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Insight into the transcriptional regulation of Msn2 required for conidiation, multi-stress responses and virulence of two entomopathogenic fungi

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ABSTRACT

Msn2/4 transcription factors in some fungi have null effects on virulence and cellular stress responses. Here we found that the transcriptional regulation of Msn2 orthologs is vital for the conidiation, virulence and multi-stress responses of *Beauveria bassiana* (Bb) and *Metarhizium robertsii* (Mr), which lack Msn4 orthologs. Compared to wild-type and complemented strains of each fungus with all similar phenotypes, *ΔBbmsn2* and *ΔMrmsn2* showed remarkable defects in conidial yield (~40% decrease) and virulence (~25% decrease). Both delta mutants lost 20–65% of their tolerances to hyperosmolarity, oxidation, carbendazim, cell wall perturbing and high temperature at 34 °C during colony growth. Their conidia were also significantly (18–41%) less tolerant to oxidation, hyperosmolarity, wet-heat stress at 45 °C and UV-B irradiation. Accompanied with the defective phenotypes, several conidiation- and virulence-associated genes were greatly repressed in *ΔBbmsn2* and *ΔMrmsn2*. Moreover, differentially expressed genes in the transcriptomes of *ΔBbmsn2* versus wild type were ~3% more under oxidative stress, but ~12% fewer under heat shock, than those in the *ΔMrmsn2* counterparts. Many stress-responsive effector genes and cellular signaling factors were remarkably downregulated. Taken together, the two entomopathogens could have evolved somewhat distinct stress-responsive mechanisms finely tuned by Msn2, highlighting the biological significance of Msn2 orthologs for filamentous fungi.

1. Introduction

Beauveria bassiana and *Metarhizium anisopliae* are classic biocontrol agents of insect pests (Feng et al., 1994; Lomer et al., 2001; Roberts and St Leger, 2004). Such entomopathogenic fungi starts infection cycle from conidial adhesion to host cuticle, followed by germination and penetration into host hemocoel, where fungal cells are rapidly propagated by budding until host death, and the cycle is terminated by the production of conidia on cadaver surface under appropriate conditions. Since unicellular conidia produced on solid substrates are the active ingredients of conventional myconsecticides (de Faria and Wraight, 2007), their responses to outdoor stresses, such as high temperature and solar UV irradiation, are of special concern for the success of a fungal formulation applied in insect control.

The transcriptional regulation of effector genes in eukaryotic cells is one of fundamental mechanisms involved in cellular responses to stressful stimuli. This mechanism requires not only the activation of the signaling cascades of mitogen-activated protein kinases (MAPKs), MAPK kinases (MAPKKs) and MAPKK kinases (MAPKKKs) (Miskei et al., 2009; Rispail et al., 2009; Saito and Tatebayashi, 2004) but also the binding of the functionally redundant zinc-finger transcription factors (TFs) Msn2/4 to stress-responsive elements (STREs) (Gasch et al., 2000; Görner et al., 1998; Martinez-Pastor et al., 1996; Roetzer et al., 2008; Schmitt and McEntee, 1996). Such TFs are known to involve in the regulation of mating and mating-type switching and of chronological aging-independent replicative life span in yeasts (Barsoum et al., 2011). STREs (CCCCT), often existing in two or more copies in the promoter regions of Msn2/4, can be recognized by C-terminal Cys_2His_2 Zinc-finger DNA-binding domains and enable to activate the transcription of downstream genes under stressful conditions (Martinez-Pastor et al., 1996; Schmitt and McEntee, 1996). Activation and shuttling of Msn2/4 are controlled by the phosphorylation and dephosphorylation of nuclear localization signal (import) and nuclear export signal respectively. Msn2/4 are usually localized in cytoplasm but become phosphorylated, accumulated and transloacted to nucleus within a few minutes under a stress (Görner et al., 1998). This nuclear translocation is controlled by protein kinase A (PKA) activity (Görner et al., 1998, 2002; Garmendia-Torres et al., 2007; Hasan et al., 2002) and rapamycin (TOR) signaling pathway (Beck and Hall, 1999; Santhanam et al., 2004). Other signaling pathways may also affect the activity of Msn2/4, including

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the Snf1 protein kinase pathway (De Wever et al., 2005; Mayordomo et al., 2002) and the high-osmolarity glycerol (HOG) pathway (Rep et al., 2000).

Although well characterized in model yeast, the roles of Msn2/4 in regulating cellular responses to stressful cues remain poorly understood for filamentous fungi and are not known at all for entomopathogenic fungi. Previously, Msn2 and Msn4 orthologues were found taking no obvious parts in the stress responses of *Candida albicans*, a human pathogenic fungus (Nicholls et al., 2004). Recently, we found only Msn2 orthologs (Msn4 absent) in the genome databases of *B. bassiana* (Xiao et al., 2012) and *Metarizium robertsii* (separated from *M. anisopliae* sensu latu) (Gao et al., 2011). This study sought to characterize the functions of *B. bassiana* Msn2 (Bbmsn2) and *M. robertsii* Msn2 (Mrmsn2) with an emphasis being placed upon a comparison of their regulative effects on the genomic and phenotypic expression of the two fungal entomopathogens. We found that *Bbmsn2* and *Mrmsn2* knockouts resulted in similar phenotypic defects in conidiation, multi-stress tolerances and virulence but distinguished transcriptomic profiles under oxidative and thermal stresses.

2. Materials and methods

2.1. Microbial strains and culture media

The wild-type strains *B. bassiana* ARSEF 2860 (Bb2860 or BbWT herein) and *M. robertsii* ARSEF 2575 (Mr2575 or MrWT) were cultured on SDAY (Sabouraud dextrose agar plus 1% yeast extract) at 25 °C. *Escherichia coli* Top10 and *E. coli* DH5α from Invitrogen (Shanghai, China) were cultured at 37 °C in Luria–Bertani broth plus kanamycin (100 μg/ml) for vector propagation. *Agrobacterium tumefaciens* AGL-1 used for fungal transformation was cultured in YEB medium (Fang et al., 2004) at 28 °C.

2.2. Disrupting and rescuing Bbmsn2 and Mrmsn2

The 5′ and 3′ flanking regions of *Bbmsn2* (1240 and 1232 bp) and *Mrmsn2* (1534 and 1408 bp) were separately amplified from BbWT and MrWT via PCR with La*Taq* DNA polymerase (TaKaRa, Dalian, China) and paired primers (Table 1) designed on the basis of their open reading frames (1467 and 1593 bp) found in the annotated genomes of Bb2860 (Xiao et al., 2012) and *M. robertsii* (previous *M. anisopliae*) ARSEF 23 (Gao et al., 2011). The deduced Bbmsn2 and Mrnsb2 proteins consist of 488 and 530 amino acids (GenBank IDs: EJP70102.1 and EFY98585.1) and share the sequence identities of 70–100% and 78–100% with other 56 fungal Msn2 orthologs in the NCBI protein database respectively. The amplified flanking regions were inserted into the *Bam*HI/*Hin*dIII and *Xho*I/*Bgl*II sites of p0380-bar (Xie et al., 2013; Zhou et al., 2012) vectoring phosphinothricin resistant *bar* marker, forming the disruption plasmid p0380-*x*up-bar-*x*dn (*x*: *Bbmsn2* or *Mrmsn2*). To rescue each target gene, the full-length sequences (4050 and 4950 bp in total) of *Bbmsn2* and *Mrmsn2* with the flanking regions were separately amplified from the two WT strains with paired primers (Table 1) and ligated into p0380-sur-gateway (Xie et al., 2013; Zhou et al., 2012) to exchange for the gateway fragment under the action of Gateway® BP ClonaseTM II Enzyme Mix (Invitrogen), resulting in the complement plasmids p0380-sur-Bbmsn2_4050 and p0380-sur-Mrmsn2_4950.

The constructed plasmids were transformed into the corresponding WT and delta mutant strains, respectively, via *A. tumefaciens* AGL-1-mediated transformation (Fang et al., 2004). Colonies grown for 5 or 6 days on selective medium at 25 °C were screened in terms of the *bar* resistance to phosphinothricin (200 μg/ml) or the *sur* resistance to chorimuron ethyl (10 μg/ml) and then sequentially identified via PCR and Southern blotting with paired primers and amplified probes (Table 1). Positive knockout mutants, *ΔBbmsn2* and *ΔMrmsn2*, were evaluated together with parental WT and complemented *ΔBbmsn2*/*Bbmsn2* and *ΔMrmsn2*/*Mrmsn2* mutants (control strains) in the following triplicate experiments.

2.3. Assessment of conidiation under normal conditions

For all WT and mutant strains, 100 μl aliquots of conidial suspension (1×10^7 conidia/ml 0.02% Tween 80; the same below unless mentioned otherwise) were evenly spread onto SDAY plates. After 8-day incubation at 25 °C and 12:12 h (light:dark cycle), conidial yields were assessed by taking three colony disc samples (5 mm diameter) from each plate, washing each sample in 1 ml of 0.02% Tween 80 via 10 min vibration, determining the concentration of the conidial suspension with microscopic counts in hemocytometer, and converting the concentration to the number of conidia per cm^2 colony.

2.4. Assaying cell responses to chemical and environmental stresses

Small hyphal mass discs (5 mm diameter) were cut off from the cultures grown for 3 days at 25 °C on cellophane overlaid SDAY

Table 1
Paired primers used for the manipulation of *Bbmsn2* and *Mrmsn2* and the identification of their mutants.

Primers	Paired sequences (5′-3′) *	Purpose
Bm-F/R	ATGGAAGCTGCAATGCTGCA/TTAGTCGGTGCGCTTGCGCT	Cloning *Bbmsn2*
Mm-F/R	ATGGACTCAAACATGATGCC/TTATTCAGAACGCTTTCGCT	Cloning *Mrmsn2*
Bm1-F/R	AAAGGATCCCTGCTGCATCGACTTTGACT/CCCAAGCTTAGGGTGCGAAGCGATGTAGA	Cloning *Bbmsn2* 5′-end
Bm2-F/R	CCGCTCGAGCTCGACTCGGAGGATGATTT/GGAAGATCTAGACCAGCCGCGCCTTATGG	Cloning *Bbmsn2* 3′-end
Mm1-F/R	CCGGAATTCACCCCAGATACACTCCCGCT/CGCGGATCCTTGTGGTGCTGCTAAGCGAG	Cloning *Mrmsn2* 5′-end
Mm2-F/R	CCGCTCGAGACGGCCACAAGGATAAGCGC/GGAAGATCTGAAAAGGCGGTCTTGAATTG	Cloning *Mrmsn2* 3′-end
pBm-F/R	GAGGCTATACTCGACTTAGT/CGAGGAGCCGGTGCAAGCGC	PCR detection of *Bbmsn2*
sbBm-F/R	AAGTCCGATGCTCGCAAGGC/AGGGTGCGAAGCGATGTAGA	Southern blotting of *Bbmsn2*
pMm-F/R	GTTCCCTCGCTTAGCAGCAC/GCGCTTATCCTTGTGGCCGT	PCR detection of *Mrmsn2*
sbMm-F/R	ACCCCATTGAACCCCATGTT/GTGCTGCTAAGCGAGGGAAC	Southern blotting of *Mrmsn2*
Sur-F/R	TCATTGGCAAGACGGGAGGA/TGCTGCGCTAATAGAAGGAA	PCR detection of *sur*
rBm-F/R	*GGGGACCACTTTGTACAAGAAAGCTGGGT*TTTATTTCTTGGCCAGCCGC/*GGGGACAAG TTTGTACAAAAAAGCAGGCT*CTTTCCATGCCTCCGATACT	Rescuing *Bbmsn2*
rMm-F/R	*GGGGACCACTTTGTACAAGAAAGCTGGGT*TGGGCACCACATTTTGACG/*GGGGACAAG TTTGTACAAAAAAGCAGGCT*CGAGGAGATACACACACACA	Rescuing *Mrmsn2*

* Underlined regions: restriction enzyme sites. Italicized and underlined regions: fragments for gateway exchange.