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A proposed adhesin AoMad1 helps nematode-trapping fungus *Arthrobotrys oligospora* recognizing host signals for life-style switching

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ABSTRACT

The nematode-trapping fungus *Arthrobotrys oligospora* is an important natural enemy of nematodes. It can capture nematodes by producing a special mycelial structure called adhesive network or trap. The trap is also a signature of the fungus switching from the saprophytic lifestyle to the predacious lifestyle. At present, little is known about the mechanism of lifestyle switch in nematode-trapping fungi. Here we describe the effect of a cell wall protein called AoMad1 on lifestyle switch. The disruption of the AoMad1-encoding gene in *A. oligospora* resulted in the formation of more traps in the presence of nematodes. Interestingly, the mutant strain was more sensitive to certain nitrogen sources as trap inducers than the wild type strain. The microscopic examinations revealed that the *AoMad1*-deletion mutant lacked cell surface adhesive materials and the cell wall structures were more porous than wild-type strains. A great of genes were differentially expressed by transcriptomic analysis when trap formation was induced by sodium nitrate compared to the wild type strain, many of them were related to nitrogen metabolism, host–pathogen interaction and mycelia development. The results suggest that AoMad1 plays an important role in life style switching in *A. oligospora*.

1. Introduction

Fungal cell wall adhesins are glycoproteins which play important roles on the cell surface. The most characterized fungal adhesins are from the FLO (flocculation) family of *Saccharomyces cerevisiea*, which confer adhesion to agar, plastic and other yeast cells (Guo et al., 2000). In pathogenic fungi such as *Candida albicans* and *C. glabrata* [in which adhesins are encoded by the *ALS* (agglutinin like sequence) genes (Hoyer, 2001) and the *EPA* (epithelial adhesin) genes, respectively (De Las Peñas et al., 2003)], these proteins are responsible for adhesion to mammalian tissues and plastics. Some of them are multifunctional. For example, ALS3 can mediate *C. albicans* iron acquisition from host ferritin, thus accelerate pathogenicity (Almeida et al., 2008). In filamentous fungi, much less is studied about cell wall adhesins than in yeasts. The cell wall protein MAD1 was firstly characterized in *Metarhizium anisopliae* (Wang and St Leger, 2007). MAD1 on the surface of *M. anisopliae* conidia or blastospores is required to orient the cytoskeleton and stimulate the expression of genes involved in the cell cycle. Knockout of *Mad1* in *M. anisopliae* resulted in delayed germination, suppressed blastospore formation, and greatly reduced virulence to caterpillars (Wang and St Leger, 2007). At present, homologous proteins of MAD1 have been identified in several fungi, including the plant parasitic fungus *Fusarium oxysporum*, the human opportunistic pathogen *Aspergillus fumigatus*, and the nematode-trapping fungus *Arthrobotrys oligospora*, but the functions of MAD1 in these fungi have not been characterized (Liang et al., 2013; Prados-Rosales et al., 2009; Upadhyay et al., 2009).

Most opportunistic pathogenic fungi have saprophytic and parasitic lifestyles, and can switch between the two lifestyles under certain conditions. Most pathogenic fungi can cause infections in regions of the body that are no longer well protected by the host's physical barriers or immunological defenses. During the switching from saprophytic to parasitic lifestyle, the fungi often secrete virulent factors like serine protease, and chitinase (Yang et al., 2013). Some of them also involve morphological changes, for example, the filamentous cell appear important in *C. albicans* infections (Hogan et al., 2004).

A. oligospora is a model fungus to research nematodes–fungi interaction. It can form a special mycelial structure, the adhesive network, to capture nematodes. There are adhesive molecules on the network to facilitate the capture (Liang et al., 2013). At present, little is known about the adhesive proteins located on the traps in the nematode-trapping fungi. Recently, the genome of *A. oligospora* was sequenced, and several adhesive proteins were identified,

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including a MAD1 homolog protein, AoMad1. Real time PCR analysis suggested that AoMad1 and related adhesive proteins play a role in the trap formation of *A. oligospora* (Yang et al., 2011), but the function of AoMad1 was unknown. In this paper, we report the knockout of the *AoMad1* gene in *A. oligopora*. To our surprise, the Δ*AoMad1* strain can form more adhesive networks and capture more nematodes, and its trap formation can be much easelier induced by some nitrogen sources besides nematodes. Our results suggest that AoMad1 may playing a role in regulating the trap formation and the life-style switching of this fungus.

2. Materials and methods

2.1. Strains and growth conditions

The wild type strain of *A. oligospora* was purchased from American Type Culture Collection (ATCC24927). It was maintained on cornmeal agar (CMA) at 26 °C. The nematodes *Caenorhabditis elegans* were grown at room temperature on oatmeal agar medium (Niu et al., 2010). The cultured nematodes were separated using the Baerman funnel technique (Gray, 1984).

2.2. Gene knockout of AoMad1

The encoding gene of the cell wall protein AoMad1 (AOL_s00076g567 GenBank no. EGX49926.1) of *A. oligospora* was knocked out following the method previously described (Colot et al., 2006) with minor modifications. Briefly, the 5′ and 3′ flanking sequence was PCR amplified with the primers 5f: GTAACGCCAGGGTTTTCCCAGTCACGACGCGGTCGAAATTAAACAGGA and 5r: ATCCACTTAACGTTACTGAAATCTCCAACGGAGCTCTTGTGGATCTTGC, 3f: CTCCTTCAATATCATCTTCTGTCTCCGACACCAGCATCCCATACGAGAC and 3r: GCGGATAACAATTTCACACAGGAAACAGCGCCATCTCTTTTACGGCAAG, using *A. oligospora* genomic DNA as template. The hygromycin resistant gene was amplified with the primers hphF: GTCGGAGACAGAAGATGATATTGAAGGAGC and hphR: GTTGGAGATTTCAGTAACGTTAAGTGGAT, using the plasmid pCSN44 as a template (Staben et al., 1989). Then the three DNA fragments as well as a pRS426 backbone were transformed into the yeast *S. cerevisiae* strain FY834 by electroporation. Total DNA of transformants was extracted using Yeast Genomic DNA Preparation Kit (Tiangen, Beijing, China), and transformed into *Escherichia coli* DH5a. Plasmids were extracted using alkaline lysis method (Green and Sambrook, 2012), and confirmed with by PCR. The knockout cassette containing hygromycin resistant gene and two homologous flanking sequences was PCR amplified using the primers 5f and 3r, and then transformed into protoplasts of *A. oligospora* according to the previously described (Tunlid et al., 1999). Transformants resistant to 200 μg/μl hygromycin were selected and verified by PCR using primers Mad-Tf: GAGCCAAGAGCGTGAAGT and Mad-Tr: GCTGGGCAAGGTAGTGTA, and then were further confirmed by Southern blotting according to the instructions provided by the North2South® chemiluminescent hybridization and detection kit (Pierce, Rockford, USA). The primer pair Mad1-Sf: GAATTGGTTCTCCGCAGCTACTATA and Mad1-Sr: TCCCCATCGTTCTGTTCCTG was used to generate a Southern hybridization probe by PCR. The restriction enzyme *Hind* III was used to digest the genomic DNA of *A. oligospora* for Southern analysis.

2.3. Indirect immunofluorescence assay

To localize the AoMad1 protein, mycelia of wild type and Δ*AoMad1* strains with traps and captured nematodes were cut from CMA agar plate, fixed in 3.7% formaldehyde, and used for indirect immunofluorescence (IIF) analysis using previously described protocols (Wang and St Leger, 2006). Control samples were incubated at the same condition but without either the primary or secondary antibody. The antibody of AoMad1 was produced using a predicted antigenic sequence of the N-terminal domains of AoMAD1 (GGRPKFEPPAYSNERC [39 to 54 amino acids]), which were synthesized commercially and used for raising antibodies in New Zealand white rabbits (Sangon, Shanghai, China).

2.4. Bioassay against nematode C. elegans

The wild type and the Δ*AoMad1* strains were cultivated on CMA plates by spreading 1×10^4 conidia per plate. After three-days of incubation at 28 °C, one hundred nematodes (*C. elegans*) were added into the middle of the plates. The traps and captured nematodes were counted under a light microscope after 12 and 24 h, respectively.

2.5. Induction by various nitrogen sources

The wild type and the Δ*AoMad1* strains were cultivated by spreading 1×10^4 conidia per plate on water agar supplemented with following types of nitrogen substrates: sodium nitrate, ammonium nitrate, urea, and yeast extract of various concentrations, respectively. The number of traps was counted after 72 h of cultivation for each treatment.

2.6. Sodium chlorate assay

Sodium chlorate is a toxic analogue of sodium nitrate to many fungi and plants (Unkles et al., 2004). To compare the uptake rates of sodium nitrate, sodium chlorate was used for growth inhibition test. The wild type and the Δ*AoMad1* strains were cultivated by spreading 5×10^5 conidia per plate or inoculating a 5-mm-diameter plug from five-day-old CMA cultures on medium containing the following ingredients (per liter): 1 g glucose, 16 g sodium chlorate, 1.2 g L-proline, and 20 g agar. The spore germination rate and mycelial growth rates were compared.

2.7. Electron microscopic investigation

To compare the cell surface of the adhesive network between the *A. oligospora* Δ*AoMad1* strain and the wild type strain, nematodes were inoculated into 3-day CMA cultures of both strains. After 12 h incubation, scanning electron microscope (SEM) was performed. To compare cell wall structures, transmission electron microscopic (TEM) investigations were performed against three-day CMA cultures of both strains. The samples were stained with ruthenium red before fixation as described earlier (Tunlid et al., 1991).

2.8. Identification of differentially expressed genes of the two strains under sodium nitrate induction

To compare differentially expressed genes of the wild type and the Δ*AoMad1* strains at the initial stage of trap induction, mycelia samples of the two strains were incubated with sodium nitrate for 30 minutes, and then were taken for digital gene expression (DGE) analysis. Three repeats were conducted for each sample. The DGE process was the same as previously reported (Guo et al., 2014). Briefly, three microgram RNA per sample with RNA integrity number values above 8.0 were used. Sequencing libraries were generated using Illumina TruSeq™ RNA Sample Preparation Kit (Illumina, San Diego, USA) following manufacturer's recommendations. The library preparations were sequenced on an Illumina Hiseq 2000

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