

## *veA* Is Required for Toxin and Sclerotial Production in *Aspergillus parasiticus*

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Received 25 February 2004/Accepted 27 April 2004

It was long been noted that secondary metabolism is associated with fungal development. In *Aspergillus nidulans*, conidiation and mycotoxin production are linked by a G protein signaling pathway. Also in *A. nidulans*, cleistothecial development and mycotoxin production are controlled by a gene called *veA*. Here we report the characterization of a *veA* ortholog in the aflatoxin-producing fungus *A. parasiticus*. Cleistothecia are not produced by *Aspergillus parasiticus*; instead, this fungus produces spherical structures called sclerotia that allow for survival under adverse conditions. Deletion of *veA* from *A. parasiticus* resulted in the blockage of sclerotial formation as well as a blockage in the production of aflatoxin intermediates. Our results indicate that *A. parasiticus veA* is required for the expression of *aflR* and *aflJ*, which regulate the activation of the aflatoxin gene cluster. In addition to these findings, we observed that deletion of *veA* reduced conidiation both on the culture medium and on peanut seed. The fact that *veA* is necessary for conidiation, production of resistant structures, and aflatoxin biosynthesis makes *veA* a good candidate gene to control aflatoxin biosynthesis or fungal development and in this way to greatly decrease its devastating impact on health and the economy.

Among the most common toxigenic fungi affecting the human food chain are *Aspergillus* spp., infecting major agricultural commodities such as corn, peanuts, cotton, sorghum, and other oil-seed sources. *Aspergillus parasiticus* produces the polyketide mycotoxin aflatoxin (AF) (26, 30, 31), one of the most mutagenic and carcinogenic natural compounds described to date. Ingestion of food or feed contaminated with AF has been associated with hepatotoxicity, teratogenicity, immunotoxicity, and even death (10, 31). For these reasons, efforts are focused on finding methods to control the production of AF or to prevent the dissemination or survival of the fungi that produce this type of mycotoxin.

A common biosynthetic pathway leads to production of both AF and a mycotoxin called sterigmatocystin (ST); ST is the penultimate intermediate in the AF pathway. One of the best genetically characterized eukaryotic systems, that of the fungus *Aspergillus nidulans*, produces ST and is especially productive for the study of the AF-ST gene clusters and signal transduction pathways governing mycotoxin production and fungal development (5, 14, 18, 19, 34). For both *A. parasiticus* and *A. nidulans* fungi, a correlation between conidiation and secondary metabolism has been demonstrated. Kale et al. (16) showed that nonaflatoxigenic variants of *A. parasiticus* had abundant vegetative mycelium and reduced conidiation. Hicks et al. (14) reported that ST biosynthesis is genetically linked with the production of asexual spores (conidia) in *A. nidulans* through the FadA G protein signaling pathway. This same study showed the conservation of this genetic linkage in *A. parasiticus*. Interestingly, certain mutations in genes involved in the FadA pathway also alter cleistothecial development (27,

28), suggesting the existence of an association not only with conidiation and secondary metabolism but also with processes leading to the development of the sexual stage.

Only a few genes are known to be involved in sexual development; one example is *veA*, the velvet gene. The *veA* gene product does not present homology with any other protein of known function; however, it is known that *veA* mediates a developmental light response in *A. nidulans* (33). In *A. nidulans* strains containing a wild-type allele of the velvet gene (referred to as *AnveA* in this work), light reduces and delays cleistothecial formation and the fungus develops asexually forming conidia, whereas in the dark, fungal development is directed towards the formation of cleistothecia. In a study focused on morphogenesis, it was reported that deletion of *AnveA* blocks cleistothecial production in *A. nidulans*, demonstrating that *AnveA* is necessary for sexual development (20). Recently, we reported that deletion of *AnveA* also blocks ST production by repression of *aflR*, a specific transcription factor that activates the ST and AF gene clusters (6, 9, 17, 25, 32, 35).

We have found a *veA* homolog in the AF producer *A. parasiticus*. Our results indicate that the *A. parasiticus veA* deletion strain is unable to produce sclerotia or the AF intermediate versicolorin A. We also show that the blockage in the production of AF intermediates in the *veA* deletion strain is related to the absence of expression of both *aflR* and the adjacent gene *aflJ*, also involved in the expression of AF structural genes (7, 23). Additionally, the *veA* deletion strain also has reduced growth and conidial production when grown on the natural substrate peanut seed. Because *veA* is required for both mycotoxin production and morphological development, we propose *veA* as a potential target gene for the implementation of a control strategy to prevent the health and economic effects of these carcinogenic mycotoxins.

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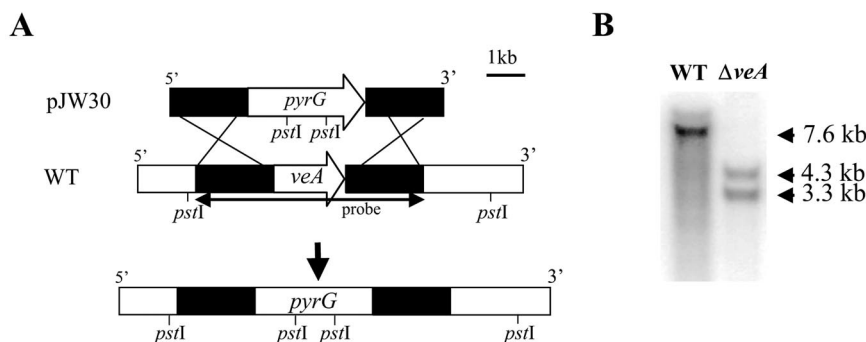


FIG. 1. Deletion of the *veA* gene in *A. parasiticus*. (A) Diagram of the *veA* open reading frame and how it was replaced with the *pyrG* selectable marker from pJW30 by homologous recombination to generate the  $\Delta veA$  mutant, TJW41.21. Solid bars indicate the 5' and 3' flanking regions of *veA*. (B) Southern analysis of wild-type (WT) strain and the  $\Delta veA$  mutant strain. Genomic DNA was digested by *Pst*I. A 6.1-kb PCR product containing *veA* was obtained with the primers 5'-AGA GAT GTC AAG TTC GAG TCG AG-3' and 5' CTC GGC ACC CAG CGT CAT CC 3' and was used as a *veA* probe (indicated by arrows in panel A). Expected hybridization band patterns: wild-type strain, 7.6-kb band;  $\Delta veA$  strain, 3.3- and 4.3-kb bands.

## MATERIALS AND METHODS

**Strains and growth conditions.** The *A. parasiticus* strains used in this study were ATCC 36537 (*wA*; *ver1*), CSS10 (*wA*; *ver1*; *pyrG*<sup>-</sup>), provided by John Linz, and also TJW41.21 (*wA*; *ver1*; *pyrG*<sup>-</sup>; *veA*:*pyrG*), generated in this study. The 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) (15). Appropriate supplements corresponding to the auxotrophic markers were added to the medium (15). Agar (15 g/liter) was added in the case of solid medium. All strains were maintained as glycerol stocks.

**Identification and sequencing of *veA*.** The identification of *A. parasiticus veA* was achieved by hybridizing an *A. parasiticus* genomic library (kindly provided by John Linz) with the *A. nidulans AnveA* gene as a heterologous probe. The *AnveA* gene was contained in a 4-kb *Xho*I fragment obtained from the plasmid pPK11 (kindly provided by Larry Yager). The screening of the *A. parasiticus* library yielded a cosmid containing *veA* (GenBank accession number AY445513). This cosmid, which we named pAMC10, was subcloned using a shotgun method and sequenced using synthetic primers and an ABI PRISM DNA sequencing kit (Perkin Elmer Life Science). This sequence information was then used for the construction of the deletion vector.

**Construction of the *veA* deletion strain.** The transformation vector, which was called pJW30 and was utilized to delete *veA*, was constructed as follows. First, a 3-kb *Eco*RI-BamHI fragment containing *A. parasiticus pyrG* from pBZ5 (29) was subcloned into pBluescript SK(-) to obtain pJW24. DNA fragments corresponding to the upstream and downstream regions of *veA* were generated by PCR from pAMC10 by the use of *Pfu* Turbo polymerase (Stratagene Co.) and primers 5'-AGAGATGTCAAGTTCGAGTCGAG3' and 5'-CAGAATTCGACGGGCC GTAAATTGAC3' and primers 5'-TTGGATCCTGCTTTTCTCTCCCGCTCA3' and 5'-CTCGGACCCAGCGTCATCC3', respectively. A 2-kb upstream region fragment was inserted into the *Eco*RI site in pJW24, and a 2-kb downstream region fragment was ligated into the BamHI site in pJW24 after blunt ending the vector in both steps. The finished deletion vector, pJW30, was used to transform *A. parasiticus* CSS10 (*wA*; *ver1*; *pyrG*<sup>-</sup>) to create TJW41.21. The *veA* deletion strain ( $\Delta veA$ ) was obtained by a double-crossover event, exchanging the *pyrG* selectable marker gene for the *veA* coding region (Fig. 1A). Southern analysis was used to confirm a single gene replacement event in TJW41.21 (Fig. 1B). On the basis of the DNA sequence, in this Southern analysis we predicted a hybridization band corresponding to a *Pst*I fragment of 7,560 bp for the wild type and two bands corresponding to two *Pst*I fragments of 3,381 and 4,256 bp in the deletion strain when the probe indicated in Fig. 1 was used. This pattern can only be obtained by a double-recombination event in the flanking regions of *veA* that results in *veA* gene replacement. Fungal transformation essentially followed that of Miller et al. (24), with the modification of embedding the protoplasts in top agar (0.75%) rather than spreading them with a glass rod on solid medium.

**Physiological studies.** The studies on fungal development were performed with the  $\Delta veA$  strain (*wA*; *ver1*; *pyrG*<sup>-</sup>; *veA*:*pyrG*) and the corresponding control strain (*wA*; *ver1*). Plates containing 30 ml of solid YGT medium were spread with 100  $\mu$ l of water containing  $10^5$  spores. The cultures were incubated at 30°C in continuous light (25 microeinsteins/m<sup>2</sup>/s) 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 spores. Conidia were counted using a hemacytometer. Identical

cores were taken to examine sclerotial production. The cores were spread with 95% ethanol to enhance visualization of sclerotia. Sclerotial production was examined under a stereo-zoom microscope. Colony growth was recorded as colony diameter measurements in point inoculation cultures on YGT medium. Experiments were carried out with four replicates.

**Mycotoxin analysis.** Four cores (16-mm diameter) or, alternatively, 2 g of fungal biomass (specified in each experiment described below) was collected from each replicate of *veA* and  $\Delta veA$  cultures and placed in a 50-ml Falcon tube. AF intermediates were extracted from these samples by adding 5 ml of CHCl<sub>3</sub> three consecutive times. Extracts were allowed to dry and then resuspended in 500  $\mu$ l of CHCl<sub>3</sub> before fractionating 15  $\mu$ l of each extract on a silica gel thin-layer chromatography (TLC) plate by the use of a benzene:glacial acetic acid (95:5 [vol/vol]) solvent system. The TLC plates were sprayed with aluminum chloride (15% in ethanol) to intensify fluorescence upon exposure to long-wave (365-nm) UV light and baked for 10 min at 80°C prior to viewing.

**mRNA studies.** Cultures of 250 ml of YGT liquid medium containing  $5 \times 10^5$  spores ml<sup>-1</sup> of either *veA* or  $\Delta veA$  strains were incubated (300 rpm and 30°C) for 24 h. At that time the mycelia from these cultures were recovered by filtration. Approximately 9 g of mycelium from either *veA* or  $\Delta veA$  strains was then shifted and spread on each petri dish (9-cm diameter) containing solid YGT medium and allowed to grow in a light incubator at 30°C. At the time of the shift ( $t = 0$  h) onto solid medium and 12 and 24 h after the shift, the mycelial samples were then harvested for RNA extraction. Total RNA was isolated from mycelia by using Trizol as described by the supplier (Invitrogen). Approximately 20  $\mu$ g of total RNA was used for RNA blot analysis. PCR products amplified from *A. parasiticus* genomic DNA with the following primer pairs were used as specific probes: for *afR*, 5'-CACCTCCACGATGGTTG-3' and 5'-GTATAGACTGTTCAAGA TTCG-3'; for *afI*, 5'-GCATGCTTGCGGTGGCTG-3' and 5'-CCGTTGCCAT TCCCAAG-3'; for *pksL1*, 5'-CTGCAGAGCATGAACACG-3' and 5'-GAAT GGTACGATGGTGAC-3'; and for *brlA*, 5'-ATGAGAGCTCAAAACAACC-3' and 5'-TTAATCGTCTAACCCTAC-3'. A *ver1*-specific 2.2-kb *Eco*RI-HindIII fragment was obtained from pBVER-1 (provided by John Linz). The identity of the probes was confirmed by sequencing. ImageQuant 5.2 software was used to perform the densitometry analysis of *brlA* expression. The results were plotted as relative band intensity values normalized to rRNA and to the highest band intensity (considered 1 U).

**Infection studies.** Seeds of the near-isogenic Florunner, Southern Runner, and 72cc peanut lines were kindly provided to us by C. Holbrook (U.S. Department of Agriculture, Atlanta, Georgia). Prior to infection by *A. parasiticus* strains, the seeds were shelled, the cotyledons were separated, and the embryos were removed. Seeds from both peanut lines were weighed so that all the peanuts used were between 0.4 and 0.6 g. The cotyledons were surfaced sterilized by immersion in 10% Clorox bleach for 1 min followed by immersion in sterile distilled water for 1 min. The seeds were inoculated as follows. For both *veA* and  $\Delta veA$  strains, four peanut cotyledons were placed in a 50-ml Falcon tube, to which was added 500  $\mu$ l of sterile distilled water containing  $10^6$  spores of the appropriate strain. Each tube was vortexed for 1 min, the caps were loosened, and the samples were placed in the light or in the dark at 30°C. These experiments were performed with four replicates. After 7 days, spores from the infected seeds were

released by adding 5 ml of Tween 80 water to each Falcon tube, vortexing for 1 min, and using an aliquot of this suspension for counting with a hemocytometer.

## RESULTS

**Deletion of *veA* affects growth and morphological development in *A. parasiticus*.** Fig. 1 shows a diagram of the strategy followed to delete *veA* as well as the results of the Southern analysis hybridization confirming the *veA* deletion. Loss of this gene resulted in reduction of colony growth (estimated as colony diameter) under either light or dark conditions (Fig. 2A). In the light the reduction was 25%, and in the dark the reduction was approximately 50%. Growth was also evaluated in liquid culture in an experiment in which 250 ml of YGT medium was inoculated with either *veA* or  $\Delta veA$  strains ( $5 \times 10^5$  spores  $\text{ml}^{-1}$ ) and incubated at 30°C and 300 rpm. After 24 h, the mycelium mass produced in the  $\Delta veA$  strain was 25% with respect to that obtained in the control strain (data not shown).

Figure 2B shows that sclerotia production only occurred in the dark in the control strain and that the *veA* deletion completely prevented the formation of sclerotia (Fig. 2B and 3). To test whether the absence of sclerotia could be a consequence of reduction in growth, we cultured the *veA* and  $\Delta veA$  strains ( $5 \times 10^5$  spores  $\text{ml}^{-1}$ ) in YGT medium at 30°C and 300 rpm for 24 h. Then, the mycelia were harvested by filtration and equal amounts of mycelium (4 g/9-cm-diameter plate) were collected and spread on solid YGT medium and cultured in the dark at the same temperature for 10 days. The results concurred with those of the experiment shown in Fig. 2B and 3, where sclerotial production occurred in the control strain but was completely abolished in the  $\Delta veA$  strain. Later examination of these cultures at 14 days of growth from the moment of the shift to solid medium showed the same results; sclerotial production was absent from the deletion strain.

The *A. parasiticus* strain with a wild-type *veA* produced more conidia (75%) in the light than in the dark (Fig. 2C). Deletion of *veA* also resulted in a reduction of conidial production in *A. parasiticus* (30% in the light and 46% in the dark) (Fig. 2C). The effect of *veA* deletion on *A. parasiticus* conidiation was further evaluated by mRNA studies (see below).

**Role of *veA* in the production of versicolorin A in *A. parasiticus*.** We evaluated the effect of *veA* deletion on AF biosynthesis in *A. parasiticus*. To facilitate the detection of cluster activation and AF pathway activity, we used the visible orange AF intermediate versicolorin A. Strains of *A. parasiticus* that produce versicolorin A have a mutation in a gene (*ver1*, encoding a ketoreductase) involved in the multistep AF pathway (21, 22). As a result of this mutation, these strains have an interrupted AF pathway in which versicolorin A accumulates instead of AF. AF biosynthetic clustered genes are simultaneously activated or deactivated; therefore, accumulation of versicolorin A is a good indicator of said activation and of AF biosynthetic pathway activity. Our TLC analysis showed that the *A. parasiticus*  $\Delta veA$  strain does not produce versicolorin A whereas it was detected in the light and in the dark in the *veA* control strain when grown on solid YGT medium for 7 days (Fig. 4A). An additional experiment was carried out to test whether the blockage of versicolorin A production was caused by a reduction in growth. The *veA* and  $\Delta veA$  strains ( $5 \times 10^5$

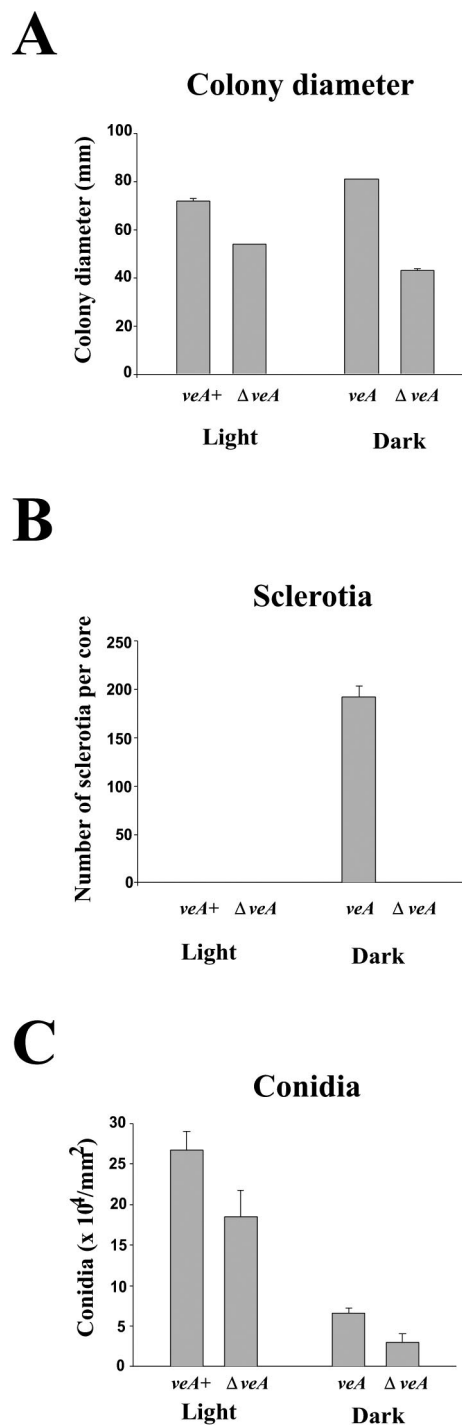


FIG. 2. *veA* is required for normal growth and morphological development in *A. parasiticus*. Wild-type and  $\Delta veA$  cultures were grown on YGT medium in the light or in the dark for 7 days. The experiment was carried out with four replicates. (A) Fungal growth, measured as colony diameter. (B) Resistant structure production, measured as number of sclerotia per core (core = 200  $\text{mm}^2$ ). (C) Asexual stage, measured as number of conidia per surface area.

spores  $\text{ml}^{-1}$ ) were cultured in YGT medium at 30°C and 300 rpm for 24 h, and then equal amounts of mycelium (6 g/9-cm-diameter plate) were shifted into solid medium and allowed to grow for 96 h. A total of 2 g of mycelium from *veA* and  $\Delta veA$

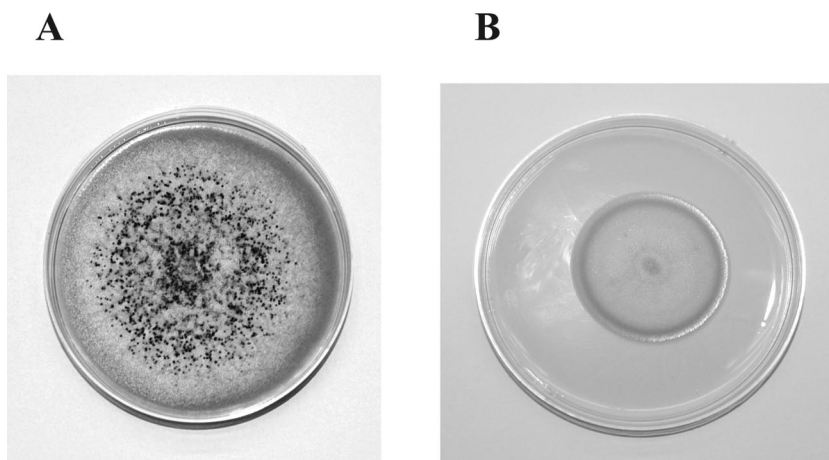


FIG. 3. *A. parasiticus* *veA* (A) and  $\Delta$ *veA* (B) strains cultured on YGT medium for 7 days in the dark. Both strains produce white conidia. The black structures observed only in the *veA* strain are sclerotia.

cultures was extracted, and versicolorin A levels were analyzed. The results of this experiment, shown in Fig. 4B, indicate that while versicolorin A was present in the control strain, it was completely absent from the  $\Delta$ *veA* strain. The effect of *veA* deletion on AF biosynthesis was also further analyzed in mRNA studies (see below).

**mRNA analysis.** In this study we evaluated the effect of *veA* deletion on regulatory genes controlling AF biosynthesis and conidiation. We looked at the expression of the regulatory AF genes *aflR* and *aflJ* and of the two structural genes *pksL1* (encoding a polyketide synthase) and *ver1* as indicators of cluster activation (Fig. 5A). Transcripts corresponding to *aflR* and *aflJ* as well as to *pksL1* and *ver1* were detected in the wild-type strain while they were completely absent from the  $\Delta$ *veA* strain.

With respect to conidiation, we also studied the effect of the *veA* deletion on the expression of *A. parasiticus* *brlA*, a key transcription factor first described in *A. nidulans* (1) and known to regulate conidiophore formation. Besides eliminating the problem that reduction of growth could cause in our analysis, shift experiments are particularly useful in the study of regulation of conidiation in *Aspergillus* species. As reviewed by Adams et al. (2), conidiophore formation is an event that is temporally regulated. The vegetative stage can be continuously maintained by growing hyphae in submerged liquid medium, a trait that provides a means for synchronous development induction by harvesting hyphae from the submerged culture and exposing them to air. During the time of vegetative growth in the submerged culture, the mycelium becomes competent for conidiation induction occurring after the shift to solid medium. The kinetics of conidiation under these conditions have been found to be very reproducible.

In our studies, *A. parasiticus* *brlA* transcripts were observed in both the control strain and the  $\Delta$ *veA* strain (Fig. 5B). Only one *brlA* transcript band was detected in the Northern analysis. At  $t = 0$  h (corresponding to the moment of the shift from liquid to solid culture) *brlA* transcripts were present in the  $\Delta$ *veA* strain while *brlA* was detected only at very low levels (6-fold lower) in the wild-type control (Fig. 5B). The densitometry analysis indicated that after the shift into solid culture

*brlA* levels increased in the wild type while they decreased in the  $\Delta$ *veA* strain. Although *brlA* transcript was present in the liquid YGT culture after 24 h in the  $\Delta$ *veA* strain, no conidiophore formation was observed using the light microscope under this condition. Furthermore, microscopic observations of these cultures revealed that after the shift to solid medium the production of conidia in  $\Delta$ *veA* was also notably lower (approximately 10%) with respect to that seen with the control.

**Role of *veA* in *A. parasiticus* conidiation on seeds.** After chemical surface sterilization, three lines of peanut seeds (Flo-

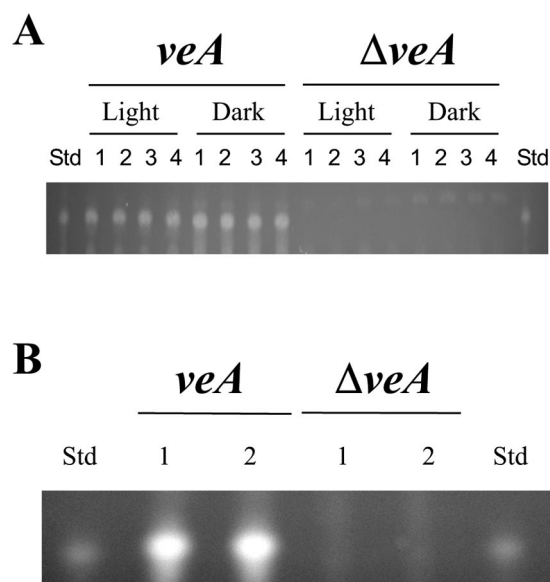


FIG. 4. TLC analysis of versicolorin A production in *A. parasiticus*. (A) The *veA* and  $\Delta$ *veA* spread plate cultures were grown in the light or in the dark on solid YGT medium for 7 days. The numbers 1 to 4 represent replicates. (B) In a shift experiment, the *veA* and  $\Delta$ *veA* cultures were incubated in liquid YGT for 24 h and then equal amounts of mycelium were shifted into solid medium (6 g/9-cm-diameter plate) and allowed to grow for an additional 96 h. The experiment was repeated twice with similar results (indicated as lanes 1 and 2). Versicolorin A standards (Std) are shown on both sides of the TLC plate.

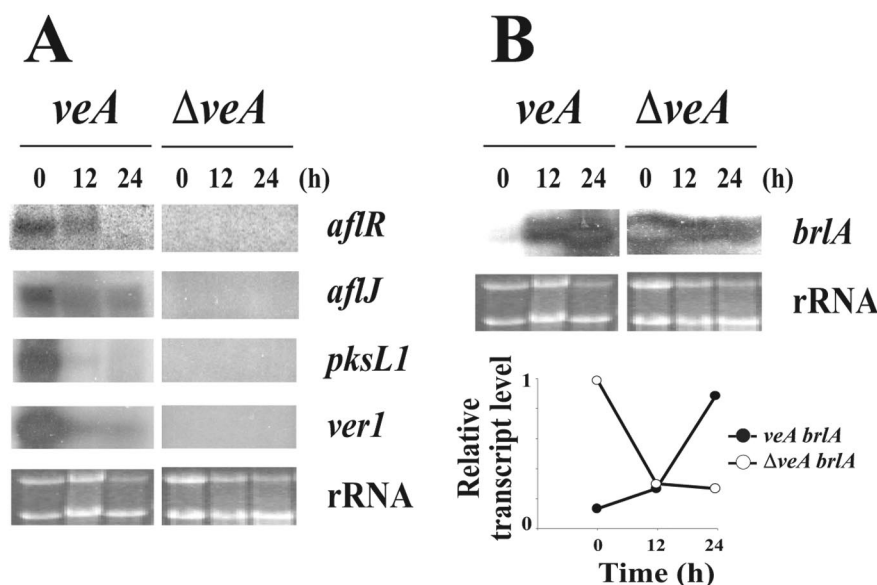


FIG. 5. Effects of the *veA* deletion on the transcription of genes required for AF biosynthesis and morphological development in *A. parasiticus*. The *veA* and  $\Delta$ *veA* strains were cultured in shaken YGT liquid medium for 24 h and then shifted onto solid YGT medium. Total RNAs of the *veA* and  $\Delta$ *veA* strains were isolated at the time of the shift ( $t = 0$ ) and 12 and 24 h after the shift to solid culture. (A) Transcriptional patterns of the AF genes *aflR*, *aflJ*, *pksL1*, and *ver1*. rRNA stained with ethidium bromide is shown to indicate RNA loading. (B) Transcriptional pattern of the developmental gene *brlA*. *brlA* mRNA, present in both *veA* and  $\Delta$ *veA*, was quantified by densitometry using ImageQuant 5.2 software and plotted as relative band intensity normalized to rRNA and to the highest band intensity (considered 1 U). Two separate repetitions of this experiment yielded similar results.

runner, Southern Runner, and 72cc) were inoculated with *A. parasiticus veA* and  $\Delta$ *veA* strains to evaluate the effect of *veA* in the production of conidia when the fungus is interacting with live seeds. The control strain produced the highest number of conidia when it was grown on Florunner peanut seeds, while it produced less conidia (40% reduction with respect to the results obtained with Florunner in both the light and the dark) when growing on 72cc peanut line seeds (Fig. 6). Conidial production in *veA* was higher in the light than in the dark (35% lower in Florunner, 55% lower in Southern Runner, and 38% lower in 72cc peanut lines). Regardless of the peanut line, deletion of *veA* resulted in a notable reduction of conidial production in both the light and the dark (Fig. 6).

## DISCUSSION

Current control strategies fail to effectively eliminate AF contamination, which continues to be a major economic problem in the United States and a serious health threat in developing countries. Researching the viability of regulation of mycotoxin biosynthesis and/or fungal development by targeting regulatory genes may help to control a worldwide threat to food supplies and human health by *Aspergillus* mycotoxin. We have found a homolog of the *A. nidulans AnveA* in the AF-producing species *A. parasiticus*. The *AnveA* gene has been described as necessary for both development (3, 4, 17, 20, 33) and mycotoxin production (17) in this model system. Here we find that *veA* is required for sclerotial and AF production in *A. parasiticus*.

We found that *veA* is relevant in *A. parasiticus* colony growth. The reduction in growth in the *veA* deletion strain is more pronounced than the slight reduction reported for the

*A. nidulans AnveA* deletion strain (17). We also found that conidiation is reduced in the *A. parasiticus veA* deletion strain (Fig. 2C). This result contrasts with the increase in conidial production in the *AnveA* deletion strain (17, 20). It is possible that *veA* regulates conidiation in *A. parasiticus* in a different manner than in *A. nidulans*. Because the reduction in conidiation was still observed when equal amounts of fungal biomass from *veA* deletion and control cultures were used, we do not think it likely that the decrease in conidiation is a consequence of a reduction in growth. Furthermore, *brlA*, encoding a transcription factor required for conidiophore formation, was found to be expressed in both strains (Fig. 5B). Under the experimental condition assayed, *brlA* was highly expressed in 24-h YGT submerged cultures in the  $\Delta$ *veA* strain; however, conidiophore formation was not observed. *brlA* expression was still detected after the cultures were shifted into solid medium, decreasing *brlA* levels in the  $\Delta$ *veA* strain and increasing levels in the control strain, in which conidiation was approximately 10-fold higher than in the deletion strain. One possibility that could explain the inverted *brlA* expression pattern observed in *veA* deletion and control strains is that *veA* lack of function could result in alteration of the sporulation temporal program (2) such that the sporulation program occurs early on, activating *brlA* prematurely and decreasing *brlA* levels when *brlA* expression is increasing in the wild type. Although the lack of an air interphase did not prevent *brlA* expression in the  $\Delta$ *veA* strain growing in submerged cultures, *brlA* expression was not sufficient to initiate conidiation in *A. parasiticus* with a *veA* deletion background under these conditions.

In *A. nidulans*, *brlA* generated two types of transcripts,  $\alpha$  and  $\beta$  transcripts, (13) and we have previously reported that *AnveA*

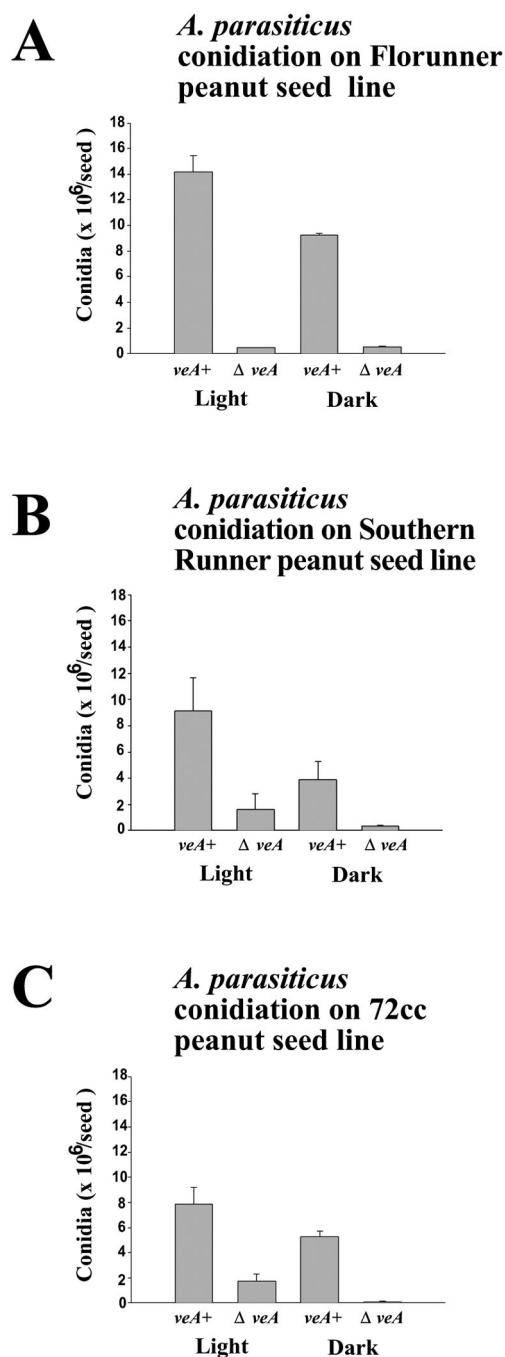


FIG. 6. Conidiation of *A. parasiticus* *veA* and  $\Delta$ *veA* strains on peanut seeds. The cultures were grown in the light or in the dark on peanut seeds for 7 days. The experiment was carried out with four replicates. (A) Florunner line; (B) Southern Runner line; (C) 72cc line.

affects the  $\alpha/\beta$  ratio that controls conidiation in this fungus. In contrast with *brlA* in *A. nidulans*, which has been studied in depth (12, 13), little is known about the *A. parasiticus* *brlA* gene. Our *A. parasiticus* *brlA* mRNA study showed only one transcript band. It is unknown whether this band corresponds to one transcript or to two or more transcripts of similar sizes. Further characterization of *A. parasiticus* *brlA* is needed to understand its functionality. Besides *brlA*, there could also be

additional factors affected by the absence of *veA* that reduce conidiation in *A. parasiticus*. We also investigated whether this reduction in conidia number holds true when the fungus is growing on peanut seed, a natural substrate for *A. parasiticus*. Our results with three different lines of peanut seeds indicated that *veA* is essential for normal conidiation in this plant pathogen.

One interesting observation was the regulation of sclerotial production by *veA*. In the *A. parasiticus* control strain, sclerotia formed only in the dark (Fig. 2B and 3), a condition that also favored the formation of cleistothecia in *A. nidulans* (33). Additionally, deletion of *veA* results in a complete blockage of sclerotial production. It is interesting that some researchers consider sclerotia as possible vestiges of cleistothecia (11, 33), taking into account that deletion of *AnveA* in *A. nidulans* results in a blockage of cleistothecial formation (17, 20).

The blockage of sclerotial production persisted when equal amounts of fungal biomass from both deletion and control strains were used, indicating that the effect of the *veA* deletion on sclerotial development is independent of the reduction of growth observed.

Besides regulating morphological differentiation, *veA* also regulates secondary metabolism. An association between sclerotial formation and AF biosynthesis in *A. parasiticus* has been reported by Chang et al. (8). These authors introduced *aflR* and *aflJ* into an *O*-methylsterigmatocystin-accumulating strain. The increase in toxin production coincided with a decrease in sclerotial size and an alteration in sclerotial shape and suggested that these alterations could be caused by competition for a common substrate such as acetate. They also described an increase in sclerotial numbers in some cases; however, this effect was medium dependent. In our studies, besides blocking sclerotial production, deletion of *veA* prevented the accumulation of versicolorin A, an intermediate indicator of AF pathway activity (21, 22, 29) (Fig. 4). We also demonstrated that this blockage is independent of the reduction in growth in the *veA* deletion strain. Furthermore, our mRNA analysis indicated that the absence of *veA* prevents the expression of the regulatory genes *aflR* and *aflJ* and consequently the expression of structural AF genes such as *pksL1* and *ver1* (Fig. 5A). These results are consistent with the fact that in *A. nidulans*, *AnveA* is necessary for ST production (17), a related polyketide mycotoxin synthesized through a highly conserved biosynthetic pathway. Because of this conservation, it is likely that a *veA* homolog also exists in *A. flavus*, another important seed-infecting fungus, and that *veA* might have a similar role in regulating AF production in *A. flavus*.

Since *veA* is required for morphological development and mycotoxin biosynthesis, *veA* is a promising potential target for a control strategy to reduce the impact of AF on health worldwide and on the economy. Further studies will include the elucidation of possible connections of *veA* with genetic elements of signal transduction pathways known to regulate morphological development and toxin production. Interestingly, the conservation of *veA* extends beyond the genus *Aspergillus*. As mentioned in Kato et al. (17), homologs of *veA* were found in other filamentous fungi, some of them plant pathogens, across genera. Importantly, *veA* has not been found in plants or animals. Due to the conserved nature of the regulatory networks among fungal species, a broader control strategy involv-

ing *veA* may well be effective against *Aspergillus* and other pathogenic fungi.

#### ACKNOWLEDGMENTS

We thank C. Holbrook (U.S. Department of Agriculture, Atlanta, Ga.) for providing us with the peanut lines; J. Linz for providing the cosmid library, ATCC 36537 and CSS10 strains, and pBVER clone; L. Yager for providing the pPK11 clone; and L. Smith for her technical support.

This study was financed by Northern Illinois University and the Plant Molecular Biology Center at the same university and by a U.S. Department of Agriculture grant (NRI 2001-35319-10996).

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