

VeA Is Associated with the Response to Oxidative Stress in the Aflatoxin Producer *Aspergillus flavus*

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Survival of fungal species depends on the ability of these organisms to respond to environmental stresses. Osmotic stress or high levels of reactive oxygen species (ROS) can cause stress in fungi resulting in growth inhibition. Both eukaryotic and prokaryotic cells have developed numerous mechanisms to counteract and survive the stress in the presence of ROS. In many fungi, the HOG signaling pathway is crucial for the oxidative stress response as well as for osmotic stress response. This study revealed that while the osmotic stress response is only slightly affected by the master regulator *veA*, this gene, also known to control morphological development and secondary metabolism in numerous fungal species, has a profound effect on the oxidative stress response in the aflatoxin-producing fungus *Aspergillus flavus*. We found that the expression of *A. flavus* homolog genes involved in the HOG signaling pathway is regulated by *veA*. Deletion of *veA* resulted in a reduction in transcription levels of oxidative stress response genes after exposure to hydrogen peroxide. Furthermore, analyses of the effect of VeA on the promoters of *cat1* and *trxB* indicate that the presence of VeA alters DNA-protein complex formation. This is particularly notable in the *cat1* promoter, where the absence of VeA results in abnormally stronger complex formation with reduced *cat1* expression and more sensitivity to ROS in a *veA* deletion mutant, suggesting that VeA might prevent binding of negative transcription regulators to the *cat1* promoter. Our study also revealed that *veA* positively influences the expression of the transcription factor gene *atfB* and that normal formation of DNA-protein complexes in the *cat1* promoter is dependent on AtfB.

Fungi are capable of colonizing a large variety of ecological niches. These organisms efficiently disseminate by producing airborne asexual spores called conidiospores or conidia that propagate long distances to different environments, where they are able to adapt and proliferate. Some fungi are also able to produce resistant structures, such as sclerotia, that allow them to survive adverse environmental conditions (1–3). The formation of these developmental structures is particularly important when considering dissemination and survival of fungi (including *Aspergillus flavus*) that have a detrimental impact on agriculture and health. *A. flavus* is a saprophyte and an opportunistic pathogen (4) that can infect a variety of oil seed crops, such as corn, peanuts, tree nuts, cotton, and sorghum. In previous work, we showed that *A. flavus* pathogenicity is influenced by the global regulator VeA (5). This cellular regulator also controls fungal morphogenesis and secondary metabolism (6–11). While VeA is necessary for sclerotial production, it is a repressor of conidiation (8). Furthermore, this regulator is required for the synthesis of numerous deleterious natural products, including the *A. flavus* mycotoxin aflatoxin, cyclopiazonic acid, and aflatoxins (AF) (8). The latter, in particular aflatoxin B1 (AFB1), is considered among the most carcinogenic natural products (12). Ingestion of foods contaminated with AF has been associated with acute toxicoses, while chronic low levels of exposure to AF can cause immune suppression and liver cancer (13, 14). The impact of AF contamination in crops is especially serious in developing countries, where a large proportion of the crop, because of the lack of legislation to control AF contamination in the grain, is directed to human consumption (15). Currently implemented control strategies fail to effectively control the *A. flavus* dissemination and survival that lead to its detrimental effects.

The development of novel control strategies to prevent or reduce the negative impact caused by *A. flavus* requires a better understanding of the effects of different environmental factors on this fungus and its adaptive response. Fungi respond to environmental changes, including biotic and abiotic stresses. Changes in nutrient type and abundance, temperature, pH, and oxidative and osmotic stresses activate signal transduction pathways that lead to adaptive responses. Most of the studies involving the molecular regulation of cellular responses to oxidative and osmotic stresses have been performed using yeast as a model organism, in particular *Saccharomyces cerevisiae* (i.e., reviewed by Hohmann et al. [16]), and the filamentous model fungus *Aspergillus nidulans* (17–22). The current availability of genome sequence databases has provided valuable tools for the identification of genetic elements putatively involved in the HOG pathway in several fungal species (17); the HOG pathway is a key signaling pathway that mediates the responses to both osmotic and oxidative stresses. In *A. nidulans*, the Hog1 homolog, Saka, has been experimentally characterized (18–21). Homolog genes of those involved in the HOG signaling pathway have also been identified in *A. flavus* (17).

In our present study, we investigated the *A. flavus* response to

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oxidative and osmotic stresses and the role of the master regulator *veA* and its gene product, VeA, in mediating such a response, which leads to physiological changes allowing the organism to survive these environmental stresses. We show that VeA plays an important role in the oxidative stress response in *A. flavus*. We also demonstrate that *hogA* is dispensable for survival in oxidative and osmotic environments, suggesting an alternative route to activate the genes necessary to counteract the stresses imposed. Importantly, VeA is a positive regulator in the expression of these genes, also affecting *atfB* expression and AtfB-dependent protein-DNA interactions in their promoter regions, particularly the promoter of the catalase gene *cat1*.

MATERIALS AND METHODS

***Aspergillus flavus* strains and culture conditions.** *Aspergillus flavus* 70S (wild type), psl82 (the transformation control), and the ΔveA mutant were used in this study. The generation of these strains has been previously described (8). *Aspergillus flavus* *hogA* deletion mutants ($\Delta hogA::pyrG$) were generated in this study as described below. Strains were cultured on potato dextrose agar (PDA) or YGT medium (0.5% yeast extract, 2% dextrose, and 1 ml of trace elements prepared as described by Käfer [23]) as indicated in each case. For osmotic and oxidative stress-induced cultures, fungal strains were grown on PDA or YGT medium supplemented with NaCl, KCl, or sorbitol for osmotic stress and H₂O₂ or menadione for oxidative stress, respectively, as described below. Appropriate supplements for the corresponding auxotrophic markers (23) were added as needed. Solid medium was prepared by adding 10 g/liter agar. Strains were stored as 30% glycerol stocks at -80°C .

Osmotic stress bioassay. The *Aspergillus flavus* 70S, psl82 control, and ΔveA mutant strains were point inoculated or top agar inoculated (approximately 10^6 spores per plate) on PDA medium supplemented with 0.6 M NaCl, 0.7 M KCl, or 1.0 M sorbitol and compared to PDA cultures without supplementation. Cultures were incubated at 30°C .

Oxidative stress bioassay. In order to elucidate whether the *A. flavus* response to oxidative stress is influenced by *veA*, we performed experiments in which hydrogen peroxide was added to the culture medium. We also performed a similar experiment in which menadione, a compound commonly used to generate reactive oxygen species (ROS) (24), was added.

(i) **Treatment with hydrogen peroxide.** *A. flavus* strains were point inoculated on YGT medium supplemented with hydrogen peroxide at the following concentrations: 0 (negative control), 5, 10, 15, 20, 25, and 30 mM. Hydrogen peroxide was added to the medium after autoclaving. The plates were incubated at 30°C in the dark. The experiment was repeated two times with three replicates each, obtaining similar results.

(ii) **Treatment with menadione.** *A. flavus* strains were point inoculated on YGT medium supplemented with menadione at the following concentrations: 0, 0.5, 0.6, 0.7, and 0.8 mM. As in the case of hydrogen peroxide, menadione was added to YGT medium after autoclaving. The plates were incubated at 30°C in the dark.

Aflatoxin analysis. Plates containing 25 ml of solid PDA were inoculated with 5 ml of top agar containing approximately 10^6 conidia/ml of the *A. flavus* 70S, psl82, and ΔveA strains. Three cores (16 mm in diameter) from each replicate plate were extracted with CHCl₃. The extracts were dried overnight and then resuspended in 200 μl of CHCl₃. Samples were analyzed by thin-layer chromatography (TLC) using toluene-ethyl acetate-formic acid (5:4:1 [vol/vol]) as a solvent system. The plates were then sprayed with aluminum chloride (15% in ethanol) and baked for 10 min at 80°C . AF bands on TLC silica plates were viewed by exposing the plates under UV light (375 nm).

Gene expression analysis. Conidia (10^6 spores/ml) from the *A. flavus* 70S, psl82, and ΔveA strains were inoculated into 400 ml of liquid YGT medium. The cultures were grown for 16 h at 30°C in a shaker incubator at 300 rpm. Mycelial samples were collected at different time points after

16 h of incubation. Approximately 2 g of wet mycelium was added to 500-ml Erlenmeyer flasks containing 250 ml of fresh liquid YGT medium with 15 mM hydrogen peroxide. Cultures were further grown for 6 and 24 h at 30°C in a shaker incubator at 300 rpm. For analysis of *hogA* expression in *A. flavus* 70S $\Delta hogA$ mutants and the *pyrG*-1 isogenic control, conidia (10^6 spores/ml) were inoculated into 250 ml potato dextrose broth (PDB) (BD Biosciences) and incubated for 36 h at 30°C with shaking at 250 rpm. Mycelia were collected by filtration through Miracloth, and 1.0 g was transferred to 125 ml PDB supplemented with 0.2 mM menadione. Cultures were incubated at 30°C with shaking for an additional 4 h. Mycelia were harvested, frozen in liquid nitrogen, and stored at -80°C for RNA extraction. Total RNA was isolated as previously described (25). Approximately 4 μg of total RNA was treated with DNase I RQ1 (Promega) and reverse transcribed with Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega) to synthesize the cDNA. Finally quantitative reverse transcription-PCR (qRT-PCR) was performed using specific primers for different genes of interest listed in Table S1 in the supplemental material. The relative expression levels were calculated by the method described by Kenneth and Schmittgen (26), and all values were normalized to the expression of the *A. flavus* 18S rRNA gene.

Sequence alignment of HogA orthologs. The protein sequence of *A. flavus* HogA (accession no. AFL2G_06243.2) was compared against those of HogA orthologs from other *Aspergillus* species. The sequences were obtained from the Broad Institute (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html). Multiple sequence alignment was performed with *A. flavus* HogA and ortholog sequences using the EMBOSS NEEDLE pairwise sequence alignment tool from EMBL-EBI (http://www.ebi.ac.uk/Tools/psa/emboss_needle/), followed by shading using Box Shade v3.2.1 (http://www.ch.embnet.org/software/BOX_form.html) for presentation purposes.

Generation of the *A. flavus* *hogA* deletion strain. A method was used to construct the *hogA* gene knockout plasmid following essentially the same protocol described by Cary et al. (27) and as depicted in Fig. S4 in the supplemental material. Briefly, 5' and 3' regions of the *A. flavus* *hogA* gene were PCR amplified using oligonucleotide primers listed in Table S1 in the supplemental material. The locations of the primers within and flanking all of the *A. flavus* genes used in this study are based on the Broad Institute *Aspergillus* Comparative Database nucleotide sequence data for the *A. flavus* 3357 genome. Following amplification of *A. flavus* genomic DNA with *Ex Taq* HS polymerase (TaKaRa), PCR products of the expected size for both the 5' (890 bp) and 3' (922 bp) *hogA* gene regions were obtained. PCR products were subcloned into TOPO pCR2.1 (Invitrogen, Carlsbad, CA) and verified by DNA sequencing. In a two-step ligation procedure, the 5' and 3' PCR products were digested with the restriction enzymes engineered onto the ends of the PCR primers and subcloned into restriction enzyme-digested pPG2.8 such that the 5' and 3' gene regions flanked the *Aspergillus parasiticus* *pyrG* gene. This generated the deletion vector pHogA-*pyrG*.

EMSA. Approximately 10^6 conidia/ml from the *A. flavus* 70S, psl82, and ΔveA strains were inoculated in 400 ml of liquid YGT medium in a 1-liter flask and grown at 30°C in the dark for 16 h in a shaker incubator at 300 rpm. Approximately 2 g of wet mycelium was then transferred to 250 ml of liquid YGT medium with or without 15 mM H₂O₂ in a 500-ml flask. The cultures were further incubated for 24 h. Preparation of cell extracts enriched in nuclear proteins from the *A. flavus* 70S, psl82, and ΔveA strains for electrophoretic mobility shift assays (EMSAs) was performed as previously described (28). Briefly, the mycelia cultured as detailed above were harvested by filtration through Miracloth, ground in liquid nitrogen with a mortar and pestle, and resuspended in lysis buffer as described previously (28). The proteins were precipitated with ammonium sulfate (10% and then 70%) and collected by centrifugation. After that, proteins were resuspended in dialysis buffer and dialyzed against the same buffer. The dialyzed protein solution was aliquoted and stored at -80°C .

EMSA and shift inhibition EMSA were performed as described previ-

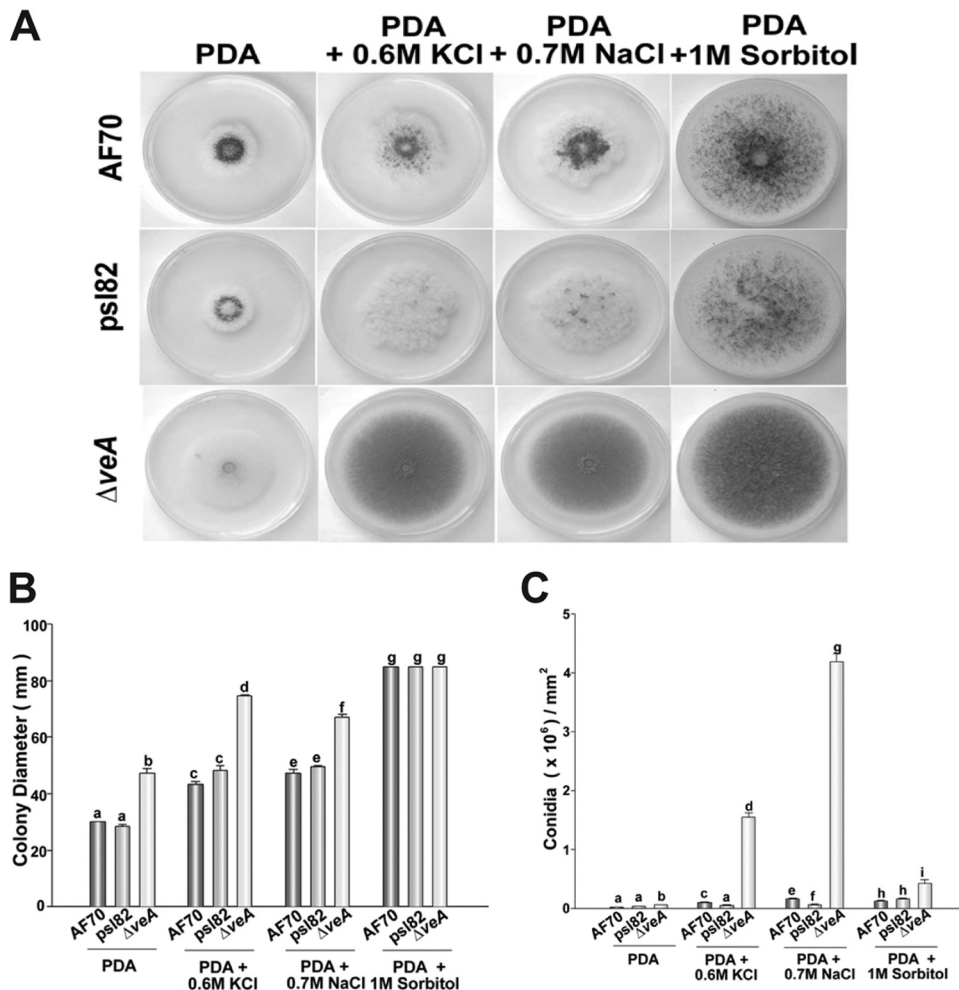


FIG 1 Effect of osmotic stress on *A. flavus* colony growth and conidiation. (A) Photographs of *A. flavus* 70S, psl82, and ΔveA point-inoculated cultures incubated for 5 days. The medium was supplemented with 0.6 M NaCl, 0.7 M KCl, or 1.0 M sorbitol to generate osmotic stress. (B) Colony growth measured as colony diameter. (C) Quantification of conidial production. Cores (16 mm in diameter) were taken 1 cm from the center of the plates and homogenized in water. Spores were counted with a hemocytometer. Error bars represent standard errors. Experiments were carried out with three replicates. Different letters indicate samples that are significantly different ($P \leq 0.05$).

ously (28). Double-stranded DNA fragments derived from the gene promoter regions were generated by PCR using the primers listed in Table S1 in the supplemental material. The DNA fragments were 5'-end labeled with [γ - 32 P]ATP using USB OptiKinase (Affymetrix, Cleveland, OH), purified using a Micro Bio-Spin P-30 chromatography column (Bio-Rad, Hercules, CA), and used as probes.

Electrophoresis on 5% nondenaturing polyacrylamide (80:1 acrylamide-bisacrylamide) gels was used to separate DNA-protein complexes. Twenty femtomoles of each 32 P-labeled probe was incubated for 15 min at 30°C with 2 μ g of poly(dI-dC) and 7.5 μ g of bovine serum albumin with 5 μ g of nuclear protein extract in a DNA binding buffer (15% glycerol, 15 mM HEPES [pH 7.9], 100 mM KCl, 1 mM EDTA, 2 mM dithiothreitol [DTT]) in 25 μ l of final binding reaction mixture. After separation, the gels were dried and exposed to X-ray film.

Polyclonal anti-AtfB antibodies (YSR) were used to block formation of DNA-protein complexes (shift inhibition EMSA) as described previously (29). Five micrograms of nuclear protein extracts was incubated in a DNA binding buffer for 15 min at 30°C with 5 μ l of anti-AtfB antibody (5.5 μ g/ μ l), or preimmune serum obtained from the same rabbit as the antibody was generated. Then a 32 P-labeled probe was added, and the mixture was incubated for additional 15 min at 30°C. Finally, DNA-pro-

tein complexes were resolved by electrophoresis. For shift inhibition EMSA with anti-VeA antibodies, VeA-specific polyclonal antibodies were used to block formation of DNA-protein complexes as described above.

RESULTS

Effect of osmotic stress. Our study showed that in *A. flavus*, the osmotic stress response is linked to colony growth and development (Fig. 1A and B). Osmotic stress did not inhibit growth; on the contrary, the colony diameter increased in all strains tested when the cultures were supplemented with 0.6 M NaCl, 0.7 M KCl, or 1.0 M sorbitol. Conidiation was also promoted under osmotic stress (Fig. 1A and C) in all the cultures, particularly in the ΔveA colonies. Formation of sclerotia and the sclerotial maturation rate decreased in a hypertonic environment (Fig. 2) in the control strains. It is known that *veA* is necessary for the production of sclerotia (7, 8). In this study, addition of 0.6 M NaCl, 0.7 M KCl, or 1.0 M sorbitol did not remediate this phenotype, and ΔveA colonies did not produce sclerotia on PDA with or without osmotic stress.

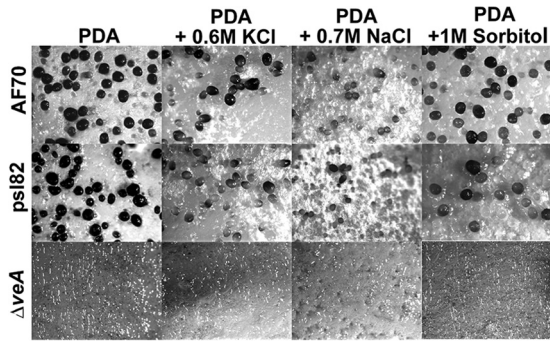


FIG 2 Effect of osmotic stress on sclerotial production. Microscopic examination of sclerotia. Sclerotial production on top agar-inoculated *A. flavus* 70S, pls82, and ΔveA cultures was observed after 5 days of incubation at 30°C. The medium was supplemented with 0.6 M NaCl, 0.7 M KCl, or 1.0 M sorbitol to generate osmotic stress. Images ($\times 32$) were captured using an upright Leica MZ75 stereomicroscope.

We previously demonstrated that *veA* is also required for the biosynthesis of several secondary metabolites, including AFB1 in *A. flavus* (5, 8). The biosynthesis of this carcinogenic mycotoxin was not recovered in the ΔveA mutant under the impact of a hypertonic environment (see Fig. S1 in the supplemental material). Both control strains produced AF under the same experimental conditions. Biosynthesis of this mycotoxin in the controls was not altered under osmotic stress. Interestingly, two unknown *veA*-dependent metabolites were also detected, and the synthesis of one of them was suppressed in a hypertonic environment (see Fig. S1).

Effect of oxidative stress. In order to elucidate whether *veA* is involved in the oxidative stress response in *A. flavus*, we exposed the *veA* deletion mutant strain and the two isogenic control strains, 70S and pls82, to increasing concentrations of hydrogen peroxide (Fig. 3). Our results revealed that addition of this compound lead to a dramatic reduction in colony growth in the ΔveA mutant cultures at a concentration of 25 mM. Furthermore, no growth was observed in ΔveA mutant cultures in the presence of 30 mM H_2O_2 , while the wild-type 70S and pls82 controls were able to form colonies.

We performed a similar experiment using menadione (see Fig. S2 in the supplemental material). Addition of this compound in-

TABLE 1 Comparison of the putative amino acid sequence of *A. flavus* HogA to those of other *Aspergillus* HogA homologs and *S. cerevisiae* Hog1

Protein and species	Accession no. ^a	Identity (%)	Similarity (%)
HogA			
<i>A. oryzae</i>	AO090701000642	84.7	85.0
<i>A. niger</i>	fge1_pg_C_5000454	54.2	69.4
<i>A. terreus</i>	ATEG_00489.1	43.7	56.0
<i>A. fischerianus</i>	NFIA_012610	54.5	68.8
<i>A. fumigatus</i>	Afu1g12940	54.4	68.8
<i>A. clavatus</i>	ACLA_022520	51.1	64.1
<i>A. nidulans</i>	ANID_04668	51.1	63.9
Hog1			
<i>S. cerevisiae</i>	SCRG_05095.1	45.5	59.5

^a Broad Institute Aspergillus Comparative Database, Broad Institute, Cambridge, MA.

hibited the growth of the *veA* deletion mutant at a concentration of 0.8 mM, a condition that still allowed the colonies of the two controls to form.

hogA is dispensable for osmotic and oxidative stress responses in *A. flavus*. The HOG pathway has been best characterized in the model yeast *S. cerevisiae* (reviewed by Hohmann et al. [16]), where the Hog1 kinase plays an important role in the activation of effector genes involved in osmotic and oxidative stress response. The deduced amino acid sequence of *A. flavus* HogA is highly conserved with respect to other HogA homologs in *Aspergillus* spp. or Hog1 in *S. cerevisiae* (Table 1; see Fig. S3 in the supplemental material). We analyzed whether *hogA* is important for *A. flavus* to survive when exposed to osmotic and oxidative stresses. With this goal, we generated $\Delta hogA$ deletion mutants. Verification of the *hogA* deletion in the fungal transformants was carried out by PCR analysis of genomic DNA from three Af70 $\Delta hogA$ mutants using primers (hog1 Up and Down) that flanked the *hogA* coding sequence (see Fig. S4A and B and Table S1 in the supplemental material). Successful disruption of the *hogA* coding region by the *pyrG* gene was confirmed by the presence of a PCR product of about 4.7 kb, while the Af70 *pyrG*-1 control gave a product of about 3.3 kb, as expected for the wild-type *hogA* gene. Real-time quantitative PCR of the $\Delta hogA$ mutants and isogenic

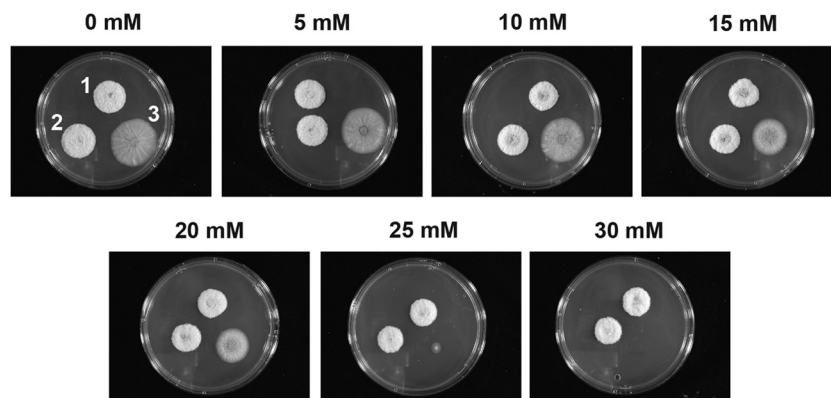


FIG 3 *veA* is necessary for a normal response to oxidative stress in *A. flavus*. Photographs of point-inoculated *A. flavus* 70S, pls82, and ΔveA strains growing on YGT supplemented with increasing amounts of hydrogen peroxide. Culture numbers: 1, 70S; 2, pls82; and 3, ΔveA mutant. Cultures were incubated at 30°C for 3 days.

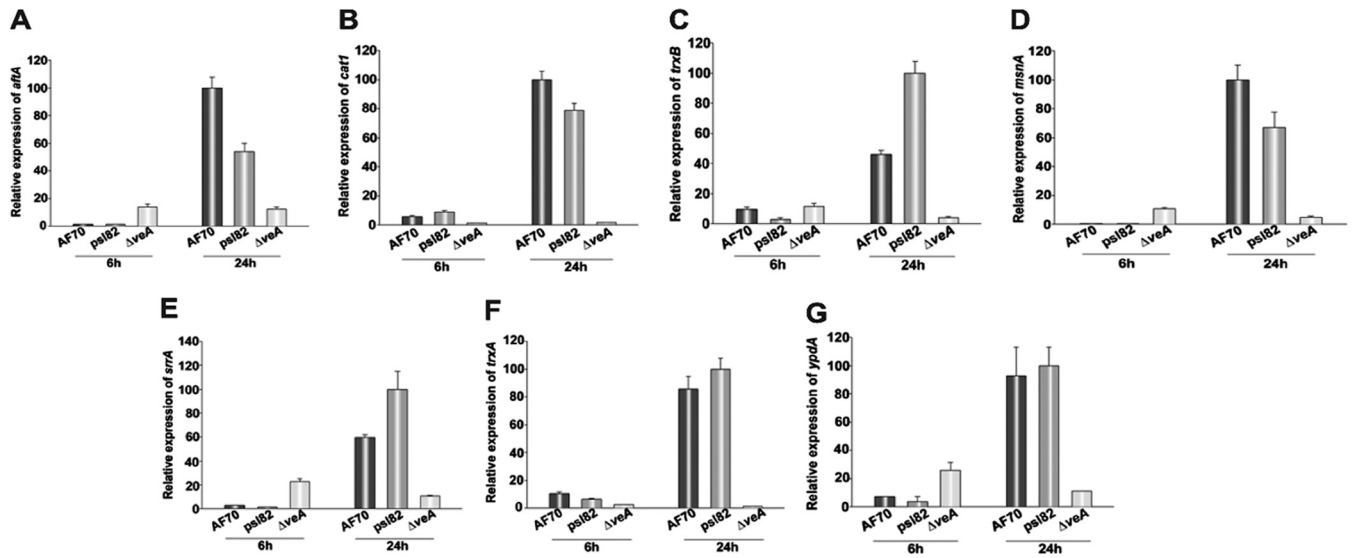


FIG 4 *veA* is required for wild-type expression levels of *A. flavus* oxidative stress response genes. Relative expression levels of *A. flavus* genes involved in the oxidative stress response 6 and 24 h after addition of 15 mM hydrogen peroxide. The relative expression levels were calculated by the method described by Kenneth and Schmittgen (26), and all values were normalized to the expression of the *A. flavus* 18S rRNA gene and to the greatest expression, considered 100.

control demonstrated that *hogA* expression was inactivated in the mutants (see Fig. S4C). Two $\Delta hogA$ mutants were selected to test their tolerance to both osmotic and oxidative stresses. *Aspergillus flavus* $\Delta hogA$ mutants and their isogenic control strain were incubated on medium containing 0.6 M NaCl, 0.7 M KCl, or 1.0 M sorbitol. The $\Delta hogA$ colonies grew as much as the wild-type control (see Fig. S5 in the supplemental material). Furthermore, when these strains were cultured on medium containing a range of menadione concentrations (0, 0.2, 0.4, and 0.6 mM), the $\Delta hogA$ mutants had similar tolerance to oxidative stress to that of the control strain (see Fig. S6 in the supplemental material). These results indicate that in *A. flavus*, the *hogA* homolog is not essential for growth under the oxidative or osmotic stress conditions tested in this study.

Expression of oxidative stress response genes is *veA* dependent. Our study showed that *veA* is necessary for oxidative stress tolerance in *A. flavus*. In order to gain further insight into the *veA*-dependent mechanism involved in the oxidative stress response, we analyzed the expression of genes known to play a role in oxidative stress tolerance in the ΔveA mutants and compared that with expression levels in the control strains. Specifically using qRT-PCR, we examined the expression of *A. flavus* *srrA*, *msnA*, *atfA*, *ypdA*, *trxA*, *trxB*, and *cat1*. Note that with regard to *catB*, the deduced amino acid sequence of *A. flavus* mycelial *cat1* is highly conserved with those of the *catB* genes from other fungal species, such as *A. nidulans*, *Histoplasma*, and *Penicillium* spp. (30–32) (Fig. 4). Our results indicated that after addition of hydrogen peroxide to the culture, the expression of all of these genes increased in the 70S and psl82 control strains; however, this was not the case in the ΔveA strain cultures, where the expression levels did not show an increase over time. These results were also verified by semiquantitative PCR (data not shown). Analysis of the effect of *veA* on the expression of *atfB*, encoding a transcription factor known to bind to the promoter regions of oxidative stress response genes (29, 33), was done separately. Our results indicated that *veA* is also necessary for the increase in the expression of *atfB*

in the presence of hydrogen peroxide over time (see Fig. S7 in the supplemental material).

Detection of DNA-protein complexes at the promoters of oxidative stress response genes. In our present studies, we screened promoter fragments of the *atfA*, *srrA*, *cat1*, *ypdA*, *trxA*, *trxB*, and *msnA* genes for complex formation with protein extracts from the *A. flavus* wild-type strain by using electrophoretic mobility shift assays (EMSA). DNA-protein complex formation was assessed by the mobility and intensity of the shifted band compared with those of the free DNA probe lanes, which contained only the DNA fragment, with no protein extract. We detected formation of prominent DNA-protein complexes with all promoters, except for the *atfA* and *srrA* promoters (data not shown). EMSA detected a strong DNA-protein complex formation between nuclear protein extracts from *A. flavus* 70S grown in YGT medium for 24 h and DNA fragments derived from the promoter regions of the *cat1* gene (518 bp upstream from ATG) and the *trxB* gene (521 bp upstream from ATG). In order to further characterize these DNA-protein interactions, the large DNA fragments were divided into smaller pieces (Fig. 5A; see Table S2 in the supplemental material). The *cat1* promoter region was divided into *cat1*-1 (259 bp) and *cat1*-2 (259 bp). The *trxB* promoter region was divided into *trxB*-1 (263 bp) and *trxB*-2 (258 bp). The *cat1*-2 and *trxB*-2 fragments demonstrated prominent DNA-protein complexes in EMSAs using nuclear protein extracts from *A. flavus* 70S and psl82. For this reason, these fragments were selected to perform the following EMSA experiments described below.

Protein extracts from the ΔveA strain show strong DNA-protein complex formation on *cat1*-2 and *trxB*-2 promoter fragments. We examined *cat1*-2 and *trxB*-2 fragments for DNA-protein complex formation using protein extracts obtained from controls and ΔveA mycelia grown with or without H_2O_2 treatment. Hydrogen peroxide treatment of 70S, psl82, and ΔveA mutant cultures did not affect DNA-protein complex formation with either *cat1*-2 or *trxB*-2 fragments compared to the same cultures without H_2O_2 treatment (Fig. 5B and C). However, ΔveA protein

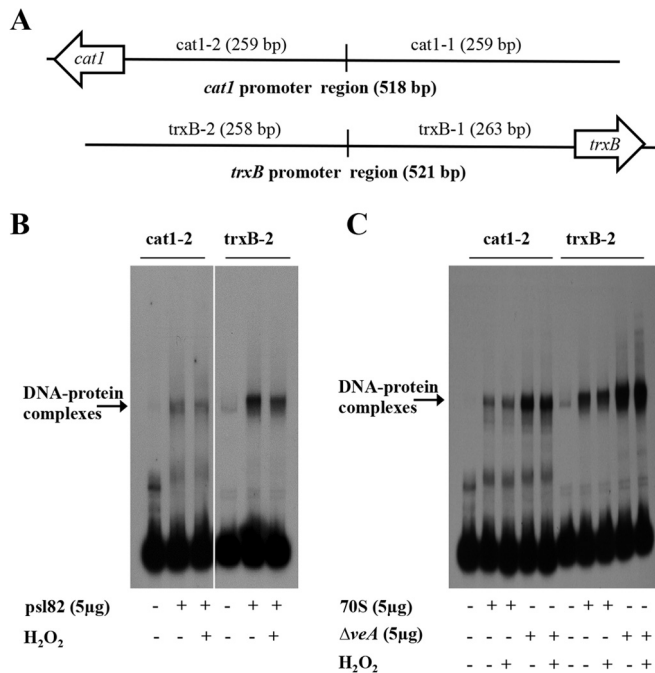


FIG 5 The formation of protein-DNA complexes at the *cat1* and *trxB* promoters is influenced by VeA. (A) Schematic representation of promoter regions of the *cat1* and *trxB* genes used in EMSA. The *cat1* promoter region was divided into two fragments designated cat1-1 and cat1-2. The *trxB* promoter region was also divided into two fragments, designated trxB-1 and trxB-2. (B and C) DNA-protein complex formation on cat1-2 and trxB-2 promoters by EMSA using 70S, psl82, or ΔveA strain protein extracts with or without H₂O₂ treatment. Cell extracts enriched in nuclear proteins were prepared as described in Materials and Methods. Five micrograms of enriched nuclear protein extracts was added to a ³²P-labeled promoter probe for each gene.

extracts showed DNA-protein complex formation with significantly higher intensity on cat1-2 and trxB-2 fragments than that for the corresponding 70S protein extracts regardless of H₂O₂ treatment (Fig. 5C). Based on the fact that these and previous studies including the 70S and psl82 controls yielded similar results, the 70S wild-type strain was selected for further promoter analyses.

AtfB binds more intensely to cat1-2 than to the trxB-2 promoter fragment. Previously we showed that AtfB, a basic leucine zipper (bZIP) transcription factor, binds not only to aflatoxin gene promoters but also to promoters of oxidative stress response genes, including mycelium-specific *cat1* and the mitochondrion-specific Mn superoxide dismutase (SOD) gene (Mn *sod*) in *A. parasiticus* (33). In addition, we also demonstrated that VeA is necessary for histone H4 acetylation and AtfB binding to occur at normal levels in the aflatoxin gene cluster (29, 34). To examine the differences between DNA-protein complexes formed on cat1-2 and trxB-2 fragments by 70S and ΔveA protein extracts, AtfB- or VeA-specific polyclonal antibodies were used to block DNA-protein complex formation (by shift inhibition EMSA). Formation of a specific DNA-protein complex was significantly inhibited by pretreatment of the enriched 70S or ΔveA protein extracts with anti-AtfB antibodies before addition of the cat1-2 fragment but not by preimmune serum obtained from the same rabbit from which the antibody was generated (Fig. 6A to C). Addition of anti-AtfB antibody alone or preimmune serum alone to the cat1-2

fragment did not produce any shifted complexes. However, DNA-protein complex formation on the trxB-2 fragment using the enriched protein extracts prepared from 70S or the ΔveA mutant was significantly less inhibited by pretreatment with anti-AtfB antibodies compared with shift inhibition on the cat1-2 fragment (Fig. 6D and E). Shift inhibition EMSA with anti-VeA antibodies demonstrated no apparent inhibition of the DNA-protein complex formation on either the cat1-2 or trxB-2 fragment using 70S and ΔveA protein extracts (Fig. 7), suggesting that VeA does not directly bind to cat1-2 or trxB-2 promoter fragments.

DISCUSSION

Fungi are versatile eukaryotic organisms capable of colonizing diverse environments and rapidly responding to changes, optimizing their survival. The negative effects of diverse types of abiotic stresses could be overcome in these organisms by the activation of signaling pathways that lead to a cellular adaptive response. Progress has been made in understanding the relationship between osmotic and oxidative stresses and the cellular response triggered by these stimuli, which is mediated by the HOG signaling pathway. The HOG pathway has been characterized in yeast in great detail (16, 35). Studies in filamentous fungi, mostly in aspergilli (20, 36, 37), suggest that the HOG pathway is semiconserved compared to that described in yeast (17). Interestingly, the regulatory output of this signaling pathway in filamentous fungi also seems to vary from that described in yeast (20). For instance, while *HOG1* is necessary for the response and survival in *S. cerevisiae*, deletion of the *sakA* homolog in *A. nidulans* does not show sensitivity to high osmolarity (20). Previous studies indicate that mutations in genes involved in the HOG pathway in filamentous fungi result in changes in morphological differentiation, suggesting a link between the osmotic stress response and fungal development. For example, the *sakA* deletion mutant showed premature sexual development. Furthermore, cleistothecial formation was prevented when the *A. nidulans steC* gene, a homolog of the yeast gene *STE11*, was deleted (38). Some osmotic stress response mutants also show an effect on asexual development, such as reduction in conidium viability in the *sakA* mutant (20) or abnormality in conidiophore formation in the *steC* mutant (38). Furthermore, a decrease in conidiation was also observed in the *A. nidulans srrA* and *sskA* mutants as well as in the *A. fumigatus MA21* mutant (*SHO S. cerevisiae* homolog) (39), further illustrating the interaction between the osmotic stress response signaling pathway and fungal morphogenesis. The effect of osmotic stress on conidiation directly influences fungal dispersal. This is especially relevant when considering fungal species with an agricultural impact, such as the aflatoxin producer *Aspergillus flavus*. This fungus is an opportunistic plant pathogen of important oil seed crops (4) that disseminates mainly by producing airborne conidia, causing large infestations in the field.

As part of the present study, we investigated first whether sub-optimal conditions caused by osmotic stress affect growth and morphogenesis in *A. flavus* and whether this could be influenced by *veA*. Our results revealed that osmotic stress positively affects vegetative growth and leads to an increase in conidiation in *A. flavus*. This is in agreement with a report from Han et al. (40) in which the authors showed an increase in conidiation in *A. nidulans* cultures with high levels of sorbitol. In contrast, Mert and Ekmekci (41) reported a reduction of conidia in *A. flavus* inoculated on NaCl medium. It is possible that this variability in conidi-

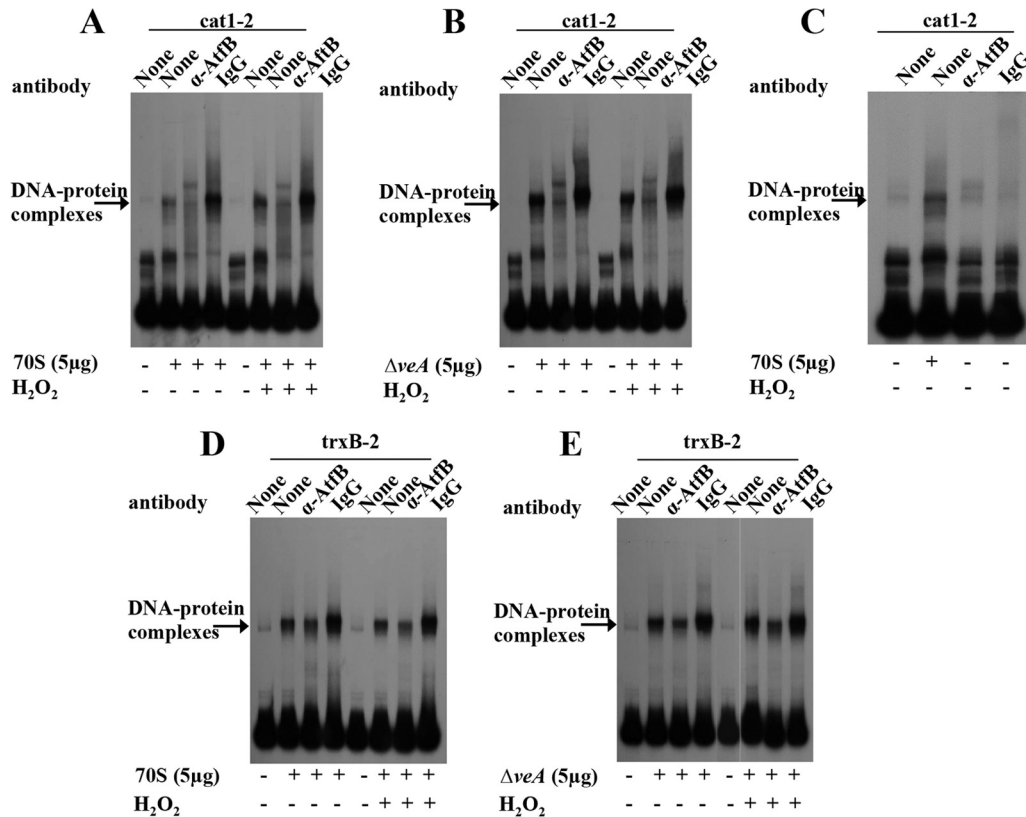


FIG 6 Analysis of AtfB binding to the *cat1* and *trxB* promoters and its relationship with VeA. Shown are results from EMSA of AtfB binding in the *cat1-2* (A to C) and *trxB-2* (D to E) promoter fragments using 70S or ΔveA mutant protein extracts with or without H₂O₂ treatment. Enriched nuclear protein extracts were prepared as described in Materials and Methods. Five micrograms of enriched nuclear protein extracts was added to a ³²P-labeled promoter probe for each gene. Anti-AtfB antibodies (YSR) or preimmune serum was added to determine whether these could block protein-DNA interaction (shift inhibition). (A) *cat1-2* probe and 70S protein extracts. (B) *cat1-2* probe and ΔveA protein extracts. (C) *cat1-2* probe and anti-AtfB antibodies alone (without protein extracts). (D) *trxB-2* probe and 70S protein extracts. (E) *trxB-2* probe and ΔveA protein extracts.

ation could result from the use different *A. flavus* strains or medium conditions in these studies. In our studies, we observed that osmotic stress further enhances hyperconidiation in the *A. flavus veA* mutant, suggesting that *veA* is involved in modulating osmotic stress-induced conidiation.

In addition to the ability to efficiently disseminate by forming conidia, *A. flavus* is able to produce sclerotia. In other fungi, such as *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, and *Sclerotium rolfsii*, salinity stress inhibits the formation of sclerotia (42). In *Botrytis cinerea*, sclerotial production was decreased as osmotic stress increased (43). However, in *Aspergillus ochraceus*, osmotic stress had little effect on sclerotial production (44). The results from our present study indicate that in *A. flavus*, hyperosmotic medium caused a delay in sclerotial production and maturation. Sclerotia are postulated to be vestiges of cleistothecia (45, 46). For this reason, it could be expected that the effects of hypertonic media on sclerotial formation could be similar to the effect on cleistothecial formation. Indeed, in *A. nidulans* cleistothecial production also decreased in the presence of 1 M sorbitol (40). As expected, based on our previous studies (7, 8), ΔveA cultures did not produce sclerotia or the carcinogenic mycotoxin AFB1, and the formation of these resistant structures and the synthesis of AFB1 were not rescued by exposure to a hypertonic environment.

Although the oxidative stress response has been extensively studied in prokaryotes and eukaryotes, it is possible that addi-

tional genetic elements unique to filamentous fungi, such as *veA*, could modulate this response in these organisms. Importantly, our study indicated that tolerance to oxidative stress decreased in the ΔveA strain compared to that in the control strains. Supplementation of medium with hydrogen peroxide or menadione resulted in complete growth inhibition of the *veA* mutant, whereas the 70S and *psl82* strains were able to form colonies. These results indicate that *veA* has an important role in protecting *A. flavus* from oxidative stress. To gain insight into the mechanism by which *veA* influences the oxidative stress response in *A. flavus*, the expression patterns of several genes encoding proteins known to play a role in the HogA-related signaling pathway were analyzed in the ΔveA strain and compared to those of the control strains under oxidative stress. Our experiments revealed that *veA* is necessary for normal expression of critical genes in this adaptive genetic response, including *atfA*, *srrA*, *msnA*, and *ypdA*, which encode transcription factors, and structural genes, such as *trxA*, *trxB*, and *cat1* (22).

To further elucidate the role of VeA in the transcription of the oxidative response genes, we performed electrophoretic mobility shift assays utilizing their promoters and protein extracts from *A. flavus veA* mutant and control strains. The formation of DNA-protein complexes in *cat1* and *trxB* promoters was particularly interesting; more prominent DNA-protein complex formation on *cat1-2* and *trxB-2* promoter fragments (Fig. 6) was detected when

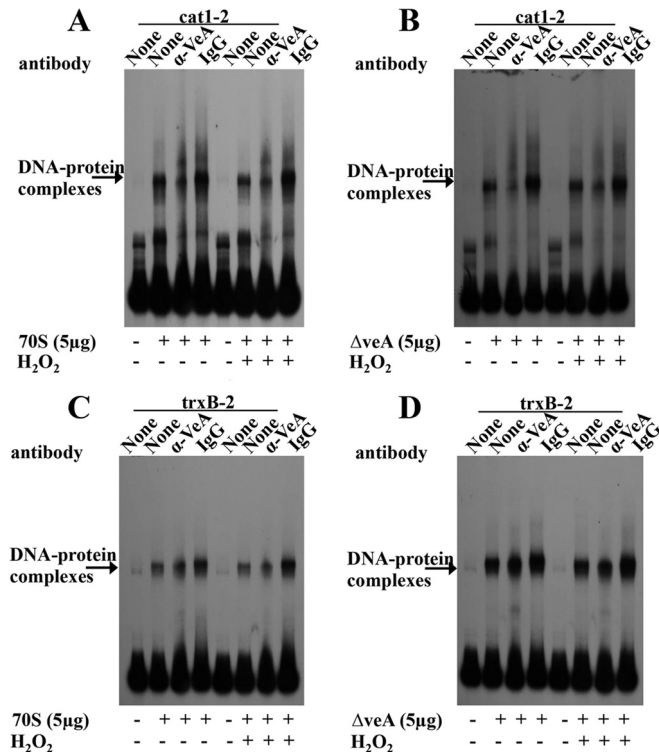


FIG 7 The presence of a VeA antibody did not prevent protein-DNA complex formation at the *cat1* and *trxB* promoters in shift inhibition EMSA. Shown are results from EMSA of VeA binding in the *cat1-2* and *trxB-2* promoter fragments using 70S or ΔveA mutant protein extracts with or without H₂O₂ treatment. Enriched nuclear protein extracts were prepared as described in Materials and Methods. Five micrograms of enriched nuclear protein extracts was added to a ³²P-labeled promoter probe for each gene. Anti-VeA antibodies or preimmune serum was added to determine whether these could block protein-DNA interaction (shift inhibition). (A) *cat1-2* probe and 70S protein extracts. (B) *cat1-2* probe and ΔveA protein extracts. (C) *trxB-2* probe and 70S protein extracts. (D) *trxB-2* probe and ΔveA protein extracts.

ΔveA protein extracts were used compared to the level of these complexes formed with wild-type protein extracts. When taken together with the lack of transcriptional induction of the oxidative stress response genes under oxidative stress in the ΔveA strain, these results suggest that the observed VeA-dependent DNA-protein complex formation on *cat1-2* and *trxB-2* promoter fragments could include repressor proteins negatively influencing the transcriptional activation of *cat1* and *trxB*. Future proteomics studies in our laboratory will provide additional information on the VeA-dependent protein complexes associated with these promoters.

Another important finding in our studies includes the requirement of the transcription factor AtfB for the formation of the DNA-protein complex at the *cat1* and *trxB* promoters. This was particularly notable in the case of the *cat1* promoter. This bZIP transcription factor, which our current study also revealed to be transcriptionally regulated by *veA*, was previously studied in *A. parasiticus* (29). AtfB is known to contribute to conidium resistance to oxidative stress and activation of the transcription of *catA* (47). Furthermore, AtfB has been demonstrated to bind to the promoter regions of oxidative stress response genes, such as the *cat1* and Mn superoxide dismutase (SOD) genes, as well as genes within the aflatoxin gene cluster (29, 33), where AtfB may aid

AflR, an endogenous transcription factor of this cluster, to activate aflatoxin biosynthetic genes (28, 48).

In conclusion, the present study revealed that while the global regulatory *veA* gene and its product have a mild effect on the response to osmotic stress, they have a critical role in survival under oxidative stress in *A. flavus*, an agriculturally important fungus with a robust oxidative stress response in which, interestingly, HogA is dispensable for growth under this stress, suggesting an alternative route for activation of the oxidative stress response genes from that described in yeast. Mechanistically, we found that VeA is a positive regulator in the expression of oxidative stress response genes. Furthermore, VeA modulates the formation of protein-DNA complexes in their promoter region of oxidative stress response genes, such as *cat1*, and perhaps contributes to coordinate production of toxin with the oxidative stress response. Also, we found that DNA-protein complex formation on the promoter is mediated by the *veA*-dependent transcription factor AtfB, known to bind to the promoters of aflatoxin genes. Due to the conserved nature of the master regulator VeA, it is likely that the role of VeA in the oxidative stress response could also be conserved in other fungal species of agriculture or medical importance. These studies could contribute to set the basis for the design of future control strategies that could decrease the negative impact of the aflatoxin producer *A. flavus* and other detrimental fungi.

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