Fungal Genetics and Biology 49 (2012) 347–357

Contents lists available at SciVerse ScienceDirect

Fungal Genetics and Biology

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

A fungal cell wall integrity-associated MAP kinase cascade in *Coniothyrium minitans* is required for conidiation and mycoparasitism

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ARTICLE INFO

Article history:
Received 1 August 2011
Accepted 27 February 2012
Available online 8 March 2012

Keywords:
Coniothyrium minitans
MAP kinase cascade
Cell wall integrity
Conidiation
Mycoparasitism
Sclerotinia sclerotiorum

ABSTRACT

Coniothyrium minitans is an important biocontrol agent against Sclerotinia diseases. Previously, a conidiation-deficient mutant ZS-1T1000 was screened out from a T-DNA insertional library of *C. minitans*. *CmBCK1*, encoding MAP kinase kinase kinase and homologous to *BCK1* of *Saccharomyces cerevisiae*, was disrupted by T-DNA insertion in this mutant. Targeted disruption of *CmBCK1* led to the mutants undergoing autolysis and displaying hypersensitivity to the cell wall-degrading enzymes. The △*CmBCK1* mutants lost the ability to produce pycnidia and conidia compared to the wild-type strain ZS-1. △*CmBCK1* mutants could grow on the surface of sclerotia of *Sclerotinia sclerotiorum* but not form conidia, which resulted in much lower ability to reduce the viability of sclerotia of *S. sclerotiorum*. Furthermore, *CmSlt2*, a homolog of *Slt2* encoding cell wall integrity-related MAP kinase and up-regulated by *BCK1* in *S. cerevisiae*, was identified and targeted disrupted. The △*CmSlt2* mutants had a similar phenotype to the △*CmBCK1* mutants. The △*CmSlt2* mutants also had autolytic aerial hyphae, hypersensitivity to cell wall-degrading enzymes, lack of conidiation and reduction of sclerotial mycoparasitism. Taken together, our results suggest that *CmBCK1* and *CmSlt2* are involved in conidiation and the hyperparasitic activities of *C. minitans*.

1. Introduction

Sclerotinia sclerotiorum is a worldwide fungal pathogen causing serious diseases on many economically important crops, such as oilseed rape (*Brassica napus*), soybean (*Glycine max*) and many vegetable crops (Boland and Hall, 1994; Bolton et al., 2006). *Coniothyrium minitans*, a sclerotial parasite of *S. sclerotiorum*, has the potential to control sclerotia diseases (Whipps and Gerlagh, 1992; Li et al., 2006; Whipps et al., 2008). *C. minitans* is a soilborne fungus; it parasites sclerotia of *Sclerotinia* spp. and produces pycnidia and conidia on them. Conidia releases into the soil are important for survival and dissemination. Bennett et al. (2006) indicated that the infected sclerotia of *S. sclerotiorum* could provide a reservoir for survival of *C. minitans* in soil.

Abundant conidial production is very important to enhance the potential of *C. minitans* as a biological control agent. Conidiation development in *C. minitans* is divided into five stages, from the hyphal growth stage to the pycnidial maturation stage (Gong et al., 2007; Li et al., 2010). The system of *C. minitans* and its host *S. sclerotiorum* is a good model system to examine the interaction between mycoparasites and their fungal hosts. A better understanding of the signaling pathways that regulate conidiation and parasitism of *C. minitans* can facilitate manipulation of the biocontrol agent for commercial use, and advance our knowledge of fungal biology.

Mitogen-activated protein (MAP) kinase is a family of serine/threonine protein kinases, involved in the transduction of various extracellular signals and the regulation of different developmental processes (Zhao et al., 2007). In the yeast *Saccharomyces cerevisiae*, there are five MAP kinase signaling pathways involved in mating, invasive growth, cell wall integrity, ascospore formation and responses to osmotic stress (Gustin et al., 1998). In *Magnaporthe oryzae*, three MAP kinases have been identified, including Pmk1, Osm1 and Mps1, which are homologous to Fus3/Kss1, Hog1, and Slt2 of *S. cerevisiae*, respectively (Xu, 2000). The MAP kinase kinase kinase MCK1 and MAP kinase Mps1 are involved in the appressorium-mediated penetration process and regulation of conidiation (Jeon et al., 2008; Xu et al., 1998). In *Botrytis cinerea*, the Δ*bmp3* mutants showed strongly impaired conidiation and have lost the ability to form sclerotia (Rui and Hahn, 2007). In *Claviceps purpurea*, the △*cpmk2* mutants could not produce conidia on complete media

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http://dx.doi.org/10.1016/j.fgb.2012.02.008

(Mey et al., 2002). In *Colletotrichum lagenarium*, the △*maf1* mutants show a significant reduction in conidiation (Kojima et al., 2002). In *Fusarium graminearum*, the *MGV1* gene is dispensable for the conidiation process, but essential for female fertility during sexual reproduction (Hou et al., 2002). In *Mycosphaerella graminicola*, *MgSlt2* is dispensable for conidiation but required for pycnidial formation (Mehrabi et al., 2006). Thus MAP kinase pathways are involved in many cellular developmental processes including conidiation in fungi and pathogenicity in fungal pathogens and their function may be variable in different fungi.

To study the genes involved in conidiation and mycoparasitizing ability of *C. minitans*, we had previously constructed a T-DNA insertional mutagenesis library using *Agrobacterium tumefaciens*-mediated transformation (ATMT), and screened out several conidiation-deficient mutants (Li et al., 2005). A conidiation-related gene *CMCPS1* has been cloned from the conidiation- deficient mutant ZS-1T2029, indicating that L-arginine is essential for conidiation in *C. minitans* (Gong et al., 2007). Recent findings showed that the cGMP signaling pathway is involved in NO-mediated conidiation in *C. minitans* (Li et al., 2010). We also found that phosphoribosylamidotransferase, the first enzyme in purine de novo synthesis, is required for conidiation in *C. minitans* (Qin et al., 2011). Here, we describe the isolation and functional analysis of another conidiation-related gene, *CmBCK1*, encoding a putative MAP kinase kinase kinase. The *CmBCK1* is homologous to the *BCK1* in *S. cerevisiae*. The *BCK1* is involved in maintaining cell wall integrity (CWI) in *S. cerevisiae*, and the cell wall integrity signaling pathway is well conserved among different fungi (Rispail et al., 2009). We also investigated the functions of *CmSlt2*, an ortholog of *Slt2*, which is a MAP kinase downstream of the CWI signaling pathway in yeast. The results of the targeted disruption and complementation of disruption mutants indicate that the CWI signaling pathway plays an essential role in the conidiation and mycoparasitism of *C. minitans*.

2. Materials and methods

2.1. Strains and culture conditions

A wild-type strain of *C. minitans* ZS-1 (CCAM 041057) isolates in Cheng et al. (2003) which regularly produces pycnidia and conidia, was used throughout this study. ZS-1T1000 is a conidiation-deficient mutant obtained from a T-DNA insertional library of wild-type strain ZS-1 (Li et al., 2005). *S. sclerotiorum* strain Ep-1PNA367, a normal virus-free strain, derived from a hypovirulent strain Ep-1PN by single ascospore isolation (Xie et al., 2006), was used to examine the mycoparasitizing ability of mutants of *C. minitans*. All strains were cultured on potato dextrose agar (PDA) at 20–22 °C and stored in PDA slants at 4 °C.

2.2. Microscopic observation of the colony morphology and mycoparasitism of C. minitans

Pycnidial development and autolytic hyphae of the mutants were observed by scanning electron microscopy (SEM Model JSM-6390/LV, NTC, Japan). The mutants were cultured on top of cellophane membranes on PDA at 20–22 °C for 14 days, and then the mycelia accompanied with cellophane was cut into 5 mm × 10 mm with scalpel. The samples were then fixed with 1% OsO_4 for 2 h, placed on the sample holder directly, and sputter coated with platinum (Model JFC-1600, NTC, Japan), the images were observed by SEM at acceleration voltage 10 kV. For the observation of mycoparasitism of *C. minitans*, the sclerotia infected by *C. minitans* for 30 days were bisected with a scalpel, and then observed under the dissection microscope (Leica S8APO, Germany) directly. The bisected sclerotia were further observed under SEM (sample preparation same as above).

2.3. DNA manipulation and Southern blot analysis

Vegetative hyphae of the mutants and wild-type strain ZS-1 which harvested 4 days after incubation on cellophane membranes in PDA plates were used for the genomic DNA isolation. Genomic DNA extraction was performed with the CTAB method (Sambrook and Russell, 2001). Plasmid DNA preparation, enzymatic manipulations of DNA and Southern blotting were carried out according to standard protocols (Sambrook and Russell, 2001).

For Southern blot analysis of T-DNA insertion in ZS-T1000 and copy number of *CmBCK1* in *C. minitans*, the protocols were performed according to Gong et al. (2007) with minor modifications. For Southern blot analysis of △*CmBCK1* mutants and △*CmSlt2* mutants, genomic DNA from ZS-1 and the mutants were digested with *Sac*I and *Pst*I, respectively. The nylon membranes were hybridized with probe P1 for *CmBCK1* and P2 for *CmSlt2*. The nylon membranes were autoradiographed and analyzed by using Bio-imaging analyzer BAS-1800II (FUJIFILM, Tokyo, Japan).

2.4. RNA manipulation and expression analysis

Total RNA was extracted from hyphae harvested after 48 h, 60 h, 72 h, 84 h, 96 h, 108 h and 120 h of incubation on PDA by using TRIzol® Plus RNA Purification Kit (Invitrogen, USA), and potential DNA contamination was removed by DNaseI treatment (RNase Free) (TaKaRa, Dalian, China) according to the manufacturer's instructions. First-strand cDNA was synthesized by using RevertAidTM First strand cDNA Synthesis kit (MBI, Fermentas, USA) following the manufacturer's instructions.

Expression of *CmBCK1* and *CmSlt2* was assessed at different time points using Reverse-Transcription Polymerase Chain Reaction (RT-PCR). Equal amounts (1.0 μl) of first-strand cDNA were used as the template for the PCR reaction. For the *CmBCK1*, a 204 bp fragment was amplified with gene-specific primers 1000CKSP and 1000CKRP (Table S1). For the *CmSlt2*, a 106 bp fragment was amplified with gene-specific primers SltexSP and SltexRP (Table S1). The *C. minitans* actin gene *CmACTIN* amplified with primers ActinFP and ActinRP (Table S1) was used as the reference gene. PCR conditions used 25 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min, and with a final extension at 72 °C for 5 min. PCR reactions were run on a PTC-200 DNA Engine Peltier Thermal cycler (BIO-RAD, USA).

For the detection of relative expression of *CmBCK1* and *CmSlt2* under the treatment of CWI inhibitors and *S. sclerotiorum* with Real-time PCR, 10^6 conidia of the wild-type strain ZS-1 were inoculated on top of cellophane membranes in PDA at 20 °C for 48 h, the germinated conidia were transformed to the PDA amended with CWI inhibitors and 2 days old mycelia of *S. sclerotiorum*, respectively. After incubation for 0 h, 4 h, 12 h and 24 h, then collected the mycelia of *C. minitans*. Total RNA were extracted from these predicated time-point mycelia and synthesized the first cDNA strand. The primers (Table S1) used in this study are: Bck-rt1 and Bck-rt2 for *CmBCK1* (111 bp), Slt-rt1 and Slt-rt2 for *CmSlt2* (168 bp), CmACT289 and CmACT419 for the reference gene *CmACTIN* (131 bp). Real-time PCR was conducted on CFX96™ Real-Time System (C1000™ Thermal cycler, Bio-Rad, USA).

2.5. Amplification of flanking sequences and cloning of related genes

To obtain the sequences flanking the T-DNA insertion site, we used genomic DNA of ZS-1T1000 as template in Thermal Asymmetric Interlaced Polymerase Chain Reaction (TAIL-PCR) following Mullins et al. (2001). Subsequent protocols were carried out