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MAP kinase Slt2 orthologs play similar roles in conidiation, trap formation, and pathogenicity in two nematode-trapping fungi

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ABSTRACT

Mitogen-activated protein (MAP) kinase Slt2 is a key player in the cell-wall integrity pathway of budding yeast. In this study, we functionally characterized Slt2 orthologs AoSlt2 and MhSlt2 from the nematode-trapping fungi *Arthrobotrys oligospora* and *Monacrosporium haptotylum*, respectively. We found that disruption of *AoSlt2* and *MhSlt2* led to reduced mycelial growth, increased sensitivity to environmental stresses such as sodium dodecyl sulfate, Congo red, and H_2O_2, and an inability to produce conidia and nematode-trapping structures. Real-time polymerase chain reaction-based analyses showed that the transcription of sporulation-related (*AbaA*, *Sep2*, and *MedA*) and cell wall synthesis-related (*Chs*, *Glu*, and *Gfpa*) genes was down-regulated in the mutants compared with the wild-type strains. Moreover, the mutant strains showed reduced extracellular proteolytic activity and decreased transcription of three homologous serine protease-encoding genes. These results show for the first time that MAP kinase Slt2 orthologs play similar roles in regulating mycelial growth, conidiation, trap formation, stress resistance, and pathogenicity in the divergent nematode-trapping fungal species *A. oligospora* and *M. haptotylum*.

1. Introduction

Nematophagous fungi are widely distributed in terrestrial and aquatic ecosystems and include more than 200 species. Nematophagous fungi infect and kill living nematodes, and have been investigated for potential use as biological control agents against harmful nematodes (Moosavi and Zare, 2012). These pathogens have been categorized into four broad groups based on their modes of action: nematode-trapping fungi, endoparasitic fungi, egg-parasitic fungi, and toxin-producing fungi (Nordbring-Hertz et al., 2011). Among these groups, nematode-trapping fungi are unique in that they can develop specific mycelial structures, called traps, to capture nematodes. The types of traps produced by these fungi are very diverse, and include adhesive nets, adhesive knobs, adhesive branches, and constricting rings (Su et al., 2017). Previous studies have investigated the morphological characteristics of traps, the evolutionary relationships among nematode-trapping fungal species, and phylogenetic distribution (Li et al., 2005; Yang et al., 2012). However, the signalling mechanism that governs trap formation in nematode-trapping fungi remains unknown.

The mitogen-activated protein kinase (MAPK) cascade, a relatively conserved signal transduction pathway, controls the fundamental aspects of growth, development, and reproduction in fungi (Rispail et al., 2009). The core elements of the MAPK cascade are MAP kinases, which are a family of serine/threonine protein kinases that are well known for transducing a variety of extracellular signals to regulate growth and differentiation processes (Nishida and Gotoh, 1993). The MAP kinases are generally activated by MAP kinase kinases, which are in turn activated by MAP kinase kinase kinases (Schaeffer and Weber, 1999). Three different MAP kinases (Hog1, Slt2/Spm1, and Fus3) have been identified in fungi (Mey et al., 2002). Slt2 was first identified in the model fungus *Saccharomyces cerevisiae*, where it was found to play a role in the cell wall integrity (CWI) pathway (Heinisch et al., 1999). Recently, the functions of Slt2 in entomopathogenic fungi have also been characterized. For example, deletion of *Bbslt2*, encoding a Slt2-family MAPK, in *Beauveria bassiana* resulted in a significant reduction in conidial production and viability, and increased sensitivity to Congo red and fungal cell wall-degrading enzymes (Luo et al., 2012). Subsequently, Bck1, Mkk1, and Slt2 orthologs in *B. bassiana* were confirmed as a three-

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module cascade essential for CWI, with deletions in these genes resulting in increased sensitivity to hyperosmotic (NaCl and sorbitol) stress (Chen et al., 2014). Further studies have revealed an interaction between the CWI pathway and the high-osmolarity glycerol (HOG) pathway in *B. bassiana* (Liu et al., 2017a, 2017b). However, little is known about the functions of MAP kinases in nematode-trapping fungi.

Arthrobotrys oligospora and *Monacrosporium haptotylum* are the two model species of nematode-trapping fungi, each producing a different type of traps to capture nematodes. *A. oligospora* produces adhesive networks, and its genome sequence was published in 2011 (Yang et al., 2011). Two years later, the genome of adhesive knob-producing species *M. haptotylum* was also sequenced (Meerupati et al., 2013). Comparative analysis indicated a high degree of similarity between several main features in the genomes of *A. oligospora* and *M. haptotylum*, such as genome size, GC content, protein-coding genes, and the number of secreted proteins. Moreover, phylogenetic analysis showed that *A. oligospora* and *M. haptotylum* are relatively closely related (Meerupati et al., 2013). Thus, we speculate that the two species may share similar mechanisms for regulating mycelial growth and differentiation, such as conidiation and trap formation. To test this hypothesis, we analyzed and compared the well-studied MAP kinase protein Slt2 in these two fungi. Specifically, we deleted *Slt2* in each of the species and compared the phenotypic properties of the mutants with their corresponding wild-type (WT) strains (AoWT and MhWT). In addition, we examined the transcriptional levels of several genes related to conidiation and cell wall synthesis in the *A. oligospora* and *M. haptotylum* mutants and WT strains.

2. Materials and methods

2.1. Fungal strains and culture conditions

A. oligospora strain ATCC 24,927 and *M. haptotylum* strain CBS 200.50 were maintained on potato dextrose agar (PDA) medium at 28 °C. *Saccharomyces cerevisiae* strain FY834 was cultured in yeast extract-peptone-dextrose broth or agar medium, with SC-Ura medium used to select recombinant strains derived from FY834 (Park et al., 2011). Plasmid pRS426 was maintained in *Escherichia coli* strain DH5α (Takara, Shiga, Japan) (Christianson et al., 1992).

2.2. Sequence and phylogenetic analyses of MAP kinases

MAP kinase Slt2 orthologs in *A. oligospora* (AoSlt2, XP_011126279) and *M. haptotylum* (MhSlt2, XP_011115715) were retrieved based on the corresponding amino acid sequences in the model fungi *Aspergillus nidulans* (AnSlt2, AAD24428) and *Neurospora crassa* (NcSlt2, XP_958040). The amino acid sequences of each of the proteins were downloaded from the GenBank database, and their isoelectric points (*p*I) and molecular weights were calculated using the online software Compute pI/Mw tool (Wilkins et al., 2005). The conserved functional domains of the MAP kinases were analyzed using Interproscan 5.0 (Jones et al., 2014). Orthologous Slt2 proteins from other filamentous fungi were also obtained from GenBank and analyzed using the DNAman software package (Version 5.2.2; Lynnon Biosoft, St. Louis, Canada). A phylogenetic tree based on the Slt2 amino acid sequences from different fungi was constructed using the MEGA6 software package (Tamura et al., 2011).

2.3. Disruption of the genes encoding AoSlt2 and MhSlt2

Slt2 gene replacement segments for *A. oligospora* and *M. haptotylum* were generated using a modified yeast cloning procedure (Colot et al., 2006; Park et al., 2011). Briefly, sequences with homology to the 5′ and 3′ regions of the target genes were amplified using primer sets AoSlt2-5f/AoSlt2-5r or MhSlt2-5f/MhSlt2-5r and AoSlt2-3f/AoSlt2-3r or MhSlt2-3f/MhSlt2-3r (Supplementary Table S1), respectively, using *A. oligospora* and *M. haptotylum* genomic DNA as template. The hygromycin resistance gene cassette (*hph*) was amplified from plasmid pCSN44 using primers hph-F and hph-R (Staben et al., 1989). The three DNA fragments along with a pRS426 backbone (Christianson et al., 1992) (digested with *Eco*RI and *Xho*I) were then transformed into *S. cerevisiae* strain FY834 (Winston et al., 1995) via electroporation. *A. oligospora* and *M. haptotylum* protoplasts were then generated according to previous reports (Tunlid et al., 1999; Zhao et al., 2014), and the newly recombined vectors, AoSlt2-hph-pRS426 and MhSlt2-hph-pRS426, were transformed into *A. oligospora* and *M. haptotylum*, respectively, using a protoplast-based protocol (Tunlid et al., 1999; Zhao et al., 2014). The transformants were cultured in regenerative PDAS medium supplemented with 200 μg/mL hygromycin B (Amresco, Solon, OH, USA) (Zhang et al., 2008; Zhao et al., 2014). Putative transformants were verified by PCR analysis using primers specific for each gene (Y-F and Y-R) (Supplementary Table S1), and the positive transformants were further confirmed by Southern blot hybridization using a North2South Chemiluminescent Hybridization and Detection Kit as per the manufacturer's instructions (Pierce, Rockford, IL, USA). Primer pairs AoSlt2-F/AoSlt2-R and MhSlt2-F/MhSlt2-R (Supplementary Table S1) were used to prepare the Southern hybridization probes, and restriction enzymes *Sac*I and *Pvu*II were used to digest *A. oligospora* and *M. haptotylum* genomic DNA, respectively, for Southern analysis.

2.4. Comparison of mycelial growth and conidial yield

To compare the growth of the WT and corresponding mutant strains under different nutritional conditions, 7-mm-dameter hyphal discs punched from the edges of plate colonies were attached to PDA plates, followed by 6 days of incubation at 28 °C (Zhao et al., 2014). Colony discs from strain were then inoculated onto PDA, TG, TYGA, and CMY agar plates (Yang et al., 2018) and incubated at 28 °C for 5–10 days. The growth rates and colony morphologies of each strain on the different media were quantified and compared. The colonies of each strain initiated as aforementioned were also incubated on CMY plates for 14 days at 28 °C. The conidial yield of each strain was then measured as described previously (Jiang et al., 2017; Zhao et al., 2014). To assay conidial germination rates, 50-μL aliquots of conidial suspension from each of the WT strains were incubated on water agar (WA) plates at 28 °C, and the numbers of germinated conidia were determined at 4 and 12 h post-inoculation (Zhao et al., 2014). These experiments were performed in triplicate.

2.5. Stress tolerance test

To examine the effects of the gene knockouts on resistance to environmental stresses, the colonies of each strain initiated with 7-mm-dameter hyphal discs were incubated at 28 °C for 5–10 days on the plates of PDA alone (control) or supplemented with each of chemical stressors: (i) NaCl (0.1, 0.2, 0.3 mol/L) or sorbitol (0.25, 0.50, 1.0 mol/L) for osmotic stress; (ii) SDS (0.005%, 0.01%, 0.02%) and Congo red (1.5, 2.0, 2.5 mg/mL) for cell wall perturbation; and (iii) H_2O_2 (5, 10, 15 mmol/L) or menadione (0.01, 0.03, 0.05 mmol/L) for oxidative stress (Chen et al., 2014; Liu et al., 2017a, 2017b). Relative growth inhibition (RGI) as a result of chemical stress was calculated using the equation $(Sc - St)/(Sc - d) \times 100$, where Sc and St denote the areas of the stressed and unstressed (control) colonies, respectively, and d is the constant area of the hyphal mass discs used to initiate colonies (Chen et al., 2014; Liu et al., 2017b). These experiments were performed in triplicate.

2.6. Protease activity assays

Hyphal cells and conidia from the colonies of the WT and mutant strains were suspended in PL-4 broth and incubated at 28 °C by shaking at 200 rpm (Yang et al., 2005). The fermentation liquid was collected