Fungal Genetics and Biology 54 (2013) 42–51

Contents lists available at SciVerse ScienceDirect

Fungal Genetics and Biology

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

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

Qian Liu [a,b], Sheng-Hua Ying [a], Jin-Gen Li [b], Chao-Guang Tian [b], Ming-Guang Feng [a,*]

[a] Institute of Microbiology, College of Life Sciences, Zhejiang University, Hangzhou, Zhejiang 310058, People's Republic of China
[b] Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, People's Republic of China

ARTICLE INFO

Article history:
Received 14 December 2012
Accepted 21 February 2013
Available online 4 March 2013

Keywords:
Entomopathogenic fungi
Msn2 transcription factor
Regulation of gene expression
Stress responses
Virulence regulation
Transcriptomes

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 translocated 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

* Corresponding author. Fax: +86 571 88206178.
E-mail address: mgfeng@zju.edu.cn (M.-G. Feng).

1087-1845 © 2013 Elsevier Inc. Open access under CC BY-NC-ND license.
http://dx.doi.org/10.1016/j.fgb.2013.02.008

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.

plates, which were spread with 100 μl aliquots of conidial suspension to initiate uniform cultures. The hyphal discs were centrally attached onto the plates (90 mm diameter) of 1/4 SDAY (SDAY nutrients diluted to 1/4) supplemented with the gradient concentrations of menadione (0–6 mM), H_2O_2 (0–140 mM), NaCl (0–1.5 M), carbendazim (0–1.5 μg/ml) or Congo red (0–1.5 mg/ml), followed by 5-day incubation at 25 °C. For heat stress assay, the drug-fee plates inoculated with the same discs were incubated at 34 °C for 5 days. All colonies were cross-measured as their growth indices under a given stress.

Assays for conidial tolerances to oxidative and hyperosmotic stresses started from spreading 100 μl aliquots of conidial suspension onto 1/4 SDAY plates supplemented with H_2O_2 (0–8 mM) and NaCl (0–1 M) respectively. After 24 h incubation at 25 °C, germinated and non-germinated conidia were counted from three microscopic fields of each plate to estimate percent germination. Conidial thermotolerance of each strain was assessed by inserting glass tubes of 1 ml conidial suspension into hot water bath at 45 °C for up to 90 min and assessing percent germinations in samples pipetted every 15 min as described previously (Wang et al., 2012). Assay for conidial UV-B resistance began from preparing conidial suspension in germination broth (2% sucrose and 0.5% peptone in 0.02% Tween 80) and spotting 10 μl aliquots centrally onto marked area (~10 mm diameter) of glass slides. Dried in air for ~10 min, the spotted slides were exposed to the irradiation of the weighted wavelength of 312 nm (280–320 nm) at the gradient doses of 0.1–1 J/cm^2 (plus zero dose as control) in Bio-Sun^{++} UV chamber (Viber Lourmat, Marne-la-Vallée, France). After exposure, the slides were incubated for up to 30 h at 25 °C under saturated humidity, followed by assessment of percent germinations as above.

The ratio of colony size or percent germination of each strain under a given stress over that in the unstressed control was defined as relative viability (V_r). The V_r trends over the concentrations (C) of each stressful chemical, the doses (D) of the UV-B irradiation, and the time lengths (T) of the heat stress at 45 °C were fitted to the equation $V_r = 1/[1 + \exp(a + bx)]$, where x denotes C, D or T. When $V_r = 0.5$, the fitted equations gave solutions ($-a/b$) to the time length required for the effective concentration (EC_{50}) of each chemical to suppress 50% colony growth or conidial germination, and the median lethal dose (LD_{50}) of the UV-B irradiation and the median lethal time (LT_{50}) of the heat stress against conidia. Relative growth inhibition (RGI) of colony after 5-day stressful incubation at 34 °C was calculated as $(C - N)/(C - 5) \times 100$ (Zhou et al., 2012), where C and N are the diameters of the control and stressed colonies of each strain, respectively. All the estimates from three repeated assays were differentiated among the WT and mutant strains of each fungus by one-way analysis of variance (ANOVA).

2.5. Bioassay of fungal virulence

All the WT and mutant strains of *B. bassiana* and *M. roberstii* were bioassayed for their virulence to *Spodoptera litura* second-instar larvae in a cabbage leaf disc system (Xie et al., 2013) and *Tenebrio molitor* third-instar larvae in a wheat bran system (Zhou et al., 2012), respectively. Briefly, batches of 30–40 larvae on cabbage leaf discs (for *S. litura*) and in lid-free Petri dishes (for *T. molitor*) were separately sprayed with 1 ml of conidial suspension (treatment) or 0.02% Tween 80 (control) in an Automatic Potter Spray Tower (Burkard Scientific Ltd., Uxbridge, UK) at uniform working pressure. After spray, all larvae were reared for 7 or 8 days (longer for *T. molitor*) at 25 °C and 12:12 h on leaf discs (renewed daily) or wheat bran in Petri dishes for their feeding and monitored daily for mortality records. The resultant mortality trends over the post-spray days were differentiated by probit analysis to generate median lethal time (LT_{50} in day) estimates of all tested strains against *S. litura* or *T. molitor* larvae in three repeated assays, followed by one-way ANOVA.

2.6. Assaying transcript levels of conidiation and virulence associated genes

Aliquots of 100 μl conidial suspension were spread onto cellophane attached SDAY plates and incubated for 4 days at 25 °C and 12:12 h. Total RNAs were extracted from the cultures of WT and mutant strains with RNAisoTM Plus Reagent (TaKaRa) and translated into cDNAs with PrimeScript® RT reagent Kit (TaKaRa). Each cDNA (20 × dilution) was used as template to assess the transcript levels of 15 genes possibly associated with fungal conidiation and virulence via quantitative real-time PCR (qRT-PCR) with paired primers (Table S1) using the fungal 18S rRNA as internal standard and the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All qRT-PCR experiments with three cDNA samples of each strain were performed under the action of SYBR® Premix Ex Taq™ (TaKaRa). The relative transcript level of each gene was expressed as the ratio of its transcript in each mutant over that in each fungal WT.

2.7. Construction and analysis of Msn2-specific transcriptomes

Conidial suspension of each fungal WT or *Δmsn2* was added to 50 ml of Sabouraud dextrose broth (SDB) at the volume ratio of 1:10 and incubated by shaking (140 rpm) at 25 °C for 2-day growth to exponential phase. The hyphal cells from each culture were harvested by filtration, washed twice in sterile water, and resuspended in the equal volume of fresh SDB. The new suspension was then shaken for 3 h at 40 °C for heat shock or supplemented with menadione to the final concentration of 2 mM, followed by shaking for 3 h at 25 °C for oxidative stress. Subsequently, harvested cells were immediately homogenized in liquid nitrogen for total RNA extraction with Trizol reagent (Invitrogen). Total RNA samples from the stress treatments of each strain were purified with Qiagen RNeasy Mini kit plus on-column treatment with DNase I and then sequenced at Beijing Genomics Institute (Shenzhen, China) for constructing their digital gene expression (DGE) libraries (transcriptomes) by means of Illumina HiSeq™ 2000 platform.

All raw DGE reads were filtered to generate clean tags. SOAP2 program was used to map all the clean tags to the genomes of *B. bassiana* and *M. robertsii* under the NCBI accessions ADAH00000000 and ADNJ00000000, respectively, at the precision level of no more than two-base mismatching. Differentially expressed genes (DEGs) were located in the paired *Δmsn2* and WT DGE libraries of each fungus shocked by oxidation or heat based on the number of reads per kilobase of exon region per million mappable reads (RPKM). A minimum of twofold expressional difference in the paired libraries (i.e., $\log_2$ ratio $\leqslant -1$ or $\geqslant 1$) was used as a standard to judge each of the DEGs at the false discovery rate (FDR) of 0.001 or less (Audic and Claverie, 1997). All identified DEGs were functionally annotated with known or putative gene information in the non-redundant NCBI protein databases of the two fungi or expressed as 'hypothetical proteins' if no information is available at the cutoff error level of less than 10^{-5}. Those responsive to both oxidation and heat shock were hierarchically clustered with HCE3.5 software using the Euclidean Distance model (Seo et al., 2004). Mean distances between clustered genes were expressed as the distances between clusters. Gene ontology (GO) term enrichment analysis (http://www.geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis (http://www.genome.jp/kegg/) were performed for all the DEGs at the threshold of corrected $P < 0.05$. The binding motifs of potential TFs in the regions of ~1000 bp upstream of the predicted translation initiation sites of those

drastically repressed under the oxidative and thermal stresses were searched by means of online WebLogo program (http://weblogo.berkeley.edu).

3. Results

3.1. Knockout and complementation of Bbmsn2 and Mrmsn2

As indicated by PCR and Southern blotting (Fig. 1) with paired primers and amplified probes (Table 1), partial coding sequences of *Bbmsn2* (350 bp) and *Mrmsn2* (470 bp) were disrupted by inserting the *bar*-inclusive cassettes into BbWT and MrWT respectively via *Agrobacterium*-mediated transformation. The disrupted target genes were well rescued by integrating the *sur* cassettes of the full-length *Bbmsn2* (4050 bp) and *Mrmsn2* (4950 bp) sequences into their delta mutants. For each target gene, several pairs of knockout and complement mutants were obtained although only one pair was presented below.

3.2. Phenotypic changes in delta mutants

3.2.1. Growth and conidiation

All WT and mutant strains were similar in colony color and sizes (3.9–4.0 cm^2 per colony for *B. bassiana* and 4.5–4.7 cm^2 per colony for *M. robertsii*) after 8-day growth on SDAY at 25 °C. In contrast, conidial yields measured from the colonies of *ΔBbmsn2* [6.5 (±0.25) × 10^8 conidia/cm^2] and *ΔMrmsn2* [5.2 (±0.08) × 10^8 conidia/cm^2] under the normal culture conditions decreased by 43% and 39% compared to the mean yields of 11.5 (±0.20) × 10^8 and 8.3 (±0.15) × 10^8 conidia/cm^2 from the WT and complement strains (control strains) of the two fungi respectively. Apparently, *ΔBbmsn2* and *ΔMrmsn2* showed similar defects in conidiation.

3.2.2. Reduced cell tolerances to chemical and environmental stresses

Both *ΔBbmsn2* and *ΔMrmsn2* were consistently much more sensitive or less tolerant to different types of stresses than their control strains, which showed similar responses to all the stressful agents tested (Tukey's HSD, $P \geqslant 0.25$). Consequently, the EC_{50}, RGI, LT_{50} or LD_{50} estimates from the paired control strains were averaged as control means (CMs) to assess percent reductions in both delta mutants. During colony growth, *ΔBbmsn2* and *ΔMrmsn2* were 36% and 30% less tolerant to NaCl (CM: 1.88 and 0.89 M; Fig. 2A), 38% and 56% less to H_2O_2 (CM: 55.5 and 134.4 mM; Fig. 2B), 45% and 65% less tolerant to menadione (CM: 4.4 and 5.3 mM; Fig. 2C), 38% and 40% in tolerance to Congo red (CM: 1.05 and 0.94 mg/ml; Fig. 2D), and ~33% in tolerance to carbendazim (CM: 1.19 and 1.06 μg/ml; Fig. 2E), respectively. When exposed to 34 °C for 5 days, both delta mutants displayed ~20% less colony growth than the control strains (CM: 52.8% and 51.6%; Fig. 2F). On the other hand, conidial tolerances to NaCl (Fig. 2G) and H_2O_2 (Fig. 2H) were reduced by 29% and 18% in *ΔBbmsn2* (CM: 1.36 M and 5.6 mM) and 29% and 24% in *ΔMrmsn2* (CM: 1.20 M and 6.6 mM), respectively. When exposed to the wet-heat stress of 45 °C for up to 90 min, the LT_{50} for conidial thermotolerance (Fig. 2I) shortened 36% in *ΔBbmsn2* (CM: 50.9 min) and 30% in *ΔMrmsn2* (CM: 68.3 min). The LD_{50} for conidial UV-B resistance (Fig. 2J) decreased 42% and 31% in the two delta mutants (CM: 0.568 and 0.583 J/cm^2).

3.2.3. Conidial virulence

In the bioassays standardized by a uniform spray of conidial suspension in the spray tower, the LT_{50}s of *ΔBbmsn2* and *ΔMrmsn2* against *S. litura* second-instar larvae and *T. molitor* third-instar larvae (Fig. 2K) were 28% and 25% longer than the estimates from the control strains of *B. bassiana* (~5.5 days) and *M. robertsii* (~4.8 days) respectively, indicating significant virulence defects in both delta mutants.

All the phenotypic defects in two delta mutants indicate that Bbmsn2 and Mrmsn2 acted as positive regulators of conidiation, multi-stress responses and virulence in the two fungal pathogens.

3.3. Repressed transcripts of conidiation and virulence related genes

In the qRT-PCR analysis of cDNA samples with paired primers (Table S1), all examined conidiation-associated genes (*FlbA-D*, *FluG*, *Atg1*, *Atg8* and *Ras1*) were repressed by 33–90% in *ΔBbmsn2* versus BbWT and 34–80% in *ΔMrmsn2* versus MrWT but showed inconspicuous changes in either complement mutant (Fig. 2L). Among those associated with conidial adhesion to host cuticle (Cho et al., 2007; Wang and St Leger, 2007), two hydrophobin genes (*Hyd1* and *Hyd2*) were repressed by 66–68% in *ΔBbmsn2* and 50–54% in *ΔMrmsn2* but the expression of two adhesion protein genes (*Mad1* and *Mad2*) was less affected except 50% *Mad2* repression in *ΔMrmsn2*. Additionally, the transcripts of three chitinase genes likely involved in cuticle degradation were repressed by 56–85% in *ΔBbmsn2* and 60–79% in *ΔMrmsn2*. The drastic repressions of these genes in the normal cultures of both delta mutants were likely causative of their defects in conidiation and virulence.

3.4. Transcriptomes of delta mutants altered by oxidation and heat shock

Eight DGE libraries (NCBI GEO accession code: GSE40765) were constructed by sequencing total RNAs from the SDB cultures of *ΔBbmsn2*, *ΔMrmsn2* and their parental WT strains exposed for 3 h to the oxidative stress of 2 mM menadione at 25 °C and the

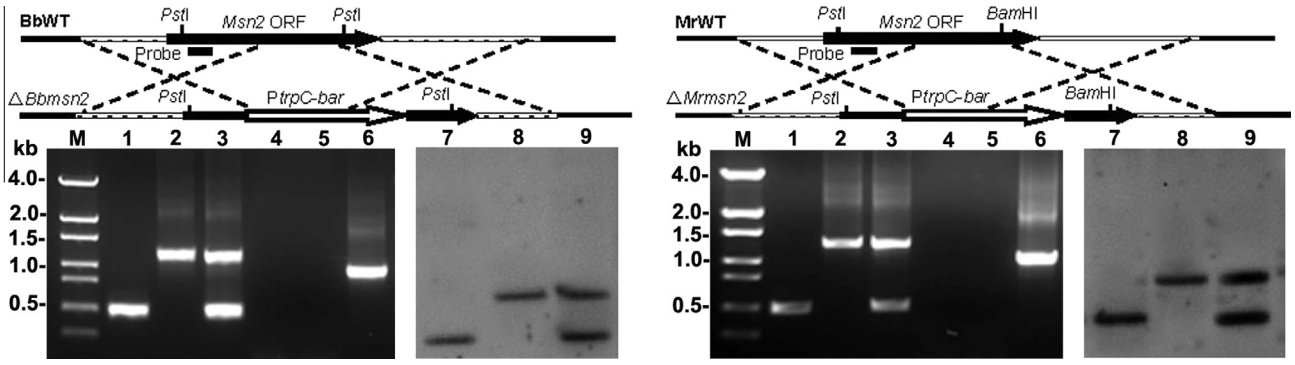

Fig. 1. Disruption of *Bbmsn2* and *Mrmsn2* from the wild-type strains *B. bassiana* (BbWT) and *M. robertsii* (MrWT). Mutants were identified via PCR (Lanes 1–6) and Southern blotting (Lanes 7–9). Lanes 1, 4, and 7: BbWT or MrWT. Lanes 2, 5 and 8: *ΔBbmsn2* or *ΔMrmsn2*. Lanes 3, 6, and 9: *ΔBbmsn2*/*Bbmsn2* or *ΔMrmsn2*/*Mrmsn2*.

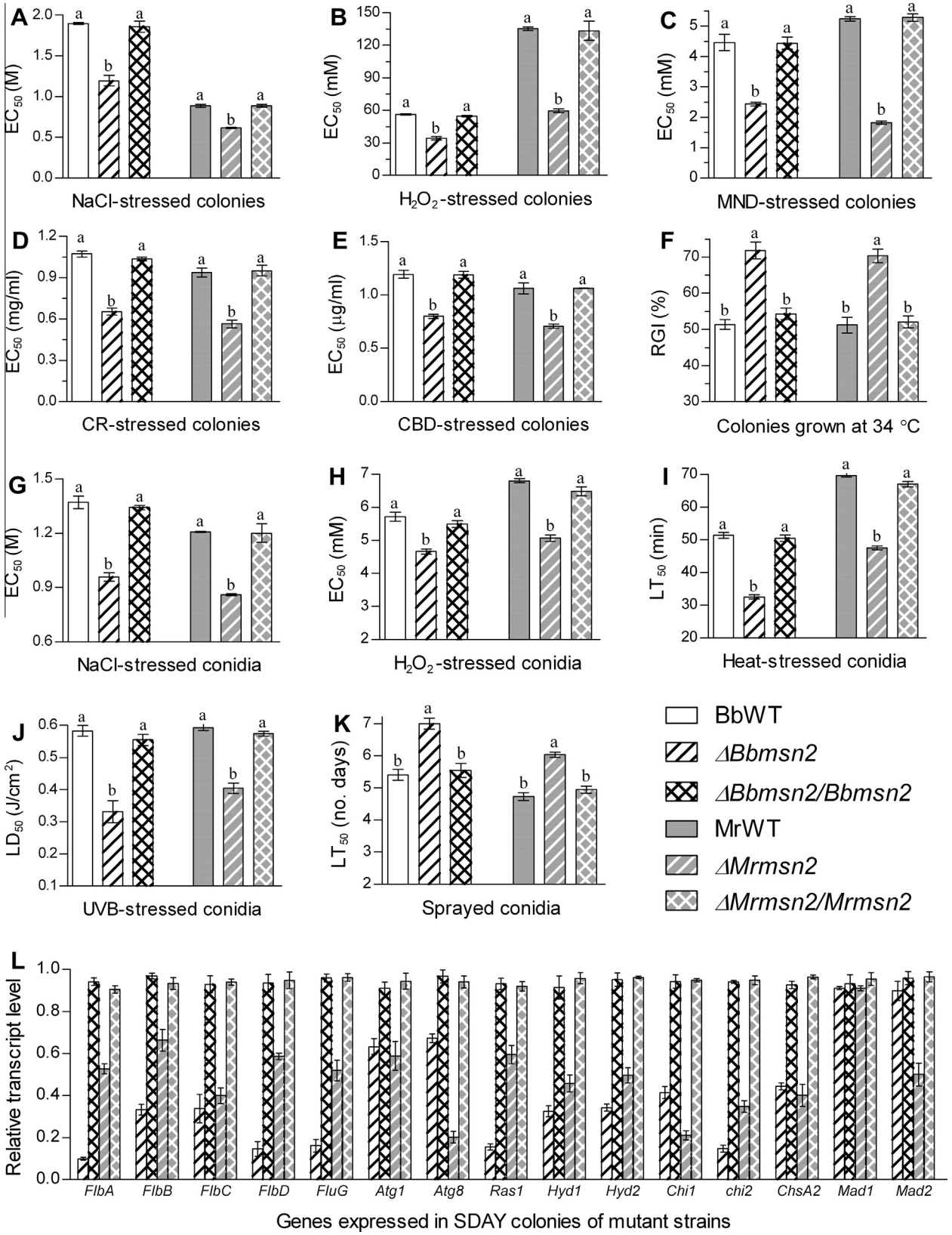

Fig. 2. Changes in multi-stress responses and virulence of *ΔBbmsn2* and *ΔMrmsn2* versus their parental WT and complemented mutants *ΔBbmsn2/Bbmsn2* and *ΔMrmsn2/Mrmsn2*. (A–E) Effective concentrations (EC_{50}s) for NaCl, H_2O_2, menadione (MND), Congo red (CR) and carbendazim (CBD) to suppress 50% colony growth on 1/4 SDAY after 5-day incubation at 25 °C respectively. (F) Relative growth inhibition (RGI) for colonies grown at 34 °C for 5 days. (G) and (H) EC_{50}s for NaCl and H_2O_2 to suppress 50% conidial germination at 25 °C. (I) Median lethal time (LT_{50}, min) of wet-heat stress (45 °C) against conidia. (J) Median lethal dose (LD_{50}, J/cm^2) of UV-B irradiation against conidia. (K) LT_{50} (no. days) estimates of fungal strains against *S. litura* and *T. molitor* larvae respectively. Different letters on the bars of each group denote significant differences (Tukey's HSD: $P < 0.05$). (L) Relative transcript levels of 15 conidiaton and virulence associated genes in mutant strains versus wild type. Error bars: SD of the mean from three repeated assays.

heat shock at 40 °C, respectively, by means of high throughput RNA-seq technique. As a result of mapping all clean tags to the annotated genomes of *B. bassiana* (Xiao et al., 2012) and *M. robertsii* (Gao et al., 2011), a large number of DEGs were found in the transcriptomes of *ΔBbmsn2* versus BbWT and *ΔMrmsn2* versus MrWT under either stress (Table 2) and most of them fell in 12 common function categories (Table 3).

The oxidative stress resulted in 970 DEGs in *ΔBbmsn2*. Of those, 212 were repressed and 267 were associated with molecular functions in terms of GO analysis. As revealed by KEGG analysis at $P < 0.05$, 169 DEGs were enriched in eight pathways such as metabolism of amino acids and nitrogen, glycan degradation, meiosis, and biosyntheses of unsaturated fatty acids and secondary metabolites (Table S2). The repressed genes were functionally classified to 17 categories and 36 of them showed $\log_2$ ratio changes from −13.73 to −1.52 (Table S3). Those drastically repressed in *ΔBbmsn2* were associated with antioxidation (cytosolic MnSOD and oxidoreductase), cell wall integrity (β-glucosidase, β-1,3-endoglucanase and cell wall-associated protein), microcycle conidiation (Mmc), and cellular signal transduction and gene transcription, such as OPT transporter, MFS transporter, transient receptor potential ion channel (TRPIC), SRF-type transcription factor, pH-response transcription factor and transferase. Many more genes repressed with $\log_2$ ratio ranging from −1.5 to −1 were functionally involved in detoxification, signal transduction and stress responses, including those encoding oxidase, oxidoreductase, cytochrome c1 heme lyase, NADPH oxidase, NADPH oxidase regulator, mannitol-1-phosphate dehydrogenase (MPD), regulator of G-protein signaling (RGS), and glycosylphosphatidylinositol (GPI) anchored serine-rich protein (Gas1p).

There were 603 DEGs in *ΔMrmsn2* under the same oxidative stress, including 159 repressed and 188 associated with molecular functions. In the KEGG pathway analysis, 411 DEGs were enriched in 21 metabolism and biosynthesis pathways of various amino acids, carbohydrates, secondary metabolites, vitamin B6 and other biochemical compounds at $P < 0.05$ (Table S4). All the repressed genes except those functionally unknown fell in 12 functional categories (Table S5). Those repressed by 3- to 21-fold were involved in stress response, detoxification, signal transduction, nitrogen metabolism and host infection, including six dehydrogenases, three MFS transporters, two oxidoreductases, acid trehalase, chitinase, adhesion protein Mad2, nitrate reductase, β-1,3-endoglucanase, glucose repressible protein (Grg1), and transferase.

Only were 275 DEGs found in *ΔBbmsn2* under the heat shock (Table 1) and 23 of them were enriched in four pathways (amino/nucleotide sugar metabolism, methane metabolism, peroxisome and phenylalanine, tyrosine and tryptophan biosynthesis) at $P < 0.05$ (Table S6). Sixteen of 90 downregulated genes were distributed in nine categories and repressed by 3- to 29-fold (Table S7). These repressed genes were involved in cellular stress responses, detoxification, conidiation, signal transduction, cell wall integrity, and protein metabolism because they encode cytosolic MnSOD, β-glucosidase, glucan 1,3-β-glucosidase, β-1,3-endoglucanase, Gas1p, Mmc, transcription initiation factor IID, C-14 sterol reductase, MFS transporter, TRPIC, cell wall ser-thr-rich galactomannoprotein Mp1, and ser/thr protein phosphatase.

Surprisingly, the same heat shock generated up to 1587 DEGs in *ΔMrmsn2*, including 708 genes downregulated by at least twofold. There were 256 DEGs enriched in the 10 pathways of DNA replication, mismatch repair, oxidative phosphorylation, base excision repair, ribosome, cell cycle, pyrimidine metabolism, nucleotide excision repair, homologous recombination, and caffeine metabolism at $P < 0.05$ (Table S8). All the downregulated genes with known functions were classified to 22 categories, including 96 genes repressed by at least fourfold (Table S9). These drastically repressed genes were involved in stress responses, carbohydrate/protein metabolism, detoxification, signal transduction, transportation, cell wall integrity, and gene transcription. Many more less repressed genes included those encoding five chitinases, seven ABC transporters, several cytochrome P450 proteins (CYPs), and three histidine kinases (HKs).

Hierarchical cluster analysis demonstrated that 75 DEGs in *ΔBbmsn2* were responsive to both oxidative and heat stresses and distributed in six major clusters with similar patterns of up- or downregulation (Fig. 3A). Clusters 1–3 included 31, 11 and 9 genes upregulated in *ΔBbmsn2* but repressed in BbWT while clusters 4–6 had 9, 3 and 6 genes repressed in *ΔBbmsn2* but upregulated in BbWT. These clustered genes were distributed in six and seven functional categories corresponding to oxidation (Fig. 3C) and heat shock (Fig. 3E) respectively. The genes significantly repressed in *ΔBbmsn2* were those associated with condiation, stress responses, signal transduction and carbohydrate metabolism because they code for cytosolic MnSOD, Gas1p, Mmc, β-1,3-endoglucanase, flocculin, β-glucosidase, TRPIC and 1,3-β-glucosidase. Responding to both oxidative and heat stresses, *ΔMrmsn2* had 166 DEGs distributed in six main clusters (Fig. 3B). Of those, 59 were upregulated in *ΔMrmsn2* but repressed in MrWT while 22 were repressed in *ΔMrmsn2* but upregulated in MrWT. All the 166 DEGs were functionally classified to nine categories in response to oxidation (Fig. 3D) or heat stress (Fig. 3F). Ten of the repressed genes were involved in stress responses, signal transduction, virulence and energy conversion, encoding ATP-binding endoribonuclease, CFEM domain protein, Gas1p, cysteine synthase B, glycerophosphoryl diester phosphodiesterase, iron transport multicopper oxidase, peptide transporter, restless-like transposase, Mad2, and nitrate reductase. WebLogo analysis indicated the existence of one or more STREs in the promoter regions of ~1000 bp upstream of all the DEGs repressed by both stresses (Fig. 3G).

All the RNA-seq data indicated that the oxidation and heat shock caused differential expression of ~9% and ~3% genes in *ΔBbmsn2* versus BbWT and of ~6% and ~15% genes in *ΔMrmsn2* versus MrWT respectively. Some of the repressed genes were likely attributable to the defective responses of *ΔBbmsn2* and *ΔMrmsn2* to different types of stressful stimuli.

Table 2
Distribution of differentially expressed genes (DEGs) in the DGE libraries of *ΔBbmsn2* and *ΔMrmsn2* versus each fungal WT under oxidative and thermal stresses..

DGE libraries	Stress*	Number of genes expressed in fold change					
		Upregulated			Downregulated		
		>2	>3	>4	>2	>3	>4
ΔBbmsn2 versus BbWT	Oxidative	758	420	273	212	72	35
	Thermal	185	49	18	90	35	17
ΔMrmsn2 versus MrWT	Oxidative	444	209	131	159	69	38
	Thermal	879	276	142	708	335	182

* SDB cultures (hyphal cells) exposed for 3 h to oxidative stress (2 mM menadione) at 25 °C or thermal stress at 40 °C after 2-day cultivation at 25 °C.

Table 3
Common functional categories of differentially expressed genes (DEGs) in the DGE libraries of *ΔBbmsn2* and *ΔMrmsn2* versus each fungal WT under oxidative and thermal stresses.

Functional category	Number of DEGs			
	Oxidative stress		Thermal stress at 40 °C	
	ΔBbmsn2 versus BbWT	*ΔMrmsn2* versus MrWT	*ΔBbmsn2* versus BbWT	*ΔMrmsn2* versus MrWT
Metabolic pathways	149	120	41	234
Biosynthesis of secondary metabolites	69	59	20	97
Amino acid metabolism	144	100	26	144
Peroxisome	16	12	7	18
Carbohydrate metabolism	69	62	15	82
MAPK signaling pathway	15	3	4	18
Fatty acid metabolism	15	14	4	12
Nitrogen metabolism	9	4	1	8
Transporters	46	25	17	50
Transcription factors	22	5	11	31
Meiosis	25	5	1	31
Cell cycle	17	2	3	50

4. Discussion

As presented above, Bbmsn2 and Mrmsn2 played similar roles in regulating the conidiation, virulence and multi-stress responses of *B. bassiana* and *M. robertsii* because *ΔBbmsn2* and *ΔMrmsn2* showed consistent phenotypic defects under normal and stressful conditions although the sizes and DEG counts of the DGE libraries were remarkably different between *ΔBbmsn2* and *ΔMrmsn2* under either oxidative or thermal stress. Phenotypic and transcriptomic alterations caused by the *Msn2* deletion are discussed below.

First of all, *ΔBbmsn2* and *ΔMrmsn2* showed similar defects in conidiation but little change in colony growth and morphology under normal culture conditions. The defective conidiation in both delta mutants is well in agreement with the repression of their eight genes (Fig. 2L) essential for conidiophore development and conidiation in many fungi (Arratia-Quijada et al., 2012; Etxebeste et al., 2009; Kikuma et al., 2007; Kwon et al., 2010; Lee and Adams, 1994; Seo et al., 2006; Xiao et al., 2010; Xie et al., 2013). Previously, the inactivation of Cag8 (RGS protein) orthologuous to *Aspergillus nidulans* FlbA, resulted in poor sporulation of *M. anisopliae* (Fang et al., 2007). Some SRF-like MADS box proteins and GPI-anchored serine-rich proteins were also found taking parts in fungal development (Nolting and Pöggeler, 2006; Ragni et al., 2007). In our transcriptomes, the factors Mmc, RGS, SRF, and/or Gas1p were remarkably repressed in *ΔBbmsn2* or *ΔMrmsn2*, indicating the control of their expression by Msn2 and the likelihood of their involvements in conidiation. Thus, Msn2 is required for the transcriptional regulation of the conidiation-associated genes in both fungi.

Moreover, *ΔBbmsn2* and *ΔMamsn2* became less tolerant to hyperosmolarity, oxidation, cell wall disturbance, carbendazim and high temperature during growth (Fig. 2A–F) and/or conidial germination (Fig. 2G–I). These phenotypic defects are well in accordance with the reduced multi-stress tolerances previously observed in yeast *msn2* delta mutants (Gasch et al., 2000; Hasan et al., 2002; Roetzer et al., 2008) but distinct from the null effect of Msn2 on the stress responses of *C. albicans* (Nicholls et al., 2004). Conidial UV resistance, an important parameter for the biocontrol potential of fungal entomopathogens, was also greatly reduced in *ΔBbmsn2* and *ΔMrmsn2*. All the phenotypic defects are strongly evident for the essential role of Msn2 in regulating the cellular responses of both fungi to chemical/environmental stresses. Our transcriptome analysis revealed possible involvements of many factors and signaling pathways in multi-stress responses, including various MAPK cascades, HKs, CYPs, MFS transporters, ABC transporters, MnSOD, MPD, trehalase, peroxisomal catalase, reductases and oxidases because oxidation and heat shock repressed their expression in *ΔBbmsn2* and *ΔMrmsn2*. These enzymes or proteins are known to take parts not only in multi-stress responses but also in xenobiotic transport, detoxification, signal transduction and carbohydrate metabolism (Enjalbert et al., 2006; Liesa et al., 2012; Miller et al., 2004; Miskei et al., 2009). For instance, MPD inactivation reduced *B. bassiana* tolerance to oxidation, heat and UV-B irradiation (Wang et al., 2012) and catalases played distinguished roles in regulating conidial thermotolerance and UV-B resistance of *B. bassiana* (Wang et al., 2013). Cytosolic and mitochodial MnSODs contributed additively to antioxidation and UV tolerance (Xie et al., 2012). Therefore, Msn2 is also required for the transcriptional regulation of those effector genes for the adaptation of the two fungi to the chemical/environmental stresses.

More interestingly, *msn2* deletion caused similar (25% and 28%) decrease in conidial virulence of both fungi (Fig. 2K). This is very different from the null effect of Msn2 on the virulence of *C. glabrata* (Roetzer et al., 2008). We examined transcript levels of some virulence-associated genes and found that two hydrophobin and three chitinase genes were remarkably repressed by *msn2* disruption (Fig. 2L). In the DGE libraries, the genes of hydrophobin, chitinases, Mad2, acid trehalase, MnSOD, MPD, HKs and cell wall-associated proteins were also repressed a great deal. Previously, inactivation of Mad2 (Wang and St Leger, 2007), hydrophobin (Zhang et al., 2011), MPD (Wang et al., 2012) or MnSOD (Xie et al., 2012) resulted in a significant reduction in the virulence of *B. bassiana* or *M. anisopliae*. The virulence of *B. bassiana* was linearly correlated to conidial antioxidant capability and UV resistance which varied with the activities of Ras1 and Ras2 GTPases (Xie et al., 2013). Thus, the repression of the virulence-associated genes and the decreased resistance to oxidation and UV-B are likely causative of the defective virulence of *ΔBbmsn2* and *ΔMamsn2*, indicating again a requirement of Msn2 for the full virulence of either fungus.

Finally, our transcriptomes provide genome-wide insight into the transcriptional regulation of Msn2 required for the responses of *B. bassiana* and *M. robertsii* to both oxidative and thermal stresses. Under the oxidative stress, ~3% more genes were differentially expressed in *ΔBbmsn2* than in *ΔMrmsn2* but those repressed by at least three- or fourfold in both delta mutants fell in common function categories and their counts were similar (Table 2). Under the heat shock, surprisingly, up to 12% more genes were differentially expressed in *ΔMrmsn2* than in *ΔBbmsn2*, showing greater diversity due to their enrichments in many more functional pathways. Previously, LT_{50} for conidial tolerance to the wet-heat stress of 48 °C differed from 20 to 150 min among host and geographically different strains of *M. anisopliae* sensu latu (Li and Feng, 2009). In this study, *M. robertsii* showed higher conidial thermotolerance than *B. bassiana* irrespective of knockout or control strains (Fig. 2I).

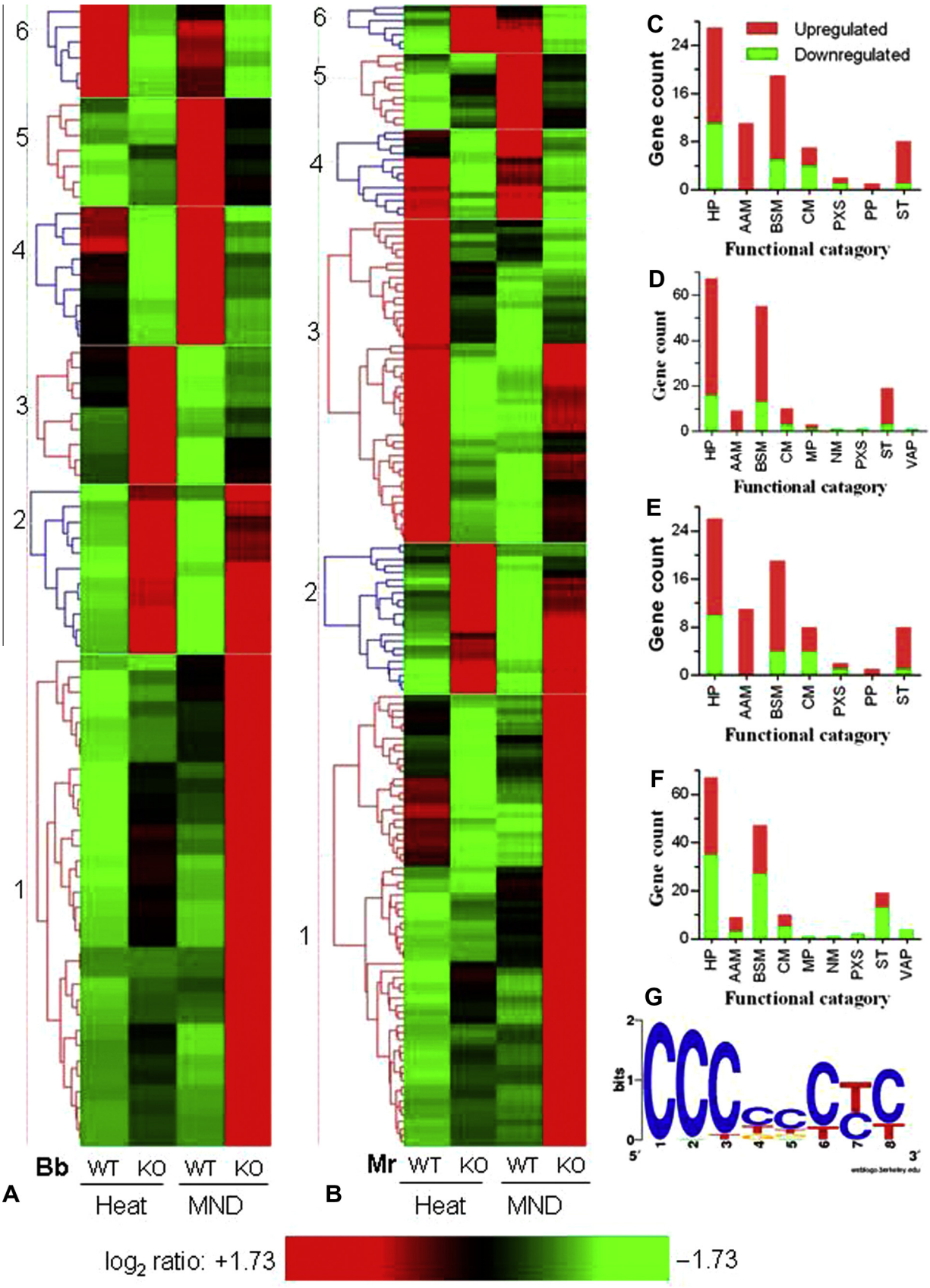

Fig. 3. Distribution of differentially expressed ($\geqslant$3.3-fold) genes in the *Msn2* knockout (KO) and wild-type (WT) strains of *B. bassiana* (Bb) and *M. robertsii* (Mr). (A and B) Hierachical clustering of differentially expressed genes (DEGs) generated from Bb and Mr responses to both menadione (MND) oxidation and heat shock respectively. (C and D) Counts of DEGs distributed in major function categories of Bb and Mr. under the oxidative stress respectively. (E and F) Counts of Bb and Mr DEGs distributed respectively in major function categories under the heat stress respectively. (G) Distribution of STRE-like sequences (located by WebLogo) in the promoter regions of the genes repressed by *Msn2* deletion. Abbreviations for functional categories: AAM (amino acid metabolism), BSM (biosynthesis of secondary metabolites), CM (carvohydrate metabolism), NM (nitrogen metabolism), MP (membrane proteins), PXS (peroxisome), PP (protein processing), ST (signal transduction), VAP (virulence associated proteins), and HP (hypothetic proteins).

Thus, mechanisms involved in conidial thermotolerance could be much more diverse and complex in *M. robertsii* than in *B. bassiana*. Interestingly, the counts of DEGs involved in the MAPK pathways of *ΔBbmsn2* and *ΔMrmsn2* were 15 and 3 under the oxidative stress but 4 and 18 under the heat shock respectively. Taken together, the two fungal entomopathogens may have evolved distinct mechanisms finely tuned by Msn2 for their adaptation to stressful cues.

Overall, Msn2 is vital for the transcriptional regulation of numerous effector genes and signaling factors involved in the conidiation, virulence and multi-stress responses of either *B. bassiana* or *M. roberstii*, thereby affecting profoundly the biocontrol potential of both fungi. Our findings provide novel insights into the complicated mechanisms of fungal stress responses and recall a special attention to the biological significance of Msn2 orthologs in filamentous fungi.

Acknowledgments

This study was supported by the Natural Science Foundation of China (Grant Nos.: 31100060 and 30930018) and the Ministry of Science and Technology of China (Grant Nos.: 2011AA10A204 and 2009CB118904).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2013.02.008.

References

Arratia-Quijada, J., Sánchez, O., Scazzocchio, C., Aguirre, J., 2012. FlbD, a Myb transcription factor of *Aspergillus nidulans* is uniquely involved in both asexual and sexual differentiation. Eukaryot. Cell 11, 1132–1142.

Audic, S., Claverie, J.M., 1997. The significance of digital gene expression profiles. Genome Res. 7, 986–995.

Barsoum, E., Rajaei, N., Åström, S.U., 2011. RAS/cyclic AMP and transcription factor Msn2 regulate mating and mating-type switching in the yeast *Kluyveromyces lactis*. Eukaryot. Cell 10, 1545–1552.

Beck, T., Hall, M.N., 1999. The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. Nature 402, 689–692.

Cho, E.M., Kirkland, B.H., Holder, D.J., Keyhani, N.O., 2007. Phage display cDNA cloning and expression analysis of hydrophobins from the entomopathogenic fungus *Beauveria (Cordyceps) bassiana*. Microbiology-(UK) 153, 3438–3447.

de Faria, M.R., Wraight, S.P., 2007. Mycoinsecticides and mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. Biol. Control 43, 237–256.

De Wever, V., Reiter, W., Ballarini, A., Ammerer, G., Brocard, C., 2005. A dual role for PP1 in shaping the Msn2-dependent transcriptional response to glucose starvation. EMBO J. 24, 4115–4123.

Enjalbert, B., Smith, D.A., Cornell, M.J., Alam, I., Nicholls, S., Brown, A.J., Quinn, J., 2006. Role of the Hog1 stress-activated protein kinase in the global transcriptional response to stress in the fungal pathogen *Candida albicans*. Mol. Biol. Cell 17, 1018–1032.

Etxebeste, O., Herrero-García, E., Araújo-Bazán, L., Rodríguez-Urra, A.B., Garzia, A., Ugalde, U., Espeso, E.A., 2009. The bZIP-type transcription factor FlbB regulates distinct morphogenetic stages of colony formation in *Aspergillus nidulans*. Mol. Microbiol. 73, 775–789.

Fang, W.G., Zhang, Y.J., Yang, X.Y., Zheng, X.L., Duan, H., Li, Y., Pei, Y., 2004. *Agrobacterium tumefaciens*-mediated transformation of *Beauveria bassiana* using an herbicide resistance gene as a selection marks. J. Invertebr. Pathol. 85, 18–24.

Fang, W., Pei, Y., Bidochka, M.J., 2007. A regulator of a G protein signalling (RGS) gene, *cag8*, from the insect-pathogenic fungus *Metarhizium anisopliae* is involved in conidiation, virulence and hydrophobin synthesis. Microbiology-(UK) 153, 1017–1025.

Feng, M.G., Poprawski, T.J., Khachatourians, G.G., 1994. Production, formulation and application of the entomopathogenic fungus *Beauveria bassiana* for insect control: current status. Biocontrol Sci. Technol. 4, 3–34.

Gao, Q., Jin, K., Ying, S.H., Zhang, Y., Xiao, G., Shang, Y.F., Duan, Z.B., Hu, X., Xie, X.Q., Zhou, G., Peng, G.X., Luo, Z.B., Huang, W., Wang, B., Fang, W.G., Wang, S.B., Zhong, Y., Ma, L.J., St Leger, R.J., Zhao, G.P., Pei, Y., Feng, M.G., Xia, Y.X., Wang, C.S., 2011. Genome sequencing and comparative transcriptomics of the model entomopathogenic fungi *Metarhizium anisopliae* and *M. acridum*. PLoS Genet. 7, e1001264.

Garmendia-Torres, C., Goldbeter, A., Jacquet, M., 2007. Nucleocytoplasmic oscillations of the yeast transcription factor Msn2: evidence for periodic PKA activation. Curr. Biol. 17, 1044–1049.

Gasch, A.P., Spellman, P.T., Kao, C.M., Carmel-Harel, O., Eisen, M.B., Storz, G., Botstein, D., Brown, P.O., 2000. Genomic expression programs in the response of yeast cells to environmental changes. Mol. Biol. Cell 11, 4241–4257.

Görner, W., Durchschlag, E., Martinez-Pastor, M.T., Estruch, F., Ammerer, G., Hamilton, B., Ruis, H., Schüller, C., 1998. Nuclear localization of the C_2H_2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. Genes Dev. 12, 586–597.

Görner, W., Durchschlag, E., Wolf, J., Brown, E.L., Ammerer, G., Ruis, H., Schüller, C., 2002. Acute glucose starvation activates the nuclear localization signal of a stress-specific yeast transcription factor. EMBO J. 21, 135–144.

Hasan, R., Leroy, C., Isnard, A.D., Labarre, J., Boy-Marcotte, E., Toledano, M.B., 2002. The control of the yeast H_2O_2 response by the Msn2/4 transcription factors. Mol. Microbiol. 45, 233–241.

Kikuma, T., Arioka, M., Kitamoto, K., 2007. Autophagy during conidiation and conidial germination in filamentous fungi. Autophagy 3, 128–129.

Kwon, N.J., Garzia, A., Espeso, E.A., Ugalde, U., Yu, J.H., 2010. FlbC is a putative nuclear C_2H_2 transcription factor regulating development in *Aspergillus nidulans*. Mol. Microbiol. 77, 1203–1219.

Lee, B.N., Adams, T.H., 1994. Overexpression of *flbA*, an early regulator of *Aspergillus* asexual sporulation, leads to activation of *brlA* and premature initiation of development. Mol. Microbiol. 114, 323–334.

Li, J., Feng, M.G., 2009. Intraspecific tolerance of *Metarhizium anisopliae* conidia to the upper thermal limits of summer with a description of a quantitative assay system. Mycol. Res. 113, 93–99.

Liesa, M., Qiu, W., Shirihai, O.S., 2012. Mitochondrial ABC transporters function: the role of ABCB10 (ABC-me) as a novel player in cellular handling of reactive oxygen species. Biochim. Biophys. Acta – Mol. Cell Res. 1823, 1945–1957.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2 (−Delta Delta C(T)) method. Methods 25, 402–408.

Lomer, C.J., Bateman, R.P., Johnson, D.L., Langewald, J., Thomas, M., 2001. Biological control of locusts and grasshoppers. Annu. Rev. Entomol. 46, 667–702.

Martinez-Pastor, M.T., Marchler, G., Schüller, C., Marchler-Bauer, A., Ruis, H., Estruch, F., 1996. The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). EMBO J. 15, 2227–2235.

Mayordomo, I., Estruch, F., Sanz, P., 2002. Convergence of the target of rapamycin and the Snf1 protein kinase pathways in the regulation of the subcellular localization of Msn2, a transcriptional activator of STRE (Stress Response Element)-regulated genes. J. Biol. Chem. 277, 35650–35656.

Miller, C.D., Rangel, D., Braga, G.U.L., Flint, S., Kwon, S.I., Messias, C.L., Roberts, D.W., Anderson, A.J., 2004. Enzyme activities associated with oxidative stress in *Metarhizium anisopliae* during germination, mycelial growth, and conidiation and in response to near-UV irradiation. Can. J. Microbiol. 50, 41–49.

Miskei, M., Karányi, Z., Pócsi, I., 2009. Annotation of stress-response proteins in the aspergilli. Fungal Genet. Biol. 46, S105–S120.

Nicholls, S., Straffon, M., Enjalbert, B., Nantel, A., Macaskill, S., Whiteway, M., Brown, A.J.P., 2004. Msn2-and Msn4-like transcription factors play no obvious roles in the stress responses of the fungal pathogen *Candida albicans*. Eukaryot. Cell 3, 1111–1123.

Nolting, N., Pöggeler, S., 2006. A MADS box protein interacts with a mating-type protein and is required for fruiting body development in the homothallic ascomycete *Sordaria macrospora*. Eukaryot. Cell 5, 1043–1056.

Ragni, E., Coluccio, A., Rolli, E., Rodriguez-Peña, J.M., Colasante, G., Arroyo, J., Neiman, A.M., Popolo, L., 2007. *GAS2* and *GAS4*, a pair of developmentally regulated genes required for spore wall assembly in *Saccharomyces cerevisiae*. Eukaryot. Cell 6, 302–316.

Rep, M., Krantz, M., Thevelein, J.M., Hohmann, S., 2000. The transcriptional response of *Saccharomyces cerevisiae* to osmotic shock. Hot1p and Msn2p/Msn4p are required for the induction of subsets of high osmolarity glycerol pathway-dependent genes. J. Biol. Chem. 275, 8290–8300.

Rispail, N., Soanes, D.M., Ant, C., Czajkowski, R., Grunler, A., Huguet, R., Perez-Nadales, E., Poli, A., Sartorel, E., Valiante, V., Yang, M., Beffa, R., Brakhage, A.A., Gow, N.A.R., Kahmann, R., Lebrun, M.H., Lenasi, H., Perez-Martin, J., Talbot, N.J., Wendland, J., Di Pietro, A., 2009. Comparative genomics of MAP kinase and calcium-calcineurin signalling components in plant and human pathogenic fungi. Fungal Genet. Biol. 46, 287–298.

Roberts, D.W., St Leger, R.J., 2004. *Metarhizium* spp., cosmopolitan insect-pathogenic fungi: mycological aspects. Adv. Appl. Microbiol. 54, 1–70.

Roetzer, A., Gregori, C., Jennings, A.M., Quintin, J., Ferrandon, D., Butler, G., Kuchler, K., Ammerer, G., Schüller, C., 2008. *Candida glabrata* environmental stress response involves *Saccharomyces cerevisiae* Msn2/4 orthologous transcription factors. Mol. Microbiol. 69, 603–620.

Santhanam, A., Hartley, A., Duvel, K., Broach, J.R., Garrett, S., 2004. PP2A phosphatase activity is required for stress and Tor kinase regulation of yeast stress response factor Msn2p. Eukaryot. Cell 3, 1261–1271.

Saito, H., Tatebayashi, K., 2004. Regulation of the osmoregulatory HOG MAPK cascade in yeast. J. Biochem. 136, 267–272.

Schmitt, A.P., McEntee, K., 1996. Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA 93, 5777–5782.

Seo, J., Bakay, M., Chen, Y.W., Hilmer, S., Shneiderman, B., Hoffman, E.P., 2004. Interactively optimizing signal-to-noise ratios in expression profiling: project-

specific algorithm selection and detection *p*-value weighting in Affymetrix microarrays. Bioinformatics 20, 2534–2544.
Seo, J.A., Guan, Y., Yu, J.H., 2006. FluG-dependent asexual development in *Aspergillus nidulans* occurs via derepression. Genetics 172, 1535–1544.
Wang, C.S., St Leger, R.J., 2007. The MAD1 adhesin of *Metarhizium anisopliae* links adhesion with blastospore production and virulence to insects, and the MAD2 adhesin enables attachment to plants. Eukaryot. Cell 6, 808–816.
Wang, Z.L., Lu, J.D., Feng, M.G., 2012. Primary roles of two dehydrogenases in the mannitol metabolism and multi-stress tolerance of entomopathogenic fungus *Beauveria bassiana*. Environ. Microbiol. 14, 2139–2150.
Wang, Z.L., Zhang, L.B., Ying, S.H., Feng, M.G., 2013. Catalases play differentiated roles in the adaptation of a fungal entomopathogen to environmental stresses. Environ. Microbiol. 15, 409–418.
Xiao, P., Shin, K.S., Wang, T.H., Yu, J.H., 2010. *Aspergillus fumigatus flbB* encodes two basic leucine zipper domain (bZIP) proteins required for proper asexual development and gliotoxin production. Eukaryot. Cell 9, 1711–1723.
Xiao, G.H., Ying, S.H., Zheng, P., Wang, Z.L., Zhang, S.W., Xie, X.Q., Shang, Y.F., St. Leger, R.J., Zhao, G.P., Wang, C.S., Feng, M.G., 2012. Genomic perspectives on the evolution of fungal entomopathogenicity in *Beauveria bassiana*. Sci. Rep. 2, 483.
Xie, X.Q., Guan, Y., Ying, S.H., Feng, M.G., 2013. Differentiated functions of Ras1 and Ras2 proteins in regulating the germination, growth, conidiation, multi-stress tolerance and virulence of *Beauveria bassiana*. Environ. Microbiol. 15, 447–462.
Xie, X.Q., Li, F., Ying, S.H., Feng, M.G., 2012. Additive contributions of two manganese-cored superoxide dismutases (MnSODs) to antioxidation, UV tolerance and virulence of *Beauveria bassiana*. PLoS One 7, e30298.
Zhang, S., Xia, Y.X., Kim, B., Keyhani, N.O., 2011. Two hydrophobins are involved in fungal spore coat rodlet layer assembly and each play distinct roles in surface interactions, development and pathogenesis in the entomopathogenic fungus, *Beauveria bassiana*. Mol. Microbiol. 80, 811–826.
Zhou, G., Wang, J., Qiu, L., Feng, M.G., 2012. A Group III histidine kinase (mhk1) upstream of high-osmolarity glycerol pathway regulates sporulation, multi-stress tolerance and virulence of *Metarhizium robertsii*, a fungal entomopathogen. Environ. Microbiol. 14, 817–829.