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Aspergillus flavus VelB acts distinctly from VeA in conidiation and may coordinate with FluG to modulate sclerotial production

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ABSTRACT

The proteins VeA, VelB and LaeA of *Aspergillus nidulans* form a heterotrimeric complex (the velvet complex) in the dark to coordinate sexual development and production of some secondary metabolites. VeA and VelB of *A. nidulans* and *Aspergillus fumigatus* also are repressors of conidiation, but VeA of *Aspergillus flavus* in studied strains acts positively on conidiation. In the present study, we show via yeast-two hybrid assays that interactions among *A. flavus* VeA, VelB, and LaeA are conserved as in the *A. nidulans* velvet complex. We found that FluG, which is required for conidiophore formation in *A. nidulans* but whose deletion in *A. flavus* delays onset of conidiation, was probably an interacting partner of VelB. Deletion of *velB* in *A. flavus* CA14 severely impaired conidiation in the dark although to a lesser extent than deletion of *veA*. In both mutants *fluG* deletion resulted in further decreased conidiation even in the light. Deletion of *fluG* in the Δ*laeA* strain, however, did not affect conidiation. All mutant types were unable to produce aflatoxin and sclerotia. Cross-complementation of the Δ*velB* strain with *gpdA::veA* restored conidiation but not aflatoxin production although *aflR*, the aflatoxin pathway regulatory gene, was expressed at a normal level. Cross-complementation of the Δ*veA* strain with *gpdA::velB* failed to restore conidiation and aflatoxin production. The Δ*velB* strain complemented with or a wild type transformed by *gpdA::velB* had elevated sclerotial production as the *ΔfluG* strain. Concerted interactions of *A. flavus* VeA and VelB with LaeA are critical for conidiation and aflatoxin biosynthesis. VelB may have a dual role and likely coordinates with FluG to modulate sclerotial production.

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1. Introduction

The homothallic fungus *Aspergillus nidulans* undergoes asexual development under light and develops sexually in the dark. The *veA* (*velvet*) gene encodes a light-dependent regulator that modulates these processes (Calvo, 2008). Wild-type *A. nidulans* displays light-dependent conidiation and strains bearing a mutation (*veA1*) conidiate vigorously regardless of illumination conditions (Kafer, 1965; Mooney et al., 1990). The *veA* gene can complement *veA1* mutation (Kim et al., 2002), thus VeA is considered a negative regulator of asexual conidiation. Because deletion of *veA* in *A. nidulans* results in a loss of production of cleistothecia and overexpression induces the formation of sexual Hülle cells and cleistothecia (Kim et al., 2002), VeA thus also is a positive regulator of sexual development. Other members of the *velvet* family have been identified including VelB (*velvet*-like protein B), VelC and a related protein called VosA, which is critical for spore viability (Ni and Yu, 2007). In the nucleus VeA interacts with VelB and LaeA, the global regulator required for production of many secondary metabolites (Bayram et al., 2008a; Bok and Keller, 2004; Butchko et al., 2012). The *A. nidulans* VelB/VeA/LaeA complex coordinates sexual development and secondary metabolite production in response to growth in the absence of light (Bayram et al., 2008a). VelB also forms a complex with VosA to repress asexual conidiation in the dark (Sarikaya Bayram et al., 2010).

Aspergillus flavus is a heterothallic fungus. A majority of field isolates (L-strain) reproduce and disseminate largely through the production of conidia. Other isolates (S-strain) produce copious sclerotia but lower amounts of conidia (Cotty, 1989). Asexual sclerotia, aggregates of melanized hyphae that serve as a survival structure, are considered to be a vestige of the sexual cleistothecia produced by other teleomorphic aspergilli (Geiser et al., 1996). Sexual reproduction has been demonstrated in *A. flavus* under laboratory conditions with strains having different mating-type genes (Horn et al., 2009). Sexual reproduction in fields, however, has not been conclusively demonstrated. For the L-strain isolates, conidiation occurs regardless of the presence or absence of light. The varied responses to light in conidiation of *A. flavus* and *A. nidulans* imply that the underlying mechanisms coordinate light signaling and development are different.

Functions of *A. flavus* VeA have been examined in strains of NRRL3357 and 70S, which are L-strain and S-strain, respectively (Amaike and Keller, 2009; Duran et al., 2007). Deletion of *veA* in

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these strains decreases conidiation and abolishes production of sclerotia and aflatoxin. Overexpression of *veA* in NRRL3357, however, also decreases conidiation, and the reason is unclear. LaeA is a global regulator of production of many secondary metabolites including aflatoxin. Deletion of *laeA* in *A. flavus* also abolishes sclerotial production (Amaike and Keller, 2009; Kale et al., 2008). Investigation of genetic interaction between *veA* and *laeA* shows that multiple copies of *laeA* in NRRL3357 greatly elevate sclerotial production, but this outcome is not affected by the *veA* copy number in the fungus (Amaike and Keller, 2009). Although published work points to the importance of a balanced partnership between VeA and LaeA, the mechanism(s) associated with the control of sclerotial production by LaeA is not well defined. *A. flavus* Δ*laeA* strains tend to have decreased conidiation on different growth media (Chang et al., 2012a; Kale et al., 2008), but inoculum amounts can change conidiation levels greatly when compared to that of the wild type. Overexpression of *laeA* in *A. flavus* NRRL3357 has been shown to affect conidial production differently (Amaike and Keller, 2009; Kale et al., 2008). The regulation of conidiation by LaeA thus appears to be indirect and is influenced by biotic and abiotic factors.

Besides VeA and LaeA, *A. flavus* FluG also is involved in the production of conidia and sclerotia. Defects in *A. nidulans fluG* result in proliferation of undifferentiated vegetative hyphae that yield fluffy cotton-like colonies (Adams et al., 1992; Wieser et al., 1994). These *A. nidulans fluG* mutants also are unable to produce the secondary metabolite, sterigmatocystin, which is the penultimate precursor of aflatoxins (Hicks et al., 1997). FluG, which consists of an N-terminal amidohydrolase domain and a C-terminal glutamine synthetase (GS) domain, was postulated to synthesize a diffusible factor that initiates conidiation (Lee and Adams, 1994). Mutations in the *fluG* mutants are primarily located in the GS domain (Lee and Adams, 1994). The *A. nidulans* strain with only the GS-containing C-terminal half conidiates normally, which indicates the N-terminal portion of FluG is dispenable (D'Souza et al., 2001). A recent study (Rodriguez-Urra et al., 2012) has concluded that dehydroaustinol is the diffusible effector that promotes conidiation, but it is missing in the *A. nidulans fluG* mutant. Two separate gene clusters in *A. nidulans* have been found to encode the complete dehydroaustinol pathway (Lo et al., 2012). Therefore, FluG in *A. nidulans* does not synthesize dehydroaustinol; its involvement in dehydroaustinol production remains unclear. For *A. flavus*, deletion of *fluG* delays and reduces conidiation, elevates sclerotial production, but does not affect aflatoxin production (Chang et al., 2012b).

Previous studies (Amaike and Keller, 2009; Lin et al., 2013) assumed that interactions among velvet components of *A. flavus* are identical to those of the *A. nidulans* velvet complex although experimental evidence were unavailable. The roles of *A. flavus velB* and *velC* in controlling development and secondary metabolism have not been investigated. In this study, we examined the effects of individual velvet gene defect and assessed the genetic relationship among the velvet complex genes and *fluG* on conidiation, sclerotial production, and aflatoxin biosynthesis in the L-strain *A. flavus*, CA14. Our results show that while interactions among the *A. flavus* velvet components are the same as those in *A. nidulans*, FluG also interacts with VelB and LaeA. The results suggest that concerted and balancing interactions among the velvet family proteins, VeA, VelB, and LaeA, together with FluG, are essential for maintaining programmed conidiation, sclerotial production, and aflatoxin biosynthesis.

2. Materials and methods

2.1. Fungal strains and culturing conditions

A. flavus PTsΔku70ΔpyrG, uracil auxotrophic, pyrithiamine-sensitive and derived from aflatoxigenic CA14 (Chang et al., 2010), was used for gene disruption. Single-gene deletion strains of Δ*veA*, Δ*velB* or Δ*velC* were generated in this study. The Δ*laeA* and Δ*fluG* strains were from previous studies (Chang et al., 2012a, 2012b). Double deletion strains of Δ*veA*/Δ*velB*, Δ*veA*/Δ*fluG*, Δ*velB*/Δ*fluG*, and Δ*laeA*/Δ*fluG* as well as a Δ*veA* strain complemented with *gpdA::veA* (self-complementation) or *gpdA::velB* (cross-complementation), a Δ*velB* strain complemented with *gpdA::veA* or *gpdA::velB* were generated. Strains were maintained on potato dextrose agar (PDA, EMD, Darmstadt, Germany) plates and supplemented with 0.5 mg/ml uracil when necessary. For the examination of culture morphology, conidia in 0.01% Triton X-100 were point-inoculated at the center of PDA plates and incubated at 30 °C in the dark and under fluorescent white light as indicated. In addition to PDA, Wickerham medium (Raper and Thom, 1968) was used for sclerotial production.

2.2. Characterization of velvet family genes, veA, velB, velC and vosA, of A. flavus CA14

BlastP search of the *Aspergillus* Comparative Database at Broad Institute (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html) using *A. nidulans* velvet family protein sequences of VeA, VelB, VelC and VosA was performed to identify orthologous genes in *A. flavus* NRRL3357 genome. The genuine coding regions and introns of *veA*, *velB*, *velC*, and *vosA* of *A. flavus* CA14 were confirmed by comparison of genomic and cDNA sequences determined in this study. Blast searches of the conserved sequence database, Conserved Domains (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), was used to locate the velvet superfamily domain. Amino acid sequence alignment was performed with DNAMAN version 5 (Lynnon Corporation, Vandreuil, Quebec, Canada).

2.3. Construction of single gene deletion and complementation vectors

Gene deletion procedures via double crossover recombination in *A. flavus* strains deficient in the nonhomologous end-joining (NHEJ) pathway have been described in detail (Chang et al., 2010). Single-gene deletion vectors were constructed with the *A. parasiticus pyrG* gene (GenBank accession number; EU817656) as the selection marker. The two flanking fragments for targeting *veA*, *velB*, or *velC* were amplified by PCR using the primers listed in Table S1. The fragments were cloned sequentially into unique restriction sites in the vector containing a reusable *pyrG* marker by standard recombinant DNA techniques. For self- and cross-complementation of Δ*veA* and Δ*velB* strains, full-length genomic fragments of the *veA* and *velB* genes were amplified using AccuPrime™ *Pfx* Taq polymerase (Invitrogen, Carlsbad, California, USA). The amplified genes including the 3′ non-translated region were placed under control of the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase gene (*gpdA*) promoter in pTR1-GPD, which contains *A. oryzae* pyrithiamine resistance gene (*ptrA*) (Kubodera et al., 2000) as the selection marker. The nucleotide sequences of *veA* and *velB* used in complementation were verified by sequencing (Iowa State University DNA Sequencing Facilities).

2.4. Generation of the double deletion mutants of ΔveA/ΔvelB, ΔveA/ΔfluG, ΔvelB/ΔfluG, and ΔlaeA/ΔfluG

The *A. oryzae ptrA*-based *velB* deletion vector was used to delete the *velB* gene in a confirmed Δ*veA* strain to generate the Δ*veA*/Δ*velB* strain. For disruption of *fluG* in the Δ*veA*, the Δ*velB* and the Δ*laeA* strains to generate double deletion strains, the uracil auxotrophy was regenerated for respective single deletion strains by spreading spores (10^6) on PDA plates that were supplemented with 2 mg uracil and 2 mg 5-fluoroorotic acid per ml to force out