A transcription factor, MrMsn2, in the dimorphic fungus *Metarhizium rileyi* is essential for dimorphism transition, aggravated pigmentation, conidiation and microsclerotia formation

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**Summary**

Microsclerotia (MS) are pseudoparenchymatous aggregations of hyphae of fungi that can be induced in liquid culture for biocontrol applications. Previously, we determined that the high-osmolarity glycerol (HOG) signalling pathway was involved in regulating MS development in the dimorphic insect pathogen *Metarhizium rileyi*. To further investigate the mechanisms by which the signalling pathway is regulated, we characterized the transcriptional factor MrMsn2, a homologue of the yeast C2H2 transcriptional factor Msn2, which is predicted to function downstream of the HOG pathway in *M. rileyi*. Compared with wild-type and complemented strains, disruption of MrMsn2 increased the yeast-to-hypha transition rate, enhanced conidiation capacity and retarded microsclerotia formation during conidia and MS development. Together, our findings confirm that MrMsn2 controlled the yeast-to-hypha transition, conidiation and MS formation, and virulence.

**Introduction**

DNA-binding and multimerization domains are often used to classify transcriptional factors (TFs) into basic leucine zipper, zinc finger motif, helix–turn–helix and helix–loop–helix types (Park *et al.*, 2008; Chai *et al.*, 2012; Jung *et al.*, 2015; Yin *et al.*, 2017). As the core of signalling pathway, fungal TFs are important for transcriptional regulation of gene expression during cellular growth, secondary metabolism, stress responses and pathogenesis (Klug, 2010; Hong *et al.*, 2013; Liu *et al.*, 2013; Marinho *et al.*, 2014; Zhang *et al.*, 2014; Huang *et al.*, 2015; Shelest, 2017; Yin *et al.*, 2017; Song *et al.*, 2018).

Filamentous fungal Msn2/4 homologues are C2H2-like zinc finger TFs that regulate the general stress response, pathogenicity, secondary metabolism and cellular growth. They are similar to ScMsn2/4 of *Saccharomyces cerevisiae* (Schmitt and Mcentee, 1996) and have been characterized in *Aspergillus parasiticus*, *A. flavus*, *Beauveria bassiana*, *Magnaporthe oryzae*, *Metarhizium robertsi* and *Verticillium dahliae* (Chang *et al.*, 2011; Liu *et al.*, 2013; Zhang *et al.*, 2014; Tian *et al.*, 2017). Under abiotic and biotic stresses, Msn2/4 is phosphorylated for translocation from the cytoplasm to the nucleus, where it drives the transcription of stress-induced genes (Hansen *et al.*, 2015; Yi and Huh, 2015; Li *et al.*, 2017). The underlying mechanism of the regulation of Msn2/4 activity by protein kinase A (PKA), the rapamycin signalling pathway, the Snf1 protein kinase pathway and the high-osmolarity glycerol (HOG) pathway have been elucidated (Liu *et al.*, 2013; Zhang *et al.*, 2014; Li *et al.*, 2017).

Microsclerotia (MS) are pseudoparenchymatous aggregations of hyphae with a diameter of 50–600 μm and become melanized during their development. As promising fungal propagules, MS can be induced in liquid culture and used for biocontrol applications such as...
biofungicides, bioherbicides, bionematicides and mycoinsecticides (Shearer, 2007; Jackson et al., 2010; Song et al., 2014, 2016a). To enhance the liquid fermentation efficiency of *Metarhizium rileyi* MS, we previously investigated the molecular mechanism of MS formation and demonstrated that internal oxidative stress triggers MS differentiation (Song et al., 2013, 2015, 2016b, 2018; Jiang et al., 2014). We found that HOG and cell wall integrity (CWI) pathways cooperate to regulate MS formation (Song et al., 2016b). We also found that *M. rileyi* MrMsn2 was predicted to function downstream of the HOG pathway and was upregulated during MS formation in comparative transcriptome analysis (Song et al., 2013). Furthermore, a bioinformatics analysis found no Msn4 orthologues in any public genome databases of *M. rileyi* (Song et al., 2013; Shang et al., 2016). These results imply a possible involvement of MrMsn2 in the regulation of MS development. However, this function has not been studied clearly.

Moreover, *M. rileyi* is a well-known dimorphic entomopathogenic fungus with yeast-like hyphal bodies and a true filamentous growth phase (Boucias et al., 2000; Fronza et al., 2017), which occurs synchronously in *in vivo* and *in vitro* (Pendland and Boucias, 1997; Boucias et al., 2016). The yeast-to-hypha transition is critical for the pathogenesis and life cycle of dimorphic fungi (Wanchoo et al., 2009; Boyce and Adrianopoulos, 2015; Gauthier, 2015; Marcos et al., 2016). Although signalling pathways related to dimorphic transition are well characterized in the model yeast *Candida albicans*, the mechanisms are not well defined (Noble et al., 2017). Thus, studies on *M. rileyi* are useful model for understanding the dimorphic transition mechanism.

This study seeks to further elucidate the role of MrMsn2 in dimorphism transition, conidiation, virulence and MS formation by phenotypic analyses of deletion/rescue mutants constructed previously (Shao et al., 2015; Song et al., 2016b). We found that the absence of MrMsn2 resulted in increased yeast-to-hypha transition rate, enhanced conidiation capacity, aggravated pigmentation and induced or suppressed expression of target genes involved in the important phenotypes of *M. rileyi*, as presented below.

**Results**

**Molecular characterization of MrMsn2**

The full-length sequence of MrMsn2 (GenBank Accession No.: MG641237) is 1752 bp, including one intron, with a calculated molecular weight of 56.7 kDa and an isolectric point of 5.06 (http://expasy.org/tools/protparam.html). Furthermore, MrMs2 contains a zinc finger double domain (Hu et al., 2014). In this study, a phylogenetic tree analysis demonstrated that the MrMs2 protein from *M. rileyi* was closely related to other *Metarhizium* spp. proteins (Fig. S1). In addition, the amino acid sequence of MrMs2 showed similarities (79–81% identity) to a cutinase G-box binding protein of *Metarhizium* spp. (Hu et al., 2014; Shang et al., 2016) and a zinc finger protein (66% identity) of *Toylpolodium ophioglossoides* (Quandt et al., 2015).

To characterize the functions of the MrMs2 gene in *M. rileyi*, gene replacement mutants and complementary transformants were generated. All recombinant strains were verified by polymerase chain reaction (PCR) and quantitative real-time PCR (RT-qPCR) screening (Fig. S2). The confirmed ΔMrMs2 mutants and the complemented (ΔMrMs2 + Ms2) strains were used in further experiments.

**MrMs2 negatively regulates yeast-to-hyphae transition and conidiation**

The *M. rileyi* CQNr01 (wild-type, WT) strain was grown on solid Sabouraud maltose agar fortified with yeast extract (SMAY). Compared with the initial results at day 0, expression of MrMs2 was found to be downregulated in the yeast-to-hypha transition at days 2 and 4 and conidiation initiation at day 6 (Fig. 1A). These results indicate that MrMs2 may be involved in the yeast-to-hypha transition and conidiation.

Further investigations showed that at day 3, the yeast-to-hyphae transition was advanced in ΔMrMs2 mutants compared to the WT and complemented (CP) strains (Fig. 1B). Furthermore, colony surfaces of ΔMrMs2 mutants were more convoluted compared to the normal smooth colony surfaces of WT and CP strains. After 6 days, the diameter of the mutant colonies was larger compared to that of the WT (Fig. 1B). Additionally, the ΔMrMs2 mutants had significantly increased conidial yields compared to the WT and CP strains (P < 0.001) (Fig. 1C). After 9 and 12 days, conidial yield had increased by 1.5- to 2.2-fold in ΔMrMs2 mutants compared to the WT and CP strains. Taken together, these transcription and phenotype growth analysis suggest that MrMs2 is involved in negative control of conidia production and yeast-to-hypha transition.

To further analyse the effect of MrMs2 on the dimorphic transition, yeast cells of test strains were grown on SMAY medium. The investigations into the switching rates and median transition time required for 50% transition of blastospores to hyphae (TT50) showed a significantly difference among the ΔMrMs2 mutants (TT50 = 5.4 ± 0.2 days), WT (TT50 = 7.3 ± 0.1 days) and CP (TT50 = 7.0 ± 0.2 days) strains (P < 0.001) (Fig. 2). This suggests that deletion of MrMs2 promoted the yeast-to-hypha transition.

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Absence of MrMsn2 leads to aggravated pigmentation

After incubation on SMAY medium for 6 days, ΔMrMsn2 mutants were found to have altered aggravated pigmentation (Fig. 3A). After 9 and 12 days, the tergal pigment of ΔMrMsn2 mutants had increased compared to WT and CP strains respectively. Furthermore, the pigment concentration in ΔMsn2 mutants was found to be significantly increased by factors of 1.8- to 4.2-fold, compared to that of WT and CP strains respectively (Fig. 3B). These data suggest that MrMsn2 had a negative influence on clone pigment biosynthesis.

To investigate the mechanism of dimorphic transition, pigment biosynthesis and conidiation regulated by MrMsn2, genes that were potentially involved were selected from transcriptome libraries (Song et al., 2013, 2018) and examined by transcriptional analysis. The following genes were selected: pigment biosynthesis-related genes (polyketide synthase, pks; polyketide synthase–non-ribosomal peptide synthetase, pks-N; polyketide synthase 6, pks-6; conidial yellow pigment biosynthesis, cyp; and laccase, lac), several chitin synthase genes (ch1, ch2 and ch4, for class I, II and IV chitin synthases respectively) and antioxidant enzyme genes (cat1 for catalase-1 and cat2 for catalase-2, sod1 for superoxide dismutase-1 and sod2 for superoxide dismutase-2). It was found that the lac, pks-N, cat1 and sod1 genes were upregulated, whereas pks, pks-6, cyp, ch1 and ch2 genes were downregulated in the WT strain during conidiation (Figs. 3C and D). Compared with the WT, all pigment biosynthesis-related gene and chitin synthase genes were significantly upregulated during conidiation in the ΔMsn2 mutants (Fig. 3C). In addition, the antioxidant enzyme genes were upregulated in aggravated pigmentation after 6 days for ΔMsn2 mutants (Fig. 3D).

MrMsn2 contributes to tolerance to abiotic stress

To examine the function of MrMsn2 on the abiotic stress response, strains were cultured under various abiotic stress conditions. Convoluted colony surfaces were more apparent, especially under cell wall perturbation and oxidative stress, for ΔMrMsn2 mutants compared to the normal smooth colony surfaces of the WT and CP strains, after 3 day incubation (Fig. S3A). Furthermore, compared to WT strain, smaller colonies were present in the ΔMrMsn2 mutants (Fig. S3A). After 12 days, the conidial yield of ΔMrMsn2 was significantly reduced between 26.6 and 91.9% on SMAY medium, under osmosensitivity, cell wall perturbation or oxidative stress ($P < 0.001$). Interestingly, the conidial yield of ΔMrMsn2 mutants was found to be significantly reduced under KCl stress, however, it was significantly increased under NaCl stress compared to the WT and CP strains.
These results indicate MrMsn2 contributes to tolerance of abiotic stress.

MrMsn2 is needed for MS development

An expression analysis showed that the relative transcriptional of MrMsn2 peaked with MS initiation (72 h) (Fig. 4A) and MrMsn2 were upregulated in liquid amended medium (AM) or MM (AM without basal salts) medium, cultured with exogenous oxidative stress (Fig. 4B). These results suggest that MrMsn2 may be involved in the regulation of MS formation.

After incubation in liquid AM for 144 h, MS produced by WT and CP strains matured and were accompanied by secondary mycelia growth, whereas the density of the induced MS in the MrMsn2 mutants was significantly decreased, with the MrMsn2 culture broth exhibiting a low degree of pigmentation (Fig. 4C). Compared to the WT and CP strains, the MS yield of MrMsn2 mutants was reduced by approximately 88.9% (Fig. 4D), and the biomass was decreased by 23.6% in the AM culture (Fig. 4E). These results indicate that MrMsn2 is needed for MS development.

Expression analysis during MS development

To investigate the genes regulated by MrMsn2 during MS formation, several groups of genes were analysed by qRT–PCR. It was found that antioxidation genes such as cat1, cat2, sod1, sod2 and the monooxygenase (mon) gene were upregulated in the MrMsn2 mutants, while the flavoprotein–ubiquinone oxidoreductase (fuo) gene was downregulated (Fig. 5A). Pigment biosynthesis genes pks, pks-6 and cyp were found to also be downregulated and pks-N and lac genes were upregulated in the MrMsn2 mutants (Fig. 5A). Additionally, chitin synthase genes ch2 and ch4 were significantly upregulated in the MrMsn2 mutants and ch1 was significantly downregulated (Fig. 5B). Interestingly, Slt2, the core gene of the CWI signalling pathway, and hog1, the core gene of the HOG signalling pathway, were significantly upregulated in the MrMsn2 mutants and ch1 was significantly downregulated (Fig. 5B). Finally, transport and storage genes for major salts, sidA (siderophore iron transporter), ct-1 (calcium-transporting ATPase 1), ct-2 (calcium-transporting p-type ATPase) and ccca (vacuolar Fe²⁺/Mn²⁺ transporter) were all found to be significantly upregulated in the MrMsn2 mutants (Fig. 5B).

MrMsn2 is required for the virulence of M. rileyi

Pathogenicity assays were conducted using third-instar Spodoptera litura larvae. These assays showed that the virulence of the MrMsn2 mutants was significantly lower than of the WT and CP strains (Fig. 6). The mean lethal time (LT₅₀) for the WT strain was 6.2 ± 0.4 days.

Fig. 2. Quantitative analysis dimorphic transition of test strains. A. Quantitative analysis of dimorphic transition (from yeast cells to hypha) rate of wild-type, complemented and MrMsn2 mutants with approximately 100 single yeast cells plated on SMAY medium. The growth morphology was observed every day. B. Median transition time required for 50% transition of blastospores to hyphae (TT₅₀) of wild-type, complemented and MrMsn2 mutants was compared. TT₅₀ was calculated using a probit analysis with the SPSS program. Error bars are standard error. * P < 0.05, ** P < 0.01, significantly different compared with wild type.
in a topical bioassay and 4.3 ± 0.5 days in the injection bioassays. The LT_{50} values for the CP strains were 6.3 ± 0.5 days in topical bioassays and 4.9 ± 0.5 days in injection bioassays, whereas the LT_{50} values for the ΔMrMsn2 mutants were 10.1 ± 0.5 (P < 0.001) in the topical bioassay and 8.6 ± 0.4 (P < 0.001) in the injection bioassays. These results show that MrMsn2 is required for virulence.

**Discussion**

In our previous investigation, we found that the HOG signalling pathway regulated the dimorphic transition and MS development (Song et al., 2016b). To better define the mechanisms of regulation, in this study, we identified and characterized the transcription factor MrMsn2, which is predicted to be downstream of the HOG pathway in
MrMsn2 belongs to a group of proteins containing C_{2}H_{2}-like Zn finger domains that are important in development, secondary metabolism and stress responses.
In this study, MrMsn2 mutants negatively controlled the yeast-to-hypha transition (Figs 1 and 2). This result was unlike the observation in Yarrowia lipolytica yeast, in which disruption of Mhy1p, an Msn2/4-like protein, restricted the dimorphic transition, and in C. albicans where it had no significant role in CaMsn2/CaMsn4 mutations (Hurtado and Rachubinski, 1999; Nicholls et al., 2004). These studies show distinct strategies for regulating the yeast-to-hypha transition using Msn2 in different fungi.

Moreover, similar to A. parasiticus and A. flavus (Chang et al., 2011; Liu et al., 2013; Zhang et al., 2014; Tian et al., 2017). In this study, △MrMsn2 mutants negatively controlled the yeast-to-hypha transition (Figs 1 and 2). This result was unlike the observation in Yarrowia lipolytica yeast, in which disruption of Mhy1p, an Msn2/4-like protein, restricted the dimorphic transition, and in C. albicans where it had no significant role in CaMsn2/CaMsn4 mutations (Hurtado and Rachubinski, 1999; Nicholls et al., 2004). These studies show distinct strategies for regulating the yeast-to-hypha transition using Msn2 in different fungi.

In eukaryotes microorganism, cellular developmental processes are reported to correlate with increased reactive oxygen species (ROS) levels (Georgiou et al., 2006; Takemoto et al., 2007). Fungi have evolved effective antioxidant mechanisms that include enzyme families that act as ROS scavengers. Previous studies have shown members of antioxidant enzymes families such as SODs and CATs may have complementary effects during the cellular developmental processes (Xie et al., 2012; Youseff et al., 2012; Wang et al., 2013; Zhang and Feng, 2018). Our investigations confirmed that antioxidant enzyme genes cat1, sod1, cat2 and sod2 had different expressions during conidia development in M. rileyi (Fig. 3). In response to ROS stress, Msn2/4

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accumulated in the nucleus, where they promoted transcriptional activation of stress-responsive genes (Hansen et al., 2015; Yi and Huh, 2015). However, deletion of MrMsn2 increased production of ROS (data not shown) and upregulated expression of antioxidant genes to protect against ROS. Upregulation of these antioxidant genes in ΔMrMsn2 mutants suggests that there may be regulated by other signalling network (Zhang and Feng, 2018).

Secondary metabolism, such as pigments production, is triggered and intensified by ROS build-up, with pigments being important for protecting the fungi against stress conditions (Cho et al., 2012; Hong et al., 2013). In the absence of MrMsn2, pigments were significantly upregulated during conidiation in ΔMrMsn2 mutants. Chitin is a main component of cell walls and is related to morphogenesis and adaptation to ecological niches (Roncero, 2002; Liu et al., 2017). Expression analysis showed that genes from the class I and II of chitin synthases were downregulated during morphogenesis and conidiation, and then in the absence of MrMsn2, these were upregulated (Fig. 3). Based on this data, we propose a link between chitin biosynthesis and MrMsn2, however, the molecular mechanism remains unknown.

Current conidia mass production methods are not cost-effective, limiting M. rileyi commercialization. MS can be used as an alternative fungal propagule for mycoinsecticide (Song et al., 2014) and has been used in large-scale production in submerged fermentation (Song et al., 2017). As for solid culture, ΔMrMsn2 mutants were defective in hyphal growth in liquid AM (data not shown). Furthermore, vegetative hyphae are the prerequisite for MS formation (Song et al., 2013; Jiang et al., 2014). Consistent with the defective MS formation in the ΔMrHog1 mutants, the ΔMrMsn2 mutants had limited ability to form MS (Fig. 4). This result was unlike the observation in V. dahliae, in which VdMsn2 deletion mutants produce more MS than wild type on solid media (Tian et al., 2017). Previously, it was found that the CWI and HOG signalling pathways cooperate to regulate MS development (Song et al., 2016b). This has been confirmed in this study, with the core genes of the CWI and HOG signalling pathway both being upregulated in the ΔMrMsn2 mutants.
Our previous study demonstrated that intracellular H$_2$O$_2$ levels fluctuated during MS development and peak at MS initiation stage (Song et al., 2018). Antioxidant enzyme genes were significantly upregulated in $\Delta$MrMsn2 mutants (Fig. 5), indicating that intracellular H$_2$O$_2$ levels were not equilibrated in the MS-initiating formation. This result confirmed our previous results that oxidative stress triggered MS formation (Song et al., 2013, 2015, 2016b; Jiang et al., 2014). As mentioned, the signalling network that regulates the chitin synthesis- and pigment synthesis-associated genes in solid SMAY and liquid AM culture is highly complicated. Major basal salts such as iron and calcium cations are necessary for MS formation (Song et al., 2014) and SidA is the major pathway of cellular iron uptake for MS formation (Li et al., 2016). We demonstrated that the iron importer and calcium transports, Ct-1 and Ct-2, were important in MS formation (Wang and Yin, unpublished data). In addition to similar transcriptional mechanisms by Msn2/4 for regulating ccc1 in yeast (Li et al., 2017), we found multiple transcriptional mechanisms for regulating genes for iron and calcium cation transport and storage by MrMsn2 during MS development (Fig. 5). However, the transcriptional mechanisms for regulating cation transport are not clear and further experiments are needed to elucidate the multiple mechanisms.

Research on the function of Msn-like transcriptional factors in pathogenicity is widespread for entomopathogenic, human pathogenic and phytopathogenic fungi (Roetzer et al., 2008; Liu et al., 2013; Zhang et al., 2014). In M. rileyi, our data indicated that $\Delta$MrMsn2 mutants were significantly less pathogenic than WT by both topical infection and injection assays (Fig. 6). Similar results were reported for $\Delta$MoMsn2 mutants of M. oryzae, $\Delta$Bbmsn2 mutants of B. bassiana, and $\Delta$Mmmsn2 mutants of M. robertii in which gene deletions cause decreased virulence (Liu et al., 2013; Zhang et al., 2014). In contrast, in C. glabrata, Msn2 was found to have no effect on virulence (Roetzer et al., 2008). One explanation for this is that $\Delta$MrMsn2 mutants counter oxidative stress from hosts in vivo (Song et al., 2016a,b) and were hypersensitive to stress. Another explanation involves morphogenic defects in the mutants. This investigation revealed that vegetative growth of $\Delta$MrMsn2 mutants was defective in haemocoeol (data not shown). These results could be reasons why the mutants had decreased pathogenicity in vivo.

In summary, this study revealed the MrMsn2 had negative effects on the dimorphic transition and conidiation and was required for abiotic stress resistance, virulence, and MS formation. Furthermore, the current transcriptional networks of MrMsn2 during conidia and MS development will enhance our ability to comprehensively understand the molecular mechanism of yeast-to-hypha transition and conidia and MS development.

**Experimental procedures**

**Strains, media and culture conditions**

The M. rileyi CQNtr01 strain was from the Engineering Research Center for Fungal Insecticides, Chongqing, China. WT and engineered strains were cultured on SMAY (Sabouraud maltose agar, fortified with 1% (w/v) yeast extract) under continuous light at 25°C for 12 days for the conidiation assays or in liquid AM (comprising of 40 g l$^{-1}$ glucose, 2.5 g l$^{-1}$ peptone, 5 g l$^{-1}$ yeast extract, 4.0 g l$^{-1}$ KH$_2$PO$_4$, 0.8 g l$^{-1}$ CaCl$_2$.2H$_2$O, 0.6 g l$^{-1}$ MgSO$_4$.7H$_2$O, 0.1 g l$^{-1}$ FeSO$_4$.7H$_2$O, 37 mg l$^{-1}$ CoCl$_2$.6H$_2$O, 16 mg l$^{-1}$ MnSO$_4$.H$_2$O and 14 mg l$^{-1}$ ZnSO$_4$.7H$_2$O) for the MS incubation assays according to previous methods (Song et al., 2016b). Escherichia coli DH5x (Invitrogen, Shanghai, China) was used for recombinant DNA manipulation and Agrobacterium tumefaciens AGL-1 (Invitrogen, Shanghai, China) for fungal transformations. Both were cultured as previously described (Shao et al., 2015; Song et al., 2016b).

**Gene cloning and bioinformatics analyses**

To study the potential function of MrMsn2, the full genomic DNA sequence of MrMsn2 was amplified using primers MrMrn2-L/MrMrn2-R (Table S1) based on sequences of a previous transcriptomic library (Song et al., 2013). The protein sequence and potentially homologues from other fungal species were aligned with Blastp (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequences were aligned with DNAMAN software (http://www.lynnon.com). Neighbour-joining tree was generated using the software MEGA 6.0 (http://www.megasoftware.net) (Tamura et al., 2013).

**Generation of deletion and complementation mutants**

The M. rileyi genome was not annotated when we constructed the targeted gene deletion plasmid. Therefore, fusion primer and nested integrated PCR (Wang et al., 2011) with primers in Table S1 were used to obtain flanking regions (data not shown). Upstream and downstream flanking sequences were amplified using primers Ms-LF/Ms-LR and Ms-RF/Ms-RR (Table S1), respectively, digested with restriction endonucleases and inserted into the plasmid pPZP-Hph-Knock, a hygromycin B-resistance vector. The resultant plasmid was named pPZP-Hph-msn2. For the mutant complementation strains, the open reading frame (ORF) of MrMsn2 with the promoter and terminator regions, was amplified based on the subsequently public annotated of the
M. rileyi genome (Shang et al., 2016) using the primers Ms-HF/Ms-HR (Table S1). PCR products were digested by restriction endonucleases and ligated into the sulfonylurea resistance vector pPZP-Sur-Knock to generate the plasmid, pPZP-Sur-msn2. Disruption and complementation vectors were transformed into Agrobacterium and transformants were screened as described previously (Song et al., 2018).

Phenotypic analyses of test strains on SMAY media

To analyse the function of MrMsn2 in yeast-to-hyphae transition, vegetative growth, conidial development and abiotic stress tolerance, conidial suspensions of the tested strains were plated on SMAY as previously described (Song et al., 2016b). Colony morphology was investigated, and images were collected using a digital camera (60-mm Macro lens; Canon Inc., Tokyo, Japan) and microscope. Conidia numbers were counted as previously described (Con et al., 2015).

In vitro, M. rileyi was grown in yeast cell form for 2–4 days on SMAY to transformed into the filamentous form. The switching rates of the tested strains were counted as previously described (Li et al., 2016). Approximately 100 simple yeast cells were pipetted onto SMAY medium and grown at 25°C. Switching rates at indicated time points were recorded and TT50 was estimated.

Purification and measurement of clone pigments

Clone pigments of tested strains were counted as described previously (Li et al., 2016). Clones from 6-day-old, 9-day-old or 12-day-old SMAY cultures were isolated with 2% NaOH. Collected clones were ground with liquid AM culture for 6 days. Biomass and MS yield were quantified for the AM cultures and determined as previously described (Song et al., 2015). MS morphologies were observed using the digital camera.

Gene expression by qRT–PCR

To assess the effect of exogenous agents on MrMsn2 expression, AM or MM was supplemented with exogenous 1 M iron or 3 mM \( \text{H}_2\text{O}_2 \) as previously described (Song et al., 2016b, 2018). Mycelia were subsequently harvested for RNA extraction. For time-specific expression patterns during conidia development, samples of WT inoculated on SMAY were collected at 0, 2, 4, 6 and 8 days for RNA extraction. To explore the impact on other genes related to dimorphic transition and melanin production during conidiation, WT or \( \Delta \text{MrMsn2} \) mutants were incubated on SMAY media and 3-, 6-, 9- or 12-day-old clones were collected independently for transcriptional analysis. For time-specific expression patterns during MS development, samples of WT inoculated in AM were collected at 36, 60, 72, 96 and 120 h for RNA extraction. To investigate the regulation of other genes during MS formation, WT and \( \Delta \text{MrMsn2} \) mutants were incubated in AM cultures. After 72 h, mycelia were collected and total RNA was prepared. Gene expression patterns were confirmed for samples of WT, \( \Delta \text{MrMsn2} \) or CP mycelia cultured in AM for 72 h.

Total RNA was collected according to previous methods (Song et al., 2015). RT–qPCR was performed using SYBR Green (Invitrogen, Shanghai, China), as per the manufacturer’s instructions. \( \beta \)-tubulin (Mrtub) and translation elongation factor (Mrtef) genes were used as internal standards. Relative expression levels were evaluated using the 2\(^{-\Delta\Delta Ct}\) method (Vandesompele et al., 2002).

Insect virulence assays

Topical infections tests and injection assays were conducted as previously described (Song et al., 2016b). Three replicate groups had 30 larvae each, and after treatment, the larvae were reared as described previously (Song et al., 2015). Larval mortality was recorded daily, and LT\(_{50}\) values were calculated using probit analysis with the software SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Data analysis

All assays were repeated three times. Data were analysed by one-way analysis of variance (ANOVA), followed by Duncan’s multiple range tests using SPSS 17.0 software. Graphs were constructed in GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). Error bars represent the standard error.

Acknowledgements

This research was supported financially by the National Science Foundation of the People’s Republic of China (No. 31701127; No. 31570073), Educational Commission of Sichuan Province of China (18ZAO515) and Joint project on Luzhou City and Southwest Medical University (2017LZXNYD-T06).
Conflict of interest

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Phyllogenetic analysis of MrMsn2 protein.

Fig. S2 Confirmation of gene disruption and complementation.

Fig. S3 Morphology analysis and conidial yield of wild-type (WT), ΔMrSwi6 mutants and complemented (CP) strains mutants under abiotic stress.

Table S1. Oligonucleotide primers used in this study.