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The *Aspergillus flavus fluP*-associated metabolite promotes sclerotial production

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ABSTRACT

Aspergillus flavus is able to synthesize a variety of polyketide-derived secondary metabolites including the hepatocarcinogen, aflatoxin B_1. The fungus reproduces and disseminates predominantly by production of conidia. It also produces hardened mycelial aggregates called sclerotia that are used to cope with unfavourable growth environments. In the present study, we examined the role of *A. flavus fluP*, the backbone polyketide synthase gene of secondary metabolite gene cluster 41, on fungal development. The *A. flavus* CA14 *fluP* deletion mutant (AfΔfluP) grew and accumulated aflatoxin normally but produced a lower amount of sclerotia than the parental strain. This was also true for the *Aspergillus parasiticus* BN9 *fluP* deletion mutant (ApΔfluP). The *A. flavus fluP* gene was positively regulated by developmental regulators of VeA and VelB but not by the global regulator of secondary metabolism, LaeA. Overexpression of *fluP* in AfΔfluP (OEfluP) elevated its ability to produce sclerotia compared to that of the parental strain. Coculture of OEfluP with CA14, AfΔfluP, ApΔfluP, or an *A. flavus pptA* deletion mutant incapable of producing functional polyketide synthases also allowed increased sclerotial production of the respective strains at edges where colonies made contact. Acetone extracts of OEfluP but not of AfΔfluP exhibited the same effect in promoting sclerotial production of AfΔfluP. These results suggest that FluP polyketide synthase is involved in the synthesis of a diffusible metabolite that could serve as a signal molecule to regulate sclerotiogenesis.

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Introduction

Fungi possess an array of diverse biochemical pathways that are capable of producing polyketide-derived, structurally heterogeneous secondary metabolites (Keller *et al.* 2005). Many of these metabolites have important pharmacological applications, such as cholesterol-lowering lovastatin (Hendrickson *et al.* 1999), immunosuppressants of dalesconols (Zhang *et al.* 2008), and anticancer agents, curvularins (Xu *et al.* 2013) and aspergiolide A (Tao *et al.* 2009). On the other hand, known mycotoxins like aflatoxins, fumonisins, ochratoxins, and zearalenone are harmful to health of humans and animals (Huffman *et al.* 2010). Polyketide compounds also are critical to the dissemination and survival of the

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producing fungi. For example, pigments deposited on fungal propagules of conidia, ascospores or sclerotia (Tilburn *et al.* 1990; Brown & Salvo 1994; Tsai *et al.* 1999; Chang *et al.* 2010; Cary *et al.* 2014) have a protective role against prolonged exposure to harsh environments.

Aspergillus flavus is able to produce a diverse array of secondary metabolites (Pildain *et al.* 2008). SMURF (Secondary Metabolite Unknown Regions Finder; http://www.jcvi.org/smurf) analysis of the *A. flavus* NRRL3357 genome sequence has predicted 55 secondary metabolite gene clusters (Georgianna *et al.* 2010; Khaldi *et al.* 2010). Approximately half of the gene clusters contain a polyketide backbone gene, but only a few polyketide-derived compounds have been confirmed to be produced by specific gene clusters. These include aflatoxins (Cluster 54), aflavarin (Cluster 39) (Cary *et al.* 2015), and conidial and sclerotial pigments by Cluster 5 and Cluster 27, respectively (Chang *et al.* 2010; Cary *et al.* 2014).

Feng & Leonard (1998), in their study of how nitrogen sources affect expression of aflatoxin biosynthesis genes, isolated a polyketide synthase gene from *Aspergillus parasiticus* NRRL2999, which they named *pksP1* (GenBank accession number: U52151). The predicted PKSP1 shares a 55 % amino acid sequence identity to that of the 6-methylsalicyclic acid synthase of *Penicillium patulum*. Zhou *et al.* (2000) later disrupted this gene in *A. parasiticus* SU-1 (=NRRL2999). The derived *pksP1* mutants are severely retarded in radial growth and exhibit a fluffy, cotton-like morphology similar to what was previously reported for the *Aspergillus nidulans* fluffy (*flu*) gene mutants (Wieser *et al.* 1994). They thus renamed the *pksP1* gene, *fluP*. The *A. flavus* polyketide gene of Cluster 41 is the orthologous gene of *fluP*. Cluster 41 is a small gene cluster, and, besides *fluP*, it putatively only includes a cytochrome P450 gene and a gene for a hypothetical protein.

The 'fluffy' phenotype, which results from proliferation of undifferentiated conidiophores that are unable to produce conidia, has intrigued many researchers for more than a decade (Adams *et al.* 1992). Several genes including *fluG* (fluffy) and *flbA-E* (fluffy with low *brlA* expression) have been isolated from *A. nidulans* and the genes, *flbA-E*, characterized (Wieser & Adams 1995; Hicks *et al.* 1997; Etxebeste *et al.* 2008; Garzia *et al.* 2009; Kwon *et al.* 2010). These corresponding genes in *Aspergillus oryzae* have been studied, and derived mutants exhibit various extents of decreased conidiation (Ogawa *et al.* 2010). Coculturing *A. nidulans fluG* mutant with wild type restores the mutant's ability to conidiate, which shows that the *fluG*-associated metabolite is extracellular and diffusible (Lee & Adams 1994). The *A. flavus fluG* mutant, although somewhat fluffy when the culture is young, produces abundant conidia as growth continues (Chang *et al.* 2012) unlike the *A. nidulans fluG* mutant. Dehydroaustinol, a hybrid polyketide and terpene molecule, has been shown to be the diffusible effector that promotes conidiation and is missing in the *A. nidulans fluG* mutant (Rodriguez-Urra *et al.* 2012). The complete dehydroaustinol biosynthesis pathway, however, is encoded by two separate gene clusters in *A. nidulans* (Lo *et al.* 2012). Therefore, FluG, a fusion protein with an amidohydrolase domain and a glutamine synthetase domain (Iyer *et al.* 2009), unlikely is able to synthesize dehydroaustinol. The function of *A. nidulans fluG* and the resulting FluG's involvement in dehydroaustinol production remains unclear.

The previous characterization of the *A. parasiticus fluP* (Feng & Leonard 1998; Zhou *et al.* 2000) suggests that FluP may produce a polyketide metabolite necessary for normal conidiation. In the present study, we found that deletion of *A. flavus fluP* did not give mature colonies with a fluffy phenotype unlike that observed for *A. nidulans* or *A. parasiticus*, but, instead gave colonies with decreased sclerotial production. This decrease appeared to be associated with the loss of the diffusible *fluP*-associated metabolite. The *fluP* deletion mutant derived from another *A. parasiticus* strain, BN9 (Ehrlich *et al.* 2008), also exhibited drastic differences from what was previously reported for the *fluP* deletion mutant of *A. parasiticus* SU-1 in growth, development, and aflatoxin production.

Materials and methods

Fungal strains and growth media

The *Aspergillus flavus* CA14PTSΔpyrG and *Aspergillus parasiticus* BN9Δku70 were used as transformation recipient strains. The *ku70* gene of both strains involved in the nonhomologous end-joining pathway was deleted, which allows a significantly increased gene-targeting frequency (Ehrlich *et al.* 2008; Chang *et al.* 2010). Media used for observing morphological changes including formation of sclerotia were potato dextrose agar (PDA, EMD, Darmstadt, Germany) and Wickerham medium (Raper & Thom 1968), which contains 2.0 g yeast extract, 3.0 g peptone, 5.0 g corn steep solids, 2.0 g dextrose, 30.0 g sucrose, 2.0 g $NaNO_3$, 1.0 g $K_2HPO_4 \cdot 3H_2O$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.2 g KC1, 0.1 g $FeSO_4 \cdot 7H_2O$, 15.0 g agar per litre (pH 5.5).

Deletion of fluP *in* Aspergillus flavus *and* Aspergillus parasiticus*, deletion of* pptA *in* A. flavus *and complementation of an* A. flavus fluP *deletion strain*

The *A. flavus fluP* sequence of NRRL3357 (AFL2G_12403.2, AFLA_114820) was retrieved from the *Aspergillus* Comparative Database at Broad Institute for primer design and synthesis. A routine double-crossover gene knockout strategy (Chang *et al.* 2010) was used to delete *A. flavus fluP*. The primer set of fluP5Sp: AGCCAAAGCCGCATGCATTCCA and fluP5S: TCTCAC TTGAGGCGTCGACTT was used to amplify a 5′ region. The set of fluP3B: CGGAGCAGATGGATCCCCAACA and fluP3Sc: TTGGAGCTCCAACTGGTGTCGA was used to amplify a 3′ region. The two PCR fragments were cloned sequentially into the vector pPG28, of which a 1 kb Xho-SphI fragment was removed leaving a 1.6-kb *pyrG* selection marker. The *A. flavus fluP* deletion vector was linearized with SphI and SacI prior to transformation. The *A. parasiticus fluP* gene (also called *pksL2*) sequence was retrieved from NCBI GenBank under the accession number of U52151. A rapid fusion PCR protocol (Szewczyk *et al.* 2006) was used to generate a linear DNA fragment for deletion of the same *fluP* region that encodes a portion of the ketoacyl synthase domain in *A. parasiticus*. The *fluP* knockout PCR fragment used the *Aspergillus oryzae* pyrithiamine resistance gene (*ptrA*) as the selection marker (Kubodera *et al.* 2000). The 5′ and 3′ flanking fragments were amplified with AGTCTATGTCCTGGCAGTTC and tgcccgtctgtcagatccccTCTCACTAGAGGCGTCGACTT, and *atcgtcacccca*

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