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Original article

PdbrlA, PdabaA and *PdwetA* control distinct stages of conidiogenesis in *Penicillium digitatum*

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Abstract

Penicillium digitatum is one of the most important citrus postharvest pathogens worldwide. Reproduction of massive asexual spores is the primary factor contributing to the epidemic of citrus green mold. To understand the molecular mechanisms underlying conidiogenesis in *P. digitatum*, we functionally characterized the *Aspergillus nidulans* orthologs of *brlA*, *abaA* and *wetA*. We showed that deletion of *PdbrlA* completely blocked formation of conidiophores, whereas deletion of *PdabaA* led to the formation of aberrant and non-functional phialides. The *PdwetA* mutant showed various defective phenotypes, such as abnormal conidia with loose cell walls, delayed germination and reduced tolerance to osmotic, detergent, heat shock and menadione stresses, but elevated resistance to H_2O_2. *PdbrlA*-influenced genes were identified by comparing global gene expression profiles between the wild-type and the *PdbrlA* deletion mutant during conidiation. Gene ontology analysis of these differentially expressed genes (DEGs) revealed the diverse roles of *PdbrlA* in metabolism, transportation and cell structure. Moreover, out of 39 genes previously reported to be involved in conidiogenesis in *Aspergillus*, mRNA levels of 14 genes were changed in Δ*PdbrlA*. Our results confirm the roles of *brlA*, *abaA* and *wetA* in *P. digitatum* conidiogenesis and provide new insights into the genetics of conidiation in filamentous fungi.

Keywords: Penicillium digitatum; *brlA*; *abaA*; *wetA*; Conidiogenesis; Gene expression profile

1. Introduction

Penicillium digitatum (Pers.:Fr) Sacc., the fungal agent of citrus green mold, is the most destructive postharvest pathogen in citrus-growing areas worldwide. It contributes to the most economically significant losses to the citrus industry during storage, transportation and marketing of orange, lemon, mandarin and other citrus fruits [1]. *P. digitatum* is a common saprophytic fungus in soil and on plant materials in orchards, as well as on organic matter in citrus packing houses. It produces large quantities of asexual spores (conidia) as the primary means of thriving. Despite recent advances in chemical control, losses caused by citrus green mold have not been significantly reduced since the emergence and spread of fungicide resistance in wild populations [1–3]. Like many plant pathogenic fungi, the infectious life cycle of *P. digitatum* begins with germination of conidia deposited on the surface of citrus fruits. Understanding the mechanisms of conidiogenesis in this fungus is of particular importance for development of new strategies for green mold control.

Conidiation is the most common reproductive mode for many filamentous fungi, and developmental mechanisms have been extensively characterized in the model organisms *Aspergillus nidulans* and *Neurospora crassa* [4–8]. These

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findings provide important clues for understanding conidiation in *P. digitatum*. In *Aspergillus nidulans,* formation of multicellular reproductive structures called conidiophores is crucial for its conidiation. Early studies indicated that formation of conidiophores in *A. nidulans* can be divided into several morphologically distinct stages [8]. Firstly, conidiophore formation begins with the thickened foot cells that branch to form aerial conidiophore stalks. After apical extension of the stalk ceases, the tip develops a swollen vesicle. The stalk, vesicle and foot cell comprise a single unit without septum. Then, a budding-like process comes into being with two layers of sterigmata, metulae and phialides, successively formed on the surface of the vesicle. Finally, phialides give rise to repeated asymmetric mitotic division to generate chains of uninucleate spores called conidia [8].

BrlA is a C_2H_2 zinc-finger transcription factor that governs the switch of the conidiophore stalk from polarized apical growth to swelling and formation of the vesicle [9]. Disruption of *brlA* fails to produce vesicles and subsequent structures for conidia formation, giving the colony a "bristle" appearance [8]. *AbaA* encodes a developmental regulator and is activated by *brlA* during the middle stages of conidiophore development; it is reported to be responsible for proper differentiation and function of phialides after formation of metulae [10,11]. A loss-of-function *abaA* mutant results in abacus-like structures with supernumerary tiers of cells with properties of metulae, but that do not form functional phialides, thus causing no conidia formation [11]. During the late phase of conidiation, *wetA* is activated by *abaA*. *WetA* is crucial for synthesis of cell wall layers that make conidia mature and impermeable [12,13]. In *wetA* mutant strains, conidia take up water and autolyze rather than undergoing the final stages of maturation [12]. *BrlA*, *abaA* and *wetA* have been proposed to comprise a central regulatory pathway that controls temporal and spatial expression of conidiation-specific genes during conidiophore development and spore maturation [6,14]. Remarkably, specific functions of the central regulatory pathway in conidiogenesis are highly conserved among the fungi in the genus *Aspergillus* and some other filamentous fungi [15–19]. Moreover, researchers have revealed that a number of upstream developmental activators (fluG, *flbA*, *flbB*, *flbC*, *flbD* and *flbE*), expressed in vegetative cells, are required for normal activation of the central regulatory pathway and play important roles in conidiation initiation [20]. Mutants from these activators result in proliferation of undifferentiated vegetative hyphae that produce fluffy cotton-like colonies [21–23]. In addition, the sensory and regulatory roles of the velvet complex that coordinates fungal growth and differentiation have been well studied and shed light on the molecular mechanisms underlying the intimate relationship between fungal development and secondary metabolism [24–26].

The genomic sequences of three strains of *P. digitatum* have been recently published [27,28]. Methodology of reversed genetics to characterize gene functions had been established earlier. To date, two genes related to virulence and other biological characteristics have been demonstrated to be associated with conidiation in *P. digitatum* [29,30]. Previous research showed that conidiation for the null *snf1* mutant was dramatically impaired due to inactivation of *brlA* expression [29]. In this study, three central regulators (orthologs *of brlA*, *abaA* and *wetA*) for conidiation in *P. digitatum* were identified and functionally characterized, and the regulatory mechanisms of *brlA* were studied through genome-wide expression profile analysis.

2. Materials and methods

2.1. Strains and culture conditions

The reference *P. digitatum* strain, PdKH8, was isolated from an infected citrus fruit from Zhejiang, China [31]. PdKH8 and its derived mutants were stored in 20% glycerol solutions at −80 °C until use. Conidia were obtained by growing the fungus on regular solid PDA (potato dextrose agar) at 25 °C, while the mycelia were obtained by growing spores in liquid PDB (potato dextrose broth) incubated on a rotary shaker with 160 rpm at 25 °C.

2.2. Identification of the *PdbrlA, PdabaA* and *PdwetA* genes

Based on the genomic sequence of *P. digitatum* [27,28], three pairs of primers (Table S1) were designed to amplify the fragments encompassing the full-length genomic sequences of *PdbrlA*, *PdabaA* and *PdwetA* (EMBL accession No.: EKV13796.1, EKV11276.1 and EKV07113.1, respectively) together with partial 5′ and 3′ untranslated regions from genomic DNA of *P. digitatum*. The amplified fragments were cloned into the pMD18-T vector according to the supplier's recommendations (TaKaRa Biotech. Co., Dalian, China) and verified by sequencing. Total RNA was extracted from mycelia using an AxyPrepTM multisource total RNA miniprep kit (Axygen, Hangzhou, China). First-strand cDNA was synthesized using the RNA PCR (AMV) 3.0 kit (TaKaRa Biotech. Co., Dalian, China). The coding region for each gene was amplified from the cDNA template and confirmed by sequencing.

2.3. Construction of *PdbrlA, PdabaA* and *PdwetA* deletion mutants

The *PdbrlA*, *PdabaA* and *PdwetA* deletion vectors were constructed by inserting two flanking sequences of the target gene into the left and right sides of the neomycin-resistance gene (neo) in the pA1300-NEO plasmid or hygromycin-resistance gene (hph) in the pTFCM plasmid (Fig. S1), as described previously [29,32,33]. The plasmids were transformed into *P. digitatum* using *Agrobacterium tumefaciens*-mediated fungal transformation (ATMT) followed by Wang and Li (2008). Putative transformants were screened aganist neomycin or hygromycin (100 mg/L) on PDA plate and then confirmed by PCR and Southern blot using the Detection Starter kit (Roche, Mannheim Germany).