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Biosynthesis of non-melanin pigment by a divergent polyketide synthase in *Metarhizium robertsii*

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ABSTRACT

Fungal polyketide synthases (PKSs) and their related gene clusters are highly diversified at both inter- and intra-specific levels. The most well characterized PKS enzymes include those responsible for the biosynthesis of polyketide pigments such as melanins. The genome of the insect pathogenic fungus *Metarhizium robertsii* contains 20 type I PKSs but none has been functionally characterized. In this study, two PKS genes (designated as *MrPks1* and *MrPKs2*) showing homologies to those counterparts for the biosynthesis of heptaketide pigments and dihydroxynaphthalene (DHN)-melanins, respectively, were deleted in two different strains of *M. robertsii*. The results indicated that disruption of *MrPks1* but not *MrPks2* impaired fungal culture pigmentation and cell wall structure. In addition to the negative effect of the DHN-melanin pathway inhibitor, it was postulated that DHN-melanin would not be produced by *M. robertsii*. Various assays revealed that the stress resistance abilities against ultraviolet radiation, heat shock and oxidants, as well as virulence against insects were not impaired in *ΔMrPks1* and *ΔMrPks2* isolates when compared with the wild-type strain. Thus, the non-melanin pigment(s) produced by the fungus do not contribute to cell damage protection and pathogenicity in *M. robertsii*. Physiological differences were evident in the two examined wild-type strains. The results from this study advance the understanding of functional divergence of fungal PKSs.

1. Introduction

Fungal polyketide synthases (PKSs) are involved in the biosynthesis of an array of polyketides including pigments and mycotoxins (Kroken et al., 2003; Chiang et al., 2010). Melanins are insoluble pigments with high molecular weights. These pigments are generally red, black or brown in color and share common physical and chemical traits (Wheeler and Bell, 1988). Two pathways of melanin biosynthesis have been elucidated in fungi, i.e., the dihydroxynaphthalene (DHN) pathway and L-3,4-dihydroxyphenylalanine (L-DOPA) pathway (Eisenman and Casadevall, 2012). In the DHN-melanin pathway, acetyl-coenzyme A (CoA) or malonyl-CoA are used as the starter substrate by type I PKS to form tetrahydroxynaphthalene (THN), followed by a series of reductions and polymerization into melanin (Langfelder et al., 2003; Chiang et al., 2010). In L-DOPA melanin synthesis, the precursor (L-DOPA or tyrosine) is catalyzed by tyrosinase or catalase into dopaquinone, which is converted into dihydroxyindole for polymerization into melanin (Langfelder et al., 2003; Land et al., 2004). Melanin pigments have multiple functions that protect cells from damage caused by ultraviolet (UV) light, oxidizing agents or ionizing radiation, and contribute to the virulence of plant and animal fungal pathogens (Eisenman and Casadevall, 2012). Fungal PKSs can also produce heptaketide pigments when the starter substrate malonyl-CoA is converted into naphthopyrone instead of THN (Watanabe et al., 1999; Chiang et al., 2010). Heptaketide and its derivative pigments differ from melanin by not contributing to fungal stress response or virulence (Malz et al., 2005; Kim et al., 2005). These two type of PKSs have not been identified and characterized in a single fungal species.

Ascomycete insect-pathogenic fungi such as *Metarhizium robertsii* and *Beauveria bassiana* have been developed as promising insect biocontrol agents (Roberts and St. Leger, 2004; Wang and Feng, 2014). The factors related to fungal virulence and environmental stress responses have close connections with fungal biocontrol efficacy (St. Leger and Wang, 2010; Liu et al., 2013). We showed that genetic engineering of *B. bassiana* to overexpress an exogenous tyrosinase gene from *Aspergillus fumigatus* could improve fungal production of melanin and thereby increase the UV resistance ability and pathogenicity against insects (Shang et al., 2012). Similarly, the transformation of *Metarhizium anisopliae*

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to express the PKS *Alm*, 1,3,4-trihydroxynaphthalene reductase (*Thr*) and scytalone dehydratase (*Scd*) genes from *Alternaria alternata* enabled *Metarhizium* to produce melanin and enhanced fungal antistress abilities, virulence gene expressions and pathogenicity (Tseng et al., 2011, 2014). However, the endogenous pathway for melanin/pigment production is still undiagnosed in insect-pathogenic fungi. The uses of different inhibitors, e.g., kojic acid and sulcotrione against the synthesis of DOPA-melanin, and tricyclazole against DHN-melanin failed to cause phenotypic changes in the *M. anisopliae* strain ARSEF 2575 (now classified as *M. robertsii*) (Fang et al., 2010), implying the presence of an unusual, yet undetermined, pathway(s) for culture pigmentation in this fungus.

Our genome analysis identified 20 type I PKSs in *M. robertsii* (with a broad host range) and 10 type I PKSs in the locust-specific pathogen *M. acridum* (Gao et al., 2011). In this study, two PKS genes were found to show homologies to functionally characterized *FgPks12* (from *Fusarium graminearum*) (Malz et al., 2005; Kim et al., 2005), *wA* (*A. nidulans*) (Watanabe et al., 1999), *Alb1* (*A. fumigatus*) (Tsai et al., 1999) and *Alm* (*Alternaria alternata*) (Kimura and Tsuge, 1993) genes for pigment or melanin biosynthesis. The genes were designated as *MrPks1* (MAA_07745) and *MrPks2* (MAA_03239). The *MrPks1* gene cluster shows conservation to the gene cluster for the production of the heptaketide pigment while the *MrPks2* cluster is highly conserved with the DHN-melanin pathway. We found that disruption of *MrPks1* but not *MrPks2* impaired fungal culture pigmentation in *M. robertsii*. However, neither null mutant demonstrated varied abilities in UV resistance, heat tolerance, oxidative stress response or insect killing when compared with the wild-type strains.

2. Materials and methods

2.1. Fungal strains and growth conditions

The wild-type (WT) and mutants of the *M. robertsii* strains ARSEF 2575 and ARSEF 23 were routinely cultured on potato dextrose agar (PDA, BD Difco, Radnor, PA) or PDA amended with 20 μg/ml of the DHN-melanin inhibitor pyroquilon (Sigma, St. Louis, MO) (Lee et al., 2003) at 25 °C. The cultures were alternatively incubated in Sabouraud dextrose broth (BD Difco) for DNA extraction or spore germination assays.

2.2. Bioinformatics and phylogenetic analyses

Homologous PKS sequences of different fungal species were retrieved from the NCBI database for those that have been functionally verified to be involved in conidial pigmentation and/or DHN-melanin biosynthesis. Modular analysis of different PKS enzymes, including the retrieval of ketosynthase (KS) domain sequences for phylogenetic analysis and prediction of *MrPks1* and *MrPks2* gene clusters, were performed using the antiSMASH 2.0 (Blin et al., 2013) and SMURF (Khaldi et al., 2010) programs. For phylogenetic analysis, the KS domain sequences from examined PKSs were aligned using Clustal X 2.0 (Larkin et al., 2007) and a maximum likelihood tree was generated using MEGA 6.0 software (Tamura et al., 2013).

2.3. Gene deletions

Targeted gene deletion was performed by homologous recombination (Duan et al., 2013). Briefly, the 5′ and 3′ flanking sequences of *MrPks1* and *MrPks2* genes were amplified using different primer pairs (Table S1) with Phanta™ Super-Fidelity DNA Polymerase (Vazyme, Piscataway, NJ). The products were digested with the restriction enzymes *Eco*RI, *Bam*HI (for deletion of *MrPks1*) or *Eco*RI (for deletion of *MrPks2*) and then inserted into the corresponding sites of the vector pDHt-bar to generate the disruption vectors pBarPKS1 and pBarPKS2 for *Agrobacterium*-mediated fungal transformation (Duan et al., 2013; Huang et al., 2014).

2.4. Pigment extraction, quantification and chromatography analysis

To determine the association of MrPks1 and MrPks2 with melanin/pigment production, the harvested fungal conidia were washed and boiled in 2% NaOH at 100 °C for 2 h. The samples were centrifuged at 12,000*g* for 15 min and filtered through a membrane (0.22 μm pore size) before spectrophotometric analysis at 459 nm (Kimura and Tsuge, 1993). A standard curve was generated using the melanin standard (Sigma) for quantification purposes. There were three replicates of each isolate. To identify the putative missing pigments, gas-chromatography (GC) analysis was conducted. The supernatants (10 μl) were derivatized by 70 μl methoxyamine at 37 °C for 2 h, followed by the treatment with 70 μl N-Methyl-N-(trimethylsilyl) trifluoroacetamide at 37 °C for 16 h. After centrifugation, 1 μl sample was injected splitlessly with an Agilent 7683 Series autosampler into an Agilent 6890 GC system (Agilent Technologies, Santa Clara, CA) equipped with a HP-5MSI column. For nuclear magnetic resonance spectroscopy (NMR) analysis, samples were diluted with 500 μl D_2O and transferred into NMR tubes. The NMR spectra were acquired using a Bruker AVANCE III 600 spectrometer (Bruker Corporation, Billerica, MA), operating at 400 MHz 1H frequency.

2.5. Transmission electron microscopy (TEM) and field emission scanning electron microscopy (FESEM) analyses

To observe cell wall melanin/pigment layer variations, the conidia of the WT strain and different mutants were harvested, washed and fixed overnight in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4 °C. The samples were analyzed under a transmission electron microscope (Hitachi, H-7650, Tokyo, Japan) operating at 80 kV (Duan et al., 2013). For the FESEM observation of the cell surface structures, fungal conidia were collected for the preparation of conidial suspensions. The spores were mounted on the surface of the forewings of adult mealworms (*Tenebrio molitro*) by immersing the wings in each suspension for 10 s. Then, the spores were incubated on water agar (1.5% w/v) for 2 h. The samples were fixed overnight in 0.1 M phosphate buffer (pH 7.2) containing a final concentration of 2.5% glutaraldehyde, and dehydrated by treatment using a series of ethanol solutions (50–100% v/v). Finally, the samples were coated with platinum and images were captured using a field emission scanning electron microscope (Carl Zeiss, MERLIN Compact, Oberkochen, Germany) operating at 3 kV.

2.6. UV resistance assays

UV resistance assays were conducted using a UV crosslinker (UVP, CX-2000, Upland, CA). Conidia were suspended in 0.05% Tween-20 (v/v) and 10 μl of spore suspension (ca. 3×10^7 conidia/ml) were inoculated on PDA medium in plastic petri plates (6 cm in diameter). After exposure to UV radiation at doses of 0.05 or 0.1 kJ/m^2, the plates were incubated at 25 °C for 24 h to determine the spore germination rates under the microscope (Olympus BX 51, Tokyo, Japan). At least six random microscopic field views of each plate and three replicate plates were counted for each treatment and the experiments were repeated twice. Non-irradiated plates were used as controls.

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