MrSkn7 Controls Sporulation, Cell Wall Integrity, Autolysis, and Virulence in *Metarhizium robertsii*

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Two-component signaling pathways generally include sensor histidine kinases and response regulators. We identified an ortholog of the response regulator protein Skn7 in the insect-pathogenic fungus *Metarhizium robertsii*, which we named MrSkn7. Gene deletion assays and functional characterizations indicated that MrSkn7 functions as a transcription factor. The MrSkn7 null mutant of *M. robertsii* lost the ability to sporulate and had defects in cell wall biosynthesis but was not sensitive to oxidative and osmotic stresses compared to the wild type. However, the mutant was able to produce spores under salt stress. Insect bioassays using these spores showed that the virulence of the mutant was significantly impaired compared to that of the wild type due to the failures to form the infection structure appressorium and evade host immunity. In particular, deletion of MrSkn7 triggered cell autolysis with typical features such as cell vacuolization, downregulation of repressor genes, and upregulation of autolysis-related genes such as extracellular chitinases and proteases. Promoter binding assays confirmed that MrSkn7 could directly or indirectly control different putative target genes. Taken together, the results of this study help us understand the functional divergence of Skn7 orthologs as well as the mechanisms underlying the development and control of virulence in insect-pathogenic fungi.

Both prokaryotes and eukaryotes have developed complicated systems to sense and adapt to changing environments. In bacteria, sensing and processing of environmental stimuli largely rely on two-component signal (TCS) transduction pathways that consist of a membrane-bound histidine kinase receptor and a corresponding response regulator (RR) (1, 2). Similar TCS systems are also present in fungi, and the mechanisms underlying their function have been well studied in the budding yeast *Saccharomyces cerevisiae* (3, 4). The TCS pathway consists of a sensor kinase, Snl1, and two response regulators, Skk1 and Skn7 (5, 6). The cytosolic Ssk1 controls the Hog1 mitogen-activated protein kinase pathway, while the highly conserved Skn7 functions as a stress response transcription factor that consists of an N-terminal HSF (heat shock factor)-type DNA binding domain and a C-terminal RR receiver domain (5). The orthologs of Skn7 in yeasts and different filamentous fungi generally contribute to cell wall integrity, sporulation, osmotic stress, and oxidative stress (5). Interestingly, deletion of MoSkn7 (*Magnaporthe oryzae* Skn7) in *M. oryzae* (7), FgSkn7 (*Fusarium graminearum* Skn7) in *F. graminearum* (8), and BcSkn7 (*Botrytis cinerea* Skn7) in *B. cinerea* (9) did not impair fungal virulence and thereby the ability to infect their respective plant hosts. In contrast, AaSkn7 (*Alternaria alternata* Skn7) is required by *A. alternata* to infect citrus (10). In mammalian pathogens, the virulence of *Candida albicans* and *Cryptococcus neoformans* were attenuated by the deletion of CaSkn7 (*C. albicans* Skn7) (11) and CnSkn7 (*C. neoformans* Skn7) (12), respectively, compared to wild-type fungi. However, the functions of Skn7 orthologs in insect-pathogenic fungi have not been investigated thus far.

The ubiquitous insect-pathogenic fungi *Metarhizium* spp. diverged from mammalian and plant fungi about 100 million years ago (13, 14). They are highly important in agriculture because, along with the other insect-pathogenic fungi such as *Beauveria bassiana*, they are being developed as environmentally friendly biocontrol agents and are used worldwide to control various insect pests (15, 16). Similar to other plant pathogens, *Metarhizium* species overcome a plethora of biotic and abiotic stresses for a successful fungus-host interaction (17, 18). One of the critical factors affecting fungal virulence is the buildup of turgor pressure in the infection structure appressorium for cuticle penetration and then adaptation to osmotic insect hemolymphs, which leads to colonizing the body cavity (19, 20). However, it is not clear whether the Skn7-like transcription factor plays a role in the regulation of stress responses in insect fungi.

In this study, we performed functional assays on the yeast Skn7 homologous protein, MrSkn7 (*Metarhizium robertsii* Skn7) (MAA_04551, 43% identity) in *M. robertsii*. Gene deletion and biochemical analyses and insect bioassays indicated that MrSkn7 is required not only for fungal conidiation, cell wall integrity maintenance, appressorium differentiation and virulence but also for cell autolysis by upregulating the extracellular proteases and chitinases that eventually lead to self-digestion of *M. robertsii*. We found that MrSkn7 did not contribute to oxidative and osmotic stress responses in *M. robertsii*, unlike other fungi.

**MATERIALS AND METHODS**

**Fungal strains and culture conditions.** The wild-type (WT) and mutant strains of *Metarhizium robertsii* ARSEF 23 were routinely maintained on
potato dextrose agar (PDA) (Difco) at 25°C in the laboratory. For liquid cultures, fungi were grown in a Sabouraud dextrose broth (SDB) (Difco) at 25°C in a rotary shaker. Appressorium induction assays were conducted using locust (Schistocerca gregaria) hind wings or minimal medium (NaNO₃ [6 g liter⁻¹], KCl [0.52 g liter⁻¹], MgSO₄ · 7H₂O [0.52 g liter⁻¹], KH₂PO₄ [0.25 g liter⁻¹]) supplemented with 1% glycerol as the sole carbon source (MM-Gly) (21). For stress response assays, fungi were grown on PDA with 40 mM H₂O₂, 1 M sorbitol, 1 M NaCl, 1 M KCl, 200 μg/ml calcofluor white (CFW) (Sigma), 250 μg/ml Congo red (Bio Basic Inc.), or 0.2% t-butyl-hydrogen peroxide (Aladdin Reagents). Genomic DNA and RNA extractions and hyphal staining were performed on fungal spores cultured in SDB at 25°C and 200 rpm for 3 days in a rotary shaker.

Saccharomyces cerevisiae strains AH109 (MATa Trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ lys2::GAL1-UAS1-GAL1-TATA-his3 MEL1 GAL2-UAS1-GAL2-TATA-ade2 URA3::MEL1-UAS1-MEL1-TATA-lacZ) and EGY48 (MATa trp1 his3 ura3 leu2-6 lexAops-LEU2) were used in this study for transcriptional activation and yeast one-hybrid tests (22), respectively. The yeast cells were cultured in YPDA (1% yeast extract, 2% peptone, 2% glucose, and 2 mg/liter adenine) or synthetic dropout (SD) agar (23, 24).

Transcriptional activation test. First, the full-length MrSkn7 cDNA was amplified with the primers Mrskn7AF (F stands for forward) and Mrskn7AR (R stands for reverse) (see Table S1 in the supplemental material), and cloned into the NdeI and BamHI sites of the yeast vector pBKT7 (Invitrogen) under the control of the Gal4 promoter. The resultant pBK7-MrSkn7 was then transformed into S. cerevisiae AH109 cells using a small-scale yeast transformation protocol (24). Briefly, yeast cells were streaked on YPDA plates, cultured for 2 days at 30°C, and then transferred to a SD lacking tryptophan (SD-Trp) medium for 2 or 3 days at 30°C. The prototrophic transformants were selected and verified by PCR. Positive transformants were transferred into a SD-Trp liquid medium at 30°C and cultured overnight in a rotatory shaker at 200 rpm to an optical density at 600 nm (OD₆₀₀) value of 0.4 to 0.6. The transformants were then moved to SD-Trp·His-Ade (SD lacking Trp, histidine, and adenine) plates at 30°C for 2 or 3 days to test transcriptional activation. A negative control was included by transforming yeast cells with the empty vector, while a positive control was generated by transforming yeast cells with the pBK7 vector containing the Gal4 activation domain sequence (22).

Gene deletion. Targeted gene deletion of the MrSkn7 gene was performed by homologous recombination via Agrobacterium-mediated fungal transformation as previously described (24, 25). Briefly, the 5' - and 3'-flanking sequences were amplified using the genomic DNA as a template with the primer pairs Mrskn7UF/Mrskn7UR and Mrskn7DF/Mrskn7DR, respectively (see Table S1 in the supplemental material). The products were digested with the restriction enzymes BamHI, PstI, and SpeI and then inserted into the corresponding sites of the binary vector pDHT-bar (confering resistance against ammonium glufoinosate) to produce the plasmid pBarskn7 for fungal transformation. The mutants were verified by PCR and reverse transcription-PCR (RT-PCR) analyses with the primers Mrskn7F and Mrskn7R (Table S1).

Ultrastructure analyses. Transmission electron microscopy (TEM) and field emission scanning electron microscopy (FESEM) were performed for ultrastructure observations of the WT and mutant strains. For TEM observation, fungal conidia and mycelia were collected and fixed overnight at 4°C in a phosphate buffer (0.1 M, pH 7.2) containing 2.5% glutaraldehyde. The samples were dehydrated in a graded series of ethanol solutions, infiltrated with a graded series of epoxy resin, and then embedded in Epon 812 resin for observation (Hitachi-7650) (21). For FESEM analysis, the mycelia were harvested from SDB cultures, washed three times, and fixed overnight in the glutaraldehyde phosphate buffer (pH 7.2). The samples were then dehydrated with ethanol solutions (50 to 100%) and finally coated with platinum (26).

Fluorescent staining. Fungal mycelia were collected from SDB cultures 4 days postinoculation, washed with phosphate buffer, and then treated in different buffers containing various fluorescent lectins. These fluorescent lectins include the Alexa Fluor 488-labeled concanavalin A (ConA) (Invitrogen) (60 μg/ml) for binding α-mannopyranosyl and α-glucopyranosyl residues, lectin GSII (from Griffonia simplicifolia) (Invitrogen) (20 μg/ml) for binding α- or β-linked N-acetyl-D-glucosamine (GlcNAc), and fluorescein-labeled Galanthus nivalis lectin (GNL) (Vector Laboratories) (20 μg/ml) for binding α-1,3-mannose (27). After treatment for 1 h, the samples were washed three times with phosphate buffer before examination with an Olympus BX51 microscope (Olympus).

Determination of free amino acids. Conidia of the WT and ΔMrSkn7 strains were inoculated in SDB for 3 days. The mycelia were harvested, washed three times with sterile water, and then transferred into minimal medium without nitrogen (MM-N) liquid medium for 24 h (24). The supernatants were collected by filtration to determine free amino acids. The mycelia were dried completely at 50°C and weighed. Quantitative assays were conducted using a ninhydrin colorimetric method (28). Briefly, to an individual sample (4 ml each) in a test tube, the following were added: 1 ml of 2% ninhydrin (wt/vol) (Sigma) and then 1 ml phosphate buffer (pH 8.0). The samples were thoroughly mixed by vortexing, and the tubes were then placed in a boiling water bath for 15 min. After the tubes were cooled to room temperature, the absorbance of each sample was recorded at 570 nm using a colorimeter (BioPhotometer plus; Eppendorf).

Extracellular enzyme activity assays. The mycelia from SDB cultures were washed twice with sterile distilled water, transferred into minimal medium containing 1% glucose (wt/vol), and incubated at 25°C and 180 rpm for 24 h. The supernatants were collected for enzyme activity assays, and the mycelia were dried and weighed for quantification. For chitinase activity assays, 100 μl culture supernatant was collected, and an equal volume of colloidal chitin (3 mg/ml) (Sigma) was added to each sample for incubation at 28°C for 30 min. The mixture was centrifuged, and the absorbance of the supernatant was measured using a colorimeter at 550 nm. One unit of chitinase activity was defined as the release of 1 μmol GlcNAc per gram of protein in 30 min at 28°C (29). Protease activity was assayed using the substrate N-succinyl-(Ala)₂-Pro-Phe-p-nitroanilide (Sigma) as described previously (30). One unit of protease activity was defined as the release of 1 μmol p-nitroanilide per gram of protein in 10 min at 28°C. All experiments were repeated twice, and each sample had three replicates.

Quantitative real-time RT-PCR analysis. Conidia from the WT and ΔMrSkn7 strains were incubated in SDB for 3 days, and the mycelia were harvested for RNA extraction. For stress challenges, the mycelia from the WT and ΔMrSkn7 strains were inoculated on glass papers covering PDA and PDA buffered with 1 M KCl for 4 days, respectively. The mycelium samples were harvested for RNA extraction using TRIzol reagent (Invitrogen) and treated with DNase I (TaKaRa) before using them for cDNA synthesis with an AffinityScript kit (Toyobo). A quantitative real-time RT-PCR (qRT-PCR) analysis was performed with an ABI Prism 7000 system (Applied Biosystems) using SYBR Premix-Ex Taq (TaKaRa), and the primers used are listed in Table S1 in the supplemental material. The PCR conditions included a 10-min denaturation at 95°C, followed by 30 cycles, with 1 cycle consisting of 30 s at 95°C, 30 s at 56°C, and 30 s at 72°C. Relative expression of each gene was determined by normalization against the expression of β-tubulin (MMAA_02081) (24).

Promoter binding assays. Yeast Skn7 binds the consensus site GCCC(G/C)(A/G) (31). For analysis of the putative MrSkn7 binding site, the promoter regions (ca. 1.5 kb upstream of each start codon) of selected genes were retrieved and analyzed using a weight matrix-based program Match (version 1.0) (Biobase, Beverly, MA). Experimentally, yeast one-hybrid tests were performed to determine the binding and activation function of MrSkn7 on different target genes (see Table S2 in the supplemental material). For this, the cDNA of the MrSkn7 strain was amplified and cloned into the SpeI and SacI sites of pPC86 to generate plasmid pPC86-Mrskn7. Promoter regions of the selected genes were individually amplified using the primers listed in Table S1. The purified products were individually inserted into the Xhol site of p178 vector to regulate the lacZ gene.
reporter gene (32). The resultant vector, pPC86-Mrskn7, and individual p178 promoters were cotransformed into the yeast EGY48 strain, and the positive colonies were identified on selective plates (SD lacking uracil and tryptophan but with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [SD/-Ura-Trp/-X-Gal]).

**Insect bioassays.** Virulence tests for the WT and ΔMrSkn7 strains were conducted using the last instar larvae of the wax moth *Galleria mellonella*. Conidial suspensions were prepared for both topical infection (1 × 10⁷ conidia/ml) and injection (1 × 10⁶ conidia/ml). Each treatment had three replicates with 15 insects each, and the experiments were repeated three times. For injection assays, each insect was injected in the second proleg with 10 μl of spore suspension. After 12 h, insects were bled at various times to examine fungal development within the insect hemocoel. Insect mortality was recorded every 12 h after the treatments, and the median lethal time (LT₅₀) was calculated using Kaplan-Meier analysis in the SPSS program (version 21.0).

**Appressorium induction.** Locust hind wings were collected, surface sterilized in 37% H₂O₂ for 5 min, washed twice with sterile water, and dipped in conidial suspensions (2 × 10⁷ spores/ml) for 20 s. The inoculated wings were placed on a 0.8% water agar at 25°C for 18 h for appressorium induction. The formation of appressorium on a hydrophobic surface was induced using a glycerol-containing medium (21). Briefly, conidia were inoculated into individual polystyrene petri dishes (5.5 cm in diameter) containing 2 ml MM-Gly medium mentioned above. The appressorium differentiation rates were recorded microscopically after incubation for 24 and 48 h.

**RESULTS**

MrSkn7 functions as a transcription factor. The complete open reading frame (ORF) of *MrSkn7* encodes a protein with 489 amino acids (aa). Sequence analysis confirmed that *MrSkn7* is present as a single-copy gene in the genome and that the protein belongs to a typical member of the Pfam 00072 family containing the RR domain (276 to 391 aa) as well as an N-terminal HSF-type DNA binding domain (22 to 44 aa). Transcription activation tests verified that yeast cells containing the *MrSkn7* gene could grow on a Trp-His-Leu dropout plate similar to the positive control containing the activation domain of Gal4 (Fig. 1), which indicates the transcription activation function of MrSkn7.

Requirement of MrSkn7 for sporulation and stress resistance. To determine the effect of *MrSkn7* on fungal development, gene deletion was performed by homologous recombination. Nine putative mutants were selected by PCR (Fig. 2A). When grown on PDA medium, the gene deletion mutants similarly failed to produce conidia. Thus, only one mutant was randomly selected for further analysis. The failure of gene expression in the ΔMrSkn7 mutant was verified by RT-PCR analysis (Fig. 2B). The null mutant demonstrated hyphal autolysis at a later growth stage compared to the WT (Fig. 2C). The Skn7 deletion mutants of other fungi were impaired in oxidative stress resistance (33–35). In contrast, the growth rates of WT and ΔMrSkn7 strains were similar when grown on PDA supplemented with H₂O₂ 15 days postinoculation (t test, *P* = 0.2254) (Fig. 3A). Interestingly, the

**FIG 1** Transcriptional activation of MrSkn7. The *S. cerevisiae* strain AH109, carrying the GAL4 DNA binding domain (BD₃GAL₄) alone (negative control) or fusion cassettes DB₃GAL₄-AD₃GAL₄ (GAL4 activation domain, as a positive control) or DB₃GAL₄-MrSkn7 were cultured on a synthetic dropout medium lacking Trp (SD/-Trp) to select for yeast transformants or SD medium lacking Trp-His-Ade (SD/-Trp-His-Ade) for transcriptional activation at 30°C for 3 days.

**FIG 2** Gene disruption and phenotype characterization. (A) PCR verification of *MrSkn7* gene deletion. WT, the wild-type DNA used as a template for PCR; M, DNA marker; PL, plasmid-cassette used for gene deletion. (B) RT-PCR verification of *MrSkn7* deletion. *Tub2*, a β-tubulin gene, was used as a reference. (C) Phenotypic characterization. In contrast to the wild type (WT), the ΔMrSkn7 mutant lost the ability to sporulate and demonstrated mycelium autolysis 15 days postinoculation on PDA. (Top) Fungal growth on PDA medium. (Bottom) Microscopic observations of the corresponding PDA cultures. Bar = 10 μm.
WT, but not the ΔMrSkn7 strain, was sensitive to t-butyl-hydrogen peroxide (P < 0.001) (Fig. 3B). This is also in contrast to the observations for other fungi that were sensitive to both oxidants (5). However, consistent with previous observations of other fungi (36–38), no obvious differences were observed when the fungi were grown under osmotic stress (1 M sorbitol [P = 0.0573], 1 M KCl [P = 0.0604], or 1 M NaCl [P = 0.4227]) or were heat challenged at 37°C (P = 0.0634) (Fig. 3A and C). In addition, we found that the null mutant was able to sporulate under salt stress (Fig. 3C), although the mutant conidia failed to generate gene complementation transformants. Together, these results reveal the functional divergence of Skn7 orthologs in different fungal species.

**Cell wall integrity defects and cell autolysis.** Skn7 and its orthologs are known to control cell wall integrity (5, 39). To examine the effect of MrSkn7 on the cell wall structures of M. robertsii, we tested fungal sensitivity to different cell wall stress factors and found that the growth of the ΔMrSkn7 strain was inhibited significantly by CR and partially by CFW compared to the WT (Fig. 4A). Lectin binding assays further indicated that the fluorescent signals of lectin GSII (specific for binding chitin component GlcNAc; Fig. 4B) and ConA (specific for binding α-glucose and α-mannose; Fig. 4C) were weaker in ΔMrSkn7 cells than in WT cells. No significant difference was observed between the WT and ΔMrSkn7 strains stained with GNL lectin (Fig. 4D), which specifically binds mannose residues. These results indicate that deletion of MrSkn7 disturbed cell wall biosynthesis, particularly the accumulation of glucan and chitin components.

Ultrastructure analysis using FESEM microscopy indicated that the ΔMrSkn7 mutant had the characteristics of self-digestion

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**FIG 3** Stress response assays. (A) Growth of the wild-type (WT) and ΔMrSkn7 strains was similar on PDA and under different growth conditions for 9 days. (B) Inhibition of the WT but not ΔMrSkn7 growth on PDA supplemented with 1% or 2% t-butyl-hydrogen peroxide (TBHP) for 15 days. (C) Resporulation under salt stress. The sporulation ability of the ΔMrSkn7 mutant was partially rescued after incubation on PDA supplemented with 1 M KCl or 1 M NaCl for 15 days. The inset panels show the microscopic images of conidiation. Bars = 10 μm.

**FIG 4** Cell wall integrity defect in the ΔMrSkn7 mutant. (A) The growth of the ΔMrSkn7 mutant (top row in each panel) was inhibited on PDA supplemented with 200 μg/ml calcofluor white (CFW) or 250 μg/ml Congo red (CR) for 2 days compared to the WT (bottom row in each panel). (B) Fluorescence staining of cell wall components with the lectin GSII (Griffonia simplicifolia) for chitin binding. (C) Staining with the lectin concanavalin A (ConA) to label the components of α-glucoside and α-mannose. (D) Staining with the lectin GNL (Galanthus nivalis lectin) to label α-mannose. Bars = 5 μm.
(Fig. 5A), which is consistent with the observed cell autolysis phenotype (Fig. 2C). Further, TEM analysis confirmed that the cell wall structures of the mutant cells were altered with a significant loss in cellular contents (i.e., the vacuolated hyphae) compared to the WT cells (Fig. 5B and C). Cell autolysis was further verified by reaction with ninhydrin to determine cell leaking and release of free amino acids in culture filtrates by \(\text{H9004} \text{MrSkn7}\) cells, especially after growth in minimal medium without a nitrogen or carbon source (Fig. 6A). In addition to cell vacuolization, fungal cell autolysis was observed with upregulation of intracellular and extracellular hydrolases (e.g., protease and chitinase) (40). Enzyme activity tests showed that the chitinase activity in \(\text{H9004} \text{MrSkn7}\) cells was significantly \((P < 0.01)\) increased in nutrient-poor conditions than in the WT cells (Fig. 6B). However, the protease activity of mutant cultures was significantly higher than that of WT cultures when the fungi were grown in nutrient-rich (SDB) or nutrient-poor media (Fig. 6C).

**Impairments in fungal virulence, appressorium differentiation, and immune evasion.** To examine the consequence of \(\text{MrSkn7}\) deletion on fungal virulence, we performed insect bioassays by both injection and immersion of wax moth larvae with fungal spores. Conidia from both WT and \(\text{ΔMrSkn7}\) strains were harvested from KCl salt stress medium and used for bioassays (Fig. 3C). The results indicated that, in contrast to the WT, the \(\text{ΔMrSkn7}\) mutant lost its ability to kill insects during topical infection (Fig. 7A). In injection assays, the \(\text{ΔMrSkn7}\) mutant could kill insects, but it took a significantly longer time \((4.19 \pm 0.27\text{ days})\) \((\chi^2 = 40.94, P < 0.0001)\) than the WT \((1.73 \pm 0.07\text{ days})\) (Fig. 7B). These data suggest that the ability to penetrate insect cuticles was attenuated in the \(\text{ΔMrSkn7}\) mutant, which was verified by appressorium induction assays both on locust hind wings and on a hydrophobic surface. Thus, the \(\text{ΔMrSkn7}\) mutant lost the ability to form appressoria and showed delayed germination compared to the WT (Fig. 7B). Further observations indicated that, relative to the WT, the \(\text{ΔMrSkn7}\) cells were susceptible to the attacks of insect hemocytes. Mutant cells were heavily encapsulated, melanized, and thus failed to produce blastospores (i.e., hyphal bodies) in insect hemocoel within 48 h after injection (Fig. 7D), which could in part contribute to the virulence defect in the \(\text{ΔMrSkn7}\) mutant in the injection assays (Fig. 7B).

**Regulation of putative target gene expression.** To examine the regulation of putative target genes involved in mediating sporulation, cell wall synthesis, and autolysis (see Table S2 in the supplemental material), we performed serial qRT-PCR analyses and compared the transcription in the WT and \(\text{ΔMrSkn7}\) strains...
Fig. 8. Consistent with the failure of the mutant strain to sporulate (Fig. 2C), transcript levels of the putative sporulation-related genes were significantly \((P < 0.05)\) downregulated in the \(\Delta MrSkn7\) strain relative to the WT when the fungi were grown on PDA (Fig. 8A). However, under salt stress, the expression of these genes was upregulated in the \(\Delta MrSkn7\) mutant compared to the WT (Fig. 8A), consistent with the restoration of sporulation ability in the mutant (Fig. 3C). For example, a homolog
(MAA_04291, 53% identity) of yeast sporulation-specific gene Spx19 (41) was downregulated >3-fold after MrSkn7 deletion, but the gene was significantly upregulated by both the WT and ΔMrSkn7 strains under salt stress. Genes involved in cell wall integrity (see Table S2 in the supplemental material) were significantly downregulated (P < 0.05) in the ΔMrSkn7 mutant compared to the WT (Fig. 8B). For example, a homolog (MAA_04270, 62% identity) of yeast oligosaccharyltransferase gene, Stt3, involved in the biosynthesis of cell wall β-1,6-glucan (22, 42) was downregulated >2-fold in the mutant cells. In addition, a homolog (MAA_08135, 55% identity) of the Aspergillus nidulans transcription factor RimA, which contributes to cell wall chitin accumulation (43, 44), was downregulated >3-fold in the ΔMrSkn7 mutant compared to the WT (Fig. 8B). These results are consistent with the reduced levels of glucan and chitin accumulations in mutant cells (Fig. 4B and C).

We also found that the expression of autolysis-related genes was differentially expressed in the WT and ΔMrSkn7 strains (Fig. 8B). MAA_06444, a putative gene encoding a carbon catabolite repressor, CreA, was significantly (P = 0.05) downregulated in the ΔMrSkn7 strain, whereas a putative GlcNAc-6-phosphate deacetylase Dac1-like gene (MAA_02207, 37% identity) was upregulated >8-fold in the ΔMrSkn7 strain compared to the WT (Fig. 8B). In A. nidulans, extracellular chitinase and protease genes were highly transcribed in CreA null mutants (44). Consistent with this, many protease and chitinase genes were evidently upregulated (P < 0.05) in the ΔMrSkn7 mutant compared to the WT (Fig. 8C and D). For example, a trypsin-like protease gene (MAA_01416) was upregulated >10-fold in the ΔMrSkn7 strain.

Transcription activation of target genes. To examine whether MrSkn7 is involved in the transcriptional control of the selected target genes (see Table S2 in the supplemental material), we first performed an in silico analysis for the putative MrSkn7 binding site. The results indicated that a conserved site, GGCC(A/G), is present in the promoter regions of all selected target genes (Fig. 9A). The motif is highly similar to the yeast Skn7 binding site GCC(C/G)(A/G) (31), suggesting that it is the putative binding site of MrSkn7. Yeast one-hybrid tests demonstrated that the genes examined were either directly or indirectly controlled by MrSkn7 (Fig. 9B). A stronger β-galactosidase activity was evident for the sporulation-related gene MAA_06431 (Spo7), autolysis-associated genes MAA_02207 (Dac1) and MAA_06444 (CreA), and cell wall integrity-associated genes MAA_08135 (RimA) and MAA_04270 (Stt3), suggesting the direct regulation of these genes by MrSkn7. Even the consensus site is similarly present in the promoter regions; however, no positive signals were detected for two proteases (MAA_08178 and MAA_08308) and two chitinases (MAA_09072 and MAA_03460), suggesting the absence of direct regulation for these genes by MrSkn7.

DISCUSSION

In this study, we characterized the functions of a yeast Skn7 homolog, MrSkn7, in the insect-pathogenic fungus M. robertsii and found that the gene is required for conidiation, maintenance of cell wall integrity, and infection structure differentiation and is therefore critical for fungal virulence. Compared to the functions of Skn7 orthologs reported in other fungi, MrSkn7 shared some similarities, such as regulating cell wall integrity, to the homologs in C. neoformans, Aspergillus fumigatus, and F. graminearum (5, 8). However, these observations were different from the gene deletion mutants of C. albicans and Schizosaccharomyces pombe where cell integrity was not affected after deletion of their respective homologs (5, 11, 45). In particular, we found that deletion of MrSkn7 impaired the proper accumulation of chitin and β-glucan during

FIG 8 Differential expression of genes. (A) Differential expression of sporulation-associated genes in WT and ΔMrSkn7 strains grown on PDA and PDA supplemented with 1 M KCl. (B) Differential expression of cell wall integrity-related genes (MAA_04270 and MAA_06444) and autolysis-related genes (MAA_02207 and MAA_06444) in WT and ΔMrSkn7 strains grown in SDB for 3 days. Relative to the WT, significant upregulations of protease (C) and chitinase (D) genes were observed in the ΔMrSkn7 mutant when the fungi were grown in SDB for 3 days. Values are the means plus standard errors (error bars) for three replicates.
cell wall biosynthesis, which is direct evidence that Skn7 plays a critical role in maintaining cell wall integrity.

We also found that the \( \Delta \text{MrSkn7} \) mutant completely lost the ability to produce conidia on PDA, while the conidiation abilities were only partially impaired in the \( Bc\text{Skn7} \) deletion mutant of \( B. \text{cinerea} \) (9), \( Fg\text{Skn7} \) mutant of \( F. \text{graminearum} \) (8), and \( \Delta \text{SrrA} \) (i.e., \( \Delta \text{AnSkn7} \)) mutant of \( A. \text{nidulans} \) (36). Salt stress could induce the upregulation of sporulation-related genes (Fig. 8A), a possible mechanism that could restore conidiation ability to the mutant. Similar to the \( Mo\text{Skn7} \) null mutant of the rice blast fungus (7), the \( \Delta \text{MrSkn7} \) mutant was not sensitive to \( \text{H}_2\text{O}_2 \) and became even more resistant to \( \text{t}-\text{butyl-hydrogen peroxide} \) than the WT strain (Fig. 3A and B). Alternatively, it could be due to the fact that \( \text{t}-\text{butyl-hydrogen peroxide} \) but not \( \text{H}_2\text{O}_2 \) inhibits cell peroxidase activity (46). The exact mechanism remains to be determined; however, this observation is in contrast to the null mutants of \( A. \text{fumigatus} \) and \( A. \text{nidulans} \) yeasts that are sensitive to both oxidants (5). Similar to the observations in \( A. \text{alternata} \) (10), \( C. \text{neoformans} \) (12), and \( C. \text{albicans} \) (11), the virulence of the \( \Delta \text{MrSkn7} \) mutant was severely impaired during topical infection. However, the infection abilities of the \( \Delta \text{AfSkn7} \) (\( \Delta \text{AfSkn7} \) stands for \( \Delta \text{A. fumigatus Skn7} \)) (47), \( \Delta \text{MoSkn7} \) (7), \( Fg\text{Skn7} \) (8), and \( \Delta \text{BcSkn7} \) (9) mutants were not affected. Consistent with their differences in virulence, the formation of appressoria failed in the \( \Delta \text{MrSkn7} \) mutant (Fig. 7C) but not in the \( \Delta \text{MoSkn7} \) mutant (7). Similarly, the responses to osmotics stresses also varied among different fungal species (5). Thus, the functional divergence of Skn7 orthologs in the different fungal species opposes the tenet of ortholog conjecture, which claims that orthologous genes perform identical or equivalent biological functions in different organisms (48).

Besides the failure to form appressorium, deletion of \( \text{MrSkn7} \) also impaired the ability of the fungus to counteract host immune responses that could contribute to virulence defects in the mutants (Fig. 7D). In general, the expression of proteases would be switched off by \( \text{Metarhizium} \) after reaching the insect hemocoel (49). Failure to do so would result in the activation of insect prophenoxidases by proteases that could lead to melanization and production of melanins, compounds that are toxic to both the fungi and insects (50). In this respect, the upregulation of proteases in the \( \Delta \text{MrSkn7} \) mutant could trigger heavy melanization responses in insects and thereby the production of melanins to attenuate fungal virulence in the injection assays.

Unlike previous studies with other fungi, we established that \( \text{MrSkn7} \) is involved in cell autolysis in \( M. \text{robertsii} \). Autolysis is a natural process that results in the self-digestion of senescent cells, which occurs due to the extracellular and intracellular hydrolyase activities and leads to cell vacuolization and disruption of cell wall structures (51). In bacteria, the TCS pathway is connected to cell autolysis (52, 53), and deletion of TCS pathway genes has been shown to accelerate cell autolysis, release more extracellular DNA, and activate autolysis-related genes (54). In filamentous fungi, autolysis is triggered by carbon starvation (55). For example, in \( A. \text{nidulans} \), the carbon catabolite repressor CreA (AN6195, homolog of \( \text{MAA}_06444 \), 52% identity) inhibits the expression of autolytic enzyme proteases and chitinases, and deletion of \( \text{CreA} \) leads to a hyperautolytic phenotype with high extracellular chitinase and protease activities (40). Upregulation of \( \text{Dac1}-\text{like} \) gene (\( \text{MAA}_02207 \)) also indicates cell death/autolysis induced by the cell wall constituents of chitin, chitooligomer and \( \text{N}-\text{acetyl-D-glucosamine} \), released by chitinase digestion (40, 56). Consistent with these observations, deletion of \( \text{MrSkn7} \) led to the upregulation of proteases, chitinases, and a \( \text{Dac1}-\text{like} \) gene, but downregulation of

**FIG 9** Analysis of putative MrSkn7 binding site and yeast one-hybrid tests. (A) Identification of the consensus binding site present in the promoter regions of target genes. (B) Yeast one-hybrid tests. Various degrees of \( \beta \)-galactosidase activities showing the differential abilities of promoter binding and transcriptional activation of different target genes by MrSkn7. The clones without \( \beta \)-galactosidase activities are evidence suggesting that the genes are likely indirectly controlled by MrSkn7.
Chen LH, Lin CH, Chung KR. MrSkn7 contributes to the regulation of fungal cell autolysis. The role of MrSkn7 in the development and control of virulence in insect-pathogenic fungus Metarhizium robertsii is still unknown. MrSkn7 is involved in the indirect control of these genes. The results of this study not only detail the function of the MrSkn7 orthologs but also provide insights into the development and control of virulence in insect-pathogenic fungi.

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