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Functional characterization of a *veA*-dependent polyketide synthase gene in *Aspergillus flavus* necessary for the synthesis of asparasone, a sclerotium-specific pigment

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ABSTRACT

The filamentous fungus, *Aspergillus flavus*, produces the toxic and carcinogenic, polyketide synthase (PKS)-derived family of secondary metabolites termed aflatoxins. While analysis of the *A. flavus* genome has identified many other PKSs capable of producing secondary metabolites, to date, only a few other metabolites have been identified. In the process of studying how the developmental regulator, VeA, affects *A. flavus* secondary metabolism we discovered that mutation of *veA* caused a dramatic down-regulation of transcription of a polyketide synthase gene belonging to cluster 27 and the loss of the ability of the fungi to produce sclerotia. Inactivation of the cluster 27 *pks* (*pks27*) resulted in formation of greyish-yellow sclerotia rather than the dark brown sclerotia normally produced by *A. flavus* while conidial pigmentation was unaffected. One metabolite produced by Pks27 was identified by thin layer chromatography and mass spectral analysis as the known anthraquinone, asparasone A. Sclerotia produced by *pks27* mutants were significantly less resistant to insect predation than were the sclerotia produced by the wild-type and more susceptible to the deleterious effects of ultraviolet light and heat. Normal sclerotia were previously thought to be resistant to damage because of a process of melanization similar to that known for pigmentation of conidia. Our results show that the dark brown pigments in sclerotia derive from anthraquinones produced by Pks27 rather than from the typical tetrahydronaphthalene melanin production pathway. To our knowledge this is the first report on the genes involved in the biosynthesis of pigments important for sclerotial survival.

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1. Introduction

Aspergilli produce a diverse array of bioactive secondary metabolites, some of which are beneficial to man, such as penicillin and lovastatin, while others are quite harmful, for example, the aflatoxins, gliotoxin and ochratoxin (Boettger and Hertweck, 2013; Sanchez et al., 2012; Schneider et al., 2008). Among the aspergilli, *Aspergillus flavus* is a frequent contaminant of agricultural commodities such as corn, peanut, tree nuts and cottonseed. Ingestion of foods contaminated with aflatoxins has been implicated in acute toxicoses while chronic, low-level exposure can lead to immune suppression and liver cancer (Probst et al., 2010; Turner et al., 2003). In addition to the health risks associated with aflatoxins there are also significant adverse economic impacts to producers due to market rejections of contaminated crops and livestock losses as well as costs associated with monitoring for aflatoxin contamination (Wu, 2006). Besides aflatoxins, *A. flavus* produces a number of other secondary metabolites including the toxic metabolites cyclopiazonic acid and aflatrem (Tokuoka et al., 2008; Zhang et al., 2004).

Analysis of the *A. flavus* genome has allowed the identification of many other putative secondary metabolic gene clusters predicted to encode metabolites derived from polyketide synthases (PKSs), non-ribosomal peptide synthetases (NRPSs), hybrid PKS-NRPS and prenyltransferases (PTRs). Such an analysis has predicted the presence of 55 gene clusters associated with secondary metabolism in *A. flavus* (Georgianna et al., 2010; Khaldi et al., 2010). To date, metabolites have only been associated with six of these clusters (Forseth et al., 2012).

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The developmental regulator VeA has been shown to not only affect sexual and sclerotial development but also the production of secondary metabolites (Calvo, 2008; Calvo et al., 2004; Chettri et al., 2012; Dhingra et al., 2012; Duran et al., 2007; Kato et al., 2003; Merhej et al., 2012). VeA, LaeA, VelB and VosA form a nuclear complex that regulates development and secondary metabolism in response to light (Bayram et al., 2010, 2008). By whole genome microarray transcript profiling we confirmed that expression of a number of genes associated with putative secondary metabolic gene clusters in *A. flavus* are affected by *veA* mutation (Cary et al., in preparation). In the course of these studies we found that among the genes significantly downregulated in a Δ*veA* mutant was a *pks* in cluster 27 [cluster numbering as designated by Georgianna et al. (2010)]. Here we present evidence that the cluster 27 *pks* gene (*pks27*) is involved in the biosynthesis of sclerotium-specific pigments that are important for thwarting damage to sclerotia by insect fungivory or abiotic stress.

2. Materials and methods

2.1. Strains and growth conditions

An *Aspergillus flavus* 70 *niaD*$^{-}$, *pyrG*$^{-}$ parental strain (referred to as Af70) was used as host for transformation. Three Δ*pks27* (Af70 *niaD*$^{-}$, *pyrG*$^{+}$, Δ*pks27,* greyish-yellow sclerotia) mutants were obtained following transformation of the parental strain with the *pks27* deletion vector PKS27-pyrG and initial characterization of these strains showed that all had a consistent phenotype. An Af70 pyrG-1 (*niaD*$^{-}$, *pyrG*$^{+}$, dark brown/black sclerotia) strain generated by transformation with the pPG2.8 vector expressing the *A. parasiticus pyrG* gene (Chang et al., 2010) was used as an isogenic transformation control (referred to as control strain). Af70 Δ*veA* and pSL82 are described in Duran et al. (2007). Cultures were point inoculated onto 2× V8 agar (per liter: 50 ml V8 juice, pH 5.2) supplemented when required with 3 mg/ml $(NH_4)_2SO_4$ and/or 1 mg/ml uracil and incubated at 30 °C in the light, a condition that promotes conidiation in Af70. Conidia were collected from plates by addition of 10 ml 0.01% Triton X-100 and gentle scraping of the colony surface with a cell scraper (BD Biosciences, San Jose, CA). Conidia were stored at 4 °C. Sclerotia were isolated from point inoculated colonies following 5 or 10 d growth at 30 °C in the dark on GMM agar supplemented with 2% sorbitol (GMMS; per liter: glucose, 10 g; ammonium tartrate, 2.0 g; sorbitol, 2.0 g; Cove's salts, 20 ml; pH 6.5). Sclerotia were collected by adding 10 ml sterile, deionized water to the agar surface and gently scraping colonies with a cell scraper. Sclerotia were washed 5 times by addition of 40 ml water to remove residual conidia and mycelia, resuspended in a final volume of 25 ml water and stored at 4 °C.

2.2. Vector construction and fungal transformation

A PCR-based method was used to construct the *A. flavus* 70 pPKS27-pyrG knockout plasmid in which a 770 bp region within the beta-ketoacyl synthase, N-terminal domain of the *pks27* coding region is replaced by the *A. parasiticus pyrG* selectable marker gene (Fig. S1A, Supplementary data). Briefly, 5′ and 3′ regions the *pks27* gene were amplified using oligonucleotide primers. The location of the primers (contig 1; in parentheses following the primer sequence) within *pks27* is based on the Broad Institute Aspergillus Genomic Database (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html; AFL2G_00935.2) nucleotide sequence data for the *A. flavus* 3357 genome. Primers used are as follows: 5′ *pks27 Eco*RI, 5′-GAATTCATCCGATTTACCACATTTGAGG-3′ (nt 2646419) and 5′ *pks27 Bam*HI, 5′-GGATCCACATGAGTCTTAACATCCCAG-3′ (nt 2647637). The 3′ portion of *pks27* was amplified using the following primers: 3′ *pks27 Sal*I, 5′-GTCGACTTGAGGATGCTAAGGCCGAC-3′ (nt 2648417) and 3′ *pks27 Hind*III, 5′-AAGCTTGATCCCTGACCGGTAAAGGCGAATGC-3′ (nt 2649459). Following PCR amplification of *A. flavus* genomic DNA with ExTaq HS polymerase (Takara Bio, Inc., Shiga, Japan), PCR products of the expected size of 1218 bp for the 5′ *pks27* amplification and 1042 bp for the 3′ *pks27* amplification were obtained. PCR products were subcloned into TOPO pCR2.1 (Life Technologies, Grand Island, NY) and verified by DNA sequencing. The 5′ and 3′ *pks27* PCR products were released from their TOPO vectors using *Eco*RI-*Bam*HI and *Sal*I-*Hind*III digestion respectively and subcloned in a stepwise manner into the analogous restriction digested pPG2.8 vector harboring the *A. parasiticus pyrG* selectable marker gene. The resulting knockout vector was designated pPKS27-pyrG (Fig. S1A, Supplementary data). The cluster 27 transcription factor (*znf27*; Broad locus AFL2G_00934.2- note that *znf27* found in the NCBI database as AFLA_082140 is not annotated properly) and scytalone dehydratase (*scd1*; Broad locus AFL2G_03259.2) gene knockout vectors, designated pZnf27-pyrG and pSCD1-pyrG respectively (Figs. S2A and S3A, Supplementary data) were generated essentially as described above using primers listed in Supplementary Table S1. Transformation was performed as described (Cary et al., 2006) using *A. flavus* 70 (Af70) as the host. Conidia were inoculated in potato dextrose broth supplemented with 1 mg/ml uracil (PDB-U) and transformants were regenerated on Czapek Solution Agar (CZ; Difco, BD, Sparks, MD) supplemented with 10 mM ammonium sulfate (CZ-AS). For genetic complementation experiments protoplasts of *A. flavus* Δ*pks27* #17 were co-transformed with plasmids pPTRI-PKS27-trpC (pyrithiamine selection) and pSL82 [harbors *A. parasiticus niaD* selectable marker; (Chang et al., 1996)] as Af70 is not sensitive to pyrithiamine. Plasmid pPTRI-PKS27-trpC was constructed by amplifying an approximate 7.5 kb region of Af70 genomic DNA representing the *pks27* coding region and 1.0 kb of upstream sequence (Fig. S4A, Supplementary data). PCR amplification was performed with PrimeSTAR® Max DNA polymerase according to the manufacturer's instructions (Takara Bio, Inc.) using primers 5′ *pks27 Hind*III and 3′ *pks27 Rsr*II (Supplementary Table S1). The PCR product was purified from a 1% agarose gel (QIAEX® II gel extraction kit, Qiagen, Valencia, CA), ligated to *Hind*III-*Rsr*II digested and gel purified pPTRI-gpd::trpC plasmid vector using the In-Fusion® HD cloning kit (Clontech, Mountain View, CA). This resulted in replacement of the *A. nidulans* glyceraldehyde-3-phosphate (*gpd*) promoter of the pPTRI-gpd::trpC vector with the 7.5 kb *pks27* gene region. Protoplasts were regenerated on CZ agar and putative genetically complemented Δ*pks27* #17 transformants were selected based on the ability to produce black sclerotia during growth on GMMS agar. Positive transformants were single-spore isolated prior to analysis for the presence of an intact copy of the *pks27* gene by PCR using primers *pks27* int1 and int2 (Supplementary Table S1). Plasmid vectors for overexpression of *veA* (OE*veA*; pUC-gpd-veA-trpC-pyrG) and *znf27* (OE*znf27*; pUC-gpd-znf27-trpC-pyrG) were generated by placing the genes under control of the *A. nidulans* glyceraldehyde-3-phosphate (*gpd*) promoter and *trpC* transcriptional terminator in pUC19 harboring the *A. parasiticus pyrG* selectable marker gene (Figs. S5A and S6A, Supplementary data).

2.3. Nucleic acid isolation and analysis

Fungal genomic DNA for PCR was prepared from mycelia following 24 h incubation with shaking (200 rpm) at 30 °C in potato dextrose broth (PDB; Difco). Genomic DNA was extracted using a ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research Corp., Irvine, CA) according to the manufacturer's instructions. To confirm successful knockout of *pks27*, *znf27* and *scd1*, transformant genomic DNA was amplified with ExTaq HS polymerase using primers