

Production of cyclopiazonic acid, aflatrem, and aflatoxin by *Aspergillus flavus* is regulated by *veA*, a gene necessary for sclerotial formation

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Abstract The plant pathogenic fungus *Aspergillus flavus* produces several types of mycotoxins. The most well known are the carcinogenic compounds called aflatoxins. In addition, *A. flavus* produces cyclopiazonic acid and aflatrem mycotoxins, contributing to the toxicity of *A. flavus* infected crops. Cyclopiazonic acid is a specific inhibitor of calcium-dependent ATPase in the sarcoplasmic reticulum that results in altered cellular Ca^{++} levels. Aflatrem is a potent tremorgenic mycotoxin known to lead to neurological disorders. Previously we showed that a gene called *veA* controls aflatoxin and sclerotial production in *A. parasiticus*. In this study in *A. flavus*, we show that the *veA* homolog in *A. flavus* not only is necessary for the production of aflatoxins B1 and B2 and sclerotia, but also regulates the synthesis of the mycotoxins cyclopiazonic acid and aflatrem. The *A. flavus* ΔveA mutant was completely blocked in the production of aflatrem and showed greater than twofold decrease in cyclopiazonic acid production. The genes involved in the synthesis of cyclopiazonic acid are unknown; however, the aflatrem gene cluster has been characterized. Northern hybridization analysis showed that *veA* is required for expression of the *A. flavus* aflatrem genes *atmC*, *atmG*, and *atmM*. This is the first report of a regulatory gene governing the production of cyclopiazonic acid and aflatrem mycotoxins.

Keywords *Aspergillus flavus* · Cyclopiazonic acid · Aflatrem · Aflatoxin · Sclerotia · Mycotoxin regulation

Introduction

Among the most common toxigenic fungi affecting the food chain are *Aspergillus* spp. These fungi infect commodities such as corn, peanuts, cotton, tree nuts, sorghum, and other oil seeds. In particular, *A. flavus* is of great importance due to its impact on agriculture and human health. *A. flavus* produces several types of mycotoxins. The most well known are the aflatoxins (AF), with AFB1 being one of the most mutagenic and carcinogenic natural compounds known (Payne and Brown 1998; Sweeney and Dobson 1999; Trail et al. 1995). Ingestion of AF-contaminated food or feed has been associated with hepatotoxicity, teratogenicity, immunotoxicity, and even death in animals and humans (Dvorackova and Kusak 1990; Trail et al. 1995). Ingestion of AF is directly correlated with incidence of human liver cancer (Wogan et al. 1992). It has also been demonstrated that AF induces mutations in the *p53* tumor suppressor gene and K-ras and H-ras protooncogenes (Denissenko et al. 1999; Lasky and Magder 1997; Riley et al. 1997; Shen and Ong 1996; Wang and Groopman 1999). Aflatoxin contamination of crops can also result in significant economic losses to both producers and processors in the USA and worldwide (Richard and Payne 2003; Wu 2004).

In addition to AF, some *A. flavus* strains produce other mycotoxins such as cyclopiazonic acid (CPA) and aflatrem. CPA is an indole tetramic acid (Fig. 1a) that is toxic to animals and humans. This compound is a specific inhibitor of calcium-dependent ATPase in the sarcoplasmic reticulum altering Ca^{++} levels, resulting in increased muscle contrac-

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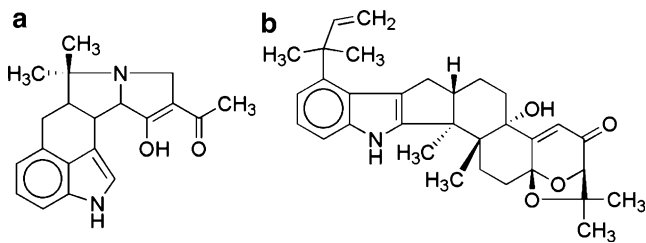


Fig. 1 Chemical structure of **a** CPA and **b** aflatrem

tion (Riley and Goeger 1992). In rats, CPA has been reported to cause lesions of the liver, kidney, pancreas, spleen, and heart (Purchase 1971). Chicken fed with rations containing 100 ppm of CPA had proventriculitis, mucosal necrosis, hepatitis, and myocardial inflammation, with a significant mortality rate (Dorner et al. 1983). The toxin accumulates in edible tissue in poultry (Norred et al. 1988; Porter et al. 1988), and it is also found in milk (in ewes) and eggs (laying hens). CPA is also produced by several *Penicillium* spp., including *P. aurantiohriseum* (*P. cyclopium*), where it was first described. *P. camembertii*, used in the production of gourmet cheese, produces CPA (Pitt et al. 1986). These findings indicate additional routes of CPA contamination in food. Aflatoxin and CPA contamination can occur together in the same food commodity (Dorner et al. 1983; Horn and Dorner 1999; Lansden and Davidson 1983; Purchase 1971; Urano et al. 1992). Both CPA and AF were isolated from ground nut meal samples related to the “turkey X disease” that caused the death of 100,000 turkey poults (Blout 1961; Goldblatt 1969), and led in large part to the initiation of AF characterization. CPA might have contributed to some of the symptoms described in this early case (Blout 1961; Cole et al. 1986).

The mycotoxin aflatrem, an indole diterpene (Fig. 1b) also produced by *A. flavus*, is a potent tremorgenic compound known to lead to neurological disorders (Valdes et al. 1985; Yao et al. 1988). Although the mechanism by which aflatrem causes tremors in animals is not well defined, clinical studies have shown that these symptoms are generated by alterations in the receptors and neurotransmitter release in the central and peripheral nervous system. Recently, a gene cluster containing three genes involved in aflatrem biosynthesis was identified (Zhang et al. 2004). These authors demonstrated that the genes *atmG*, *atmC*, and *atmM* are necessary for aflatrem biosynthesis in *A. flavus*.

Our previous studies in *A. parasiticus* showed that the *veA* gene is required for the production of AF intermediates as well as for the morphogenesis of resistant structures termed sclerotia (Calvo et al. 2004). The *veA* gene product does not present homology with any other protein of known function. Our chemical analysis showed that the synthesis of additional unknown compounds could also be affected by the *veA* mutation. The possible role of *veA* as a regulator

of additional secondary metabolic pathways leading to the production of other mycotoxins in AF producers has not been investigated. In *A. flavus*, the genes involved in the CPA biosynthetic pathway or the regulatory mechanisms controlling CPA production are unknown. Furthermore, the regulatory mechanisms controlling expression of the aflatrem biosynthetic gene cluster have yet to be elucidated. This is the first report of a gene, *veA*, controlling the synthesis of the mycotoxins CPA and aflatrem. These findings add to the significance of *veA* as a possible genetic target to control mycotoxin contamination of food and feed commodities.

Materials and methods

Fungal strains and growth conditions The *A. flavus* strain used in this study was ATCC MYA384 (a *niaD*⁻ *A. flavus* 70S strain). This *A. flavus* strain produces abundant small sclerotia. ATCC MYA384 was also used as host strain for fungal transformation (see below). ATCC MYA384 and the transformant strains were inoculated on YGT medium (0.5% wt/v yeast extract, 2% wt/v glucose, and 1 ml of trace element solution per liter), unless otherwise indicated. Agar (15 g l⁻¹) was added in the case of solid medium. Strains were maintained as glycerol stocks at -80 °C.

Identification of the *A. flavus veA* gene A 3.6-kb polymerase chain reaction (PCR) product representing the *A. flavus veA* coding region and flanking DNA sequences was obtained by PCR amplification of *A. flavus* genomic DNA template using the primers *FveA* and *RveA* (Table 1)

Table 1 Primers used in this study

| Primer name | Sequence |
|----------------|-------------------------------------|
| <i>FveA</i> | 5'-TACCTCACAGCTGGCTGAGTTCAC-3' |
| <i>RveA</i> | 5'-GGTGTCTCTCCCTGGAATGTTCCCT-3' |
| <i>FajfR</i> | 5'-GATAGCTGTACGAGTTGTGCCAGCTCA-3' |
| <i>RajfR</i> | 5'-CATTCTCGATGCAGGTAATCAATAATGTC-3' |
| <i>FajfD-1</i> | 5'-ATGAACGGATCACTTAGCCAGCACGGTC-3' |
| <i>RajfD-1</i> | 5'-CTACCAGGGGAGTTGAGATCCATCCGT-3' |
| <i>FajfM-1</i> | 5'-ATGTCCGACAACCACCGTTTAGATGGCA-3' |
| <i>RajfM-1</i> | 5'-CAATGATCTTCCACTTACCCATTCGGCTG-3' |
| <i>FajfPA</i> | 5'-ATGACGGGCCCTGACATGGAATCATC-3' |
| <i>RajfPA</i> | 5'-GTGGCTCGAAGAAATTGTGCTCCATGAAC-3' |
| <i>FatmC</i> | 5'-CCTCTCTCGGCTACAAC-3' |
| <i>RatmC</i> | 5'-AGCTCCTTTGGCTTTTGC-3' |
| <i>FatmG</i> | 5'-TTTCAGGTGTGCCGATC-3' |
| <i>RatmG</i> | 5'-AGTTGTTGATTACCCGGG-3' |
| <i>FatmM</i> | 5'-GGATCTGTCGCTGGTCTC-3' |
| <i>RatmM</i> | 5'-GACGTAACCTCCCTGTTTC-3' |

designed from the available *A. parasiticus veA* nucleotide sequence (Calvo et al. 2004; GenBank accession no. AY445513). The PCR product was cloned in TOPO-pCR 2.1 vector and sequenced using synthetic primers and an ABI PRISM DNA sequencing kit (Perkin Elmer Life Science). The *A. flavus veA* sequence was deposited in GenBank with accession no. DQ296645. This sequence information was then used for the construction of the deletion vector.

Generation of the *A. flavus* ΔveA mutant strain The 3.6-kb PCR product was subsequently used in the engineering of a plasmid vector for the insertional inactivation of the wild-type *A. flavus* ΔveA gene. The transformation vector pBS-*veA-niaD* was generated as follows: The 3.6-kb *veA* region was released from the TOPO-pCR 2.1 vector by *EcoRI* digestion. The *veA* fragment was then ligated into *EcoRI* digested, dephosphorylated plasmid vector pBluescript SK⁻ (Stratagene) in which the *XhoI* site present in the multiple cloning site had been previously inactivated by *XhoI* digestion followed by a Klenow end-fill reaction. The pBS-*veA* vector was then digested with *XhoI*, which cuts at nt 65 and 638 of the *veA* ORF, and dephosphorylated. A 7.4-kb *Sall*-*XhoI* fragment containing the *A. parasiticus*

niaD gene present in the vector pSL82 (Horng et al. 1990) was ligated into the *XhoI* digested pBS-*veA*, creating the plasmid pBS-*veA-niaD*. This construct now harbored the *veA* gene that was disrupted by insertion of the *niaD* gene region. The pBS-*veA-niaD* plasmid DNA was used to transform ATCC MYA384 (*A. flavus* 70S *niaD*⁻ strain). Protoplasts of *A. flavus* 70S *niaD*⁻ were prepared as previously described by Cary et al. (2005). Transformants in which the wild-type *veA* gene had been insertionaly inactivated via double homologous recombination with the *veA-niaD* gene region (Fig. 2a) were initially identified as those colonies that did not form sclerotia during growth. Five transformants of *veA-niaD* (ΔveA) demonstrated this phenotype and were selected for further analysis. Three control transformants were also obtained after transformation of MYA384 with pSL82. Two different selectable markers, required for complementation, are not available in *A. flavus* S strains. However, the ΔveA mutants showed the same phenotype and were similar to those generated in other *Aspergillus* spp. with respect to aflatoxin/sterigmatocystin (ST) production and sclerotial formation (Calvo et al. 2004; Kato et al. 2003). Southern analysis confirmed the *veA* disruptions via recombinational inactivation (Fig. 2b).

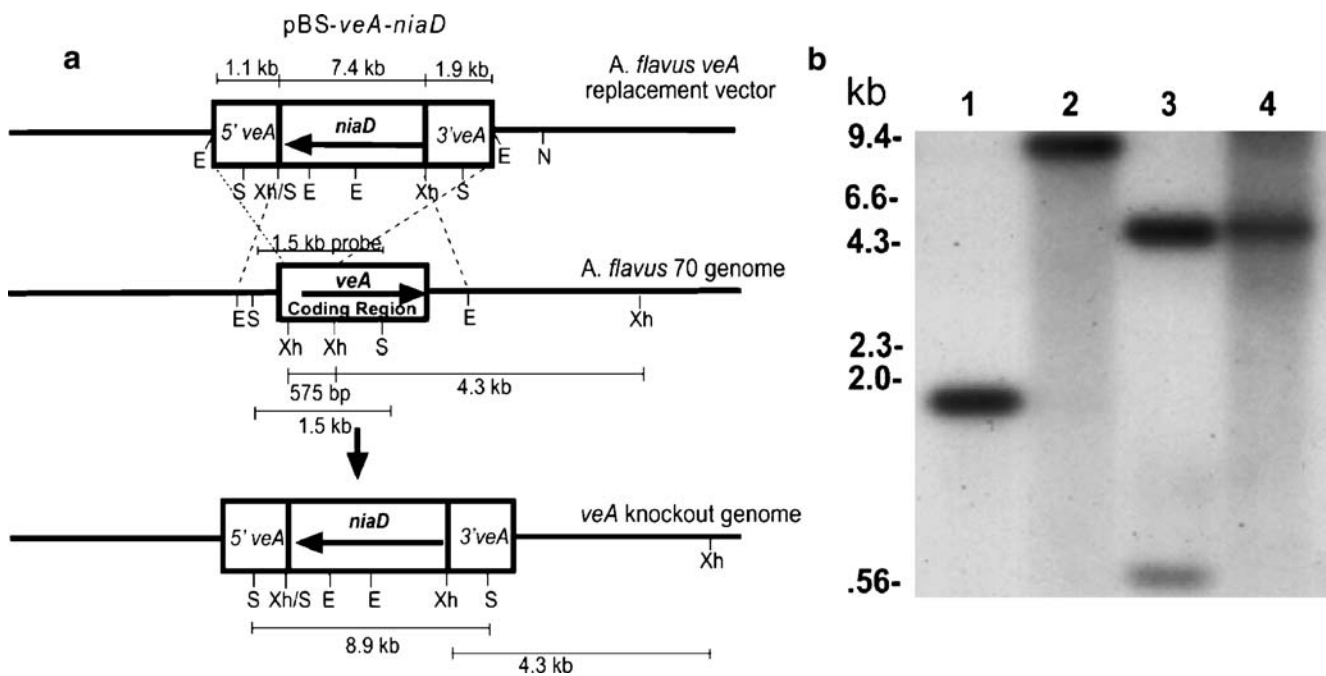


Fig. 2 Construction of the *A. flavus veA* knockout mutant. **a** Schematic diagram of the knockout vector pBS-*veA-niaD* used to generate the *veA* knockout mutants. The dashed lines show the results expected by replacement of the wild-type DNA with DNA containing the *niaD* transformant selection cassette. Direction of transcription is indicated by horizontal arrows. The lengths of expected *Sall* or *XhoI* DNA fragments upon restriction enzyme digestion of either the wild-type strain or the *veA* knockout transformant genomic DNA are shown

under the horizontal lines. Abbreviations: Xh, *XhoI*; S, *Sall*; E, *EcoRI*; N, *NotI*; Xh/S, *XhoI/Sall* site inactivated by Klenow end-fill ligation. Distances depicted are not to scale. **b** Results of Southern hybridization. Lanes: 1, *veA*, *Sall*; 2, ΔveA , *Sall*; 3, *veA*, *XhoI*; 4, ΔveA , *XhoI*. All the transformants were examined by Southern analysis, obtaining the sample band patterns indicating gene replacement in the ΔveA strains or presence of the wild-type *veA* allele in the control strains. Molecular size markers are Lambda *HindIII* digested DNA

Physiological studies Studies on fungal development were performed with the *A. flavus* pSL82 control strains (*veA* wild-type allele and *niaD+*; herein referred to as control strains) and the *A. flavus* ΔveA mutant strains. Plates containing 30 ml of solid YGT medium were point inoculated and incubated in the light ($25 \mu\text{E m}^{-2} \text{s}^{-1}$) or in the dark at 30 °C for 7 days. Conidia were harvested with equal amounts of water and counted using a hemacytometer. Sclerotia were numerous and homogeneously distributed on the colony surface in the control strains. To evaluate sclerotial production, three cores of 1-cm diameter were collected from different areas of each replicate plate surface. Experiments were carried out with four replicates. Sclerotia found in those cores were counted under a stereozoom microscope (Lieder). Total sclerotial production per colony was estimated taking into account the colony diameter. Colony growth was recorded as colony diameter.

Aflatoxin analysis Approximately 10^6 spores per milliliter were inoculated into 500-ml Erlenmeyer flasks containing 250 ml YGT media and incubated in the dark for 14 h at 30 °C with shaking at 200 rpm. Mycelia from each flask were harvested onto a sterile 87-mm nylon membrane (Schleicher & Schuell, Inc.) and placed onto the surface of a YGT agar plate and incubated at 30 °C in the light. A nylon filter was removed from the surface of a plate at 2-, 12-, 24-, or 36-h time points. The filter and macerated agar were placed in a 250-ml Erlenmeyer flask, and 100 ml sterile water and 50 ml acetone were added. The sample was gently agitated at room temperature for a minimum of 1 h. AF was extracted and analyzed by thin layer chromatography (TLC) as previously described (Cleveland et al. 1987). The residue was resuspended in acetone and spotted on a TLC plate (Si250F, J.T. Baker). The plate was then developed in toluene/ethyl acetate/formic acid (5:4:1, v/v/v), air-dried, and examined for the presence of AF under ultraviolet (UV) light.

CPA analysis Twenty sterile vials containing 2 ml of YGT media were inoculated using a final concentration of 10^6 spores per milliliter. The samples were vortexed following inoculation and before they were placed in the incubator. The cultures were grown statically at 30 °C for 7 days in the light or in the dark. The experiment was carried out with five replicates. Following incubation, the cultures were harvested, homogenized, extracted with chloroform, and allowed to dry overnight. Samples were analyzed by high performance liquid chromatography (HPLC) on a Waters 600E HPLC system with Empower software in conjunction with a Waters 717 auto sampler and a Waters 486 tunable absorbance detector unit adjusted to a UV wavelength of 280 nm. The extracts were resuspended in 200 μl of mobile phase solvent mixture that consisted

of *n*-haptane:2propanol:water:40% tetrabutylammonium hydroxide (2,560:1, 120:32:8, v/v/v/v) (Sobolev et al. 1998). Twenty-five microliters was injected into a Luna 100A (Phenomenex) normal phase silica column (150 \times 4.6 mm) of 5- μm particle size. A flow rate of 1 ml min^{-1} was used. The elution time of the samples was compared with pure CPA standard (Sigma) and quantified on the basis of the ratio of the peak area of CPA to that of the standard.

Aflatrem analysis Seed cultures of 25 ml of YEPGA liquid medium (Zhang et al. 2004) containing approximately 5×10^6 spores per milliliter (final concentration) were incubated in 125-ml Erlenmeyer flasks at 200 rpm at 30 °C for 48 h. Two milliliters of the *A. flavus* seed cultures was then transferred to 250-ml Erlenmeyer flasks containing 50 ml of aflatrem production medium (Zhang et al. 2004). Cultures were incubated statically under light or dark conditions at 30 °C, and the mycelia were harvested after 7 days of incubation by filtering through Miracloth and removing the excess liquid with paper towels. One-gram aliquots were lyophilized and homogenized in 10 ml of a 2:1 mixture of chloroform–methanol and vortexed. The samples were placed at room temperature and vortexed every 10 min for 1 h. The samples were centrifuged at 2900 $\times g$ at room temperature for 10 min to pellet insoluble material. The supernatants were transferred to new tubes and the solvent allowed to evaporate overnight. Five hundred microliters of acetonitrile:water (9:1) was added to each of the dried extracts and vortexed until the samples were totally resuspended. Samples were centrifuged at 150 $\times g$ at room temperature for 20 min, and the supernatants were analyzed by HPLC using the Waters system described above. Aflatrem was detected at 230 and 280 nm as previously described (McMillan et al. 2003). A Luna (Phenomenex) C18 100A (46 \times 25 cm) with a particle size of 5 μm was used for the aflatrem analysis. The mobile phase was composed of acetonitrile:water (9:1) with a flow rate of 1.0 ml min^{-1} . Elution time and quantification of the samples were obtained by comparing with that of pure aflatrem standard (a gift provided by Merck).

Northern analysis For Northern analysis of AF gene expression, the fungal cultures were grown as described for aflatoxin analysis above. Mycelia were carefully scraped from the nylon membrane, frozen with liquid nitrogen, and stored at -80 °C. The samples for Northern analysis of aflatrem gene expression were obtained using the same culture conditions described for aflatrem analysis above, but in this case, mycelia were harvested at 72, 96, 120, and 144 h for RNA extraction. Total RNA was isolated from fungal mycelia using Triazol reagent (Invitrogen) according to the manufacturer's instructions. Approximate-

ly 20 μg of total RNA for each sample was separated by agarose gel electrophoresis and blotted as previously described (Sambrook et al. 1989). Agarose gel purified DNA fragments representing the *A. flavus aflR*, *aflD* (*nor-1*), *aflM* (*ver-1*), and *aflP* (*omtA*) gene coding regions were used to generate [α - ^{32}P]dCTP labeled probes using the RediPrime II DNA labeling system (Amersham Biosciences). The primers used to PCR amplify those fragments from *A. flavus* genomic DNA to generate these probes are listed in Table 1 and were based on the *A. flavus* AF gene cluster sequence (GenBank accession no. AY510453). The primers used to PCR fragments corresponding to the aflatoxin genes *atmC*, *atmG*, and *atmG* are also listed in Table 1 and were based on the *A. flavus* aflatoxin cluster sequence (GenBank accession no. AY559849).

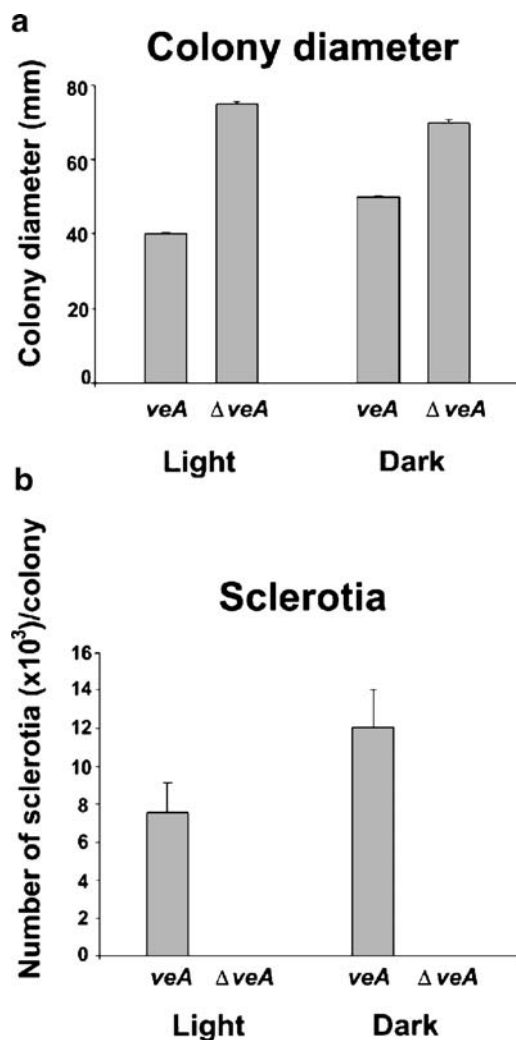


Fig. 3 Effect of the *veA* mutation in *A. flavus* development. *veA* and ΔveA mutant cultures were grown on YGT in the light or in the dark for 7 days. The experiment was carried out with four replicates. **a** Fungal growth, measured as colony diameter. **b** Sclerotial production

Results

veA regulates morphogenesis in *A. flavus*

Evaluation of the ΔveA morphological phenotype revealed a slight increase in vegetative growth (estimated as colony growth) compared with the control strains, particularly in the dark (Fig. 3a, data not shown). Cultures exposed to light showed higher variation among control strains (approximately ± 14 mm).

In addition, all the *A. flavus* ΔveA mutants were unable to form sclerotia under conditions where the control strains abundantly produced these resistant structures (Figs. 3b and 4). Although conidiation varied among the transformants analyzed, a general decrease in aerial mycelia was observed in all the ΔveA mutants compared with the control strains (Fig. 4 and data not shown).

veA controls AF production in *A. flavus* at transcriptional level

We examined AF production in both ΔveA and control *A. flavus* strains in a time course experiment in which the mycelia were shifted to solid medium after growing in liquid culture for 14 h (Fig. 5a). Our results indicate that ΔveA was unable to produce either AFB1 or AFB2, whereas both aflatoxins were clearly detected in the *veA* control at 12, 24, and 36 h after the shift to solid medium.

The effect of *veA* deletion on AF biosynthesis was further analyzed by Northern analysis (Fig. 5b). In the control strain,

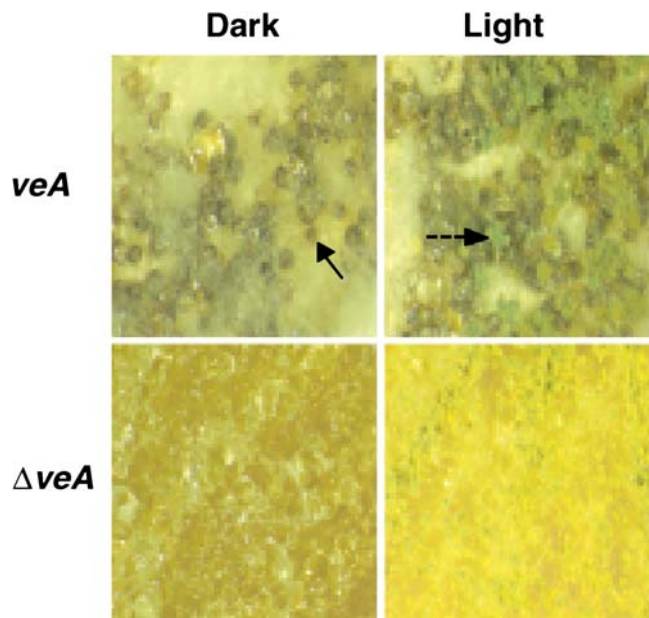
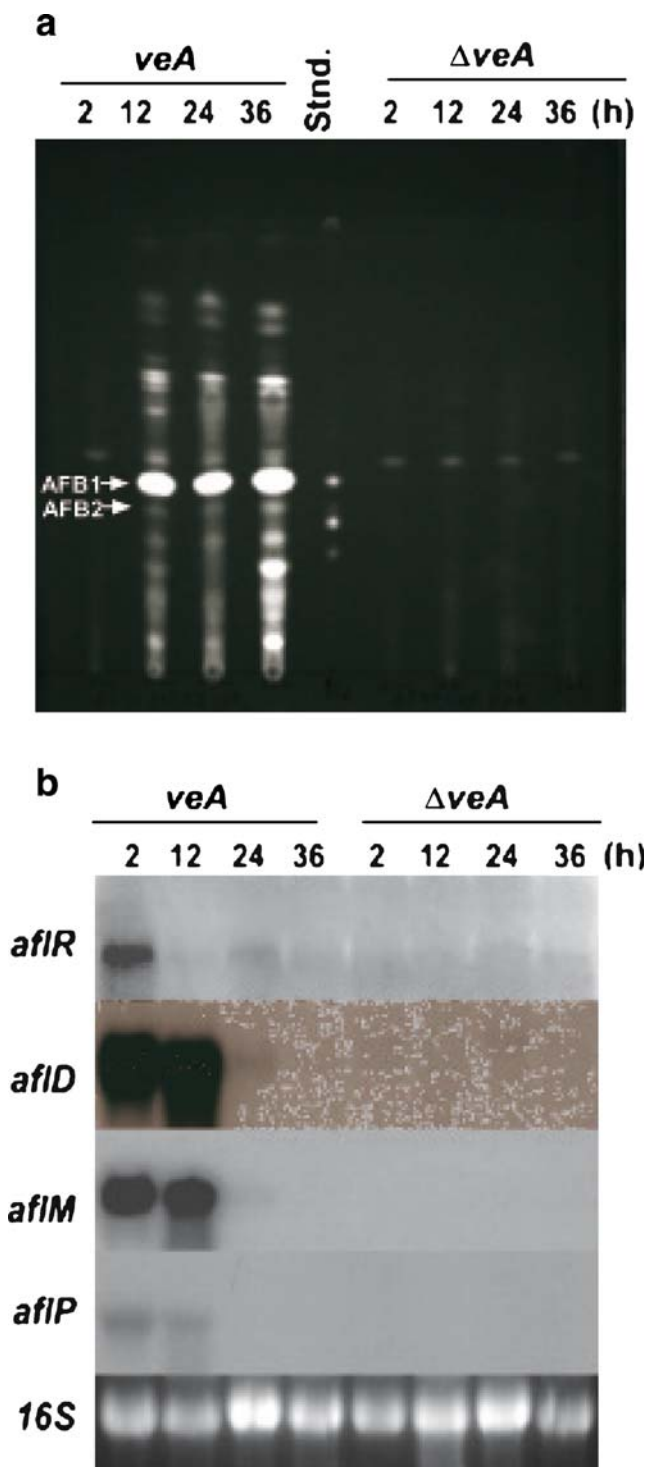


Fig. 4 Microscopic examination of *A. flavus* ΔveA and *veA* strains after a 7-day incubation on YGT. The black structures only observed in the *veA* control strain are sclerotia, indicated with a continuous arrow. Conidiophores are indicated with a discontinuous arrow



transcripts of the AF-specific regulatory transcription factor gene *afIR* (Woloshuk et al. 1995; Yabe and Nakajima 2004) were detected 2 h after the shift, and transcripts corresponding to the enzymatic genes *afID*, *afIM*, and *afIP* (Yabe and Nakajima 2004; Yu et al. 2004) were detected mainly at 2 and 12 h after the shift, indicating cluster activation. At 24 h, the accumulation of these transcripts decreased, and at 36 h, they were not detected. In the ΔveA , *afIR*, *afID*, *afIM*, and *afIP*

Fig. 5 TLC and Northern hybridization of ΔveA mutant and control strain for AF production and AF gene expression, respectively. **a** TLC analysis of AF production in the *A. flavus* ΔveA mutant and control strain. Metabolites were extracted from cultures grown on YGT agar medium in the light and harvested at 2, 12, 24, and 36 h. Abbreviation: Std., AF standard. The experiment was repeated, with similar results. Results were also confirmed by HPLC (data not shown). **b** Northern hybridization of total RNA from the ΔveA mutant and control strain using ^{32}P -labeled AF gene probes. The ethidium-bromide-stained 16S rRNA region of each gel is shown to indicate RNA loading

transcripts were not detected at any time point analyzed. These results were confirmed by quantitative reverse transcriptase (qRT)-PCR (data not shown). The experiment was repeated twice, obtaining similar results.

CPA production is controlled by *veA*

Our TLC studies (Fig. 5a) indicated that the biosynthesis of additional metabolites is also affected by the *veA* deletion. For this reason, we explored the possible role of *veA* as a global regulator controlling other mycotoxins produced by *A. flavus* besides AF. Our CPA HPLC analysis revealed that the production of this *A. flavus* mycotoxin is also *veA*-dependent (Fig. 6). The ΔveA mutant presented a reduction in CPA production of 48% in the light and 66% in dark culture conditions with respect to the control strain. An additional compound was consistently detected eluting at 3.1 min (Fig. 6b). Remarkably, this unknown compound was more abundant in the ΔveA strain than in the control strain (approximately fivefold higher). The results were consistent in all replicates.

veA is necessary for aflatrem production in *A. flavus*

Results from our HPLC analysis indicate that the *veA* deletion completely prevented the synthesis of aflatrem under conditions that allowed production of this mycotoxin in the *A. flavus* control strain (Fig. 7). The amount of aflatrem in the control strain was higher in the light than in the cultures in the dark (approximately 3.6-fold higher) in all the samples analyzed. Besides the peak corresponding to aflatrem (the elution time coinciding with pure aflatrem standard elution time), we detected other peaks corresponding to unknown compounds of lower abundance. Some of those compounds were only observed in the control strain (Fig. 7b). Other compounds were detected at higher levels in the ΔveA mutant.

veA is a positive regulator for the activation of the aflatrem gene cluster

The discovery of the aflatrem gene cluster (Zhang et al. 2004) allowed us to further study the possible role of *veA* in

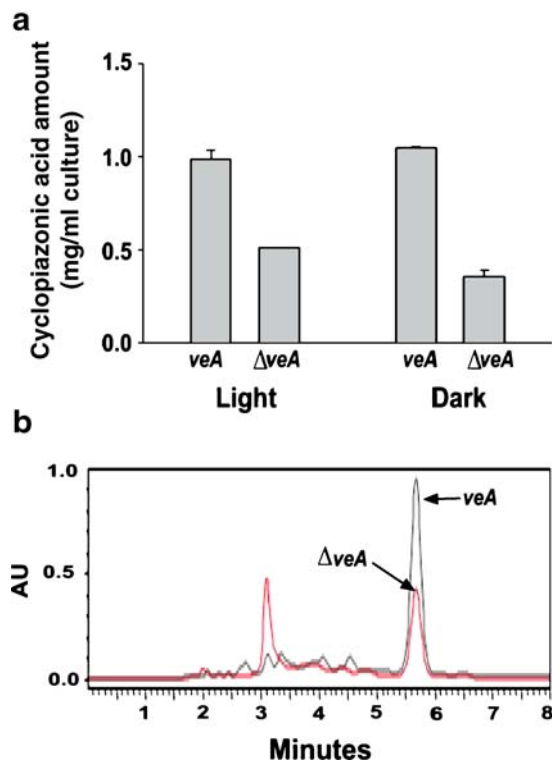


Fig. 6 Effects of the *veA* mutation on CPA production in *A. flavus*. **a** HPLC quantification of CPA in the ΔveA mutant and control samples obtained from cultures grown in YGT liquid medium in light or dark conditions for 7 days. The experiment was carried out with five replicates and repeated twice, with similar results. **b** Overlaid chromatograms showing the production of CPA in one of the samples analyzed from the *A. flavus* ΔveA mutant and control strain. Total time corresponding to the void volume at 1 ml min^{-1} flow rate was 1 min. The CPA standard elution time coincided with the peaks indicated by arrows in all the replicates

the transcriptional regulation of enzymatic genes responsible for the synthesis of this tremorgenic mycotoxin. We examined the expression of *atmC*, *atmG*, and *atmM* in a time course experiment (Fig. 8). Transcripts of these three genes were detected in the control strain but not in the ΔveA mutant. Our Northern analysis indicated that accumulation of *atmC*, *atmG*, and *atmM* transcripts in the control strain occurred earlier in the light than in the dark (96 and 120 h, respectively, after culture shift to aflatrem producing medium) (Fig. 8). The experiment was repeated twice, obtaining similar results.

Discussion

Effect of the *veA* mutation in *A. flavus* development and aflatoxin production, and comparison with previous findings

Previous studies support that mycotoxin production in *Aspergillus* is associated with morphogenesis (Calvo et al.

2002). Specifically, AF/ST production in *Aspergillus* spp. has been closely linked to asexual development (conidiation). Most studies of genetic regulation of fungal development have been performed in the model system *A. nidulans* and have focused on conidiation (Adams et al. 1998). *A. nidulans* also develops resistant structures called cleistothecia where sexual spores (ascospores) are produced. *A. flavus* and *A. parasiticus* form resistant structures called sclerotia, which are similar to cleistothecia but are unable to form spores. One role of cleistothecia and sclerotia is to survive environmental extremes (Coley-Smith and Cooke 1971; Malloch and Cain 1972; Wicklow 1987). Interestingly, evidence presented by Geiser et al. (1996) indicates that asexual *Aspergilli* are often derived from meiotic lineages and that sclerotia might be vestigial cleistothecia that lost spore-producing capacity. We hypothesize that conserved signaling pathways could control both cleistothecial and sclerotial development. Molecular studies on regulation of fungal sexual development or formation of resistant structures are still limited, and only a few genes

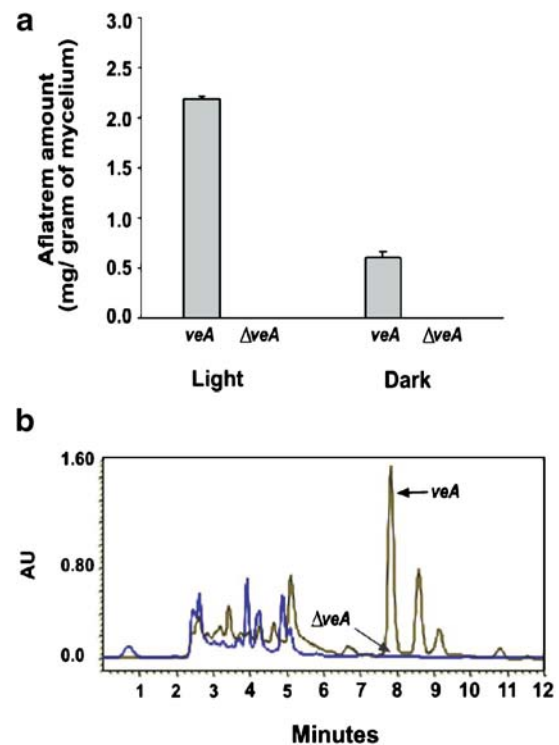


Fig. 7 Effects of the *veA* mutation on aflatrem production in *A. flavus*. **a** HPLC quantification of aflatrem in ΔveA and control cultures grown in aflatrem-inducing medium (Zhang et al. 2004). The experiment was carried out with three replicates and repeated twice, with similar results. **b** Overlaid chromatograms showing the aflatrem analysis results of one sample obtained from the *A. flavus* ΔveA mutant and control strain. Total time corresponding to the void volume at 1 ml min^{-1} flow rate was 1.7 min. The aflatrem standard elution time coincided with the peaks indicated by arrows in all the replicates

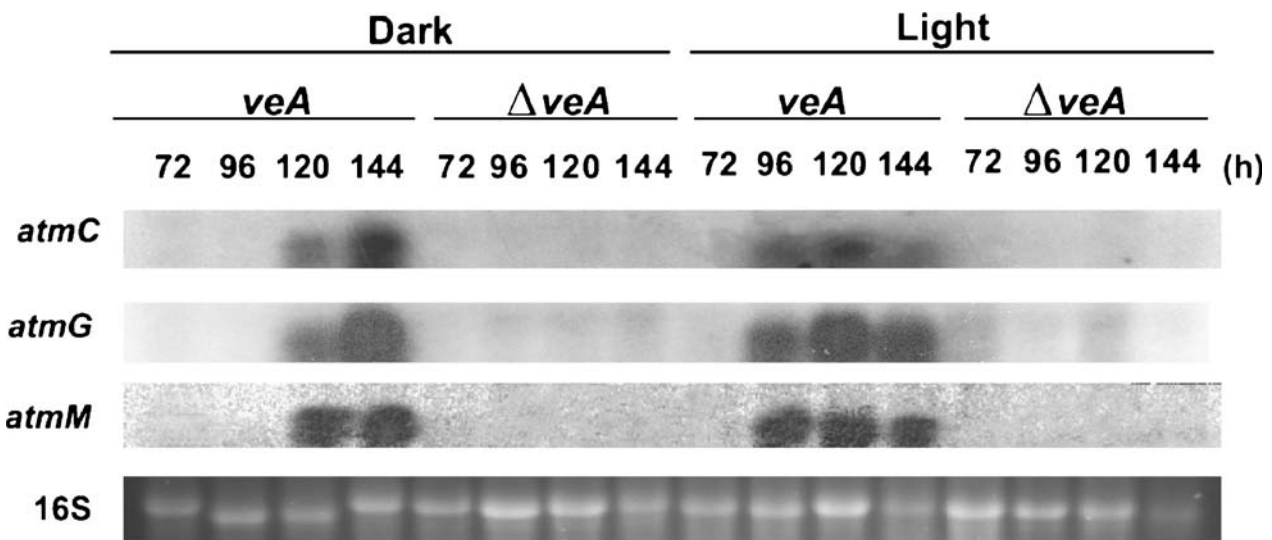


Fig. 8 Effect of *veA* mutation on the transcription of the aflatrem gene cluster. Northern blots were hybridized with *atmC*, *atmM*, and *atmG* probes. Approximately 20 μ g of total RNA from the Δ *veA* mutant and control samples was used for the RNA blot analysis. RNAs were

extracted from mycelial samples during a time course experiment (72-, 96-, 120-, and 144-h time points). The ethidium-bromide-stained 16S rRNA region of each gel is shown to indicate RNA loading

involved have been identified, mainly in *A. nidulans*. One of these genes is called velvet, or *veA*. In previous studies in *A. nidulans*, we showed that *veA* not only regulates morphogenesis but also is necessary for the production of ST (Kato et al. 2003), the penultimate precursor in the AF biosynthetic pathway. We also found a *veA* homolog in *A. parasiticus* and generated a *veA* deletion mutant unable to produce AF intermediates (Calvo et al. 2004) or sclerotia. Our TLC analysis indicated that *A. nidulans* and *A. parasiticus* mutants present a different profile compared with the wild-type controls with respect to other metabolites besides ST and AF intermediates, suggesting that the *veA* gene could have a broader effect, perhaps over additional metabolic pathways. We found further evidence analyzing penicillin (PN) production in *A. nidulans* (Kato et al. 2003), where the deletion of *veA* resulted in alteration of the PN gene expression and concomitant reduction in PN production. In the current study, we investigated the possibility that additional metabolites, specifically mycotoxins, are also regulated by *veA* in *A. flavus*, the predominant *Aspergillus* species responsible for AF contamination of crops in the USA and worldwide. In addition, we tested if the requirement for *veA* in AF production and normal morphogenesis observed in *A. parasiticus* also holds true in *A. flavus*.

The morphological analysis of the *A. flavus* Δ *veA* mutant revealed a slight increase in vegetative growth compared with the control strain, mainly in the dark (Fig. 3). The results obtained from cultures in the dark differ from those observed in the *A. parasiticus* and *A.*

nidulans Δ *veA* mutants (Calvo et al. 2004; Kato et al. 2003), where deletion of *veA* caused a reduction in colony growth, particularly in the *A. parasiticus* Δ *veA* mutant (only a slight reduction was observed in the *A. nidulans* mutant).

A reduction in conidiation was previously shown in *veA* deletion mutants in *A. parasiticus* (Calvo et al. 2004) and in *A. fumigatus* (Krappmann et al. 2005). In contrast, an increase in conidiation that was medium-dependent was observed in the *A. nidulans* *veA* mutant (Kato et al. 2003; Kim et al. 2002). In addition, differences in conidiation levels among *A. parasiticus* and *A. flavus* have commonly been observed even in natural isolates, e.g., between L and S *A. flavus* strains (unpublished observations). The differences in genetic background that determine the variation in conidiation levels in these isolates are unknown. Our results showed variation in conidiation, particularly among the control transformants. However, all the control strains presented more aerial mycelia than the *veA* mutants (Fig. 4, data not shown), suggesting that deletion of *veA* has an effect on vegetative growth in *A. flavus*.

At this time, the *veA* mode of action is unknown and the gene product does not present homology with any known protein to help in predicting its functionality. Further research on the *veA* mechanism is needed to understand how *veA* regulates fungal growth and development. Despite some differences in the role of *veA* in growth in different *Aspergillus* species, the effect of the *veA* mutation in *A. flavus* on sclerotial production was consistent among the transformants, with previous findings in *A. parasiticus*

(Calvo et al. 2004), and with the blockage of cleistothecial production in *A. nidulans* (Kato et al. 2003). The mutation in *A. flavus veA* completely prevented the formation of sclerotia in all five deletion mutants under conditions that allowed the production of these structures in all control strains tested, indicating that *veA* is a necessary gene for the production of these resistant structures (Figs. 3b and 4). This is relevant since the role of sclerotia is to survive environmental extremes, even during long periods (Coley-Smith and Cooke 1971; Malloch and Cain 1972; Wicklow 1987). When the conditions are again favorable, fungal sclerotia give rise to new mycelia and the infection process is reestablished. These results also strengthen the hypothesis that sclerotia and cleistothecia might have a common origin (Geiser et al. 1996). In addition, in *A. flavus*, wild-type sclerotia were produced in both light and dark, whereas in *A. parasiticus* and in *A. nidulans*, sclerotial/cleistothecial production takes place mainly in the dark (Calvo et al. 2004; Kato et al. 2003; Yager 1992), suggesting that the light-dependent regulation of the formation of these structures in *A. flavus*, *A. parasiticus*, and *A. nidulans* is not identical.

Besides regulating morphological differentiation, *veA* also regulates secondary metabolism in *A. flavus*. Our TLC results demonstrated that in *A. flavus*, the deletion of *veA* eliminated the production of AF, whereas AFB1 and AFB2 were present in strains with the *veA* wild-type allele (Fig. 5a). This result was confirmed in all the transformants and also by HPLC analysis (data not shown). Furthermore, the expression of *aflR* (encoding a specific transcription factor that simultaneously activates the AF gene cluster) and AF biosynthetic genes *aflD*, *aflM*, and *aflP* was not detected in the ΔveA mutant by Northern hybridization analysis (Fig. 5b). The blockage of AF biosynthesis in the *A. flavus* ΔveA mutant is consistent with previous finding on AF regulation in *A. parasiticus* (Calvo et al. 2004) and ST production in *A. nidulans* (Kato et al. 2003), suggesting that the mechanism through which *veA* regulates AF/ST production is conserved among *Aspergillus* spp. Furthermore, based on our results that the *veA* mutation causes alteration in morphogenesis as well as in AF production, it is possible that the VeA protein could be part of a signaling pathway controlling both processes. Further studies on this matter are under way.

A. flavus veA controls CPA and aflatrem biosynthesis

We also examined if *veA* is involved in regulating the production of other toxins in *A. flavus*, specifically CPA and aflatrem. CPA is a specific inhibitor of calcium-dependent ATPase and induces alterations in ion transport across cell membranes (Riley and Goeger 1992). The results of our chemical analysis showed that the ΔveA

mutation results in a decrease in CPA production in either light or dark conditions (Fig. 6). In this analysis, we also detected a metabolite that was produced in much higher levels in ΔveA compared with the control. These results indicate that *veA* not only can act as a positive regulator in the biosynthesis of a variety of metabolites in *A. flavus*, but it could also have a role as a negative regulator in the synthesis of other compounds. It is also possible that some of the precursors utilized in the metabolic routes blocked by the *veA* mutation accumulate or are available in higher abundance for other routes, thus enhancing the production of other metabolites.

Another mycotoxin produced by *A. flavus* is the potent tremorgen aflatrem (Zhang et al. 2004). Our HPLC studies revealed that deletion of *veA* results in a complete blockage of aflatrem production, indicating that *veA* is also required for the biosynthesis of this mycotoxin (Fig. 7). In addition to the peak corresponding to aflatrem, we also observed a number of other peaks in extracts of both strains that correspond to other metabolites. These metabolites are found in differing abundance in the ΔveA mutant and in the control strain. In some cases, their levels are higher in the control strain with respect to the ΔveA mutant or vice versa, supporting the hypothesis that *veA* has a differential effect on the production on a variety of *A. flavus* metabolites.

veA regulates aflatrem production at the transcriptional level

The enzymatic genes necessary for aflatrem production have been previously described (Zhang et al. 2004). The aflatrem genes *atmC*, *atmG*, and *atmM* are clustered. Until this study, no regulatory gene(s) controlling the aflatrem cluster activation had been described. Our time course Northern analysis revealed that *veA* is necessary for the activation of the aflatrem biosynthetic genes *atmC*, *atmG*, and *atmM* (Fig. 8). We also noticed that illumination had an effect, inducing earlier expression of the aflatrem genes (Fig. 8) and, consequently, higher aflatrem production in light cultures (Fig. 7). Further research is needed to elucidate the influence of additional environmental factors on aflatrem gene expression and subsequent production of this potent tremorgenic compound.

This study represents the first report of a gene that regulates the biosynthesis of CPA and aflatrem mycotoxins. The fact that *veA* regulates CPA and aflatrem biosynthesis in addition to AF suggests the involvement of *veA* in a global regulatory mechanism controlling secondary metabolism in *A. flavus*. Our previous findings of *veA*-mediated regulation of both ST and penicillin production in *A. nidulans* further support this hypothesis (Kato et al. 2003). Another global regulator gene, *laeA*, has been described to control the expression of several gene clusters

involved in secondary metabolism in *Aspergillus* spp. (Bok and Keller 2004; Bok et al. 2006; Keller et al. 2005). *LaEA* is postulated to be involved in the conversion of heterochromatin to euchromatin (Bok and Keller 2004; Keller et al. 2005). We are currently studying the possible connection between *veA* and *laeA*. The fact that *veA* controls the production of AF, CPA, and aflatrem adds to the importance of *veA* as a potential target to control *Aspergillus* mycotoxin contamination. In addition, inhibiting the activity of *veA* could also affect *A. flavus* survivability since *veA* is required for sclerotial formation, thus making *veA* a desirable candidate to target in control strategies.

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References

- Adams TH, Wieser JK, Yu JH (1998) Asexual sporulation in *Aspergillus nidulans*. Microbiol Mol Biol Rev 62:35–54
- Blout WP (1961) Turkey disease. Turkeys 9:52, 55–58, 61, 77
- Bok JW, Keller NP (2004) *LaEA*, a regulator of secondary metabolism I *Aspergillus*. Eukaryot Cell 3:527–535
- Bok JW, Hossmeister D, Maggio-Hall LA, Murillo R, Glasner JD, Keller NP (2006) Genomic mining for *Aspergillus* natural products. Chem Biol 1:31–37
- Calvo AM, Wilson RA, Bok JW, Keller NP (2002) Relationship between secondary metabolism and fungal development. Microbiol Mol Biol Rev 66:447–459
- Calvo AM, Bok J, Brooks W, Keller NP (2004) *veA* is required for toxin and sclerotial production in *Aspergillus parasiticus*. Appl Environ Microbiol 70:4733–4739
- Cary JW, Ehrlich KC, Bland JM, Montalbano BG (2005) The aflatoxin biosynthesis cluster gene, *aflX*, encodes an oxidoreductase involved in conversion of vesicolorin A to demethylsterigmatocystin. Appl Environ Microbiol 72:1096–1101
- Cleveland TE, Bhatnagar D, Foell CJ, McCormick SP (1987) Conversion of a new metabolite to aflatoxin B₂ by *Aspergillus parasiticus*. Appl Environ Microbiol 53:2804–2807
- Cole RJ, Hill RA, Blankenship PD, Sanders TH (1986) Color mutants of *Aspergillus flavus* and *Aspergillus parasiticus* in a study of preharvest invasion of peanuts. Appl Environ Microbiol 52:1128–1131
- Coley-Smith JR, Cooke RC (1971) Survival and germination of fungal sclerotia. Annu Rev Phytopathol 9:65–92
- Denissenko MF, Cahill J, Koudriakova TB, Gerber N, Pfeifer GP (1999) Quantitation and mapping of aflatoxin i DNA damage in genomic DNA using aflatoxin B₁-8,9-epoxide and microsomal activation systems. Mutat Res 425:205–211
- Domer JW, Cole RJ, Lomax LG, Gosser HS, Diener UL (1983) Cyclopiazonic acid production by *Aspergillus flavus* and its effects on broiler chickens. Appl Environ Microbiol 46:698–703
- Dvorackova I, Kusak V (1990) Hepatocellular carcinoma (a 28-year necropsy review). J Environ Pathol Toxicol Oncol 10:220–224
- Geiser DM, Timberlake WE, Arnold ML (1996) Loss of meiosis in *Aspergillus*. Mol Biol Evol 13:809–817
- Goldblatt L (1969) Aflatoxin: scientific background, control, and implications. Academic, New York, NY
- Horn BW, Domer JW (1999) Regional differences in production of aflatoxin B₁ and cyclopiazonic acid by soil isolates of *Aspergillus flavus* along a transect within the United States. Appl Environ Microbiol 4:1444–1449
- Horng JS, Chang PK, Pestka JJ, Linz JE (1990) Development of a homologous transformation system for *Aspergillus parasiticus* with the gene encoding nitrate reductase. Mol Gen Genet 224:294–296
- Kato N, Brooks W, Calvo AM (2003) The expression of sterigmatocystin and penicillin genes in *Aspergillus nidulans* is controlled by *veA*, a gene required for sexual development. Eukaryot Cell 2:1178–1186
- Keller NP, Turner G, Bennet JW (2005) Fungal secondary metabolism—from biochemistry to genomics. Nat Rev Microbiol 12:937–947
- Kim H, Han K, Kim K, Han D, Jahng K, Chae K (2002) The *veA* gene activates sexual development in *Aspergillus nidulans*. Fungal Genet Biol 37:72–80
- Krappmann S, Bayram O, Braus GH (2005) Deletion and allelic exchange of *Aspergillus fumigatus veA* locus via a novel recyclable marker module. Eukaryot cell 4:1298–1307
- Lansden JA, Davidson JI (1983) Occurrence of cyclopiazonic acid in peanuts. Appl Environ Microbiol 45:766–769
- Lasky T, Magder L (1997) Hepatocellular carcinoma *p53* G>T transversions at codon 249: the fingerprint of aflatoxin exposure? Environ Health Perspect 105:392–397
- Malloch D, Cain RF (1972) The Trichocomataceae: ascomycetes with *Aspergillus*, *Paecilomyces* and *Penicillium* imperfect states. Can J Bot 50:2613–2628
- McMillan LK, Carr RL, Young CA, Astin JW, Lowe RGT, Parker EJ, Jameson GB, Finch SC, Miles CO, McManus OB, Chmalhofer WA, Garcia L, Kaczorowski GJ, Goetz MA, Tkacz JS, Scott B (2003) Molecular analysis of two cytochrome P450 monooxygenase genes required for paxilline biosynthesis in *Penicillium paxilli* and effects of paxilline intermediates on mammalian maxi-K ion channels. Mol Genet Genomics 270:9–23
- Norred WP, Porter JK, Doner JW, Cole RJ (1988) Occurrence of the mycotoxin cyclopiazonic acid in meat after oral administration to chickens. J Agric Food Chem 36:113–116
- Payne GA, Brown MP (1998) Genetics and physiology of aflatoxin biosynthesis. Annu Rev Phytopathol 36:329–362
- Pitt JI, Cruickshank RH, Leistner L (1986) *Penicillium commune*, *P. camembertii*, the origin of white cheese mould, and the production of cyclopiazonic acid. Food Microbiol 3:363–371
- Porter JK, Norred WP, Cole RJ, Domer JW (1988) Neurochemical effects of cyclopiazonic acid in chickens. Proc Soc Exp Biol Med 187:335–340
- Purchase IFH (1971) The acute toxicity of the mycotoxin cyclopiazonic acid to rats. Toxicol Appl Pharmacol 18:114–123
- Richard JL, Payne GA (2003) Mycotoxins: risks in plant, animal and human health. CAST Report 139. Council for Agricultural Science and Technology, p 199
- Riley RT, Goeger DE (1992) Cyclopiazonic acid: speculations on its function in fungi. In: Bhatnagar D, Lillehoj EB, Arora DK (eds) Handbook of applied mycology. Mycotoxins in ecological systems. Marcel Dekker, New York, NY, pp 5:385–402
- Riley J, Mandel HG, Sinha S, Judah DJ, Neal GE (1997) In vitro activation of the human Harvey-ras proto-oncogene by aflatoxin B₁. Carcinogenesis 18:905–910
- Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

- Shen HM, Ong CN (1996) Mutations of the p53 tumor suppressor gene and *ras* oncogenes in aflatoxin hepatocarcinogenesis. *Mutat Res* 366:23–44
- Sobolev VS, Horn BW, Dorner JW, Cole RJ (1998) Liquid chromatographic determination of major secondary metabolites produced by *Aspergillus* species from section Flavi. *J AOAC Int* 81:57–60
- Sweeney MJ, Dobson AD (1999) Molecular biology of mycotoxin biosynthesis. *FEMS Microbiol Lett* 175:149–163
- Trail F, Mahanti N, Linz JE (1995) Molecular biology of aflatoxin biosynthesis. *Microbiology* 141:755–765
- Urano T, Trucksess MW, Beaver RW, Wilson DM, Dorner JW, Dowell FE (1992) Co-occurrence of cyclopiazonic acid and aflatoxins in corn and peanuts. *J AOAC Int* 75:838–841
- Valdes JJ, Cameron JE, Cole RJ (1985) Aflatrem: a tremorgenic mycotoxin with acute neurotoxic effects. *Environ Health Perspect* 62:459–463
- Wang JS, Groopman JD (1999) DNA damage by mycotoxins. *Mutat Res* 424:167–181
- Wicklow DT (1987) Survival of *Aspergillus flavus* sclerotia in soil. *Trans Br Mycol Soc* 89:131–134
- Wogan GN, Househam KC, Hundt HK (1992) Aflatoxins as risk factors for hepatocellular carcinoma in humans. Aflatoxin exposure and its relationship to kwashiorkor in African children. *Cancer Res* 52:2114–2118
- Woloshuk CP, Yousibova GL, Rollins JA, Bhatnagar D, Payne GA (1995) Molecular characterization of the *afl-1* locus in *Aspergillus flavus*. *Appl Environ Microbiol* 61:3019–3023
- Wu F (2004) Mycotoxin risk assessment for the purpose of setting international regulatory standards. *Environ Sci Technol* 38:4049–4055
- Yabe K, Nakajima H (2004) Enzyme reactions and genes in aflatoxin biosynthesis. *Appl Microbiol Biotechnol* 64:745–755
- Yager LN (1992) Early developmental events during sexual and asexual sporulation in *Aspergillus nidulans*. *Biotechnology* 23:19–41
- Yao I, Peter AB, Baur R, Sigel E (1988) The tremorgen aflatrem is a positive allosteric modulator of γ -aminobutyric acid A receptor channel expressed in *Xenopus* oocytes. *Mol Pharmacol* 35:319–323
- Yu J, Chang PK, Ehrlich KC, Cary JW, Bhatnagar D, Cleveland TE, Payne GA, Linz JE, Woloshuk CP, Bennett JW (2004) Clustered pathways genes in aflatoxin biosynthesis. *Appl Environ Microbiol* 70:1253–1262
- Zhang S, Monahan JB, Tkacz JS, Berry S (2004) Indole diterpene gene cluster from *Aspergillus flavus*. *Appl Environ Microbiol* 70:6875–6883