aflR* nor *stcU* was detected in the Δ*veA* strain without the *alcA*(p)::*aflR* fusion, resulting in no ST production (Fig. 5). As expected, high *aflR* transcript accumulation was detected in both *alcA*(p)::*aflR* strains 24 h after the shift into TMM. Under these conditions, *stcU* expression was partially restored in the Δ*veA* strain by the overexpression of *aflR*, leading to ST production.

Deletion of *veA* alters the expression of the PN biosynthetic genes *ipnA* and *acvA* in *A. nidulans*. The TLC analysis of the *veA*$^+$ and Δ*veA* strains also indicated a different profile with respect to other metabolites (Fig. 3C and D and 4B), suggesting that *veA* could have a broader effect, perhaps over additional metabolic pathways. For this reason, we looked at the possible effect of *veA* deletion on PN biosynthesis at the transcriptional level; specifically, we first examined the expression of *ipnA*. The *ipnA* gene encodes an isopenicillin N synthetase, an enzyme required for PN biosynthesis. In contrast to the effect observed on *aflR* and *stcU* expression, the *ipnA* transcripts were abundant in the Δ*veA* strain (Fig. 3A and 4A). PN production was analyzed by using a bioassay method with *B. calidolactis* as the test organism. Surprisingly, the *veA* deletion produced less PN than the *veA*$^+$ strain (Fig. 3E). For this reason, we also looked at the expression of the gene involved in the first step of the PN biosynthesis pathway, *acvA*, in the *veA*$^+$ and Δ*veA* strains cultured in the light and in the dark. Because of the large size of the *acvA* transcript (11,313 nucleotides), we chose RT-PCR for its analysis. Our RT-PCR indicated that *acvA* transcripts were detected only in the *veA*$^+$ strain, in both light and dark cultures (Fig. 3F) (the rRNA control generated similar amounts of PCR products in all *veA*$^+$ and Δ*veA* samples [data not shown]).

DISCUSSION

In this study, we discovered a genetic connection between sexual development and secondary metabolism in *A. nidulans* mediated by the regulatory gene *veA*. Our most interesting

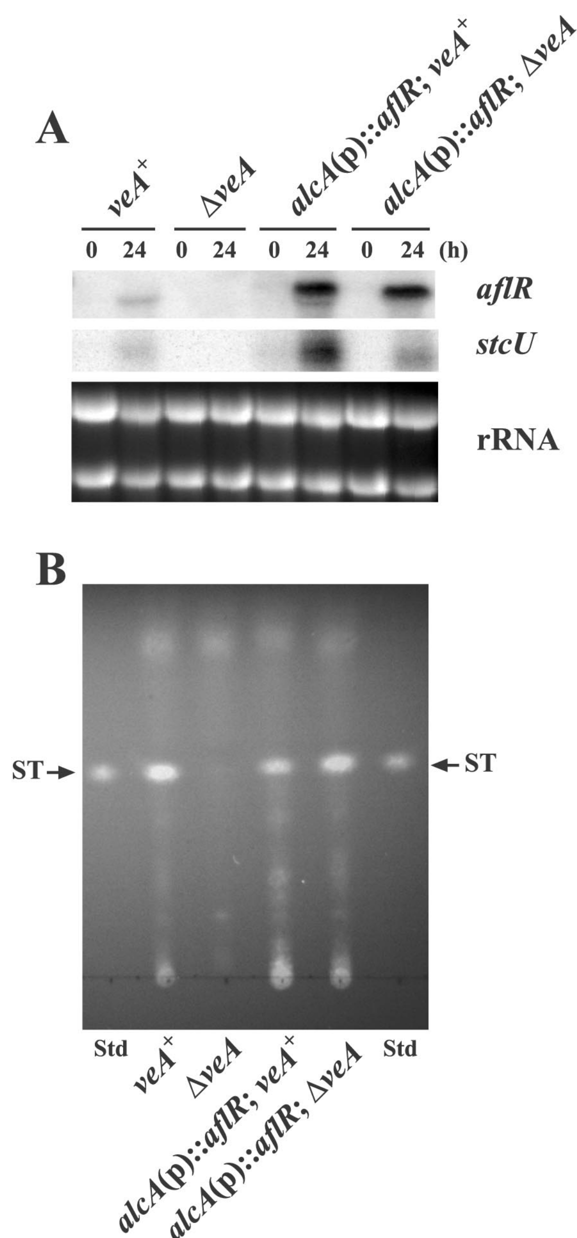

FIG. 5. Effects of the overexpression of *aflR* on ST biosynthesis in the *A. nidulans veA*$^{+}$ and Δ*veA* strains. (A) The expression of *aflR* and *stcU* in FGSC4 (*veA*$^{+}$), RNKT1 (Δ*veA*), RNKT6 [*alcA*(p)::*aflR veA*$^{+}$], and RNKT4 [*alcA*(p)::*aflR* Δ*veA*] strains was evaluated by mRNA analysis. Expression of the *alcA*(p)::*aflR* construct results in an *aflR* functional transcript slightly larger than the endogenous transcript (24). Total RNA was isolated from the cultures at zero h and 24 h after transfer from liquid GMM into liquid TMM. rRNA stained with ethidium bromide is shown to indicate RNA loading. (B) ST analysis by TLC. The uncharacterized compound observed in Δ*veA* near the ST R_f is not ST (in this assay, ST fluoresces bright yellow while the unknown compound fluoresced blue). Std, standard.

finding is the control of *veA* over the expression of genes leading to the production of two different secondary metabolites, the mycotoxin ST and the antibiotic PN. Deletion of *veA* repressed transcription of the ST-specific transcription factor *aflR*, and consequently ST gene expression (Fig. 3A, 4A, and 5A), indicating that *veA* is required for normal *aflR* expression and ST biosynthesis. On the other hand, deletion of *veA* enhanced levels of *ipnA* mRNA (Fig. 3A and 4A), an enzymatic gene in the PN gene cluster. The opposite regulation of ST and PN production has been described for the G-protein α-subunit FadA (45). *A. nidulans* strains containing the dominant activating allele, *fadA*^{G42R}, also lost ST gene expression and showed an increase in *ipnA* expression that led to greater levels of PN biosynthesis. In contrast, the higher *ipnA* expression found in the *veA* deletion in our studies did not result in an increase but in a reduction of PN production (Fig. 3E), indicating that although *veA* is a repressor of *ipnA*, the *ipnA* expression level has little effect on PN production. Consequently, these results are in line with those of Fernández-Cañón and Peñalva (19), in which overexpression of *ipnA* increased isopenicillin N synthetase activity 40-fold yet resulted in only a modest increase in PN production. Subsequently, Kennedy and Turner (32) demonstrated that the expression of *acvA*, another gene in the PN cluster encoding δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase, is the rate-limiting step in PN biosynthesis. Furthermore, a reduction in *ipnA* caused by deletion of the *hapC* gene (which encodes a component of the wide-domain-regulatory CCAAT-binding complex AnCF/AnCP/PENR1 [28, 37, 40]) resulted in only a moderate reduction in PN, since *acvA* expression was only slightly affected by the deletion (37). Our results showed a correlation between *acvA* expression levels and the bioassay results (Fig. 3E and F), supporting *acvA* (not *ipnA*) as the limiting step in PN biosynthesis.

Varying the production of secondary metabolites such as ST and PN is a logical strategy by organisms to adapt to their changing environments. One of the environmental factors is light. In *A. nidulans*, *veA* has been shown to mediate a light-dependent developmental response (50). Furthermore, results reported by Yager et al. suggest a genetic interaction between *veA* and *fluG*, a gene linking development and ST production that, according to these authors, could be a light photoreceptor (51). For this reason we examined the possible effect of light on secondary metabolism in the *veA*$^{+}$ strain. Greater accumulation of *aflR* and *stcU* transcripts, and consequently higher production of ST, was observed in the *A. nidulans* wild-type strain incubated in the light than in the same strain incubated in the dark when the fungus was grown on the rich YGT medium (Fig. 3A and C). Higher levels of ST in the light than in the dark were also found when the fungus was growing on another rich medium containing yeast extract called YES (1% sucrose, 2% yeast extract [20]). Interestingly, we found that when the fungus was cultured on the synthetic medium GMM (Fig. 3D), ST production was higher in dark cultures than in those exposed to light. These findings suggest that the effect of light on ST biosynthesis is the result of a complex interaction with other factors, such as the abundance and type of nutrients (i.e., carbon and nitrogen sources) available in the environment. Although light stimulated *aflR* expression in the absence of *veA* (to levels still lower than in the *veA*$^{+}$ strain in the light), *stcU* expression was not detected (Fig. 3A), suggesting a possible additional role for *veA* in regulating *aflR* at a posttranscriptional level or perhaps affecting additional genetic elements that could affect ST cluster activation. Consistent with these hypotheses, we found that although forced expression of *aflR*

was able to restore *stcU* expression and ST production in the Δ*veA* genetic background (Fig. 5), *stcU* transcript accumulation was still lower than those achieved by *aflR* overexpression in the *veA*$^+$ background (Fig. 5A). Shimizu and Keller (43) showed that *aflR* is negatively regulated at both the transcriptional and posttranscriptional levels by the cyclic-AMP-dependent kinase PkaA, one of the components of the FadA G-protein signaling pathway regulating ST biosynthesis and morphological development (Fig. 1) (25, 43). Whether the role of *veA* in regulating *aflR* is connected with the PkaA-mediated mechanism is still unknown but is being investigated as part of our current studies.

Our results indicate that *veA* constitutes a genetic link between secondary metabolism and morphological development in *A. nidulans*. As previously mentioned, it is known that light influences fungal development in a response mediated by VeA. Specifically, in *A. nidulans* strains with a *veA* wild-type allele (*veA*$^+$), light decreases and delays cleistothecial formation and the fungus mainly produces conidia, whereas in the absence of light, fungal development is directed toward the sexual stage (50). Δ*veA* had a mild effect on colony growth, and therefore it is likely that the morphological phenotype observed is not a consequence of a pleiotrophic effect caused by deficient growth. As we confirmed, even under conditions inducing sexual development, Δ*veA* is unable to form any type of sexual structure: Hülle cells, cleistothecia, or ascospores (data not shown and reference 33), verifying that *veA* is indeed required for the sexual stage in this fungus. However, the mechanism by which *veA* regulates cleistothecial production is unknown. This is due in part to the fact that *veA* is constitutively expressed (33) and its gene product does not present homology with any other protein of characterized function that could indicate a possible mechanism of action for *veA*. We carried out mRNA analysis to investigate whether the effect of *veA* on the sexual stage is mediated by the transcription factors *nsdD* and *steA*, which are required for normal sexual development in *A. nidulans* (21, 48). The expression levels of the *nsdD* and *steA* genes were similar in the *veA*$^+$ and Δ*veA* strains; only slight variations were observed (Fig. 2). It is likely that the moderate differences in the expression levels of these transcription factor genes in the Δ*veA* strain with respect to the wild type might not be responsible for its complete absence of a sexual stage. Alternatively, whether *veA* could affect *nsdD* and *steA* posttranscriptionally or affect the sexual stage through an *nsdD*- and *steA*-independent mechanism is unknown. However, we do know that sexual development and ST production are coregulated by *veA* at a step previous to the *nsdD* regulation point, since the Δ*nsdD* strain produced wild-type levels of ST (data not shown). The case of *nsdD* in the sexual development-ST relationship is similar to that of *brlA* in the conidiation-ST relationship. Although *brlA* deletion prevents conidiation (2), the deletion does not affect ST production (20). Nevertheless, it is known that conidiation and ST production are genetically linked by the FadA signaling pathway at a point preceding *brlA* (Fig. 1) (25).

In addition to the *veA* roles discussed above, *veA* has also been postulated to inhibit conidiation (3, 42, 50). We found that the role of *veA* in conidiation is medium dependent. While the production of conidia decreased twofold in the Δ*veA* strain with respect to the control when the strains were grown on GMM (data not shown), conidial production by the Δ*veA* strain was higher than in the wild type when the strains were grown on YGT medium (~3,000-fold higher in the dark and 30-fold higher in the light [Fig. 3B]). The higher conidial production in the Δ*veA* strain on YGT (our results) coincides with results on complete medium (33). However, the possible role of *veA* in controlling the transcription of conidiation-regulatory genes has not been investigated. Our experiments showed that the increase in conidiation in the Δ*veA* strain on YGT medium was mediated by an alteration of *brlA* expression. *brlA* generates two types of transcripts called *brlA*α and *brlA*β, and its expression is subject to complex regulation at the transcriptional and translational levels (22, 23). There are two open reading frames (ORFs) in the *brlA*β transcript; one of them is a small ORF called μORF found upstream of the BrlA coding region. According to Han and Adams (22), translation of μORF inhibits the translation of the second and larger *brlA*β ORF (BrlA). The translation of this larger *brlA*β ORF is necessary for *brlA*α transcription. The transcription of *brlA*α causes the activation of a series of genes that lead to conidiation. In our experiments, *brlA*β was detected in the *veA*$^+$ and Δ*veA* strains (Fig. 2 and 3A). However, most of the *brlA* transcript was α type in the Δ*veA* strain (Fig. 2 and 3A), particularly when exposed to light (Fig. 3A). The fact that *brlA*α is more abundant in the light in the Δ*veA* strain coincides with higher conidial production under these conditions (Fig. 3B). Consequently, our data indicate that the deletion of *veA* affects the α/β *brlA* transcript ratio. Several possibilities emerge as to how this regulation takes place: (i) *veA* is required for the translation of μORF, which inhibits *brlA*β translation; (ii) *veA* might negatively regulate *brlA*β translation; or (iii) *veA* might negatively regulate *brlA*α transcription. Further investigation is needed to elucidate which of these alternatives is correct. In addition, the increase in *brlA*α transcription in the light in the Δ*veA* strain suggests the existence of other light-responsive genetic factors controlling *brlA* expression.

In conclusion, the results of this study indicate that *veA* is a global regulator controlling both morphological development and secondary metabolism. A *veA* homolog in *A. parasiticus*, a producer of the carcinogenic mycotoxin AF, has been identified (A. M. Calvo, J.-W. Bok, W. Brooks, and N. P. Keller, submitted for publication). The observation that deletion of *veA* suppresses the production of ST without increasing antibiotic production in *A. nidulans* could be important from an applied perspective as a control strategy for AF because it would avoid the risk of enhancing antibiotic resistance in bacteria in the food chain (36, 45). We have also found *veA* homologs in *A. fumigatus* (http://www.tigr.org/tdb/e2k1/afu1) and in other fungal genera, including several mycotoxin-producing *Fusarium* spp., in *Neurospora crassa* (http://www.genome.wi.mit.edu/annotation/fungi/neurospora), and in *Magnaporthe grisea* (http://www.genome.wi.mit.edu/annotation/fungi/magnaporthe). Therefore, the widespread distribution of *veA* possibly indicates that this important regulator is conserved across fungal genera. These studies contribute to the understanding of the regulatory networks that control fungal development and the production of secondary metabolites. This knowledge will be useful in reducing the detrimental effects of these natural products and in

enhancing the production of secondary metabolites that are beneficial.

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