Microbiological Research 179 (2015) 1–11

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Melanin is required for the formation of the multi-cellular conidia in the endophytic fungus *Pestalotiopsis microspora*

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ARTICLE INFO

Article history:
Received 11 February 2015
Received in revised form 5 June 2015
Accepted 14 June 2015
Available online 26 June 2015

Keywords:
Polyketide synthase
Melanin
Conidial development
Germination tube
Pestalotiopsis microspora

ABSTRACT

Melanin plays an important role in regulating various biological processes in many fungi. However, its biological role in conidiation remains largely elusive. We report here that conidia production, morphogenesis, integrity, germination and their viability in *Pestalotiopsis microspora* require the polyketide-derived melanin. A polyketide synthase gene, *pks1*, was identified and demonstrated responsible for melanin biosynthesis in this fungus. A targeted deletion mutant strain *Δpks1* displayed a defect in pigmentation of conidia and had an albino colonial phenotype. Interestingly, *Δpks1* produced approximately 6-fold as many conidia as the wild type did, suggesting a negative modulation of melanin on conidia production in this fungus. Moreover, the conidia failed to develop into the normal five-cell morphology, rather the three main-body cells separated via constriction at the original septum position to generate three independent mutant conidia. This result suggests a novel role of melanin in the formation of the multi-cellular conidia. Germ tubes could develop from the three different types of mutant conidia and kept elongating, despite a significantly lower germination rate was observed for them. Still more, the unpigmented conidia became permeable to Calcofluor White and DAPI, suggesting the integrity of the conidia was impaired. Deliberate inhibition of melanin biosynthesis by a specific inhibitor, tricyclazole, led to a similar phenotypes. This work demonstrates a new function of fungal melanin in conidial development.

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Introduction

Melanins are widely spread high molecular weight dark brown or black pigments produced by many fungi, in particular, in the reproduction structure such as conidia (Bell and Wheeler, 1986; Butler and Day, 1998). Despite that chemical structure of fungal melanin is complex and remains unresolved at this stage, it has lately drawn attention to researchers for its ubiquity in nature and unique chemical property. Some works suggest, for instance, that the biopolymers notably promised environmental and medical applications (Plonka and Grabacka, 2006). Owing to superficial hydrophobic and negatively charged functional groups, melanins can bind to a broad spectrum of substances, e.g. heavy metals and dyes which are contaminating agents in waters and soils. Thus, melanins can be a great scavenger in bioremediation as proposed (Fogarty and Tobin, 1996; Butler and Day, 1998). A couple of studies bring about a thought that melanins may be applied for shielding cancer patients and radioimmunotherapy receivers from side effects in radiation therapy (Dadachova et al., 2007; Schweitzer et al., 2010).

Despite ubiquitously synthesized by fungi, the biological roles of melanins, in particular in the production and development of conidia the spore have received little meticulous investigation. It seems a view that melanins are not essential for the growth and development of the producer. Observations suggest that melanins are frequently associated with the cell wall, which can provide a protective function for conidia against harms caused by ultraviolet (UV) light, oxidants, radiation and extreme climate conditions (Butler and Day, 1998; Calvo et al., 2002; Eisenman et al., 2005; Zhong et al., 2008). For example, an L-dopa-derived melanin, a virulence factor of the human pathogenic yeast *Cryptococcus neoformans*, could be abandoned for its growth under the normal conditions (Zhu et al., 2001; Eisenman et al., 2005). Thus, in many if not all cases, fungal melanin is believed dispensable for the normal growth of the fungi.

Fungi in the form genus *Pestalotiopsis* are among the most encountered in the tropical and temperate regions. Based on some induction of the sexual stages, namely, the teleomorphs of the fungi, they have been identified taxonomically as ascomycetes *Pestalotiosphaeria* spp. Most of them are known as successful plant endophytes, while some of them cause diseases on rainforest

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http://dx.doi.org/10.1016/j.micres.2015.06.004
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plants such as banana or tea trees (Maharachchikumbura et al., 2011; Yang et al., 2012). Lately, this group of fungi has spurred great interest amongst researchers for their ability to produce a wealth of bioactive secondary metabolites (Weber, 2009; Xu et al., 2010; Maharachchikumbura et al., 2011; Yang et al., 2012). Notably, chemicals with novel structure, chloropupukeananin and pestalamide A, isolated from *P. fici* and *P. theae* exhibited strong anti-HIV-1 effects at the stage of viral replication (Ding et al., 2008; Liu et al., 2008). A novel class of cholesterol ester transfer protein inhibitors (CETP) – dibenzodioxocinones, which are derivatives of the polyketide penicillides and pestalotiollides, were found in *Pestalotiopsis* spp. (Brückner et al., 2005; Xu et al., 2011). Still more, the antitumor drug taxol (paclitaxel) has been reported, by a number of laboratories from all over the world, in the culture of over thirty *Pestalotiopsis* spp., name a few, *Pestalotiopsis microspora, P. guepin*, and *P. malicola* (Strobel et al., 1996; Kumaran et al., 2010; Bi et al., 2011; Yang et al., 2012). As one of the most commonly isolated taxol-producing fungi, the endophytic *P. microspora* attracts immense interests due to its potential as a model organism for the research of the biosynthesis and metabolite of this important natural product (Strobel et al., 1996; Xu et al., 2010; Niu et al. 2015). Despite its prevalence, the research of evolutionary and genetics about fungi remains insufficiency and may block the way to promote taxonomically understood and to elucidate the molecular basis of fungal bioactive metabolites pathways (Xu et al., 2010; Maharachchikumbura et al., 2011).

Morphologically, this group of fungi is featured for their unique asexual spores (conidia) that usually consist of five main-body cells, three hyphal filaments (appendages) on the top end cell plus a short one on the bottom end cell. The three median cells among the five main body cells are heavily pigmented with melanin on both the wall and the septa, but not on the two end cells and the appendages (Strobel et al., 1996; Maharachchikumbura et al., 2011). On the opposite, there is little detectable pigment in the vegetative mycelium of the fungi. These features make the fungi a potential ideal model for the study of conidiation considering that many model fungi, e.g. *Aspergillus* only have single-cell conidia that are relatively less differentiated. Besides, conidiation, pigmentation and morphology of conidia are particularly important criteria in the taxonomy for this group of fungi (Maharachchikumbura et al., 2011; Yang et al., 2012). Unfortunately, little attention has been paid, by far, to the formation of the multi-cellular conidia and their biological advantage of this type of conidia in nature.

Our laboratory previously isolated a strain, *P. microspora* NK17, which can produce a taxol-like secondary molecule and a high yield pestalotiollide B (a derivative of dibenzodioxocinones) in liquid culture (Bi et al., 2011; Niu et al., 2015). We attempt to initiate the molecular study in this fungus starting with the identification of PKS-encoding genes for melanin biosynthesis and to observe their roles in the development of the pigmented conidia. Since the majority of fungal melanins are made through the aromatic polyketide biosynthetic pathway, we demonstrated one of the sixteen PKS-like genes in the genome of NK17, designated as *pks1*, was responsible for the biosynthesis of the pigment melanin in the conidia of NK17. We report here several unexpected effects of melanin biosynthesis in this fungus, including conidiation, conidial morphogenesis and viability, which will enrich our understanding on the biological importance of the biopolymer melanins, and provide a novel link between melaninization and conidial morphogenesis.

Materials and methods

Fungal strains and culture conditions

P. microspora NK17, isolated by this laboratory (Niu et al., 2015), was used as the wild-type strain in this study. Unless otherwise specified, PDA (20% peeled and sliced potato, 1.0% glucose and 1.5% agar, natural pH) and PDB (PDA without agar) were the media for routine growth of the fungus. The liquid culture was shaken at 180 rpm, 28 °C under normal illumination of the indoor light condition. The inhibitor of polyketide-melanin biosynthesis, tricyclazole, was dissolved in ethanol and added to the medium to a final concentration of 40 μM. The inhibition assay was conducted in triplicate (Pihet et al., 2009).

Table 1
PCR primers in this study. Under lines indicate restriction sites..

Primer name	Sequence (5′–3′)
Pks-up(s)	AAAAAGAGCTCCCCGCTTTACCCTAGATGTG
Pks-up(as)	AAGGGTCTAGAAGCTTCACGTGGCGACATAT
Pks-down(s)	ACGCGTCGACGACCGCCGCTACCGTGGAAT
Pks-down(as)	CCCCAAGCTTGCTACCGCGTCGGCACTTTC
Hyg(s)	CGTTGCAAGACCTGCCTGAA
Hyg(as)	GGATGCCTCCGCTCGAAGTA
Actin(s)	GTCGCTGCCCTCGTTATC
Actin(as)	CGAGAATGGAACCACCGAT
Pks(s)	GGCTTGCACGTCACTCTATG
Pks(as)	GTTTGCGGGAGCGAATACAT
Up-hyg	CGTCTCTTGCGGAAAGGCTG
Down-hyg	TTGACCTCATCCTTCTCACTCTCG
Parp2(s)	GTCTCCCTCCGCAACCCA
Parp2(as)	CGCTTCTTTCCGCAGTCG
Pabr1(s)	ACGACGGGTCTTCACTTT
Pabr1(as)	TGGATTGTCTCCCTCTGC

Disruption construct of pks1

A protocol for gene targeting with *Agrobacterium*-mediated transformation of T-DNA in NK17 was previously created (Hao et al., 2012). Briefly, to disrupt *pks1*, a disruption cassette was constructed from the T-DNA based binary vector pBI121-HPH which contained a hygromycin B resistant gene (*hph*) for selection. An 846-bp *pks1* 5′-fragment harboring restriction sites of *Sal* I and *Hin*d III on the ends, was acquired by PCR amplification with primers Pks-up (s)/Pks-up (as) (Table 1). This fragment was double digested with *Sal* I and *Hind* III and inserted into the corresponding sites of pBI121-HPH. Similarly, a 785-bp *pks1* 3′-fragment containing *Sac* I and *Xba* I restriction sites on the termini, was also cloned to make the final construct, pBI-HPH-pks. The disruption cassette on T-DNA would be linearized and transferred by *Agrobacterium tumefaciens* via conjugation with the conidia of NK17 (see section below). A 3.0-kb region of *pks1* was expected to be replaced by *hph* via homologous recombination, resulting in a deletion of a portion of the gene between nucleotide 1520 and 4554 (numbering starts from ATG codon). Transformants were selected on 100 μg/ml hygromycin B.

Agrobacterium-*mediated transformation to create* pks1 *disruption mutants*

The bacterial strain *A. tumefaciens* LBA4404, host of pBI121-HPH-pks, was grown in 5-ml LB (Luria Both) containing antibiotics: 100 mg/l streptomycin and 25 mg/l rifampicin, at 28 °C, overnight. The vector pBI-HPH-pks was introduced into LBA4404 by $CaCl_2$-mediated transformation (Hao et al., 2012). Approximately 100 μl of the bacterium culture (10^8 CFU/ml) was mixed with an equal volume of NK17 conidia (10^7) and spread on nitrocellulose filter (45-mm in diameter). The membrane was incubated on LB agar in the presence of 200 μM acetosyringone (AS, Sigma, St. Louis, USA), at 24 °C, 48 h. The filter was then transferred to PDA containing hygromycin B at 24 °C for 2 weeks for sporulation. Fungal transformants were single-spore purified on PDA supplemented with 100 μg/ml hygromycin B, and 200 μg/ml cefotaxime to kill the bacteria.

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